Retinal ischemia/reperfusion injury is mediated by Toll-like receptor 4 activation of NLRP3 inflammasomes

Yun Qi, Min Zhao, Yujing Bai, Lvzhen Huang, Wenzhen Yu, Zongmei Bian, Mingwei Zhao, and Xiaoxin Li

Key Laboratory of Vision Loss and Restoration, Ministry of Education; Beijing Key Laboratory of Diagnosis and Therapy of Retinal and Choroid Diseases; Department of Ophthalmology, Peking University People’s Hospital, Beijing, China

Department of Ophthalmology and Visual Sciences, University of Michigan Kellogg Eye Center, Ann Arbor, Michigan, United States

Purpose. Retinal ischemia/reperfusion (IR) injury is common in eye disorders. Pattern-recognition receptors (PRRs) are reported to initiate sterile inflammatory response. The role of PRRs in retinal IR injury is currently unknown. Thus, we investigated the expression and function of membrane and cytoplasmic PRRs during retinal IR.

Methods. Retinal IR was induced in adult Brown Norway rats by clipping the retinal vessels for 30 minutes. RNA and proteins were extracted during the course of reperfusion, and the expression levels of the following proteins were determined: Toll-like receptor 2 (TLR2), TLR4, myeloid differentiation factor 88 (MyD88), TNF receptor-associated factor 6 (TRAF6), nuclear factor-κB (NF-κB), nucleotide-binding oligomerization domain-like receptor with pyrin domain protein 1 (NLRP1), NLRP3, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), caspase-1, IL-1β, and IL-18. TLR expression in the retina was studied using immunohistochemistry. In addition, a TLR4 inhibitor was injected into the vitreous body as a therapeutic agent. After the treatment of TLR4 inhibitors, the levels of the above factors were evaluated, the apoptosis of cells in the retina, expression of cleaved-casp-3 (c-casp-3), death of retinal ganglion cells, and the retina electroretinography was assessed.

Results. After releasing the artery clamp, the retinal vessels were reperfused in 5 minutes. During the reperfusion, TLR4, MyD88, TRAF6, NF-κB, NLRP1, NLRP3, mature IL-1β, and IL-18 were upregulated, but not TLR2. In the IR model, TLR4 was highly expressed in ganglion cell and glia cell. Additionally, the inhibition of TLR4 significantly downregulated the activation of NLRP3, but not NLRP1, and the secretion of mature IL-1β and IL-18 also were inhibited. Moreover, the TLR4 inhibitor partially attenuated the injury of the retina, including alleviated retina apoptosis, downregulated c-casp-3 expression, rescued retinal ganglion cells death, and restored retina function.

Conclusions. These findings suggest that TLR4-signaling activation, triggered by damage-associated molecular patterns, regulates the activation of the NLRP3 inflammasomes and is responsible for the function of the retina in retinal IR injury.

Keywords: retina, ischemia-reperfusion, Toll-like receptor 4, inflammasomes, IL-1β, IL-18

Retinal ischemia/reperfusion (IR) injury is associated with many ocular diseases, including acute glaucoma, diabetic retinopathy, and retinal vascular occlusion. Retinal ischemia/reperfusion injury leads to the death of retinal ganglion cells (RGCs), morphological degeneration of the retina, the loss of retinal function, and ultimately, vision loss. The mechanisms underlying retinal damage induced by IR injury have been widely investigated; however, seldom have studies reported the role of the immune and inflammatory response in retinal IR injury.

Recent evidence suggests that pattern-recognition receptors (PRRs) are required for initiating a profound sterile inflammatory response and play an important role in central nervous system (CNS) IR injury. Toll-like receptors (TLRs) and NOD-like receptors (NLRs) are very well characterized PRRs. They are responsible for sensing damage signals outside of the cell and intracellular noninfectious signals, which initiate the innate immune response thereafter. Studies have shown the expression of TLR2 and TLR4 is increased in mouse brain after cerebral ischemia. After TLR2 and TLR4 gene deficiency, brain damage was attenuated in the presence of cerebral ischemia. In addition, a significantly higher number of RGCs in TLR4-deficient mice survived after retinal IR injury compared with TLR4-positive mice. However, the mechanisms underlying the involvement of TLR4 in retinal IR injury are unknown.

The NOD-like receptor proteins are innate immune sensors that respond to pathogen and damage signals. On injury, some NLR proteins together with the adaptor proteins, apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and caspase 1, form large multimeric protein complexes, called inflammasomes. Inflammasomes are key signaling platforms that detect microbial and sterile stressors and activate the highly proinflammatory cytokines IL-1β and IL-18.
For the treatment groups, the experimental eye (right eye) was injected with 2 μl of a TLR4 inhibitor (2 mg/2 μl; anti-TRL4 antibody [HTA125], ab30667; Abcam, Cambridge, MA, USA) or 2 μl of a control agent (IgG) into the vitreous body 1 hour before clipping the retinal vessel.

**Fundus Examination**

The cessation and reperfusion of retinal blood flow was confirmed by fundus photography (FP) and fluorescein angiography (FA) with a Phoenix Micron IV Retinal Imaging Microscope (Phoenix, Pleasanton, CA, USA) according to the manufacturer’s illustration. For FA detection, 0.3 ml fluorescein sodium (FLUORESCITE Injection; Alcon, Fort Worth, TX, USA) was injected through the intraperitoneal space just before removing the clip. Eyes that were confirmed to be reperfused within 5 minutes after removing the clip were used. A sham procedure performed in the left eye without clamping the retina vessels served as the control.

**Gene Expression in BN Rats Using the QuantiGenePlex (QGP) Reagent System**

Retinal tissue for analysis of RNA expression was harvested at 0 minute (at the time of removing clip), 30 minutes, 1 hour, 4 hours, 8 hours, 12 hours, and 24 hours after reperfusion (each group had three rats). Target-specific RNA molecules (Table 1) were detected using the QGP 6.0 Reagent System according to the manufacturer’s protocol (Affymetrix, Fremont, CA, USA). Briefly, RNA from the retina lysates was captured by fluorescent microspheres. Signals of the cascade amplification were detected by the LumineX 100 xMAP technology and Bio-Plex 5.0 software (Bio-Rad Laboratories, Hercules, CA, USA). The geometric means of two housekeeping genes, PPIB (NM_012583) and HPRT1 (NM_012583), in each sample were used for normalization. Fold-changes are expressed as the relative ratios between the normalized values of the four treated groups and the values of the untreated group. All experiments were repeated three times.

**Western Blot Analysis**

Retinal tissue for analysis of protein expression was harvested at 0 minute (at the time of removing clip), 30 minutes, 1 hour, 4 hours, 8 hours, 12 hours, and 24 hours after reperfusion (each group had three rats). The entire retina was extracted and processed for Western blot analysis. NuPAGE Bis-Tris gels (10%; Life Technologies, Carlsbad, CA, USA) were used according to the manufacturer’s instructions. The membranes were blocked with 10% fat-free milk and incubated with primary antibodies overnight at 4°C. The following primary antibodies from Abcam were used at a 1:1000 dilution: TLR2 (ab108998), TLR4 (ab22048), TNF receptor-associated factor 6 (TRAF6) (ab33915), NLRP3 (ab91525), cleaved caspase-1 (c-casp-1) (ab108632), ASC (ab127537), IL-1β (ab9787), and IL-18 (ab106939). The following primary antibodies from Cell Signaling Technology (Beverly, MA, USA) were used at a 1:1000 dilution: NLRP1 (#4990s), myeloid differentiation factor 88 (MyD88) (#4283), phosphorylation nuclear factor-kB (NF-kB) (#3033s), cleaved caspase-3 (c-casp-3) (#9661), and beta-actin (#4970). Relative changes in protein expression were calculated in relation to normal retinas and expressed as fold change. Each experiment was repeated at least three times.

**Immunohistochemistry Staining**

The eyes were enucleated and embedded in paraffin. Eye sections (5-μm thick) were de-paraffinized and dehydrated.
After blocking, the sections were incubated with CD68 (ab125212) or TLR4 (ab22048) primary antibody. The slides were incubated with biotin-conjugated secondary antibody (Envision-Detection Kit, GK500705; GenTech, San Francisco, CA, USA) at room temperature. After being rinsed in PBS, peroxidase activity was revealed by incubating the sections in a solution of DAB (3,3-diaminobenzidine tetrahydrochloride). Slides were counterstained with hematoxylin. Images of slides were captured on digital microscope (DM4000B; Leica, Wetzlar, Germany).

**Retina Apoptosis Assay Using TUNEL Staining**

The TUNEL assay was used to evaluate apoptosis of cells within the retina after IR injury; TUNEL staining (In Situ Cell Death Detection Kit; Roche Applied Science, Indianapolis, IN, USA) was performed on 10-μm frozen sections. Ten sections of each eye sample (1.5–2.0 mm away from ON head) were included in the count of the TUNEL staining assay.

**Superior Colliculus (SC) Retrograde Labeling**

Retinal ganglion cells were retrograde labeled with a 4% Fluoro-Gold (FG) (Fluorochrome, Englewood, CO, USA) applied bilaterally to the SC. Rats were mounted on a stereotactic apparatus (Kopf Instruments, Tujunga, CA, USA) and holes were drilled in the skull 1.5 mm laterally to the sagittal suture and 2.5 mm anteriorly to the lambda suture on each side. Fluoro-Gold (3 μl) was injected into the SC at a depth of 6.0 mm below the skull, and the holes were filled with Gelfoam soaked with FG. In the IR model, retrograde labeling was performed 7 days before clamping the retinal vessels.

**Retinal Ganglion Cell Survival Quantification**

Retinas were fixed and flat-mounted on a glass slide with the vitreous side up at 24 hours after retina reperfusion (each group had six rats). Pictures for each freshly flat-mounted retina were taken with a Zeiss fluorescence microscope (Carl Zeiss Meditec, Jena, Germany), with 12 pictures per retina at ×20 magnification. For each quadrant, three pictures were taken at the distance of 1 mm, 2 mm, and 3 mm radially from the ON head. In all cases, RGC counting was performed manually by two independent persons. The standardization of RGC survival in each rat was calculated as the ratio of the experimental OD versus the control OS, which was the contralateral eye (RGC_{experimental}/RGC_{contralateral}). The ratios were averaged for all rats in each group and were presented as the mean ± SEM.

**Electroretinogram Measurement of Retina Function**

Rats were dark-adapted overnight and under dim red illumination (λ > 600 nm). Topical proparacaine hydrochloride eye drops (Alcon Laboratories) and tropicamide eye drops (Alcon Laboratories) were administered after deep anesthesia. A gold-tipped electrode was placed centrally on the cornea, a reference electrode was placed subcutaneously between the two ears, and the ground electrode was placed in the tail. The coordination of ERG stimulation and the recording of electrical responses was completed using the Phoenix Micron IV Retinal Imaging Microscope, according to the manufacturer’s instruction manual.

To elicit an ERG response, the Ganzfeld rat default setting was used (green light, 8.0E±4 cd/m² intensity, 1 ms duration, 0 ms offset, 80 cd sec/m²). Specifically, two flashes were delivered with a 15-second interval, and five responses were used as an average. The amplitude of the a-wave and b-wave and the total amplitude (a-wave + b-wave) were recorded. The mean values obtained from each eye were averaged and represented as the mean ± SEM. The ERG recordings were collected 24 hours after retina reperfusion (each group had six rats).

**Statistics**

Statistical analyses for the QuantiGene analysis were performed using GraphPad software (La Jolla, CA, USA). Differences between groups were compared with the nonparametric Kruskal-Wallis test, and paired comparisons were made using the Mann-Whitney test. All other statistical analyses were performed using Student’s t-test. Data are expressed as the mean ± SEM. A P value less than 0.05 was considered statistically significant.

**RESULTS**

**The Rat Retinal IR Model was Validated by FP and FA Examination**

Cessation and reperfusion of rat retinal blood flow in the IR model was confirmed using retinal imaging systems. As shown in FP and FA images (Fig. 1), the retinal blood flow was completely stopped after clamping the retinal vessels (Fig. 1; 0 minutes), which were reperfused 5 minutes (Figs. 1, 2; 5 minutes) after removing the clip. Gradually, the blood flowed back into the retinal vessels within 20 minutes (Fig. 1; 20 minutes). In the normal rat retina, the fundus vessels were filled within 30 seconds after an intraperitoneal injection of 0.3 mL sodium fluorescein (data not shown).
Toll-like Receptor 4 Signaling Was Involved in the Development of Retinal IR Injury

Toll-like receptor 4 is an important membrane damage-associated molecular pattern (DAMP) receptor that is involved in acute and chronic nerve system ischemia. In the present study, we found that retinal IR induced the upregulation of TLR4, but not TLR2. The expression of TLR4 peaked at 1 hour after reperfusion (Figs. 2A, 3A, 3B) and then decreased gradually. Proteins downstream of TLR4, such as MyD88, TRAF6, and p-NF-kB, were also highly expressed (Figs. 2A, 3C–E). Among these, p-NF-kB was expressed the highest at 8 hours, whereas MyD88 and TRAF6 remained at almost the same expression level throughout the study time course. The differences between the gene and protein levels detected in our study could be the results of the process in protein posttranscription and translation.

Toll-like Receptor 4 Was Highly Expressed in the Ganglion Cell Layer and Inner Nuclear Layer of IR Retina

To identify the TLR4-expressed location in the IR retina, immunohistochemistry studies of IR model were performed. As shown in Figure 4, 6 hours (Fig. 4B), 12 hours (Fig. 4C), and 24 hours (Fig. 4D) after vessel occlusion in the IR rat model, TLR4 was highly expressed in both ganglion cell layer (GCL) and inner nuclear layer (INL) throughout different time points. The results indicated that both ganglion cell and glia cell were activated during acute ischemia, and TLR4 can be detected in both cell types. Correspondence with Western blot results, the expression of TLR4 was downregulated in 24-hour retina section.
TLR4 Regulates NLRP3 Activation in Retinal IR

Inflammation in the IR model treated with TLR4 inhibitor using the QGP Reagent System. The indicated genes were detected using the QGP 6.0 Reagent System. The results show that after treatment with the TLR inhibitor, the tested genes were downregulated. (A) Toll-like receptor-related genes include Myd88, TRAF6, and Nf-xB. (B) Inflammasome-related genes: NLRP1, NLRP3, ASC, and caspase-1. (C) Interleukin-related genes: IL-1β, IL-1β, and IL-18. The data were compared with normal controls. The data are represented as the mean ± SEM of fold changes compared with the controls.

**Figure 5.** Gene expression in the IR model treated with TLR4 inhibitor using the QGP Reagent System. The indicated genes were detected using the QGP 6.0 Reagent System. The results show that after treatment with the TLR inhibitor, the tested genes were downregulated. (A) Toll-like receptor-related genes include Myd88, TRAF6, and Nf-xB. (B) Inflammasome-related genes: NLRP1, NLRP3, ASC, and caspase-1. (C) Interleukin-related genes: IL-1β, IL-1β, and IL-18. The data were compared with normal controls. The data are represented as the mean ± SEM of fold changes compared with the controls.

**FIGURE 4.** Toll-like receptor 4 expression in IR retina sections by immunohistochemistry. (A) The normal retina stain for TLR4, which showed no obvious expression; (B–D) retina sections after 6 hours, 12 hours, and 24 hours of reperfusion, which indicated that TLR4 can be highly expressed in both GCL and INL. Morphometric analysis of TLR4-stained sections was performed under ×400 magnification. IPL, inner plexiform layer; OPL, outer plexiform layer; ONL, outer nuclear layer; Rods & Cones, photoreceptor layer.

**Nucleotide-Binding Oligomerization Domain-Like Receptor With Pyrin Domain Protein 1 and NLRP3 Inflammasomes Are Activated in Retinal IR Injury**

Nucleotide-binding oligomerization domain-like receptor with pyrin domain protein 1 and NLRP3 inflammasomes are important cytoplasmic DAMP receptors and have been demonstrated to play a key role in ischemia injury. The potential role of these inflammasomes in retina IR injury is still unknown. The present study showed that NLRP1 and NLRP3 inflammasomes (Figs. 2B, 3G–I) are activated in the rat retinal ischemia model and induced the release of mature IL-1β and IL-18 (Figs. 2C, 3J, 3K) after perfusion, which further mediates other inflammatory effects. In our study, the expression of NLRP3, ASC, and c-casp-1 peaked at 4 to 8 hours after reperfusion, and NLRP1, IL-1β, and IL-18 were expressed stably at a high level throughout the study time course.

**Nucleotide-Binding Oligomerization Domain-Like Receptor With Pyrin Domain Protein 3 Inflammasomes Are Regulated by TLR4 Signaling in Retinal IR Injury**

To elucidate the relationship between TLR4 signaling and NLRP1/3 inflammasomes in retinal IR injury, an intravitreal injection of a TLR4 inhibitor was used in this study. Our findings showed that the TLR4 inhibitor downregulated Myd88, TRAF6, and p-Nf-xB in a time-dependent manner. Additionally, Myd88, TRAF6, and p-Nf-xB were all upregulated after 8 hours of reperfusion in the absence of the TLR4 inhibitor. Furthermore, the TLR4 inhibitor also downregulated NLRP3, ASC, c-casp-1, IL-1β, and IL-18 but not NLRP1 (Figs. 5, 6). The above findings provide evidence that TLR4 induced the activation of NLRP3 inflammasomes in retinal IR injury.

**Inhibition of TLR4 Partially Halted the Development of Retinal IR Injury**

In addition to the detection of RNA and protein levels, the apoptosis of cells in the retina (including RGCs), apoptosis-related factor c-casp-3, RGC survival, and retinal function were also evaluated 24 hours after retinal reperfusion. Figure 7 shows that the TLR4 inhibitor significantly decreased the number of apoptotic cells in the retinal cryosection (IR group compared with TLR4 inhibitor group, p < 0.001). Figure 8 reveals that TLR4 inhibitor reduced the activation of apoptosis-related protein c-casp-3 at different time points after IR injury. Figure 9 demonstrates that the TLR4 inhibitor increased the survival of RGCs in the flat-mounted retina from 73.0% ± 2.1% in the IR model to 81.1% ± 2.2% in the treatment group (IR group compared with TLR4 inhibitor group, p = 0.021).

Visual function in animals can be assessed by recording evoked potentials of the retina and cortex. The ERG measures retinal activity and allows for partial differentiation of cells within the different layers of the retina. In our study, IR induced a reduction in both a- and b-wave amplitude, which are representative of the retinal photoreceptor cells and Müller cells and the bipolar cell layer. The in vivo ERG measurements showed that the TLR4 inhibitor increased the b-wave amplitude in IR-injured animals compared with control animals (Table 2; Fig. 10).

**DISCUSSION**

Retinal ischemia is a major cause of visual impairment and blindness and plays an important role in the pathophysiology of various retinal disorders, such as ischemic optic neuropathies, obstructive arterial and venous retinopathies, diabetic retinopathy, and glaucoma. Retina ischemia can result in reduced blood supply, leading to energy depletion and cell death, both of which are mediated by intermediate factors, such as the release of excess excitatory amino acids, free radical formation, inflammation factors, and ultimately ending in damage to the retinal neurons. As an extended part of the brain, the retina has similar characteristics to the CNS, making the retinal ischemia model an ideal model for studying the mechanisms of cerebral ischemia. In addition, studies designed...
Figure 6. Western blot analysis of proteins in the TLR4 inhibitor–treated IR model. Representative blot images are shown on the left, and results of the statistical analysis are shown on the right (A–I). As shown in this figure, MyD88, TRAF6, p-NF-κB, NLRP3, ASC, c-casp-1, IL-18, and IL-1β were downregulated compared with the IR model group. These results indicate that NLRP3 inhibition is regulated by the TLR4 receptor. Data are represented as the mean ± SEM of fold changes compared with the controls.

Figure 7. Effects of TLR4 inhibitor on apoptosis 24 hours after retinal IR injury. (A-1–C-1) The apoptotic nuclei detected by TUNEL staining (green); (A-2–C-2) the total nuclei that were labeled with propidium iodide (PI; red). (D) The quantitative analysis of TUNEL-positive cells per field of view. Morphometric analysis of TUNEL-stained sections was performed under ×20 magnification. n = 10 per group. Data are shown as the mean ± SEM. ***P < 0.001.
TLR4 Regulates NLRP3 Activation in Retinal IR

**Figure 8.** Western blot analysis of c-casp-3 proteins in the IR model and TLR4 inhibitor-treated IR model. (A) Representative blot images of c-casp-3 in the IR model and TLR4 inhibitor-treated IR model. (B) The statistical analysis results. These results indicated that TLR4 inhibitor can reduce the activation of apoptosis-related protein c-casp-3. Data are represented as the mean ± SEM of fold changes compared with the controls. *P < 0.05, **P < 0.01.

To develop treatment strategies for the retina also can provide fundamental guidance for the treatment of CNS ischemia.

There are two representative models of retinal ischemia/reperfusion: raising the IOP above the systolic arterial blood pressure and ligating the central retinal artery together with the ON. The former model will induce mechanical damage to the ocular structures, whereas the latter model will result in damage to the ON. To avoid the defects of the two models, in the present study, we clamped only the retina vascular without ligating the ON by separating the sheath from ON parenchyma. The IR model in the present study exhibited obvious structural and functional changes (Figs. 1, 7, 9, 10). Using this retina central vessel ligation animal model, we showed that IR injury triggered TLR4 signaling through MyD88, TRAF6, and NF-κB, leading to the activation of NLRP1 and NLRP3 inflammasomes and the release of mature IL-1β and IL-18. Additionally, the inhibition of TLR4 significantly reduced the activation of NLRP3 and downstream signals and partially halted the apoptosis of cells in the retina and rescued the function of the retina (Fig. 11).

Retinal IR belongs to a class of sterile inflammation diseases. Accumulating evidence suggests that PRRs play an important role in IR injury in the CNS. Pattern recognition receptors are proteins expressed by innate immune cells to identify specific pathogen-associated molecular patterns present in microbial molecules or by DAMPs exposed on the surface of, or released by, damaged cells. Pattern recognition receptors include membrane-associated TLRs, C-type lectin receptors, NLRs, RIG-I-like receptors, and AIM2-like receptors (ALRs). In most cases, ligand recognition by PRRs triggers an intracellular signal transduction cascade that results in the expression of chemokines, proinflammatory cytokines, and antiviral molecules. In contrast, the activation of some NLRs and ALRs leads to the formation of multiprotein inflammasome complexes that serve as platforms for the activation of caspase-1. Caspase-1 promotes the maturation and secretion of IL-1β and IL-18, which further activates the NF-κB signaling pathway and induces the production of inflammatory molecules and amplifies the immune response. Crosstalk between different receptors may also play a role in enhancing or inhibiting the immune response. Therefore, the tight regulation of PRR signaling is required to prevent aberrant or excessive PRR activation, which can lead to the development of inflammatory disorders.

In the present study, retinal IR induced the expression of TLR4, but not TLR2, at the mRNA and protein levels (Figs. 2, 3). We also verified that TLR4 was mainly expressed in the GCL and INL after IR injury (Fig. 4), which indicated that neuron and glia cell in the retina is the primary source of TLR4 expression. Toll-like receptor 4 has been shown to play a central role in retinal and CNS IR injuries and may involve sensing ischemic damage. The activation of TLR4 signaling leads to the recruitment of several adapter proteins that activate NF-κB, which induces the expression of proinflammatory genes and inflammatory cytokines. It has been demonstrated that TLR4-deficient mice are protected against ischemic brain damage. In the present study, our findings showed that IR induces the activation of TLR4 and the downstream signals MyD88 and TRAF6. The expression peak of TLR4 in our IR model was between 30 minutes and 4 hours after perfusion, and was gradually reduced until 24 hours (Figs. 3, 4). This could be due to the death of retinal cells after 4 hours of reperfusion (TUNEL stain in retina section, data not shown). However, MyD88 and TRAF6 were gradually upregulated after the activation of TLR4, which can be explained by multiple TLR signals being activated by these two adaptors. We also showed that inhibition of the TLR4 receptor restores retinal function and partially attenuates RGC death. In previous studies, it was suggested that TLR2 is involved in retinal function in DR and uveitis.20,21 But in our short-term study, we did not detect any changes in the expression of this receptor. A long-term study will need to be performed to further comment on the role of TLR2. There is one interesting phenomenon that the QGP detected results were not fully correspondent to the protein results. This inconsistency of the gene and protein expression displayed the complex of the process of posttranscription and translation. In addition, the exact mechanisms of TLR4 activation in ganglion cell and glia cell needed to be explored in further study by primary culture of RGCs and Müller cells.

Toll-like receptor 4 inhibition has been shown to have a protective effect on ischemic organs, including the retina.22 However, the molecular mechanisms underlying the protective effects of TLR4 remain unknown. As stated previously, TLR4 is a membrane-spanning receptor, and the inflammasome is a cytoplasmic sensor; the relationship between these two types of PRRs in the retina remains unclear. Our findings revealed that IR induced TLR4-signaling activation and triggered NLRP3 inflammasomes sequentially, shedding new light on how TLR4 mediates ischemic inflammation. The expression peak of the NLRP3 inflammasome and the release of IL-1β and IL-18 occurred 4 hours after removing the clip, which was posterior to the activation of TLR4. Moreover, the neutralization of TLR4 can inhibit the activity of the NLRP3 inflammasome, which further reduced the production of IL-1β and IL-18 and attenuates tissue damage in retinal IR. All of the above results indicated that TLR4 and NLRP3 inflammasomes have a close association in the retinal IR model. However, TLR4 inhibition was found to inhibit only NLRP3 inflammasome activation transitory with NLRP3 being highly expressed again 24 hours after TLR4 inhibition. On one hand, this may be the short-term effects of the neutralizing antibody, and on the other hand, the NLRP3 may be regulated by another unknown signal, such as noncanonical inflammasomes signal pathways. Considering the inflammasome activation could be from infiltrating immune cells, we performed an immunohistochemistry study that showed no CD68-positive cells were seen in the retina sections after IR injury (Supplementary Fig. S1). This indicated that the activated NLRP3 inflammasome is mainly from intrinsic retina cells. In addition to NLRP3 inflammasomes,
we also monitored NLRP1 inflammasomes. However, the TLR4 inhibitor did not alter the expression of NLRP1 inflammasomes; therefore, the intrinsic mechanism of NLRP1 inflammasomes in retinal IR requires further investigation. Besides, one of the limitations of the present study is lacking of the results from TLR4 knockout mice, which would be interesting additional information and strengthen our conclusion. The studies on TLR4 knockout mice will be performed in the further study for verifying the relation between IR-TLR4-NLRP3 inflammasomes in the IR model.

Over the past decade, there has been a surge of information regarding inflammation in the CNS, such as stroke, multiple sclerosis, and Alzheimer's disease. Among these studies, it was recently discovered that multiprotein inflammasomes, which were initially described by Martinon and colleagues in 2002, are thought to play a central role in immune and inflammatory processes. The NLRP3 inflammasome is by far the most widely studied complex in neurological diseases. Investigations have shown that NLRP3 inflammasomes are involved in CNS IR through the production of reactive oxygen species and mitochondrial dysfunction, endosomal rupture, and K⁺ efflux. In addition, Nlrp3-knockout mice have a delayed onset of CNS injury. Our present study not only reveals that the NLRP3 inflammasome is involved in retinal IR but that it also establishes the relationship between TLR4 and NLRP3 inflammasomes, providing some new ideas for future research in retina IR.

### Table 2. Electroretinogram Calculations

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>IR Model</th>
<th>TLR4 Inhibitor</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>a-wave</td>
<td>204.1</td>
<td>5.1</td>
<td>115.8</td>
</tr>
<tr>
<td>b-wave</td>
<td>318.1</td>
<td>17.1</td>
<td>129.5</td>
</tr>
<tr>
<td>Amplitude</td>
<td>522.2</td>
<td>16.9</td>
<td>245.3</td>
</tr>
</tbody>
</table>

Fig. 9. Effects of a TLR4 inhibitor on RGCs 24 hours after retinal IR injury. The above three panels are representative pictures of FG-labeled RGCs, which represent normal group, ischemia/reperfusion group, and TLR4 inhibitor treatment group. (1-1-1-3) Pictures taken from a distance of 1 mm, 2 mm, and 3 mm radially from the ON head in the nasal superior quadrant of the retina. The lowest panel is the statistical analysis result and shows that the number of RGCs in the TLR4 inhibitor group is significantly higher compared with the IR model group. n = 6 per group. Data are shown as the mean ± SEM. *P < 0.05.
Figure 10. Effects of a TLR4 inhibitor on retinal function 24 hours after retinal IR injury. Representative pictures of ERG in the normal group (A), IR group (B), TLR4 inhibitor group (C), and statistical analysis result (D). The results show that the b-wave and the amplitude of both the b-wave and the a-wave in the TLR4 inhibitor group is higher compared with the IR model group; the result is statistically significant. n = 6 per group. Data are shown as the mean ± SEM. *P < 0.05.

Figure 11. Schematic summary of the putative signaling mechanism of retina IR injury. Rat retina IR injury leads to the activation of the TLR4 signaling pathway through MyD88 and TRAF6, which further induces the expression of p-NF-κB and triggers the activation of NLRP3 inflammasomes and the release of mature IL-18 and IL-1β.
**Acknowledgments**

We thank Youzhi Yu, technician, for the help in performing the immunohistochemistry study in the resubmitted manuscript. Supported by the Beijing Nova Program (Z131102000413004), National Basic Research Program of China (973 Program, 2011CB510200), and the National Natural Science Foundation of China (81200690). The organizations that funded this study had no role in the study design, data collection and analysis, or the decision to publish or prepare the manuscript. The authors alone are responsible for the content and writing of the paper.

Disclosure: **Y. Qi**, None; **Z. Bian**, None; **M. Zhao**, None; **Y. Bai**, None; **L. Huang**, None; **W. Yu**, None; **L. Xu**, None.

**References**


