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Interleukin-33 Prevents Retinal Ischemia Reperfusion Injury in Rats by Preventing Apoptosis

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Abstract

Background: Retinal ischemia reperfusion (RIR) injury is a common pathological process that can result in visual impairment in many ophthalmic diseases. Inflammation and apoptosis play an important role in RIR injury.
Objectives: This experimental study was designed to explore the ability of a new cytokine, IL-33, to attenuate RIR injury via an apoptosis-inhibitory mechanism.
Methods: From June, 2015 to October, 2015, 40 Sprague-Dawley (SD) rats from Wuhan university in China were divided into the following four groups: normal control group (NCG), RIR injury model group (MG), IL-33 pretreatment group (IL-33), and PBS group (PBS) according to random number tables. Rats in the IL-33 and PBS groups received an intravitreous injection of 2 μg of recombinant IL-33 (rIL-33) or PBS one hour before the induction of ischemia. Histological evaluation, inflammatory cell infiltration, and apoptosis of retinal cells were examined. The expressions of apoptotic-related proteins (Bcl-2 and Bax) were quantified by immunohistochemistry and western blotting. The presence of NF-κB p65 in the retina was assessed by western blotting.
Results: Our data revealed that IL-33 pretreatment maintained a better retinal structure, inhibited leukocyte infiltration (IL-33 vs. MG with $P < 0.01$ and IL-33 vs. PBS group with $P < 0.01$), and reduced the apoptosis of retinal ganglion cells (IL-33 vs. MG with $P < 0.05$ and IL-33 vs. PBS group with $P < 0.05$). Furthermore, IL-33 upregulated the expression of Bcl-2and decreased the expression of Bax (IL-33 vs. MG with $P < 0.01$ and IL-33 vs. PBS group with $P < 0.01$). In addition, IL-33 attenuated NF-κB p65 levels in the retina and inhibited the activation of NF-κB (IL-33 vs. MG with $P = 0.021$ and IL-33 vs. PBS group with $P = 0.025$).
Conclusions: IL-33 may be a potential new agent to attenuate RIR injury by reducing inflammatory cell infiltration and preventing apoptosis.

Keywords: Retinal Ischemia Reperfusion Injury, Interleukin-33, Apoptosis, Cytokine

1. Background

Retinal ischemia reperfusion (RIR) injury is a common pathological process, which leads to the death of retinal ganglion cells (RGCs), serious degeneration of the retina, and in some cases loss of vision (1). The pathogenesis of RIR injury is complicated, and the mechanisms involved in neuronal degeneration have not yet been completely elucidated. However, it is known that excessive activation of the apoptotic pathway plays an important role in disease pathogenesis (1, 2). In addition, the upregulation of inflammatory cytokines and the recruitment of leukocytes into the retina are also involved (3, 4). Many studies have confirmed that inflammatory factor-mediated immune inflammation is particularly involved in RIR injury.

IL-33 is a recently identified multifunctional cytokine and a newly identified member of the IL-1 family (5). IL-33 participates in diverse processes, including infectious diseases (6), allergic diseases (7, 8), and autoimmune disorders (9, 10) when combined with its specific receptor, ST2. IL-33 also functions as an inflammatory cytokine to induce Th2 cytokine production (5).

Previous studies have demonstrated that IL-33 has diverse effects in immune and inflammatory responses. IL-33 has a protective effect in tissues and organs undergoing ischemia/reperfusion (I/R) injury. In a model of liver I/R injury, IL-33 pretreatment relieved symptoms and protected hepatocytes. These observations might be related to the inhibition of NF-κB activation by IL-33, which suppresses the expression of inflammatory cytokines and neutrophil activation related to infiltration (11). IL-33 significantly improved acute stage and delayed acute cerebral I/R injury in mice, and the protective role of IL-33 has been suggested to involve an induced immuno-shift of Th cells from a Th1 to a Th2-type response, as well as suppression of Th17 immune responses (12). Many studies have shown that IL-33 prevents myocardial I/R injury by inhibiting inflammation and cardiocyte apoptosis (13, 14). However, the role and mechanisms of IL-33 in RIR injury have not yet been stud-

ied.

2. Objectives

The present study investigated whether IL-33 is a protective factor in RIR injury; the underlying mechanisms were also investigated.

3. Methods

3.1. Grouping and Preparation of the RIR Animal Model

From June, 2015 to October, 2015, a total of 40 SPF male Sprague-Dawley (SD) rats weighing 200 g - 250 g were purchased from the experimental animal center of Wuhan university in China. All of the experimental and animal-handling procedures conformed to the association for research in vision and ophthalmology statement for the use of animals in ophthalmic and vision research and were approved by the committee on the Use of living animals in teaching and research at Wuhan university in June, 2015 (ID 20150617Q172).

Before modeling, all rats were observed carefully by slit lamp and ophthalmoscope to ensure that they were without eye diseases. RIR injury was induced by increasing the intraocular pressure in the right eye, as previously described (15). Rats were deeply anesthetized with 10% chloral hydrate solution (3 mL/kg, intraperitoneal). The anterior chamber was cannulated with a 27-gauge needle attached to an infusion line of 250 mL of sterile saline. Intraocular pressure was elevated to 110 mmHg by lifting the infusion saline bottle to a height 150 cm over the eyeball for 60 minutes, causing retinal ischemia. Retinal ischemia was confirmed by a whitening of the iris and fundus via fundus examination. Intraocular pressure was then reverted to a normal level by reducing the bottle height to allow for reperfusion of the retinal vasculature. The right eyes of all rats were used for modeling. Rats were divided into the following four groups using a random number table: (1) normal control group (NCG), (2) RIR injury model group (MG), (3) IL-33 pretreatment group (IL-33), (4) PBS group (PBS), (n = 10 per group). Rats in the IL-33 and PBS groups received an intravitreous injection of 2 μg of recombinant IL-33 (PeproTech, Rocky Hill, USA) (IL-33 pretreatment group) or an equal amount of PBS (PBS group) with a microinjector one hour before RIR injury modeling. Rats were sacrificed 24 hours after reperfusion, and the right eyeball of each animal was immediately enucleated. Five eyeballs were fixed with 4% paraformaldehyde for histopathological and immunohistochemical evaluations, and the other five retinal tissues were collected for western blotting.

3.2. Hematoxylin and Eosin Staining and Morphological Examination

At 24 hours after reperfusion, the eyeballs were fixed with 4% paraformaldehyde overnight at room temperature and examined with hematoxylin and eosin (H&E) staining to evaluate histopathology and inflammatory cell infiltration in the retina. Fixed retinal tissues were dehydrated, embedded in paraffin, and cut into 5-μm sagittal sections (the retina near the optic disc). The sections were treated with toluene for two hours to remove the paraffin, then hydrated in a series of alcohol solutions, and stained with H&E. The retinal structure was evaluated, and the infiltrated inflammatory cells in the ganglion cell layer (GCL) were counted over an area of 200 μm in four retinal sections of each group.

3.3. TUNEL Staining

Apoptotic cells were identified using terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) (TUNEL kit; 11684817910, roche applied science, indianapolis, IN, USA). Retinal sections (5-μm thick) were obtained as described above, and the deparaffinized sections were treated with the kit in accordance with the manufacturer's instructions. The sections were then stained using a DAB staining kit (DA1010, Solarbio Technology Co. Ltd., Beijing, China). The sections were examined under 400x magnification. Apoptotic cells on tissue sections appeared brown under the microscope. TUNEL-positive cells in the GCL and inner nuclear layer (INL) were counted in five vision fields randomly selected through each 100-mm retina length on both sides of the optic nerve head, and the apoptotic index (AI) was calculated. AI was defined as (number of brown nuclei per field/total number of nuclei per field) $\times$ 100%.

3.4. Immunohistochemistry for Bcl-2 and Bax in the Retina

Retinal sections (5-μm thick) were obtained as described above, and immunohistochemistry was performed on paraffin sections using standard protocols to detect Bcl-2 and Bax protein levels in the retina at 24 hours after RIR injury. After routine dewaxing, hydration, antigen retrieval, and blocking of endogenous peroxidase, the sections were incubated with primary antibody against Bcl-2 (1:100, ab7973, Abcam, Cambridge, UK) or Bax (1:50, ab32503, Abcam, Cambridge, England) overnight at 4°C. The sections were then incubated with biotinylated goat anti-rabbit IgG (1:1, K5007, Dako, Denmark) at 37°C for 50 minutes. Finally, slides were examined under a microscope (Nikon E100, Japan) with a digital camera (Canon 600D, Japan). Cells with cytoplasmic yellow or brownish-yellow staining were considered to be positive, and the

integrated optical density (IOD) values of the staining for retinal Bcl-2 and Bax in each group were analyzed using Image-Pro Plus 6.0 Software (Media Cybernetics, USA).

3.5. Western Blotting Assay of Bcl-2, Bax and p-P65 Expression in the Retina

The expression of Bcl-2, Bax, and NF-κB p65 was assessed by western blotting, as described previously (16). Briefly, 24 hours after ischemia, the retina tissue was harvested and homogenized for 30 minutes on ice in a buffer containing proteinase inhibitors. Supernatant was collected after five minutes of centrifugation at 12,000 $\times$ g at 4°C. The amount of protein was determined using the Bradford assay (Bio-rad, Hercules, CA, USA). The samples were boiled for five minutes in a water bath, and the retinal proteins (30 mg per sample) were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% (w/v) acrylamide gels and transferred onto a nitrocellulose membrane.

After being blocked with 5% skim milk in Tween20/PBS, the membranes were probed with primary antibodies, including anti-Bcl-2 (diluted 1:100, ab7973, Abcam, Cambridge, UK), anti-Bax (diluted 1:2,000, ab32503, Abcam, Cambridge, UK) and anti-p65 (diluted 1:2,000, ab16502, Abcam, Cambridge, UK) overnight at 4°C. As a loading control, β-actin (Wuhan Boster Bioengineering Co. Ltd., Wuhan, China) was measured in each western blotting. Then the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1.5 hours at room temperature, and the specific bands were visualized with an ECL detection system (Pierce Biosciences, Rockford, IL) according to the manufacturer's instructions. Relative changes in the optical intensity of each band were semi quantitatively calculated with Image Pro Plus 6.0 software (Media Cybernetics, USA). The band densities of each sample were normalized to the β-actin band.

3.6. Statistical Analysis

The data are presented as mean $\pm$ standard deviation. The results were statistically analyzed using a computerized statistical program (SPSS version, 17.0). LSD-t test and one-way ANOVA were applied for intergroup comparisons. A value of $P < 0.05$ was considered statistically significant.

4. Results

4.1. Changes in Retinal Structure and Leukocyte Infiltration after Different Treatments

Retinal histological evaluation at 24 hours was used to determine the morphology and GCL leukocyte infiltration of rat retinas following different treatments (Figure 1). In the NCG, the retinal structure was clear, and cells in every layer appeared normal. In the MG and PBS-treated groups, retinal edema and cell interrupted distribution in various layers was observed. In addition, obvious leukocyte infiltration was observed in the GCL and other inner layers in the MG- and PBS-treated groups. There was no difference in terms of morphology and leukocyte infiltration between the PBS group at 24 hours and the MG group at 24 hours. However, in the IL-33 pretreated group, retinal edema and cellular arrangement disorder were not obvious compared to the MG and PBS treated groups. In addition, the infiltrating cell rate in the GCL in the IL-33 group was significantly lower than that of the MG group and PBS group ($P < 0.01$, Table 1). These results suggest that RIR injury increased inflammation reaction and IL-33 pretreatment protected the retina via the inhibition of leukocyte infiltration after RIR injury.

4.2. IL-33 Treatment Inhibited Apoptosis in the Retina Following Ischemic Injury

Previous studies have shown that apoptotic cells reached a peak at 24 hours after RIR injury. In this study, the TUNEL method was used to detect apoptotic retinal cells, and the results indicated no obvious apoptosis cells in the retina of the NCG. In the model and the PBS groups, a large number of nuclei were dark brown and were mainly located in the retinal GCL and INL. In the IL-33 pretreatment group, the apoptotic cells showed low positive expression, and the apoptotic index was significantly lower than that of the model group and the PBS group ($P < 0.05$, Figure 2, Table 1). The results showed that IL-33 pretreatment inhibited the apoptosis of retinal neurons in the retina after RIR injury and protected the retina.

4.3. IL-33 Increases Bcl-2 and Decreases Bax Levels in the Retina

Immunohistochemistry results showed that Bcl-2 and Bax expressions were low in the retinas of the NCG. In the model, IL-33 pretreatment and PBS group, Bcl-2, and Bax positive cells were mainly present in the RGC layer and the INL (Figures 3 and 4). The expression of Bcl-2 and Bax in the four groups of retinal average optical density values are shown in Table 1. The eyes that received IL-33 pretreatment had a significantly higher level of Bcl-2 and a lower level of Bax protein than those in the model or PBS groups (all $P < 0.01$). No significant difference in the level of Bcl-2 and Bax was found between the model and PBS groups ($P > 0.05$).

The levels of Bcl-2 and Bax in rat retinas receiving IL-33 or PBS treatment 24 hours after RIR were also detected by western blotting (Figure 5). These results also demonstrate that Bcl-2 expression was higher and Bax expression was lower in the IL-33 pretreatment group compared with the

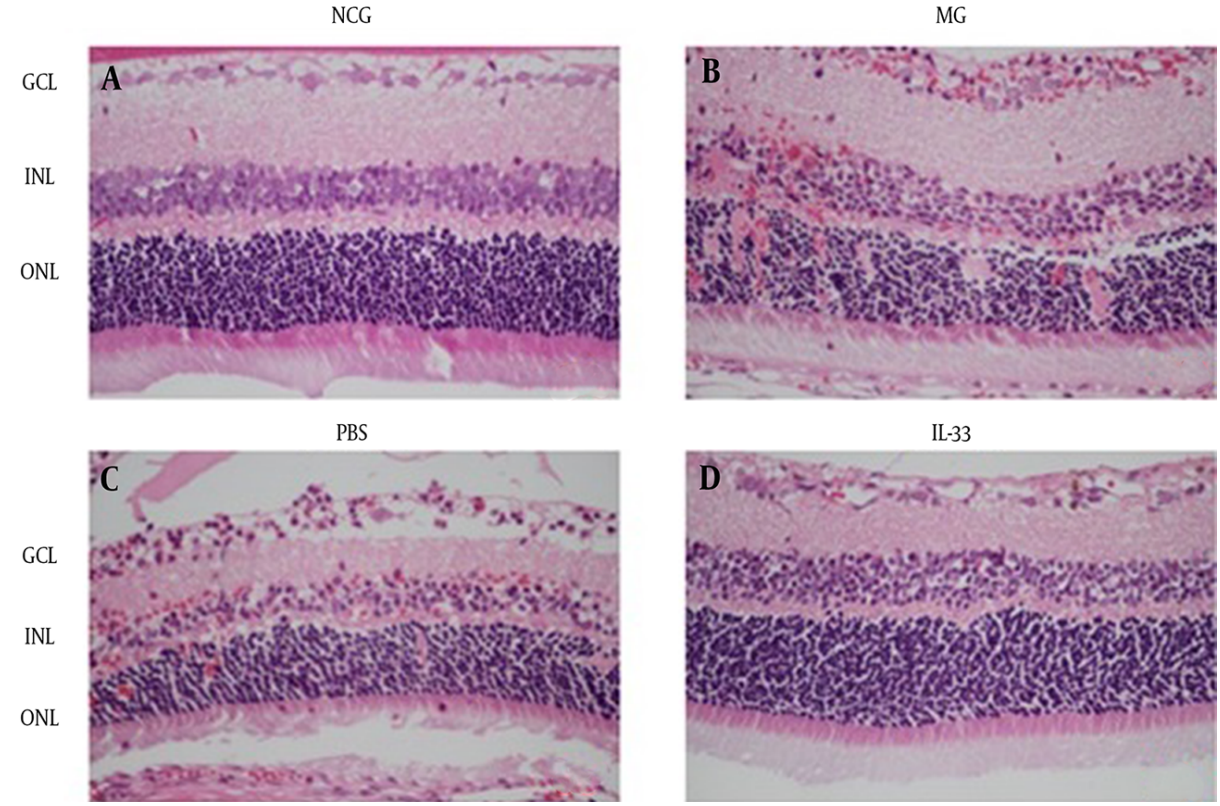


Figure 1. Retinal Sections Were Subjected to H&E Staining to Evaluate the Histopathology and Inflammatory Cell Infiltration in the Retina at 24 Hours after Different Treatments

Table 1. The Number of Infiltrating Cells, AIm and the Retinal Expression of Bcl-2 and Bax[a]

Group	Infiltrated Cells	AI (%)	Bcl-2	Bax
NCG	12.48 ± 2.16	4.35 ± 0.82	0.124 ± 0.009	0.117 ± 0.011
MG	56.32 ± 6.84[b]	67.88 ± 17.34[b]	0.233 ± 0.026[b]	0.239 ± 0.025[b]
PBS	63.45 ± 10.21[b,c]	70.35 ± 15.86[b]	0.252 ± 0.024[b]	0.254 ± 0.030[b]
IL-33	42.33 ± 9.82[b,d,e]	37.38 ± 12.11[b,d,e]	0.308 ± 0.067[b,d,e]	0.169 ± 0.021[b,d,e]

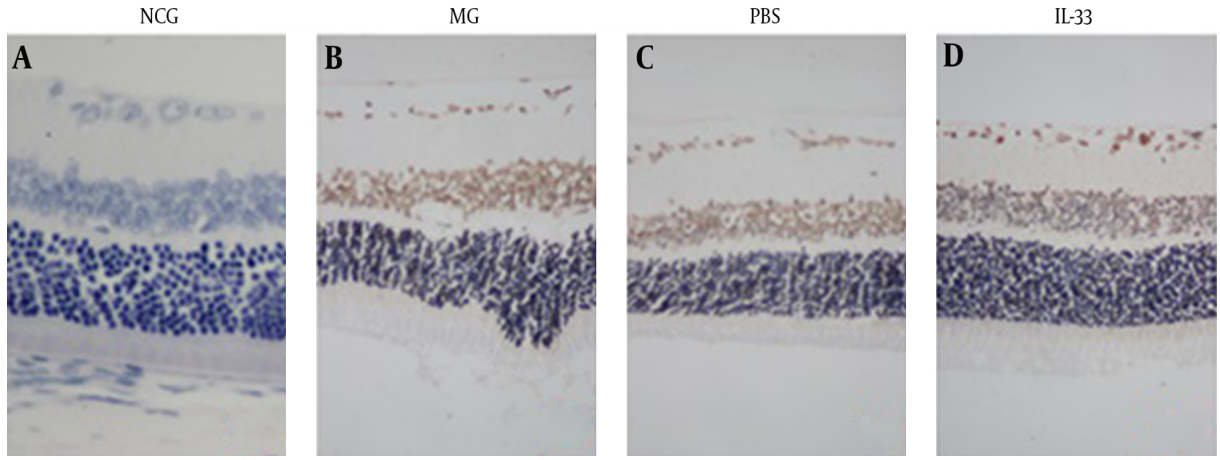
[a]Data are the mean ± standard deviation; n = 5 per group.
[b]P < 0.01 vs. NCG.
[c]P < 0.05 vs. MG.
[d]P < 0.01 vs. MG.
[e]P < 0.01 vs. PBS.

model and PBS groups (Table 2). Western blotting demonstrated that IL-33 can regulate the ratio of Bcl-2/Bax expression to inhibit the apoptosis of retinal tissues after RIR injury.

4.4. IL-33 Inhibits the Activation of NF-κB in the Retina

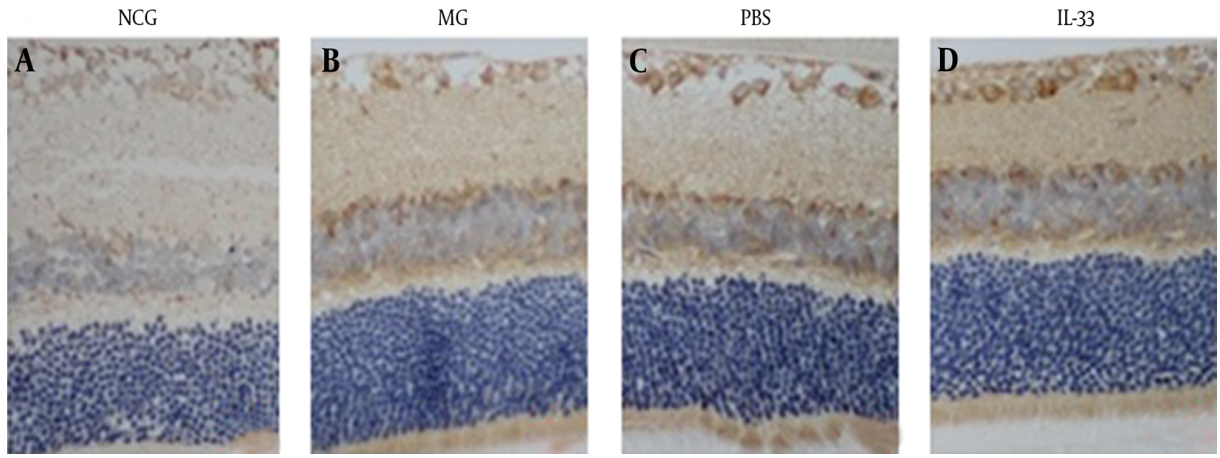
The level of NF-κB p65 in rat retinas with or without IL-33 treatment 24 hours after RIR is shown in Figure 5 (n = 5 in each group). Retinal ischemia markedly upregulated p65 protein expression at 24 hours (P < 0.0001 between the NCG and MG groups, P < 0.0001 between the NCG and PBS groups and P = 0.001 between the NCG and IL-33 groups). Significant differences in p65 protein levels were observed after IL-33 treatment (P = 0.021 between the MG and IL-33 groups and P = 0.025 between the IL-33 and PBS groups). These results suggest that IL-33 inhibited the activation of NF-κB in RIR.

Figure 2. IL-33 Treatment Inhibits Apoptosis in the Retina at 24 Hours Following Ischemic Injury



Representative photomicrographs showing TUNEL-labeled cells in retinal sections from various treatment groups (2A-2D). Barely visible TUNEL-reactive cells were present in the retinas of the NCG (2A). A robust increase in TUNEL positive cells was present in the ganglion cell layer (GCL) and the inner nuclear layer (INL) of the MG and PBS groups (2B and 2C). Treatment with IL-33 significantly reduced TUNEL positive cells in both the GCL and the INL (2D).

Figure 3. IL-33 Upregulates Bcl-2 Expression in the Retina at 24 Hours Following Ischemic Injury

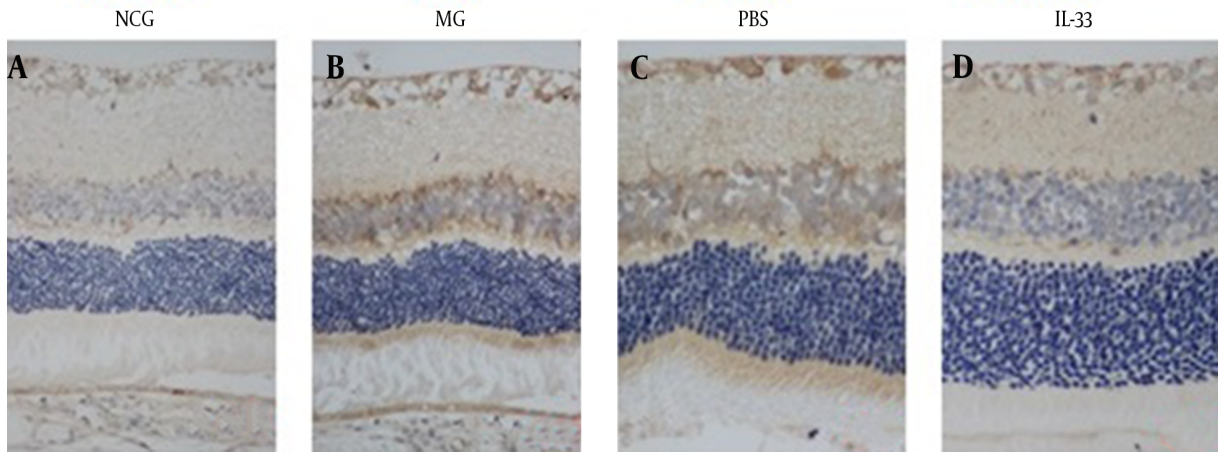


Low positive expression in the retinas of the NCG group (3A), moderate positive expression in MG and PBS groups (3B and 3C), and strong positive expression in the IL-33 group (3D).

5. Discussion

I/R injury refers to the phenomenon that occurs when rebuilding the blood supply after ischemia, which usually aggravates the function of cells, causing structural damage and metabolic disorders. RIR injury is a common clinical condition that occurs in a variety of ocular pathologies, including retinal vascular occlusion, acute glaucoma, diabetic retinopathy, and ophthalmic operations that affect the retinal blood flow, and which represents the main cause of irreversible vision loss in humans (17). The pathogenesis of RIR injury is complicated and is not completely understood. However, it is known that apoptosis plays an important role (18). One study revealed that cells in the inner retina especially RGCs are more vulnerable to ischemic damage compared to cells of the outer retina (19). Apoptosis, as detected by TUNEL staining, has been observed in the GCL, INL, and outer nuclear layer of the retina (20). Apoptosis of the ganglion cells is progressive and irreversible (20); therefore, anti-apoptosis therapy in retinal ischemic diseases may be beneficial to improve retinal functions. Re-

Figure 4. IL-33 Treatment Downregulated Bax Expression in the Retina at 24 Hours Following Ischemic Injury



Low positive expression in the retinas of the NCG and IL-33 group (4A and 4D), and moderate positive expression in the retinas of the MG and PBS groups (4B and 4C).

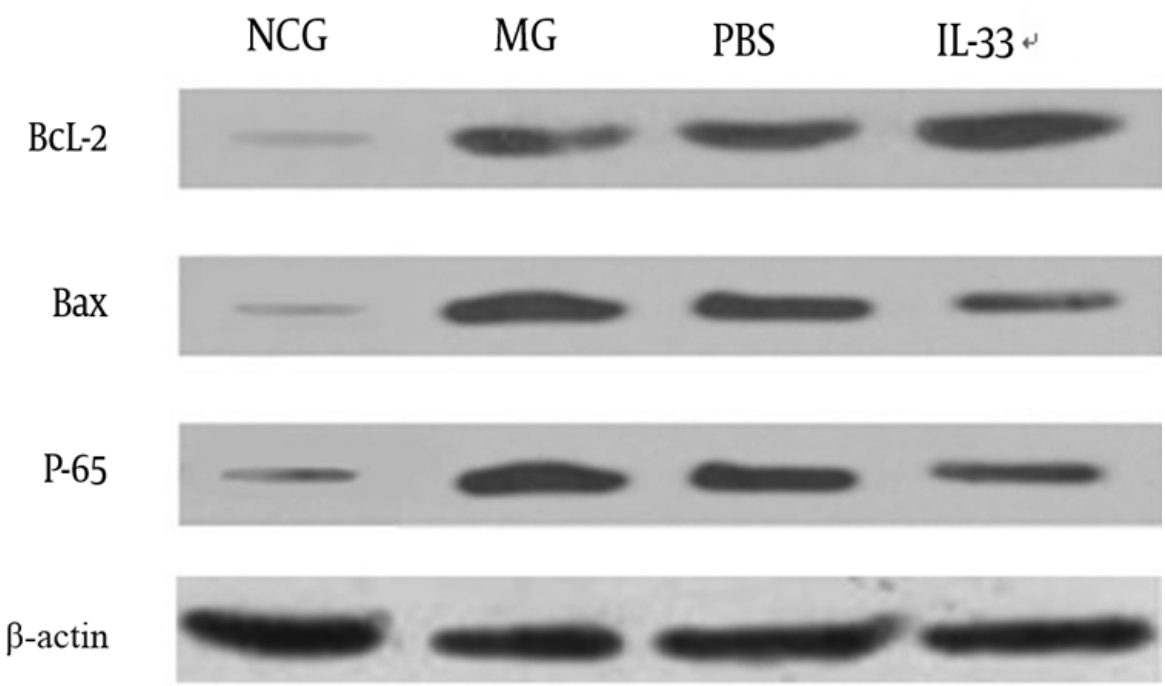


Figure 5. Western Blotting Assay of Bcl-2, Bax, and p65 Protein Expression in the Retina at 24 Hours after Different Treatments

search on RIR injury is a current hot topic in basic and clinical research.

It was reported that in the subacute phase of RIR injury (several hours to several days after RIR), inflammatory factor-mediated immune inflammation is particularly involved (21, 22). Tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), and other inflammatory mediators, through a series of cascade reactions, may induce leukocyte infiltration to the lesions, block the capillaries, and damage the microvascular endothelium, leading to low perfusion. The infiltrating leukocytes further produce large amounts of cytokines, chemokines, and matrix met-

Table 2. The Protein Expression of Bcl-2, Bax, and p65 in the Retina (Western Blotting)[a]

Group	Bcl-2	Bax	p65
NCG	0.09 ± 0.02	0.07 ± 0.01	0.12 ± 0.02
MG	0.83 ± 0.15^{b}	0.94 ± 0.21^{b}	1.03 ± 0.38^{b}
PBS	0.88 ± 0.27^{b}	0.89 ± 0.13^{b}	0.93 ± 0.27^{b}
IL-33	$1.24 \pm 0.38^{b,c}$	$0.42 \pm 0.20^{b,c}$	$0.39 \pm 0.18^{b,c}$

[a] Bcl-2, Bax and p65 protein expression changes measured by the optical intensity of each band were normalized to the β-actin band; Data are expressed as mean $\pm$ standard deviation; n = 5 per group.
[b] $P < 0.001$ vs. NCG.
[c] $P < 0.001$ vs. MG or PBS.

alloproteinases (MMPs), causing blood-retinal barrier destruction and aggravating the injury, ultimately resulting in retinal neuronal cell death (23, 24).

However, there are also some protective cytokines in RIR injury. For example, IL-6 may have protective effects on GCL neurons (25), and exogenous IL-6 is neuroprotective in RIR injury (26). IL-33 is a new multifunctional cytokine discovered in 2005 (5). It is well known that IL-33 has significant effects on inflammatory, infectious, and immunological responses when it binds and signals through its specific receptor, ST2L. Previous studies have demonstrated that IL-33 is a significant protective factor in the I/R injury of the heart, liver, brain, and other organs; however, the role of IL-33 in RIR injury has not yet been reported. Therefore, in the current study, we determined whether IL-33 has the same protective effect in RIR injury.

Apoptosis is a special kind of cell death and is regulated by multiple genes and proteins. The Bcl protein family plays an important regulatory role in the process of cell apoptosis, in which Bcl-2 is the most important anti-apoptotic protein, whereas Bax plays a role in promoting apoptosis (27). The ratio of Bcl-2 to Bax plays an important role in cell fate (i.e., survival or death) following an apoptotic stimulus (16, 28). In this experiment, through the establishment of the RIR injury model, we observed the effects of rIL-33 on the expression of Bcl-2/Bax in the retina. It is known that in RIR, RGCs are rapidly (< 2 day) damaged following reperfusion (29), and the number of apoptotic cells reaches a peak at 24 hours after reperfusion (28); therefore, we chose this time point to perform our analyses. The results of immunohistochemistry and western blotting confirmed that IL-33 promoted the expression of Bcl-2 and inhibited the expression of Bax. Therefore, it appears that IL-33 administration contributes to protection against RIR injury by inhibiting the apoptosis of ganglion cells. This result is similar to findings in other organs. A growing body of evidence has suggested that IL-33 plays a protective role in I/R injury by inhibiting apoptosis. IL-33 can increase the Bcl-2/Bax ratio and inhibit the apoptosis of myocardial cells in myocardial I/R injury (13). A recent study showed that IL-33 has direct protective effects on hepatocytes associated with the activation of Bcl-2 to limit liver-injury and reduce the stimulus for inflammation (11).

NF-κB is a multifunctional nuclear transcription factor and a key regulator in inflammation and apoptosis in all cells, and it has been proposed as a potential target to treat neuronal ischemia (30). The activation of NF-κB results in the expression of related inflammatory factors such as TNF-α and IL-1β and inhibition of NF-κB, which may result in the downregulation of Bcl-2-family proteins (31). Previous studies have shown that the activation of NF-κB may mediate apoptosis in RGCs (32), which are closely related to RIR injury. In the present study, the expression of the phosphorylated p65-NF-κB subunit was attenuated by IL-33. Our results provide evidence that IL-33 can inhibit the activation of NF-κB, and subsequently inhibit the expression of TNF-α and IL-1β at the transcriptional level. A previous study suggested that IL-33 reduced HMGB1 expression in myocardial I/R injury by suppressing the NF-κB signaling pathway, which is also the downstream signaling pathway of IL-33/ST2 (33-35).

5.1. Conclusions

Our results revealed that IL-33 pretreatment can attenuate retinal inflammation and prevent apoptosis. Therefore, IL-33 may be a new promising agent for the treatment of RIR-related diseases. However, RIR injury is a complex pathological process involving numerous factors, and the exact mechanisms of IL-33 in RIR injury remain to be further elucidated.

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Footnotes

Authors' Contribution: Proposal design: Zhi-Qin Wu, Shang-Wu Nie, Yan-Ning Yang and Yi-Qiao Xing; material preparation: Zhi-Qin Wu, Jing Yuan; carrying out the experiment: Zhi-Qin Wu, Shang-Wu Nie; data analysis: all authors; manuscript preparation: Zhi-Qin Wu and Shang-Wu Nie.

Conflicts of Interest: The authors declare no conflicts of interest with any financial organization regarding the material discussed in this manuscript.

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