Article

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TLR4 Inhibition Protects against Retinal Ganglion Cell Damage in Rats with Chronic Ocular Hypertension

Zhongjing Lin[1], Li Ru[1], Hongliang Wang[1], Ke Mao[1,*]

[1]Department of Ophthalmology, Renji Hospital, School of Medicine, Shanghai Jiaotong University, 200127 Shanghai, China
*Correspondence: maoke@renji.com (Ke Mao)
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Background: This study aims to investigate the protective effect of Toll-like receptor 4 (TLR4) inhibitor Resatorvid (TAK-242) on retinal ganglion cells (RGCs) in a chronic ocular hypertension (COH) rat model, as well as to explore the potential involved mechanisms.

Methods: COH model was built up in rats with a single intracameral administration of cross-linking hydrogel. The expression levels of TLR4, NLR family pyrin domain containing 3 (NLRP3), microglial activation and pro-inflammatory cytokines were evaluated in COH retinas and COH retinas treated with TAK-242 using immunofluorescence staining and Western blot. Additionally, retrograde labeling and neuronal nuclear protein (NeuN) staining were performed to count RGCs.

Results: Activated microglia and increased TLR4 expression were observed in the retinas of COH rats. This was accompanied by upregulated expressions of NLRP3, tumor necrosis factor alpha (TNF-α), cytokine interleukin-1β (IL-1β) and Interleukin-6 (IL-6). Intravitreal injection of TAK-242 promoted the survival of RGCs by attenuating microglial activation, interfering with the TLR4-NLRP3 pathway and regulating pro-inflammatory cytokines.

Conclusions: Targeting TLR4 inhibition could be a potential therapeutic strategy to protect RGCs from COH damage.

Keywords: chronic ocular hypertension; retinal ganglion cells; microglial activation

Introduction

Glaucoma is a complex neurodegenerative disease that is characterized by elevated intraocular pressure (IOP), which ultimately leads to the chronic dysfunction of retinal ganglion cells (RGCs) and irreversible visual disorders. Although the complex and multifactorial mechanisms remain to be fully elucidated, retinal microglial activation may be involved in the regulation of neuroinflammation and RGC apoptosis after glaucomatous injury. Microglial reactivation has been associated with RGC loss in human glaucoma and the experimental models of glaucoma [1–3]. Multiple lines of evidence have confirmed that inhibition of the excessively activated microglia exerts a neuroprotective effect, which may provide an effective strategy to improve the survival of RGCs for alleviating glaucoma [4,5].

Toll-like receptor (TLR) is a pattern-recognition receptor that activates the immune response [6]. TLRs play a crucial role in regulating the balance of proapoptotic and protective effects, which may lead to the degeneration of RGCs in glaucoma, especially TLR4 [7]. Specific gene mutations of TLR4 have been found in glaucoma patients, increasing glaucoma susceptibility [8]. Proteomic profiling of donated retina from glaucoma patients showed significant upregulation of TLR4 signaling, and positive TLR4 staining was also identified to be colocalized with microglia in human retina [9]. These observations highlighted the important link between TLR4 and glaucoma. Highly expressed TLR4 was actively involved, and TLR4 deficiency showed a neuroprotective effect in the mouse model of acute IOP-induced ischemic injury [10]. However, little is known about how TLR4 interacts with microglial activation in rats with chronic ocular hypertension (COH).

In this study, COH model was achieved with a single intracameral administration of cross-linking hydrogel. This study aimed to identify whether the TLR4 inhibitor Resatorvid (TAK-242) can protect RGCs from COH-associated damage and to explore the potential mechanisms.

Materials and Methods

Animals

Animals used in this study were adult male Sprague-Dawley rats weighing 180–220 g (Zhejiang Vital River Laboratory Animal Technology Co., Ltd.). The animals were kept in a regular rhythm with light or darkness every 12 hours, and were provided with a plenty supply of food and water. All procedures were performed following the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Establishment of the COH Model

A rat COH model was built up by injection of cross-linking hydrogel into the anterior chamber [11,12]. Briefly, rats were anesthetized with ketamine hydrochloride (25 mg/kg) and xylazine (10 mg/kg) via intraperitoneal injection. Subsequently, the operated eyes were locally anesthetized with 0.5% proparacaine hydrochloride eye drops (Alcon, Puurs, Belgium). IOP rise was achieved by intracameral injection using a cross-linking hydrogel (HyStem Cell Culture Scaffold kit, Sigma-Aldrich, St. Louis, MO, USA). During the experiments, 66 rats received the injection of cross-linking hydrogel. However, 4 eyes failed to keep sustained IOP elevation due to the leakage of corneal incision and 2 eyes had postoperative endophthalmitis. Thus, the success rate for COH induction was 90.9%. IOP was measured before the surgery and every other day after the operation using a rebound tonometer (Icare, Vantaa, Finland). IOP was obtained between 1 PM and 3 PM so as to avoid the potential diurnal variations.

Experiment Groups and Drug Administration

Sprague-Dawley (SD) rats were randomized into control, sham, COH 1w, COH 2w, COH 4w. For the sham groups, an equal volume of phosphate-buffered saline (PBS) was administered intracamerally. SD rats were then divided into 4 groups: control, control+TAK-242, COH, COH+TAK-242. TAK-242 (HY-11109, MedChemExpress, Monmouth Junction, NJ, USA) was dissolved in 1% DMSO (Nacalai Tesque, Kyoto, Japan) and then diluted in sterile saline. Intravitreal injection of TAK-242 was administered to the control+TAK-242 group and COH+TAK-242 group. TAK-242 was intravitreally injected at a dosage of 0.01 µg/2 µL/eye using a sterile 31-gauge needle. The injection was performed 3 days before the surgery and repeated every week until 4 weeks after the surgery. 120 rats were involved in our experiments, including 4 eyes failed to maintain IOP elevation and 2 eyes had postoperative endophthalmitis. Thus, samples were collected from 114 rats with n = 6 for each separate experimental procedures and n = 12 for IOP measurements.

Retrograde Labeling and Quantification of RGCs

The procedure of retrograde labeling of RGCs was conducted as previously described [12]. Briefly, 1 mL of 4% Fluoro-Gold (Biotium, Hayward, CA, USA) was injected into the bilateral superior colliculus through a predefined hole. 7 days after the injection, rats were sacrificed using isoflurane followed by cervical dislocation, and then the retinas were extracted. To quantify labeled RGCs, areas of approximately 1/2 retinal radius from the optic disc were captured using a Zeiss Axio Imager M1 microscope (Carl Zeiss, Jena, Germany).

Immunofluorescence Staining

After removing the anterior segments of the eyeballs, the eyecups were preserved and frozen in an optimum cutting temperature compound (OCT; Sakura Finetek, Torrance, CA, USA). Cryo-sections of 10 µm were obtained. The retinal tissues were permeated with 0.25% of Triton X-100 for 30 min. After being blocked in 1% of BSA, the tissue sections were treated with the targeted primary antibodies at 4 ℃ overnight: anti-ionized calcium-binding adapter molecule 1 (Iba-1) (1:500 dilution, ab178846; Abcam, Cambridge, UK), TLR4 (1:200 dilution; Santa Cruz Biotechnology, sc-293072, Dallas, TX, USA), neuronal nuclear protein (NeuN) (1:500 dilution; Invitrogen, 702022, Waltham, MA, USA). On the following day, they were incubated with the following corresponding secondary antibodies at room temperature for 1 h: Fluorescein isothiocyanate (FITC) conjugated donkey anti-rabbit (1:1000 dilution; Abcam) targeting for Iba-1, Texas-red conjugated donkey anti-mouse (1:1000 dilution; Abcam) targeting for TLR4, Texas-red conjugated donkey anti-rabbit (1:1000 dilution; Abcam) targeting for NeuN. The samples were further treated with 4′,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich) for 5 min. Finally, images were acquired with confocal scanning laser microscope (Leica TCS SP8 STED 3X, Leica, Wetzlar, Germany).

Western Blotting

Retinas were lysed in radio-immunoprecipitation assay (RIPA) buffer supplemented with a protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany). Protein concentration was measured using a BCA assay kit (Boster, Wuhan, China). All protein samples were boiled at 100 ℃ for 10 min and then stored at –80 ℃. The extracted protein samples (30 µg) were separated by 12.5% SDS-PAGE and transferred onto PVDF membranes (Millipore, Burlington, MA, USA). After blocking the membranes, they were incubated with primary antibodies including glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:10,000 dilution; Abcam, ab8245), Iba-1 (1:1000 dilution; Abcam, ab178846), TLR4 (1:1000 dilution; Santa Cruz Biotechnology, sc-293072), NLR family pyrin domain containing 3 (NLRP3) (1:1000 dilution; Novus Biologicals, NBP2-12446, Centennial, CO, USA), tumor necrosis factor alpha (TNF-α) (1:1000 dilution; Abcam, ab205587), cytokine interleukin-1β (IL-1β) (1:500 dilution; Abcam, ab205924), and Interleukin-6 (IL-6) (1:2000 dilution; Abcam, ab9324). On the following day, after adequate washing, the membranes were incubated with the corresponding second antibodies for 90 min: horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (1:2000; Abcam, ab97051) or anti-mouse IgG (1:2000; Abcam, ab97023). The signals were detected using the ECL Plus reagents (Pierce Scientific, Waltham, MA, USA). The band densities of each target protein and corresponding GAPDH

were quantified using ImageJ software. The relative expression level of each protein was analyzed with the application of gray value ratio (target protein/GAPDH).

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 7.0 (GraphPad Software Inc., San Diego, CA, USA). The one-way analysis of variance (ANOVA) was used to analyze statistical differences between groups, followed by Tukey's post hoc test. $p < 0.05$ was deemed statistically significant.

Results

Alteration of TLR4 and Activated Microglia in a COH Rat Model

The COH rat model was successfully established, and IOP fluctuations were documented (Fig. 1A). After the cross-linking hydrogel was injected intracamerally, the IOP of the operated eyes rapidly increased and remained steadily higher than that in the control group (all $p < 0.05$). The average IOP was 28.3 $\pm$ 2.5 mmHg at 1 week, 26.3 $\pm$ 1.5 mmHg at 2 weeks, and 21.5 $\pm$ 2.4 mmHg at 4 weeks ($p < 0.05$).

The expression of Iba-1 was used to evaluate the activation of retinal microglia cells in the retina tissues. The expression levels of Iba-1 proteins were significantly upregulated during the COH process. The protein expression of TLR4 in the retina also increased gradually at 1, 2, and 4 weeks after initial IOP increase (all $p < 0.05$). Meanwhile, compared to the control and sham groups, NLRP3 protein in COH groups showed significantly higher expression levels (all $p < 0.0001$). Moreover, the protein expressions of TNF-α and IL-1β in retina tissue also increased after the induction of COH (all $p < 0.05$). At one week after IOP elevation, IL-6 expression in COH retinas was 1.4-fold higher than the control ($p < 0.05$). Thereafter, the levels of IL-6 gradually diminished over time (Fig. 1B,C).

TAK-242 Attenuates TLR4, Activated Microglia and Pro-Inflammatory Cytokines in the Retina of a COH Model

At 4 weeks after IOP elevation, the average IOP in the COH group was 22.0 $\pm$ 1.9 mmHg, while the average IOP in the COH+TAK-242 group was 21.4 $\pm$ 2.3 mmHg ($p < 0.05$). Notably, comparisons of IOP at each time point showed no significant differences between the two groups (all $p > 0.05$), suggesting that TAK-242 treatment did not markedly affect the IOP fluctuations (Fig. 2A).

Intravitreal injection of TAK-242 significantly reduced the protein expression of TLR4 (by 71.3%, from 64.6% to 18.5%, $p < 0.001$) (Fig. 2B,C), as well as NLRP3 (by 39.0%, from 68.8% to 42.0%, $p < 0.001$) (Fig. 2B,C), in the retina of COH model rats. Intravitreal injection of TAK-242 in normal rats caused changes in these protein levels similar to those controls (all $p > 0.05$). The effect of TAK-242 on pro-inflammatory cytokines were determined by measuring the protein levels of TNF-α, IL-1β and IL-6 in the retina (Fig. 2B,C). There was no difference in the expression of all proteins between the control and sham groups (all $p > 0.05$). TNF-α and IL-1β levels were significantly downregulated in the COH+TAK-242 group (both $p < 0.05$), while higher expression of IL-6 was detected as compared to the COH group ($p < 0.05$).

Furthermore, co-localization of TLR4 and Iba-1 staining was observed in inner plexiform layer (IPL), inner nuclear layer (INL) and outer plexiform layer (OPL) at 4 weeks after COH induction (Fig. 3). This suggests that the expression of TLR4 was upregulated after microglia were activated when IOP elevation was induced. COH eyes treated with TAK-242 showed a decrease in the expression levels of Iba-1 and TLR4 (Figs. 2B,3), indicating that TAK-242 inhibited TLR4-mediated microglial activation in the COH model.

TAK-242 Promoted RGCs Recovery after COH

To determine the effects of TLR4 inhibitor TAK-242 on retinal damage after COH, we assessed the number of labeled RGCs using retrograde Fluoro-Gold technology (Fig. 4A–C). The number of RGCs significantly decreased in COH-treated retinas compared to the control group (2245 $\pm$ 179 vs. 2743 $\pm$ 155 cells/mm^2) ($p < 0.05$). However, intravitreal injection of TAK-242 considerably alleviated the loss of RGCs after COH-induced damage (2011 $\pm$ 112 vs. 2245 $\pm$ 179 cells/mm^2) ($p < 0.05$) (Fig. 4D). Moreover, RGCs loss in the ganglion cell layer (GCL) was evaluated by directly counting cells using immunostaining with the neuron-specific marker NeuN (Fig. 4E–G). In comparison with the control group, the number of RGCs in the COH group decreased by 24.9% ($p < 0.05$). However, COH eyes treated with TAK-242 demonstrated significantly smaller reductions in the number of RGCs (72.3% vs. 84.5%) ($p < 0.05$) (Fig. 4H). Overall, these results suggest that TAK-242 may protect RGC against COH-induced glaucomatous injury.

Discussion

Recent research has shown that chronically activated glia can impact the pathology of various neurodegenerative diseases, including glaucoma [13]. As elevated IOP is strongly associated with the progression of glaucoma, a COH model was established to mimic the clinical and physiological changes in experimental glaucoma. Our preliminary work has shown that TAK-242 can protect RGCs against COH-associated injury by inhibiting retinal microglial activation and the associated inflammation response. Specifically, TAK-242 treatment effectively alleviated COH-induced microglial activation and release of inflammatory cytokines, and intravitreal injection of TAK-

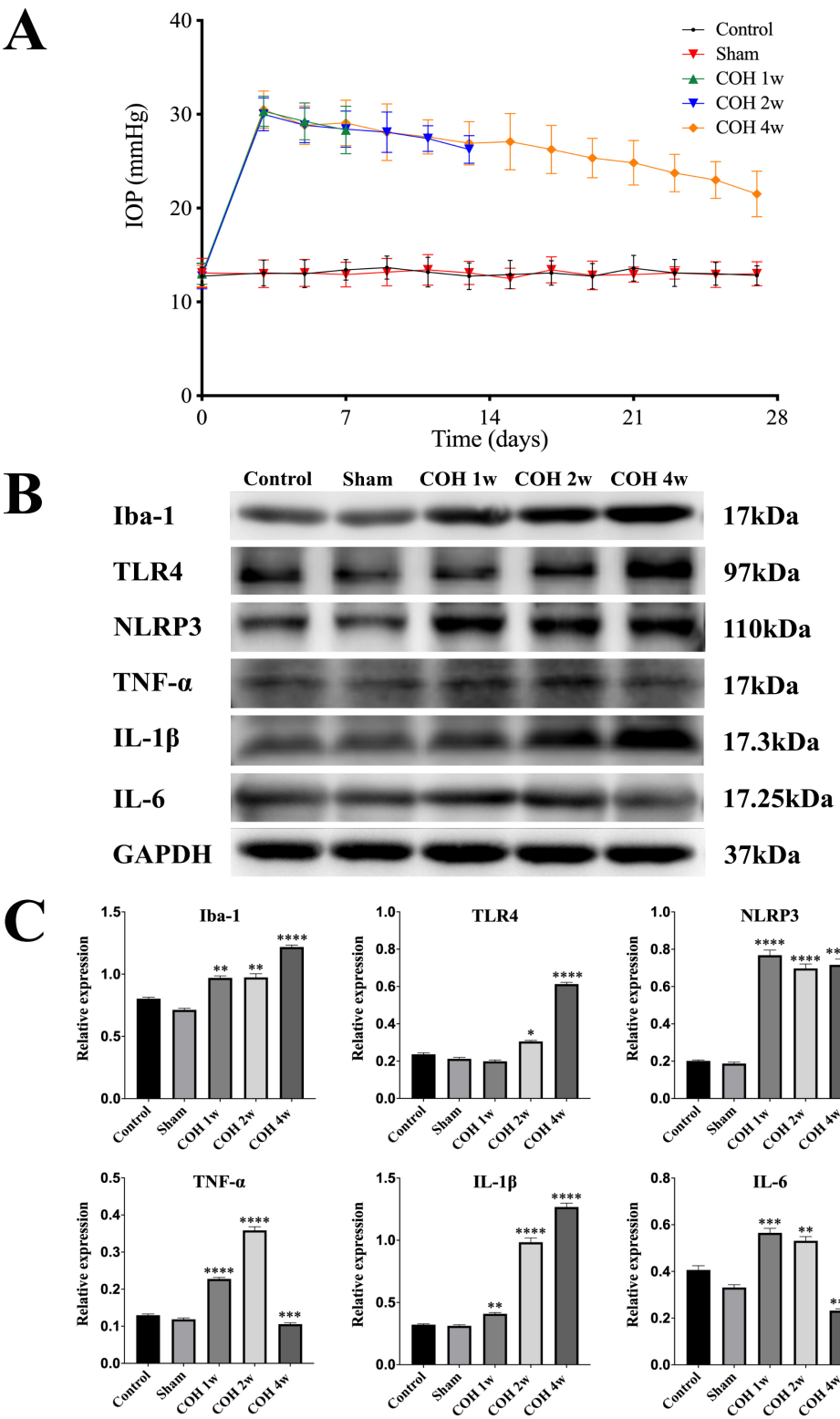

Fig. 1. Alterations of ionized calcium-binding adapter molecule 1 (Iba-1), Toll-like receptor 4 (TLR4), NLR family pyrin domain containing 3 (NLRP3) and pro-inflammatory cytokines during chronic ocular hypertension (COH) process. (A) Intraocular pressure (IOP) fluctuations among different groups throughout four weeks (n = 12). The error bars represent mean ± SD. (B) Bands of western blotting showing protein expressions of Iba-1, TLR4, NLRP3 and pro-inflammatory cytokines during COH process. (C) Quantitative analysis of grey values as detected with Image J (version 1.53, National Institutes of Health, Bethesda, MD, USA) among different groups. n = 6, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$ vs the control group. Bars represent mean ± SEM. TNF-α, tumor necrosis factor alpha; IL-1β, cytokine interleukin-1β; IL-6, Interleukin-6; GADPH, glyceraldehyde-3-phosphate dehydrogenase.

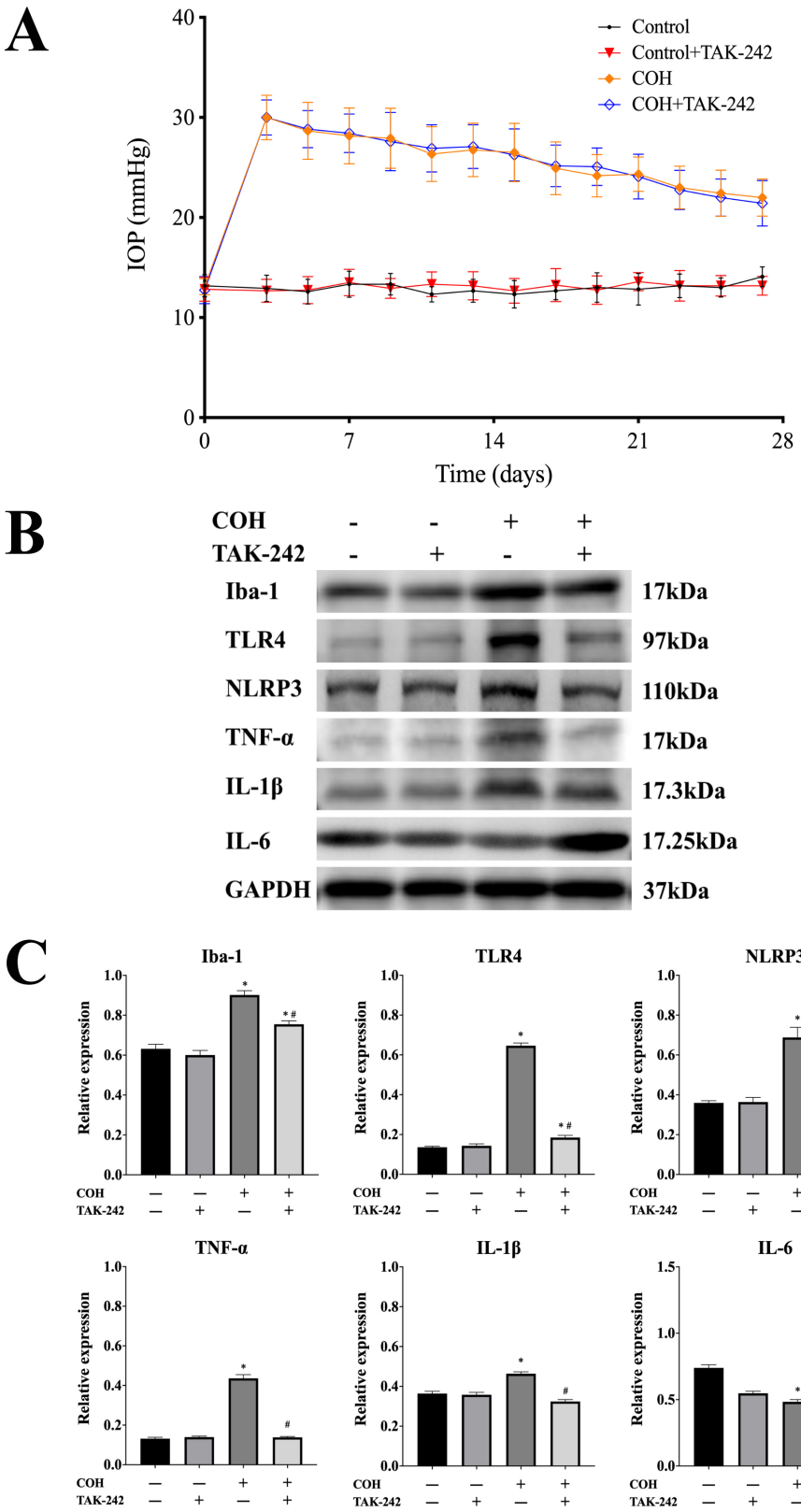

Fig. 2. Impact of Resatorvid (TAK-242) administration on the alterations of Iba-1, TLR4, NLRP3 and pro-inflammatory cytokines in rats of COH 4 weeks. (A) IOP fluctuations among different groups throughout four weeks (n = 12). The error bars represent mean ± SD. (B) Bands of western blotting showing protein expressions of Iba-1, TLR4, NLRP3 and pro-inflammatory cytokines in the control, control+TAK-242, COH, COH+TAK-242 groups. (C) Quantitative analysis of grey values as detected with Image J among different groups. n = 6, *$p < 0.05$ versus the control group, #$p < 0.05$ versus the COH group. Bars represent mean ± SEM.

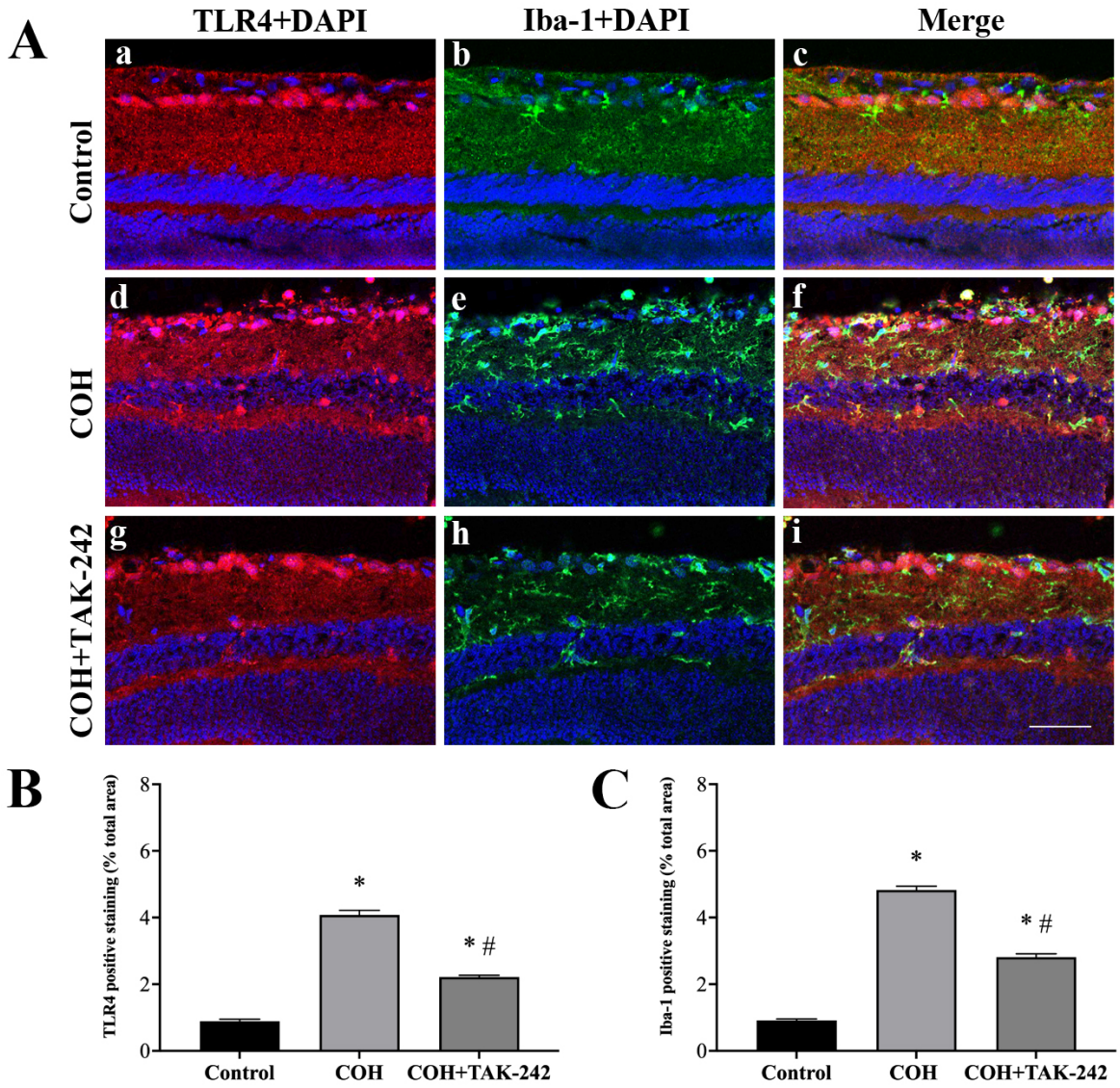

Fig. 3. Impact of TAK-242 administration on TLR4 and microglial activation in rats of COH 4 weeks. (A) Representative images showing double immunofluorescence staining of Iba-1 (green), TLR4 (red) and DAPI (blue) in the retina in control, COH and COH+TAK-242 rats (scale bar = 50 µm). (a) showed staining for TLR4 (red) in the control group. (b) showed staining for Iba-1 (green) in the control group. (c) Merged images showed faint staining for co-localization of TLR4 and Iba-1 in the control group. (d) showed increased TLR4 staining in the COH group. (e) showed increased Iba-1 staining in the COH group. (f) Merged images revealed massive co-localization of TLR4 and Iba-1 staining at 4 weeks after COH induction. (g) showed TLR4 staining in the COH+TAK-242 group. (h) showed Iba-1 staining in the COH+TAK-242 group. (i) Merged images revealed COH eyes treated with TAK-242 showed decreased expressions of TLR4 and Iba-1. (B,C) Quantitative analysis of TLR4 (B) and Iba-1 (C) positive staining (% total area) as calculated using ImageJ among different groups (n = 6, *$p < 0.05$ versus the control group, #$p < 0.05$ versus the COH group). The error bars represent mean ± SEM.

242 considerably attenuated the decreased number of RGCs in the retina tissues of COH rats. Additionally, we found that TAK-242 treatment did not markedly affect the IOP fluctuations. However, a recent *in vitro* experiment examined the anterior segments of human donor eyes in the perfusion culture model and revealed that treatment with transforming growth factor beta 2 (TGFβ2) could elevate IOP, while co-treatment with TAK-242 produced significantly lower IOP values [14]. The potential mechanistic property of TAK-242 in perfusion cultures and COH rat model might be different, which still awaits further confirmation.

Retinal microglia are activated by various injurious signals, which may be mediated by TLRs, especially TLR4 [6]. Researches have revealed that the excessive activation of retinal microglia is highly associated with RGCs apoptosis. Novel therapeutic options targeting activated microglia could be a viable approach to managing glaucomatous dam-

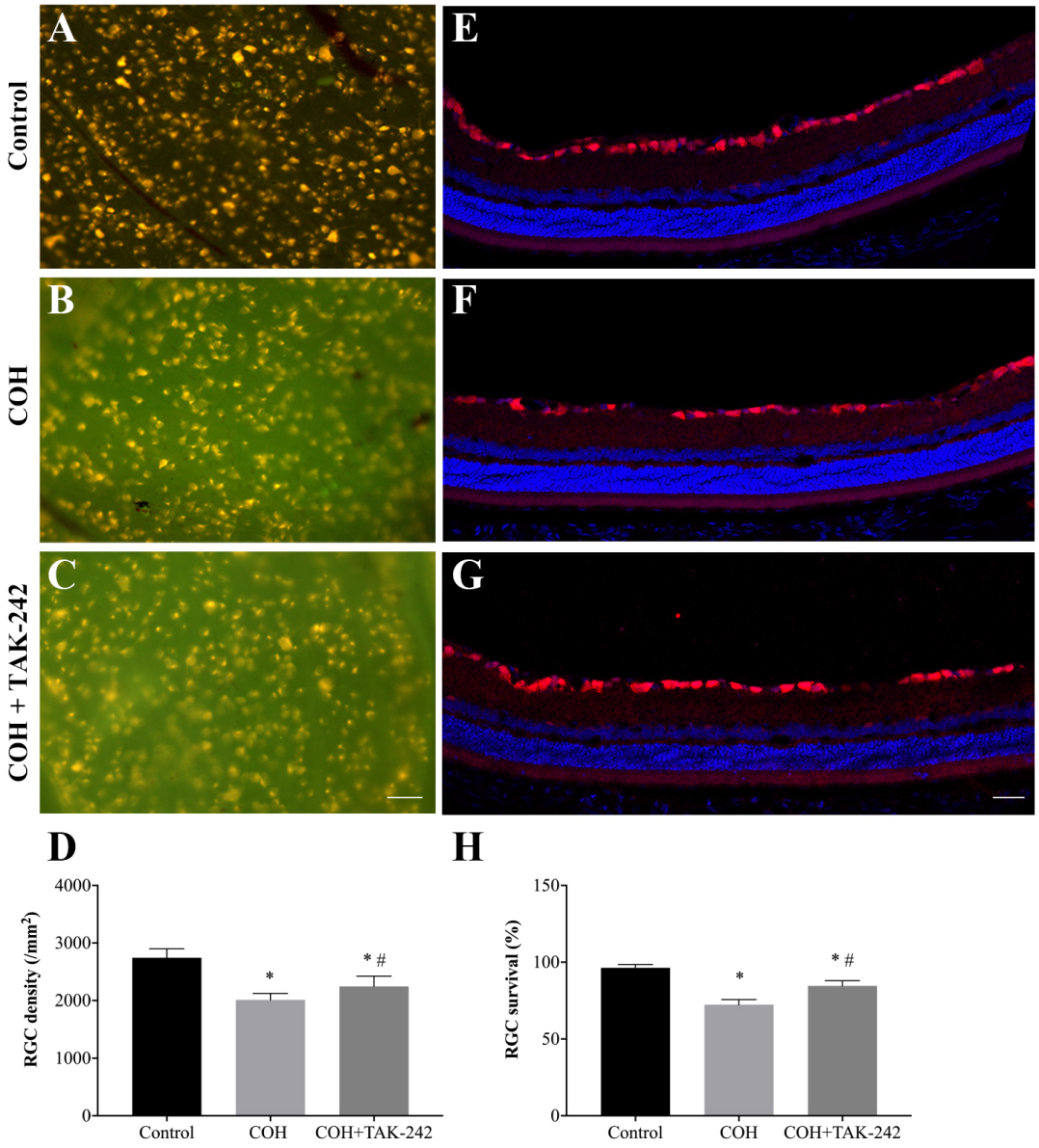

Fig. 4. Impact of TAK-242 administration on RGCs survival in COH rats. (A–C) Retrograde labeling of retinal ganglion cells (RGCs) with Fluoro-Gold in different groups (scale bar = 100 μm). (D) Quantitative analysis of RGCs counting in areas of approximately 1/2 retinal radius from the optic disk (n = 6, *$p < 0.05$ versus the control group, #$p < 0.05$ versus the COH group). (E–G) Immunolabeling with neuronal nuclear protein (NeuN) staining in the ganglion cell layer (GCL) in different groups (scale bar = 50 μm). (H) The percentage of NeuN-positive cells in the GCL in different groups (n = 6, *$p < 0.05$ versus the control group, #$p < 0.05$ versus the COH group). The error bars represent mean ± SD.

age [15,16]. In this study, early activation of microglia was induced by continuous elevated IOP in a rat COH model. TLR4 expression was also upregulated after the induction of COH, whereas treatment with TAK-242 efficiently suppressed TLR4-mediated microglial activation and significantly promoted the survival of RGCs. Retinal damage is aggravated through TLR4, and TAK-242 exhibits neuroprotective properties by attenuating the activation of microglia. A previous study has shown that gene expression of TLR4 was markedly upregulated at 28 days after IOP elevation in a microbead-induced mouse model of glaucoma [17]. Wang *et al.* [18] also reported that overexpression of TLR4 was obviously detected in activated microglia of the COH rat model. Meanwhile, Dvoriantchikova *et al.* [10] revealed that TLR4 deficiency reduced the retinal inflammation and significantly increased the survival of RGCs. Moreover, we detected increased protein expression of NLRP3 in COH rats and TAK-242 treatment resulted in a significant attenuation in NLRP3 protein levels, which confirmed that the activation of NLRP3 occurred under high IOP at least partially in a TLR4-independent fashion. Additionally, accumulating evidence suggests that

NLRP3-induced neuroinflammation amplifies the inflammatory signals and contributes to RGCs loss in glaucomatous injury [19]. To sum up, the beneficial effects of TAK-242 are achieved by interfering with the TLR4-NLRP3 pathway.

Mounting evidence suggests that activated microglia can induce the release of various inflammatory cytokines, such as complement factors, TNF-α and IL-1β, resulting in subsequent magnified local inflammatory response [20,21]. A robust inflammatory response in COH animals can exacerbate neural death by overexposing neurons to neurotoxic cytokines. In the present study, the increased production of cytokines, including IL-1β and TNF-α, is involved in the inflammatory response and tissue injury in the COH model, which has previously been reported in other experimental glaucoma models [4,22,23]. Moreover, increased levels of IL-1β and TNF-α were detected when microglia were activated [12]. Interestingly, the expression trend of IL-6 showed a different pattern, which was not consistent with IL-1β and TNF-α. IL-6 level was low in normal rats but was upregulated after raising IOP at 1 week and diminished over time. TAK-242 treatment significantly upregulated the expression of IL-6 in the retina during experimental glaucoma. Similar to our data, Husain *et al*. [22] demonstrated increased IL-6 mRNA expression and immunofluorescence staining in the retina of ocular hypertensive animals at day 7. Chidlow *et al*. [24] found that IL-6 was also synthesized by injured RGCs and transported within axons after induction of elevated IOP, and it was actively involved in regenerating their axons. Additionally, IL-6 is increasingly identified to play a vital role in the regulation of neuroprotective, neurotrophic and neuro-regenerative activities [25]. The possible explanation for our results may be related to the interplay among different cell types responsible for IL-6 synthesis and transportation.

Taken together, COH-induced microglial activation and activated TLR4-NLRP3 pathway induced a series of dysregulated neuroinflammatory processes, eventually contributing to RGCs death. TAK-242, through inhibiting microglial activation, exhibits the neuroprotective and anti-inflammatory properties. Therefore, targeting TLR4 holds promise as a potential therapeutic strategy for glaucoma in the future. However, further explorations are necessary to explore the regulatory mechanisms mediating these actions.

Conclusions

Targeting TLR4 inhibition could be a potential therapeutic strategy to protect RGCs from COH damage.

Availability of Data and Materials

All data reported in this paper will be shared by the lead contact upon reasonable request. All materials are fully available in the paper without restriction.

Author Contributions

ZJL and KM designed the study. ZJL, LR and HLW performed the research, analyzed the data, prepared the figures and drafted the manuscript. KM supervised the research and critically reviewed the manuscript. All authors contributed to the article and approved the submitted version. All authors agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

The animal procedures were performed in accordance with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and with approval from the institutional review board of Renji Hospital, affiliated with Shanghai Jiao Tong University School of Medicine, Shanghai, China.

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Conflict of Interest

The authors declare no conflict of interest.

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