Resveratrol Alleviates Retinal Ischemia-Reperfusion Injury by Inhibiting the NLRP3/Gasdermin D/Caspase-1/Interleukin-1β Pyroptosis Pathway

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Purpose. The purpose of this study is to investigate the anti-pyroptotic effect of resveratrol in the context of ischemia-reperfusion (I/R)-induced retinal injury, with a particular focus on Müller glial cells (MGCs) and to elucidate the underlying molecular mechanisms.

Methods. The retinal I/R model was constructed in mice and pyroptotic markers were measured at six, 12, 24, 48, and 72 hours after I/R injury to determine the peak of pyroptotic activity. The effects of resveratrol on pyroptosis, inflammasomes, and the activation of MGCs after I/R injury were observed on the retina of mice. Moreover, induction of pyroptosis in rat Müller glial cells (r-MC) via lipopolysaccharide was used to explore the effects of resveratrol on pyroptosis of r-MC in vitro.

Results. After the induction of retinal I/R injury in mice, the intricate involvement of pyroptosis in the progressive degeneration of the retina was observed, reaching its zenith at the onset of 24 hours after I/R injury. Resveratrol treatment alleviated I/R injury on the retina, relieved retinal ganglion cells death. In addition, resveratrol inhibited Caspase-1 activation, gasdermin D (GSDMD-N) cleavage, the inflammasome assembly, and the release of inflammatory cytokines, simultaneously relieving the MGCs activation. Furthermore, resveratrol inhibited the pyroptosis-related NLRP3/GSDMD-N/TMS1/ASC/Caspase-1/IL-1β pathway in r-MC cells, and mitigated cells death in vitro.

Conclusions. Pyroptosis plays an important role in the pathogenesis of retinal I/R injury. Resveratrol can attenuate pyroptotic-driven damage in the retina and MGC by inhibiting the NLRP3/GSDMD-N/TMS1/ASC/Caspase-1/IL-1β pyroptosis pathway.

Keywords: glaucoma, pyroptosis, NLRP3, resveratrol, müller glial cells

Glaucoma is a multifactorial, progressive, degenerative disease of the optic nerve and is currently the most common cause of irreversible blindness. Epidemiological statistics estimate that the number of glaucoma patients worldwide was approximately 76.5 million in 2020 and will rise to 111.18 million patients by 2040 because of an increasingly aging population.[1] The important risk factors for glaucoma include intraocular pressure (IOP), age, race, family history, corticosteroid sensitivity, and more.[2] Acute glaucoma remains the most common type of glaucoma among Asians, with episodes characterized by a large acute increase in IOP (often greater than 50 mm Hg), severe eye pain, and recurrent chronic progression to permanent blindness.[3] The main treatment for glaucoma remains the reduction of IOP to normal levels via various drugs or surgical procedures[4]; however, traditional pressure-lowering therapy does not effectively alleviate retinal nerve cell damage and the subsequent retinal degeneration process after an acute glaucoma attack in patients. Therefore it is particularly urgent to find crucial therapeutic targets in acute glaucoma attacks.

Glaucoma is currently considered a neurodegenerative disease, akin to Alzheimer disease and Parkinson disease and is closely associated with immune inflammation. Studies have shown that during glaucomatous ocular hypertensive episodes, ischemia and hypoxia as well as mechanical traction can induce inflammation of the retina and optic nerve.[5] This is demonstrated by the activation of retinal glial cells, such as the proliferation of microglia, astrocytes, and Müller cells, and the subsequent release of proinflammatory factors such as TNF-α, IL-1β, and prostaglandin E2,[6] which induce retinal ganglion cells (RGCs) death and optic neurodegeneration.

Potential mechanisms of RGC death, including cell autophagy, apoptosis, necrosis, pyroptosis, iron death[7] and copper death, have been implicated by recent studies.[8] Among these, pyroptosis is a programmed mode of inflammatory cell death[9] without the morphological manifestations of cellular crumpling and necrotic cell disintegration presented by apoptosis. In classical pyroptosis, Caspase-1 is activated by inflammasomes such as that of NLRP3,

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and inflammatory cleavage of gasdermin D (GSDMD) forms water channels in the cytoplasmic membrane, which promotes local swelling of cells, induces cell lysis and releases cell contents and inflammatory factors such as IL-1β and IL-18.[10] Interestingly, the NLRP3 inflammasome is known to be activated by retinal pigment epithelium, astrocytes, microglia and Müller cells,[11] and activation of inflammatory vesicles and production of IL-1β have been reported to be more pronounced in astrocytes and Müller cells,[12] suggesting that pyroptosis could contribute to demise and degeneration of RGCs.[11] Müller cells are unique in spanning the entire width of the retina, allowing them to maintain retinal homeostasis through close contact with both retinal blood vessels and neurons.[13] Substantial experimental results conducted by our group have demonstrated that Müller cells involved in glaucoma pathogenesis and could be considered as a potential therapeutic target in glaucoma treatment.[14,15] However, the proinflammatory role of Müller cells in the development and progression of glaucoma remains unclear.

Sirtuin family proteins are a class of NAD(+)-dependent protein lysine deacylases containing SIRT1-7 human isoforms. SIRT1 exerts anti-inflammatory and anti-stress effects, thereby delaying cellular senescence and death.[16] Resveratrol, a natural polyphenol, has been shown to be a potent agonist of SIRT1.[17] Previous studies by our research group have revealed that resveratrol can effectively upregulate decreased SIRT1 expression after retinal ischemic-reperfusion (I/R) injury, alleviate retinal injury as well as gliosis and inflammation,[18] activate the Ampk/SIRT1/Pgc1α pathway, inhibit the Akt/mTOR pathway in the zebrafish retina,[19] refine mitochondrial dynamics, diminish apoptosis of RGCs by moderating Bax and Caspase-3 and prevent RGCs death.[20] In addition, relevant experiments have demonstrated that SIRT1 deficiency exacerbates I/R injury in the liver and stimulates inflammasome activation in the pyroptosis pathway in vivo.[21] However, there are no relevant studies to substantiate the role of resveratrol in the occurrence and development of retinopathy through the pyroptosis pathway.

In summary, resveratrol, as a SIRT1 agonist, has exhibited a beneficial effect on the retina in both animal and cellular models. However, how resveratrol regulates pyroptosis in the pathogenesis of glaucoma model remained to be adequately explored. This study aims to further investigate if resveratrol protects against retinal damage and MGC injury by regulating the pyroptosis process of cells through NLRP3/GSDMD-N/Caspase-1 signaling. In addition, we explore the potential involvement of SIRT1 in mediating the aforementioned protective mechanisms of resveratrol. This study provides valuable insights into the therapeutic potential of resveratrol as a means to mitigate pyroptotic-induced damage in MGCs and therefore to foster retinal neuroprotection.

Material and Methods

Animal and Drug Treatment

Male 6- to 8-week-old wild-type (WT) C57BL/6 mice were obtained from SPF Biotechnology Co., Ltd. (Beijing, China) (license number: SYXK2021-0001). All mice had free access to water and food and were maintained at a controlled temperature (22°C-24°C) with 12-hour dark/light cycles. All animals were treated in accordance with the Guide for the Care and Use of Laboratory Animals and the ARVO Statement for Use of Animals in Ophthalmic and Vision Research and monitored with approval from the Institutional Animal Care and Use Committee of Nanchang University, China.

There are 2 means of grouping animals used in this study: (I) The mice were divided into six groups: (1) Control (normal mice); (2) I/R 6h (six hours after the establishment of the mouse retinal I/R model); (3) I/R 12h (12 hours after the establishment of the mouse retinal I/R model); (4) I/R 24h (24 hours after the establishment of the mouse retinal I/R model); (5) I/R 48h (48 hours after the establishment of the mouse retinal I/R model); and 6) I/R 72h (72 hours after the establishment of the mouse retinal I/R model). Each group consisted of 3 to 6 mice. The retina was removed six, 12, 24, 48, and 72 hours after the end of I/R for follow-up experiments.

(II) The mice were divided into four groups: (1) control (normal mice); (2) I/R (mouse models of retinal I/R were constructed); (3) I/R + NS (dimethyl sulfoxide [DMSO] [D8370; Solarbio Life Science, Beijing, China] diluted with normal saline was given intraperitoneally for five days before I/R and again immediately after I/R); and (4) I/R+RES (5 mg resveratrol [R5010; Sigma-Aldrich Corp., St. Louis, MO, USA] was dissolved in 1 mL of DMSO to prepare a 5 mg/mL resveratrol solution. This solution (25 mg/kg) was given for five days before I/R and administered again immediately after I/R, following the protocol established in our research group's previous study).[14] Each group consisted of three to six mice. The retina was removed one day after I/R for follow-up experiments.

Retinal I/R Model

Through anterior chamber intubation, transient retinal ischemia was induced in mice whereby the retinal I/R model was constructed.[19] Under anesthesia by intraperitoneal injection of 3.6% chloral hydrate at a ratio of 0.1 mL/10 g, unilateral transient retinal ischemia was established. Then the cornea was locally anesthetized with 0.5% arcaine eye drops, and the pupil was dilated with 1% tropicamide. A 30-gauge needle was inserted into the anterior chamber of the right eye with a balanced salt solution. The IOP of the right eye was maintained above the systolic pressure (~110 mm Hg) for 60 minutes, whereas the IOP of the opposite eye was maintained at a normal level. The observed blanching of the retina suggested completion of retinal ischemia. At the end of treatment, the mice were sacrificed six, 12, 24, 48, and 72 hours after retinal ischemia. Resveratrol (3,4,5-trihydroxytrans stilbene) was dissolved in DMSO at a concentration of 5 mg/mL. C57BL/6 mice aged two months were intraperitoneally injected with RES (25 mg/kg, diluted with normal saline solution) for five consecutive days before I/R and then intraperitoneally injected again immediately after I/R. The control group was intraperitoneally injected with the same amount of DMSO (NS diluted with normal saline solution).

Cell Culture and Drug Treatment

The rat Müller Glia cell line, rMC-1, were purchased from Shanghai Yubo Biotechnology Co., Ltd. (Shanghai, China). The rMC-1 cells were cultured in DMEM containing 10%

FBS and 1% penicillin-streptomycin at 37°C in a humidified incubator containing 5% CO_2. When the cells reached 70% confluence, they were subjected to the described treatments for 24 hours.

There are two means of grouping cells used in this study: (I) The cells were divided into six groups: lipopolysaccharide (LPS) 0, 1, 5, 10, 50, and 100 mg/mL. Cells were cultured with cell medium containing the corresponding concentration of LPS (1 mg LPS was dissolved in 1 ml cell medium, which was diluted to the desired concentration at administration; L4391; Sigma-Aldrich Corp.) for 24 hours, and then 5 mM ATP medium solution (50.718 mg of ATP powder was dissolved in 1 mL of culture medium to make 100 mM ATP solution, which was diluted with the medium to 5 mM at administration; A9130; Solarbio Life Science) was given for 30 minutes. The combination of LPS and ATP treatment to induce pyroptosis was a method previously described in a published article.[22]

(II) The cells were separated into four groups: Control, LPS, LPS + DMSO, and LPS + RES. In addition to the control group, cells were cultured with 50 mg/mL LPS for 24 hours and then given 5 mM ATP medium solution for 30 minutes. The medium of the LPS + RES group contained 5 µM resveratrol (11.412 mg of resveratrol was dissolved in 1 mL DMSO and prepared into 50 mM resveratrol solution. When administered, the solution was diluted to 5 µM with medium.). The same dose of DMSO was administered to the LPS + DMSO group.

Retinal Paraffin Section

Mouse eyeballs were implanted in FAS eyeball fixation solution immediately after removal and were sent to Servicebio Company in Wuhan, Hubei Province, for paraffin embedding and sectioning. The sections were cut along to the sagittal plane of the eyeball, and the passage of the optic plexus was observed under an optical microscope. The thickness of the slices was 5 µm.

Retinal H&E Staining

Paraffin sections of the retina were incubated at 85°C for 40 minutes and dewaxed by conventional methods: immersion in xylene twice for 15 minutes each time, anhydrous ethanol twice for five minutes each time, and 95%, 80%, and 75% ethanol successively for three minutes each time. After staining with hematoxylin for one minute, the sections were rinsed with running water for one minute, stained with eosin for 30 seconds, and rinsed with running water for 30 seconds. Then the sections were successively placed into 75%, 80%, 95%, and 100% ethanol from low to high concentration for one minute. After drying, the sections were sealed with neutral resin. Under a microscope, retinal images magnified × 400 were taken 1 mm away from the optic nerve.

Cell Viability Assay

The r-MC cells were inoculated into 96-well plates at a density of 1×10^4 cells/well and cultured for 24 hours. The cells were treated in different groups for 24 hours. Cell viability was detected using a Cell Counting Kit-8 (CCK-8; Yeasen, Shanghai, China); r-MC cells were treated with CCK-8 at 37°C for one to four hours. Absorbance at 450 nm was measured using an enzyme label to quantify cell viability.

Cellular Lactate Dehydrogenase (LDH) Release Assay

The r-MC cells were inoculated into 96-well plates at a density of 1×10^4 cells/well and cultured for 24 hours. The cells were treated in different groups for 24 hours. The release of LDH was determined with a LDH detection kit (microenzyme labeling method). Cell medium 20 µL was taken from each well and transferred to a new 96-well plate. Then, 25 µL of matrix buffer and 5 µL of coenzyme I were added, and the reaction was carried out at 37°C for 15 minutes. Next, 25 µL of 2,4-dinitrophenylhydrazine was added and incubated at 37°C for 15 minutes. Then, 250 µL of 0.4 M NaOH solution was added and incubated for five minutes at room temperature (RT). Absorbance at 450 nm was measured using an enzyme label to quantify the amount of LDH leakage from cells.

Dead/Live Cell Staining

The dead/live cell staining was performed using a Calcein/PI kit (C2015S; Beyotime Institute of Biotechnology, Jiangsu, China). The r-MC cells were seeded onto 24-well plates at a density of 1×10^5 cells/well and cultured for 24 hours. The cells were treated in different groups for 24 hours. Then, the culture medium was aspirated, and the cells were rinsed with PBS. Calcein AM/PI working solution (250 µL) was added and incubated for 30 minutes at 37°C in the dark. Then the cells were viewed under a fluorescence microscope, and images of cells magnified × 200 were taken.

TUNEL Staining

To detect apoptotic retinal cells, staining was performed using the one-step TUNEL In Situ Apoptosis Kit (Green, Elab Fluor 488; Elabscience, Wuhan, China). Paraffin sections were dewaxed as described above, immersed in PBS, and rinsed three times for five minutes each time. Then, protease K solution was added to each sample at 37°C for 30 minutes. Terminal deoxynucleotidyl transferase equilibration buffer was added at 37°C for 10 to 30 minutes. The labeled working liquid was added and placed in a wet box at 37°C for two hours in the dark. DAPI working solution (G1012; Servicebio, Wuhan, China) was added and incubated for five minutes at RT in the dark. Retinal images magnified × 200 were taken under a Zeiss laser confocal microscope (Zeiss, Oberkochen, Germany).

The r-MC cells were seeded onto 24-well plates at a density of 1×10^5 cells/well and cultured for 24 hours. The cells were treated in different groups for 24 hours. The cell-crawling tablets were immersed in PBS, rinsed once, immersed in 4% paraformaldehyde, and fixed at RT for 15 minutes. The samples were immersed in PBS containing 0.2% Triton X-100 (T8200; Solarbio Life Science) and permeabilized at 37°C for 10 minutes. Follow-up incubation procedures refer to retinal TUNEL staining above. Images of cells magnified × 200 were taken under a fluorescence microscope.

Immunofluorescence Staining

Paraffin sections were dewaxed by the above method, immersed in boiling citrate repair solution (pH = 6.0),

placed in a microwave oven for eight minutes, and cooled to RT. PBS containing 5% donkey serum and 0.1% Triton X-100 was added to seal and permeabilize tissues at RT for one hour. The tissue was incubated with primary antibody at a ratio of 1:100 and placed in a wet box at 4°C overnight (14-18 hours). The corresponding secondary antibody was incubated for one hour at RT in the dark. DAPI was used to stain for 10 minutes. The section was sealed with a tablet sealer containing an anti-fluorescence quenching agent (G1401; Servicebio). Retinal images magnified 200× were taken under a Zeiss laser confocal microscope.

r-MC cells were inoculated into 24-well plates at a density of 1×10^5 cells/well and cultured for 24 h. The cells were treated in different groups for 24 h. The procedures of cell-crawling fixation, blocking, primary antibody, secondary antibody, and DAPI incubation were performed as described above. Images magnified 200× were taken under a Zeiss laser confocal microscope.

Western Blotting Analysis

Retinal tissues and r-MC cells were lysed in RIPA buffer containing protease inhibitor (1:100), and the lysates were treated with ultrasound on ice for 30 min. Then, the supernatant was collected via centrifugation. The protein samples were analyzed in 10% SDS-polyacrylamide gels by electrophoresis and transferred to PVDF membranes. Then, the blots were blocked in 5% skimmed milk (GC310001; Servicebio) at RT for one hour and incubated with the appropriate primary antibodies at 4°C overnight. The membrane was rinsed with TBST three times for 10 minutes each time, followed by incubation with corresponding secondary antibodies for one hour. The following antibodies were used: β-tubulin (J10715, 1:2500; TransGen Biotech, Beijing, China), β-actin (L0117, 1:2500; Santa Cruz, CA, USA), GAPDH (HC301-01, 1:2500; TransGen Biotech), SIRT1 (no. 9475, 1:1000; Cell Signaling Technology, Danvers, MA, USA), NLRP3 (WL02635, 1:1000; Wanleibio Co., Ltd., Shenyang, China), GSDMD (ab209845, 1:1000; Abcam, Cambridge, MA, USA), Caspase-1 (WL02996a, 1:1000; Wanleibio Co., Ltd.), TMS1/ASC (WL02462, 1:1000; Wanleibio Co., Ltd.), IL-1β (WL00891, 1:1000; Wanleibio Co., Ltd.), RBPMS (ab152101, 1:1000; Abcam, Cambridge, MA, USA), GFAP (G3893, 1:1000; Sigma-Aldrich Corp.), GS (ab49873, 1:1000; Abcam), anti-mouse HRP-linked antibody (no. 7076, 1:2500; Cell Signaling Technology), and anti-rabbit HRP-linked antibody (no. 7074, 1:2500; Cell Signaling Technology). The targeting proteins were detected using an EasySee Western blotting Kit, and digital images were collected. The gray value of the images was quantitatively analyzed by ImageJ software.

Statistics

GraphPad Prism (version 8.0; GraphPad, La Jolla, CA, USA) was used to analyze and graph data. All data are expressed as the means ± SEMs. Comparisons between two groups were performed using unpaired *t* tests. The differences among three or more groups were analyzed using one-way ANOVA followed by Dunnett's multiple comparison test. Statistical significance was considered at $P < 0.05$, and all experiments were repeated at least three times.

Results

Retinal I/R Injury Induces Pyroptosis in Retinas of Mice

To investigate whether pyroptosis is involved in retinal injury induced by I/R and the time point of its occurrence, we examined the expression level of key proteins related to pyroptosis, including GSDMD-N and Caspase-1p20. The results of Western blotting showed that the expression of pyroptosis-related proteins increased significantly within 24 hours after I/R induction and returned to normal levels immediately (Figs. 1A, 1C–E). Meanwhile, the expression trend of the NLRP3 inflammasome was consistent with that of pyroptosis-related proteins, with its expression remarkably increasing and peaking 24 hours after injury, followed by a decrease (Figs. 1A, 1B). In addition, SIRT1 expression in the retina dropped markedly 24 hours after retinal injury (Figs. 1A, 1F), indicating that SIRT1 expression may be associated with I/R retinal injury. The results suggest that pyroptosis may be involved in the loss of retinal neurons induced by retinal I/R, which peaks within 24 hours.

Resveratrol Protects the Mouse Retina From I/R Injury

Our previous study showed that resveratrol, a powerful agonist of SIRT1, has significant anti-inflammatory properties and ameliorates retinal I/R injury; thus H&E staining was performed to verify the neuroprotective effect of resveratrol on I/R-induced retinal cell injury.

According to previous findings, which indicated that the peak of retinal pyroptosis occurs 24 hours after I/R injury, we chose to observe the effect of resveratrol intervention 24 h after injury. As shown in Figures 2A and 2B, the retina was clearly edematous after I/R modeling, with disorganized cell arrangement and reduced thickness in all layers, and visible vacuoles were observed in the GCL compared with those of wild-type mice. Interestingly, resveratrol treatment recovered retinal edema caused by I/R and restores retinal thickness with neatly arranged ganglion layers. In addition, immunofluorescence staining of RGCs showed that the number of RGCs declined to 20% after I/R, while the number of surviving RGCs reached 55% after resveratrol treatment (Figs. 2C, 2D). These results suggest that resveratrol administration can significantly prevent retinal damage in mice with retinal I/R compared with mice in the I/R group.

Resveratrol Attenuates Pyroptosis in Retinas Undergoing I/R Injury

We further applied Western blotting to detect the expression of pyroptotic markers GSDMD-N and Caspase-1p20, inflammasome markers NLRP3 and TMS1/ASC, and inflammatory factor IL-1β. Consistent with the previous results, the pyroptotic signals as well as inflammasome markers were significantly increased in the mouse retina one day after I/R (Figs. 3A–E), while the level of SIRT1 was sharply decreased (Fig. 3F). In contrast, resveratrol treatment significantly upregulated SIRT1 expression and attenuated the upregulated levels of pyroptosis, as well as inflammatory protein expression levels (Figs. 3A–F).

As mentioned previously, DNA damage also occurs during pyroptosis, which presents as positive TUNEL staining. Therefore we performed TUNEL staining to identify

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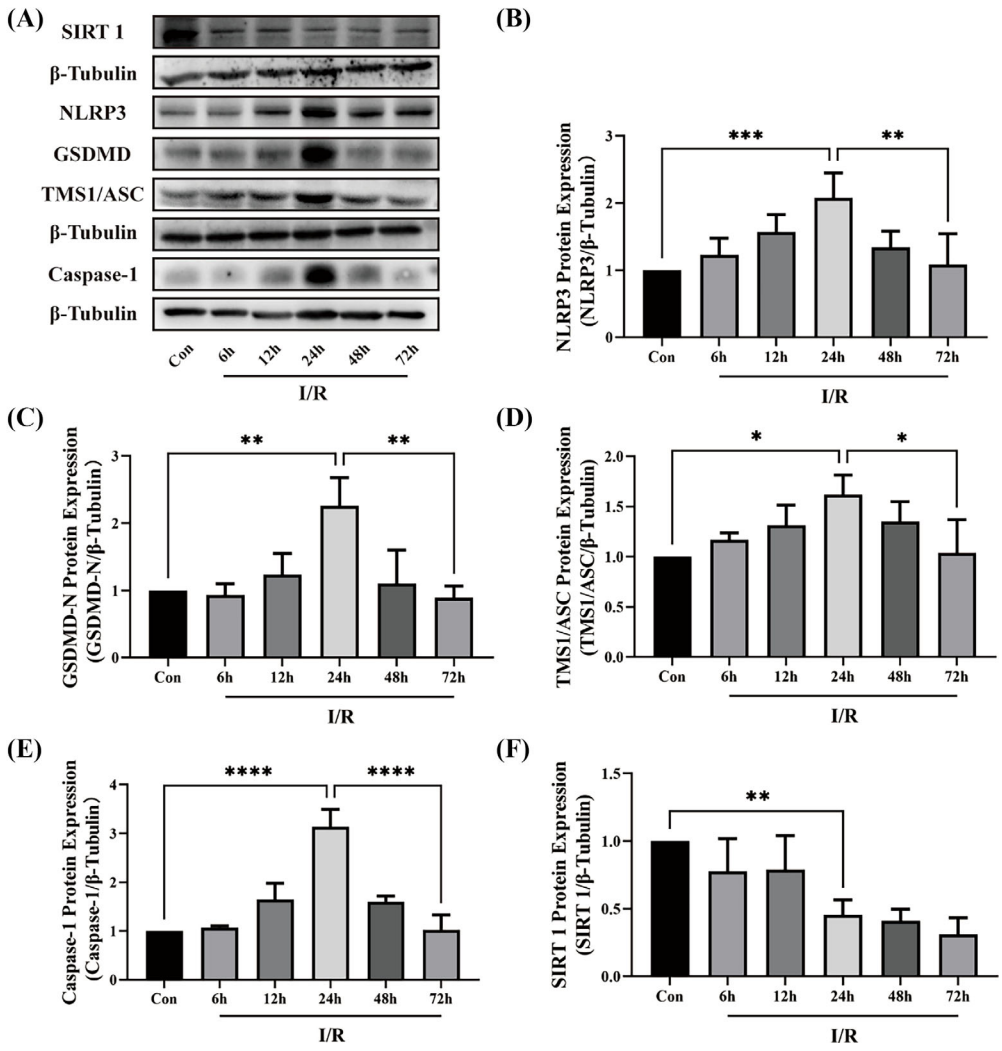

Figure 1. Retinal I/R injury induces pyroptosis in the retinas of C57 mice. (**A**) Expression of NLRP3, GSDMD-N, TMS1/ASC, Caspase-1 and SIRT 1 in the retinas of C57 mice at six,12,24,48, and 72 hours after I/R injury compared with the control group. β-Tubulin was used as the loading control. (**B–F**) Quantitative analysis of proteins as shown in **A**. Con, control group; 6h, 6 hours after I/R injury; 12h, 12 hours after I/R injury; 24h, 24 hours after I/R injury; 48h, 48 hours after I/R injury; 72h, 72 hours after I/R injury. Data represent the mean ± SEM for groups of three to six mice. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; $^{****}P < 0.0001$.

pyroptotic-positive cells. Compared to the control groups, TUNEL-positive signals were observed in the retinas of mice in the I/R group from the GCL to the INL layer and were mainly distributed in the INL layer. However, the TUNEL-positive signal was reduced by approximately 55% after resveratrol treatment (Figs. 3G, 3H). These results indicate that resveratrol can protect the retina by reducing the inflammatory factors in the retina after I/R, thus preventing pyroptosis in RGCs.

Resveratrol Reduces Glial Cell Proliferation and Activation After Retinal I/R Injury

Glial cell proliferation is the trigger that drives the inflammatory response occurring after retinal damage. Our research team has been dedicated to studying the role of glial cells in the occurrence and progression of glaucoma.[14] We therefore further examined the effect of resveratrol intervention on retinal glial cell proliferation and activation after I/R. Glial fibrillary acidic protein (GFAP), which normally remains quiescent in the retina, is highly expressed in activated astrocytes and Müller cells in the presence of injury. Western blotting identifying GFAP levels were consistent with previous results, showing that GFAP was elevated immediately one day after I/R, whereas the level of GFAP expression decreased after resveratrol intervention (Figs. 4A, 4B). Furthermore, immunofluorescence staining of the retina revealed changes in the GFAP pattern. One day after I/R modeling, GFAP was in a filamentous pattern and then turned into thinner filaments with weaker fluorescence after resveratrol treatment (Fig. 4D).

Glutamine synthetase (GS) is secreted by Müller glial cells and can be used as a specific marker of Müller glial cells. To further examine whether Müller glial cells participate in retinal I/R injury, Western blottings were performed and showed that GS increased immediately one day after I/R modeling but decreased after resveratrol intervention (Figs. 4A, C). Moreover, immunofluorescence staining demonstrated that the density of GS in the retina considerably increased

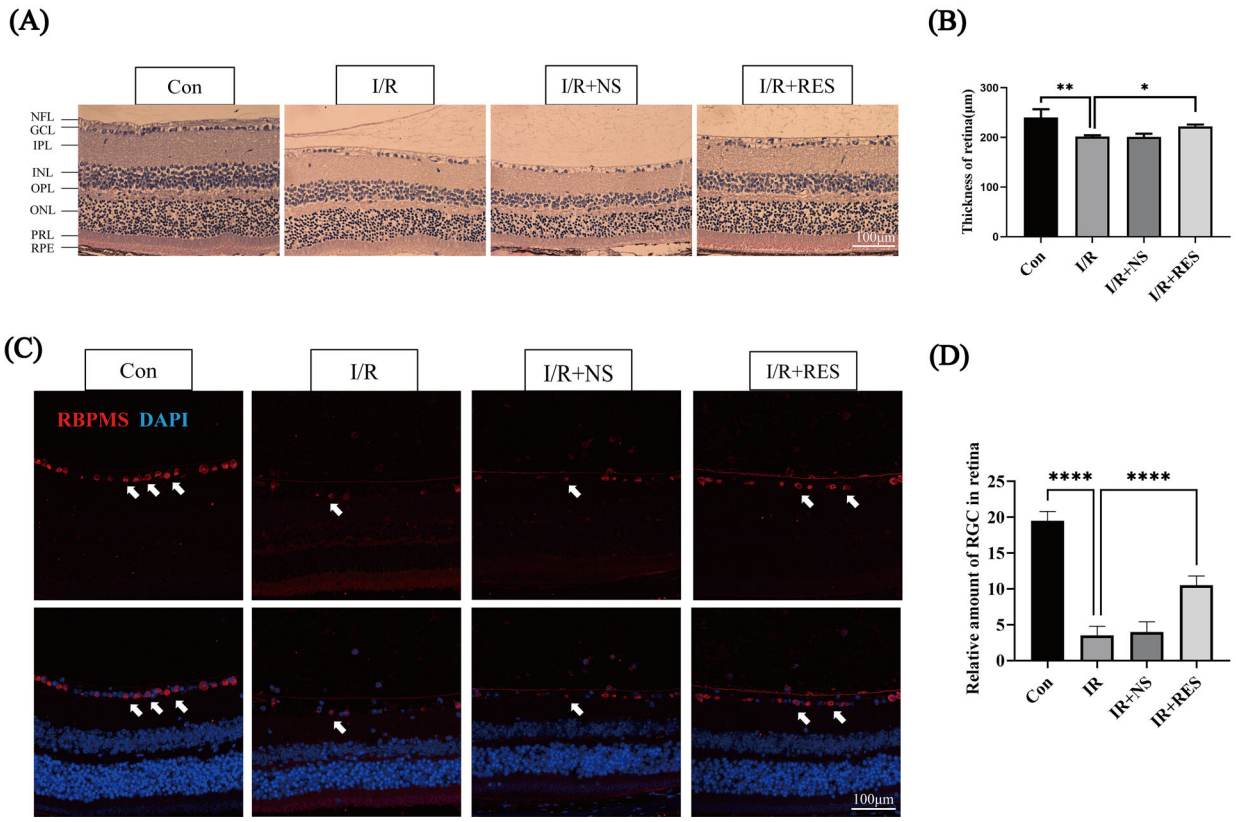

Figure 2. Resveratrol protects the mouse retina from ischemia/reperfusion injury. (**A**) H&E staining of retina obtained from wild-type mice, I/R mice, I/R+NS mice and I/R+RES mice. *Scale bars*: ×400. (**B**) Retinal thickness analysis after H&E staining of paraffin sections. (**C**) Double immunostaining of RBPMS (*red*) and DAPI (*blue*) in the retina. *Scale bars*: ×200 . (**D**) Quantitative analysis of RGCs in the retina as shown in **C**. Data represent the mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$ $^{****}P < 0.0001$. NFL: nerve fiber layer; GCL: ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer; RPE: retinal pigment epithelium.; NS: normal saline; RES: resveratrol.

with a disorganized distribution and loss of the original filamentous structure. Although the fluorescence intensity after resveratrol intervention was stronger than that of the control groups, it was markedly lower than that of the I/R group, and its original pattern was restored (Fig. 4E). These findings demonstrated the proliferation and activation of Müller glial cells in response to retinal damage after I/R. This alteration was alleviated by resveratrol intervention, which further reduced the development of retinal inflammation and protected the survival of RGCs.

LPS Induces Pyroptosis in Retinal Müller cells

Since we observed that activation of Müller cells plays an important role in retinal I/R injury, we used an in vitro experiment for further verification. We used LPS with 5 mM/ml ATP to induce the classical pyroptotic pathway in a mouse retinal Müller cell line (r-MC) to build a cellular model.

First, to determine the appropriate concentration of LPS for establishing the pyroptotic model in Müller cells, we treated r-MC with concentration of 0 μg/mL, 1 μg/ml, 5 μg/mL, 10 μg/mL, 50 μg/mL, and 100 μg/mL LPS for 24 hours and then added 5 mM/ml ATP for 30 minutes. A slight increase in cell density was observed under the light microscope when LPS was 1 μg/mL, which may be related to its reactive gliosis, and then a gradual decrease in cell density was observed with an increase in the concentration of LPS (Fig. 5A). Data from the CCK-8 test showed that 50 μg/mL was a concentration under which cell viability significantly decreased to 60% compared with that of the control group (Fig. 5B). In addition, LDH leakage from the cells gradually increased with increasing LPS concentration, reaching a peak at a concentration of 50 μg/mL (Fig. 5C). Therefore we chose 50 μg/mL LPS as the concentration for the Müller cell pyroptosis model.

We also examined the expression levels of NLRP3/Caspase-1/GSDMD-N in retinal Müller cells, and the Western blotting results were consistent with previous findings. The expression levels of the proapoptotic markers Caspase-1p20 and GSDMD-N and the NLRP3 inflammasome in Müller cells were significantly increased when the concentration of LPS reached 50 μg/mL (Figs. 5D–G). Taken together, these results suggest that the activation of retinal Müller cells can generate pyroptotic signals, which may be involved in retinal degeneration.

Resveratrol Attenuates LPS-Induced Pyroptosis in Retinal Müller Cells

To understand the effect of resveratrol on pyroptosis of r-MC, we added 5 μM/ml resveratrol to the r-MC pyroptotic model for intervention. The CCK-8 results showed that LPS treatment resulted in a significant decrease in Müller cell

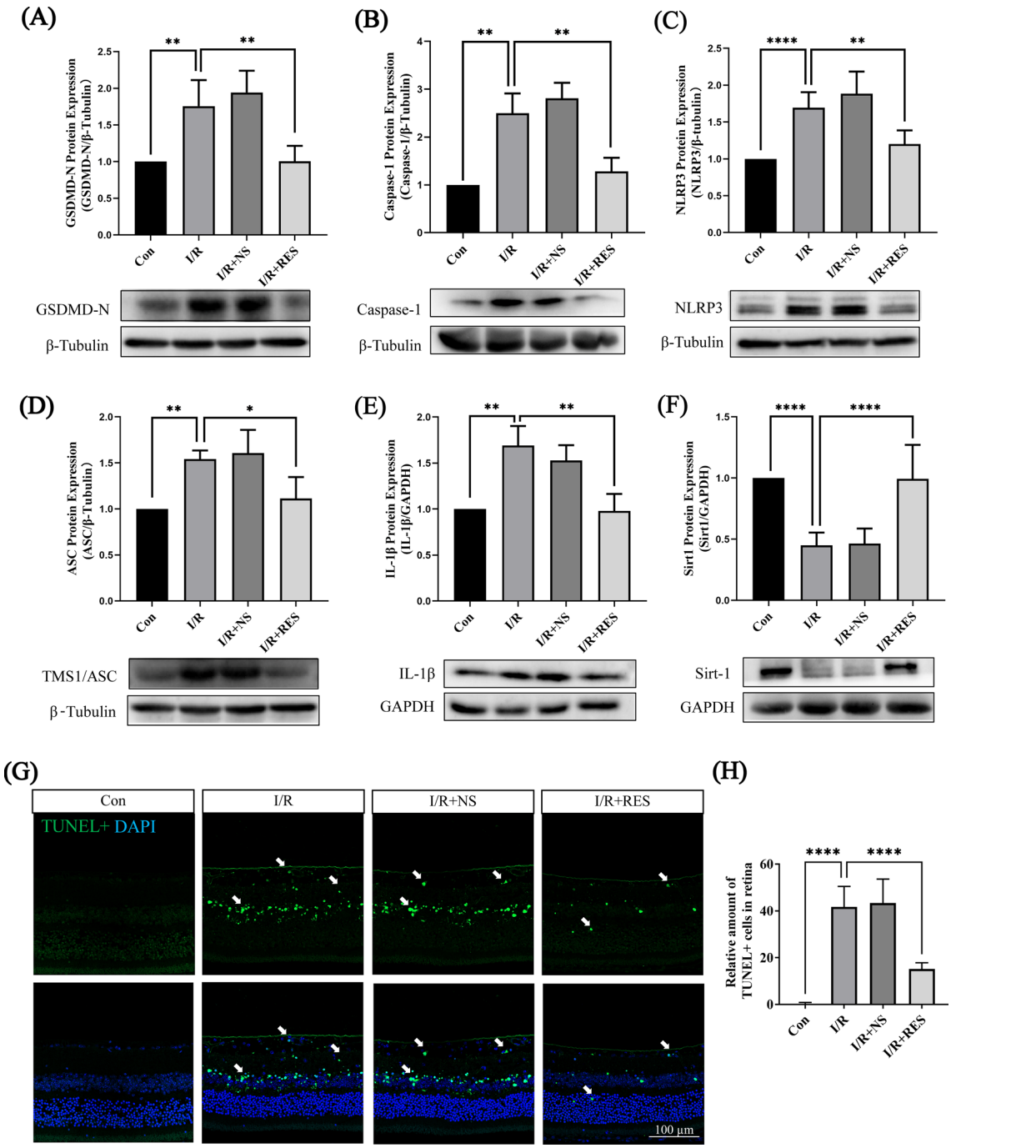

Figure 3. Resveratrol attenuates pyroptosis in retinas suffering ischemia-reperfusion injury. (**A–F**) Retinal expression and quantitative analysis of GSDMD-N, Caspase-1, NLRP3, TMS1/ASC, IL-1β, SIRT 1 in wild-type mice, I/R mice, I/R+NS mice and I/R+RES mice. (**G**) TUNEL staining of TUNEL (*green*) and DAPI (*blue*) in the retina. *Scale bar*: 200×. (**H**) Quantitative analysis of TUNEL-positive cells in the retina. Data represent the mean ± SEM for groups of three to six mice. *$P < 0.05$; **$P < 0.01$; ****$P < 0.0001$. I/R: ischemia/reperfusion; NS: normal saline; RES: resveratrol.

viability compared to that of the control group, while resveratrol treatment restored the LPS-induced decrease in cell viability (Fig. 6A). Moreover, as shown in Figure 6B, resveratrol treatment inhibited LPS-induced LDH release.

As mentioned above, propidium iodide (PI), a substance of low molecular weight, can enter pyroptotic cells through GSDMD-N pores but cannot penetrate normal cells with intact cytosolic membranes because of pore size. Calcein AM can penetrate the membranes of living cells and is retained in the cell to emit intense green fluorescence. Therefore calcein AM can be used in combination with PI to detect membrane pore formation in pyroptotic cells. Our results showed that the number of PI-positive Müller cells was remarkably elevated after LPS modeling compared to the control group, although there was a decrease in the number of PI-positive cells and fluorescence intensity after

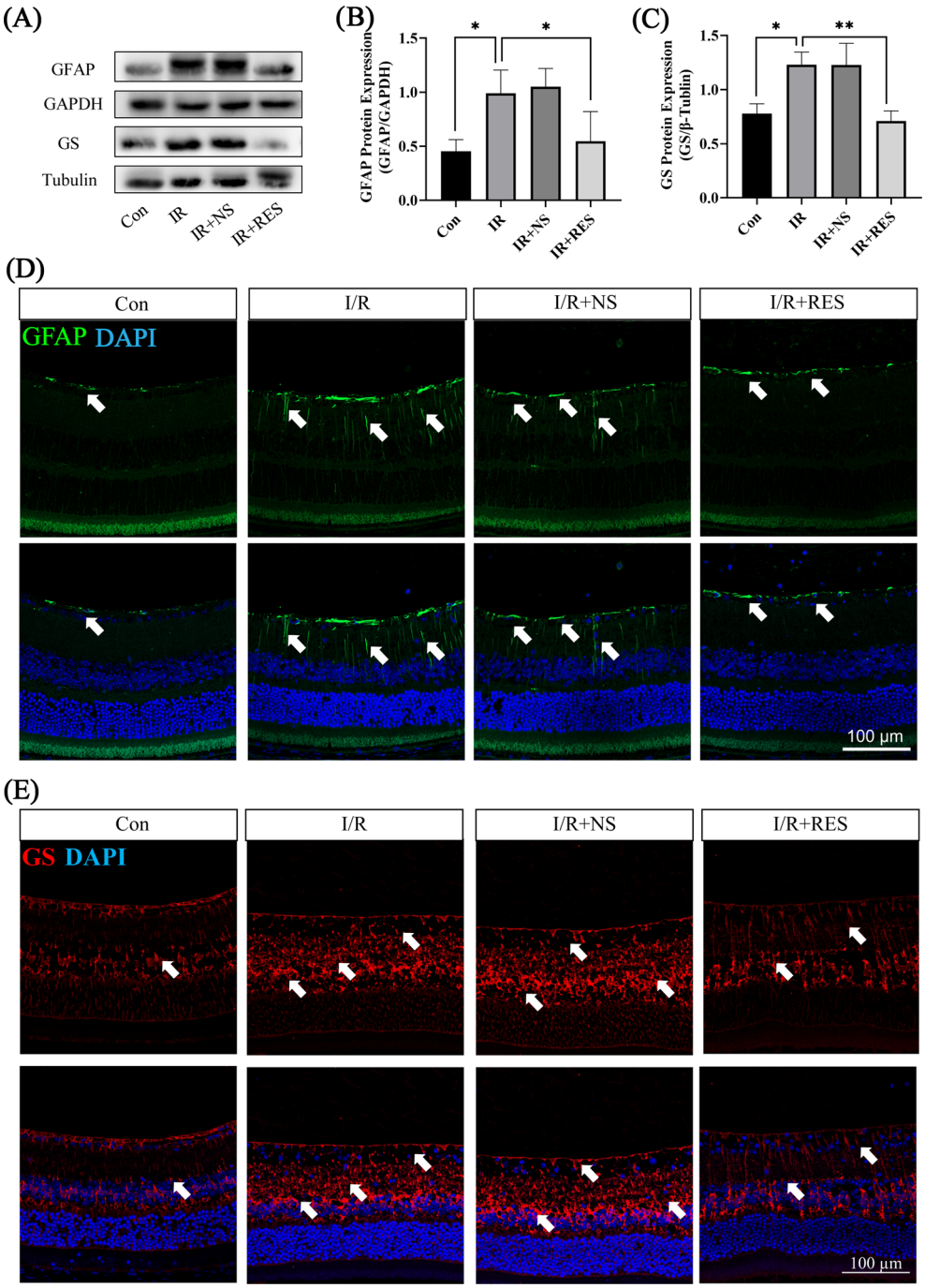

Figure 4. Resveratrol reduces glial cell proliferation and activation after retinal ischemia/reperfusion injury. (**A**) Retinal expression of GFAP and GS in wild-type mice, I/R mice, I/R + NS mice and I/R + RES mice. (**B**, **C**) Quantitative analysis of proteins in the retina as shown in **A**. (**D**, **E**) Immunofluorescent staining of GS (*red*), GFAP (*green*), and DAPI (*blue*) in the retina. Data represent the mean ± SEM for groups of three to six mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$. *Scale bars*: ×200. I/R: ischemia/reperfusion; NS: normal saline; RES: resveratrol.

resveratrol intervention was given under the same conditions (Figs. 6C, 6E). TUNEL staining was also performed on the r-MC model. An increase in TUNEL-positive cells was seen in the LPS group and a decrease after resveratrol intervention was seen compared to the former group (Figs. 6D, 6F).

Consistent with the results shown in the mouse retina, the expression of NLRP3, Caspase-1p20 and GSDMD-N as well as the levels of TMS1/ASC and IL-1β were increased, indicating the elevation of pyroptotic signal levels. In contrast, resveratrol intervention significantly reduced the LPS-induced pyroptotic signal produced by Müller cells (Figs. 7A–F). As displayed in Figures 8A–C, TMS1/ASC, NLRP3 and Caspase-1p20 inflammasomes punctuated the cytoplasm after LPS modeling, whereas the number and fluorescence intensity of NLRP3, TMS1/ASC, and Caspase-1p20

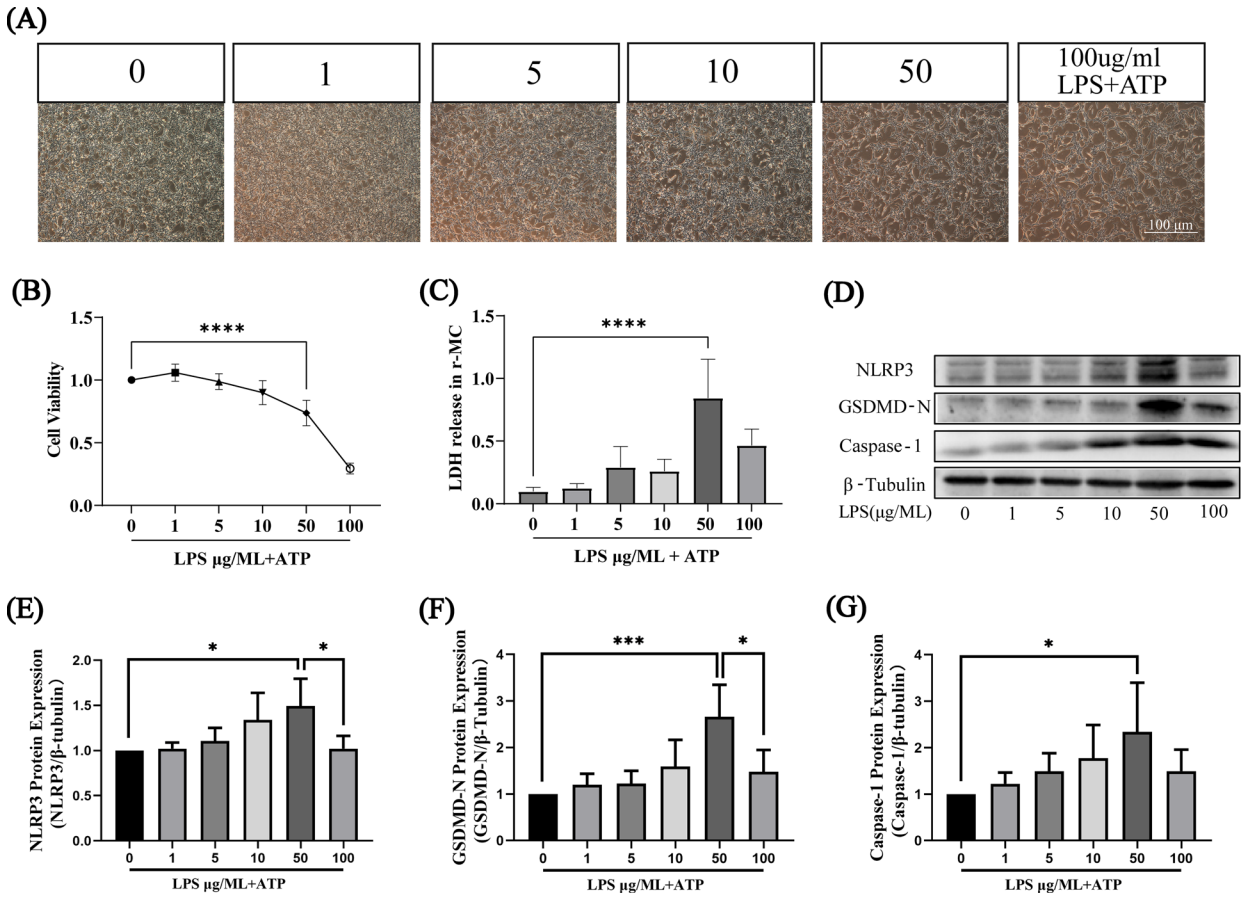

Figure 5. The optimal concentration of LPS-induced pyroptosis in r-MC. (**A**) The density of r-MC after 24 hours' treatment with different concentrations (0, 1, 5, 10, 50 and 100 μg/mL) of LPS under the microscope. *Scale bars*: ×200. (**B**) Quantitative analysis of cell viability after treatment with different concentrations of LPS in CCK-8 test. (**C**) The degree of LDH release after treatment with different concentrations of LPS. (**D–G**) Expression and quantitative analysis of NLRP3,GSDMD-N,Caspase-1 in r-MC after 24 hours' treatment with different concentrations of LPS. Data represent the mean ± SEM for groups of three to six mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$; ****$P < 0.0001$.

inflammasomes in the cytoplasm decreased significantly after resveratrol intervention. The above results indicated that resveratrol can alleviate LPS-induced Müller cell pyroptosis and the production of inflammasomes and inflammatory factors, ultimately protecting Müller cell viability.

Discussion

For retinal I/R injury, our aim is to diminish retinal inflammation and salvage RGCs from death. In this study, we demonstrated that cellular pyroptosis plays a significant role in retinal I/R injury and that the administration of resveratrol is effective in alleviating retinal inflammation and pyroptosis. Further experiments confirmed that this phenomenon may be attributed to the fact that resveratrol is able to reduce Müller glial cell activation, which in turn reduces inflammasome activation and the occurrence of pyroptosis (Fig. 9).

Pyroptosis, an emerging programmed cell death pathway, is dependent on inflammasomes and mediated by activated Caspase-1. It is characterized by the aggregation of GSDMD N-termini to form pores in the cell membrane.[23] Detection of proteins associated with the pyroptosis signaling pathway is convincing evidence that pyroptosis occurs after retinal I/R injury in mice. As previously shown, the expression levels of GSDMD-N, Caspase-1p20 and NLRP3 peaked 24 hours after retinal I/R injury. Not surprisingly, Dvoriantchikova et al.[24] and Pronin et al.[11] found that the upregulation and peak of pyroptosis signals were 24 hours after I/R in mice. Meanwhile, in another study, a spike of proteins associated with pyroptosis was detected six hours after I/R injury in rats, possibly because the use of a different species. It is generally accepted that pyroptosis arises in the early stages after I/R injury and is an effective target for early intervention in acute glaucoma attacks. In addition, the results of Qin et al.[25] showed that during retinal I/R injury, RGCs first undergo cell necrosis, and apoptosis occurs approximately 12 hours later, whereas iron death is involved in the entire process; however, their study did not consider cell scorching. Therefore the temporal sequence of the various forms of cell death that occur during retinal I/R injury requires additional research for better intervention and treatment of retinal I/R injury.

Several studies have been demonstrated the potential of resveratrol in the treatment of glaucoma[26] because of its anti-inflammatory and antioxidant properties,[5] which can also reduce the expression of NLRP3, the inflammatory vesicle receptor, in retinal endothelial cells[27] and retinal pigment epithelial cells.[28] However, to our knowledge, the present study is the first to recommend that resveratrol is beneficial in alleviating cellular pyroptosis in retinal I/R

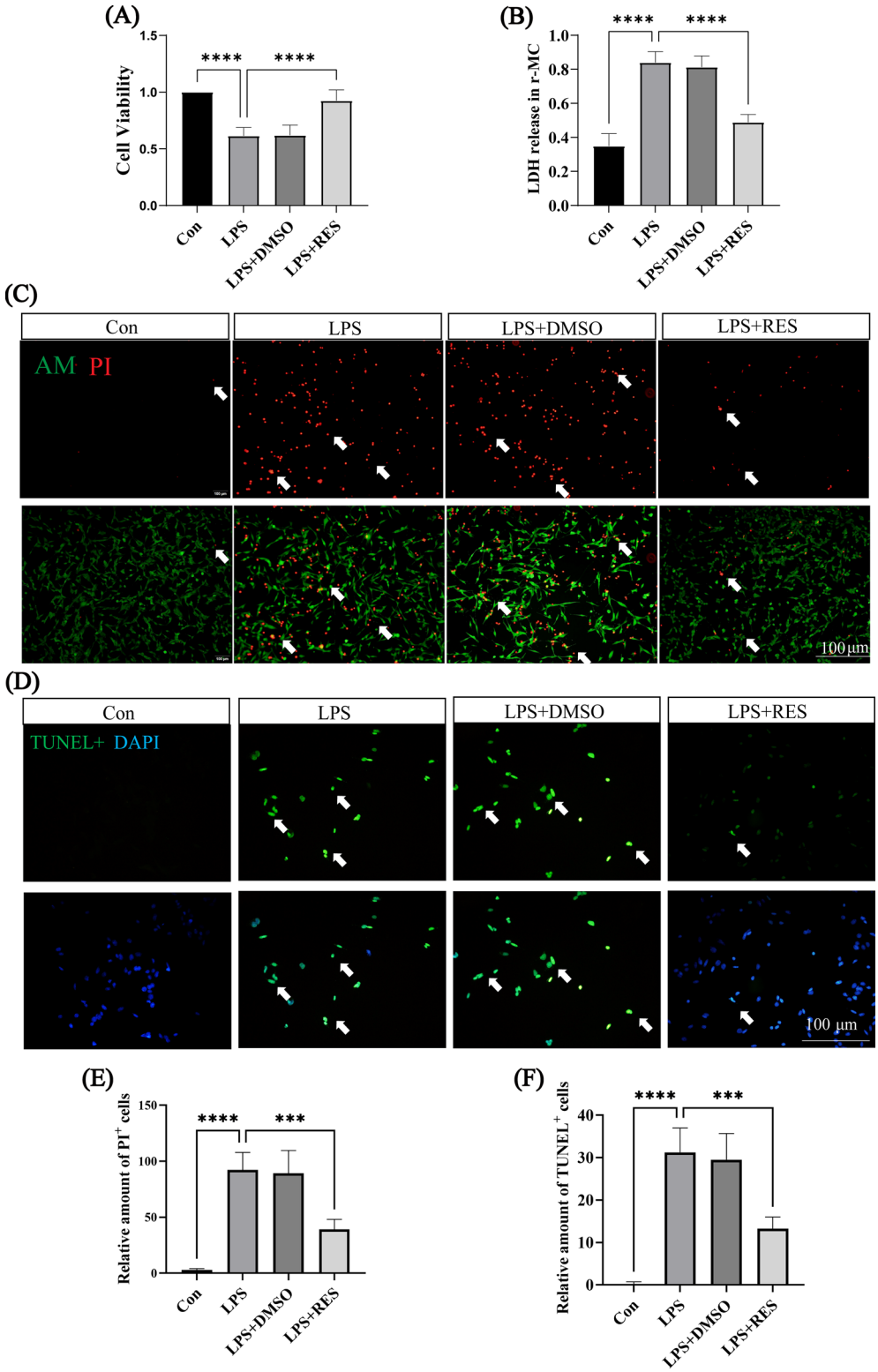

Figure 6. Resveratrol enhances the cell viability of r-MC after LPS treatment. (**A**) Quantitative analysis of r-MC viability of control group, LPS group, LPS+DMSO group and LPS+RES group in CCK-8 test. (**B**) Quantitative analysis of the degree of LDH release in r-MC of different groups. (**C**) PI staining of PI (*red*) and AM (*green*) in r-MC. (**D**) TUNEL staining of TUNEL (*green*) and DAPI (*blue*) in r-MC of different groups. (**E**) Quantitative analysis of PI-positive cells as shown in **C**. (**F**) Quantitative analysis of pyroptotic-positive cells as shown in **D**. Data represent the mean ± SEM for groups of three to six mice. ***P < 0.001; ****P < 0.0001. *Scale bar*: ×200. Con, control; RES, resveratrol.

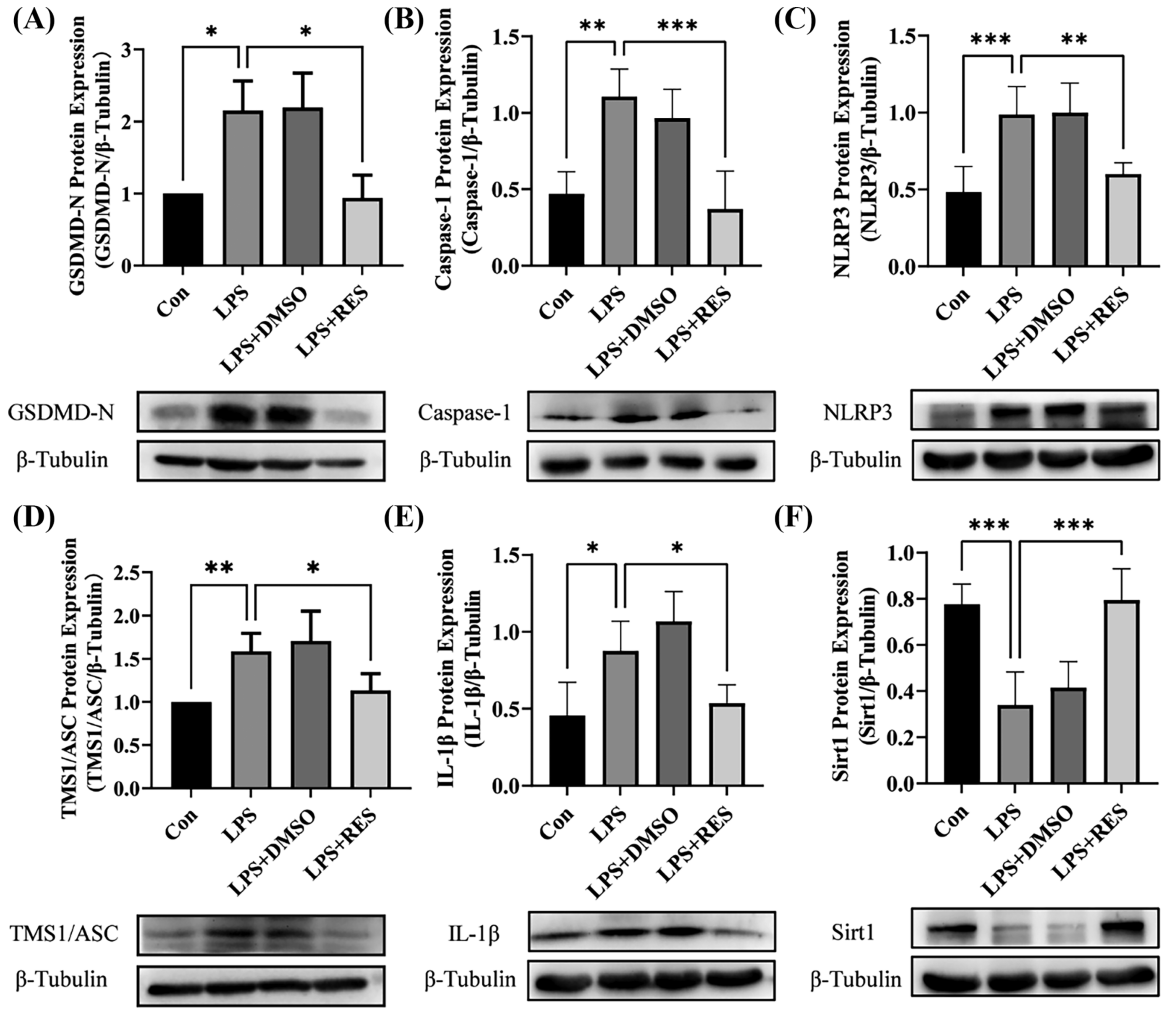

Figure 7. Resveratrol attenuated LPS-induced pyroptosis in retinal Müller cells. (**A–F**) Expression and quantitative analysis of GSDMD-N, Caspase-1, NLRP3, TMS1/ASC, IL-1β, SIRT 1 in r-MC of control group, LPS group, LPS + DMSO group and LPS + RES group. Data represent the mean ± SEM for groups of three to six mice. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$. Con, control; RES, resveratrol.

injury. The results of our study revealed that resveratrol diminished the loss of retinal ganglion cells after I/R and restored retinal structure and thickness, which is consistent with previous studies.[18] This conservational effect is partly achieved by mitigating retinal focal death, which is reflected by the significant decline in GSDMD-N and Caspase-1p20 expression after resveratrol treatment and the significant elevation of NLRP3, the TMS1/ASC inflammasome and the inflammatory factor IL-1β in the retinas of mice. Notably, the expression of SIRT1 was enhanced as well. Similarly, our TUNEL staining results revealed that resveratrol significantly downregulated the number of TUNEL-positive cells after I/R in the retinas of mice, further illustrating its potential to relieve pyroptosis. A study on cardiac ischemia and hypoxia demonstrated that resveratrol protects against ischemia-induced cardiac injury in mice in vivo by inhibiting NLRP3-mediated inflammasome activation via SIRT1/p53,[29] from which we hypothesized that after retinal I/R injury, resveratrol also suppresses the NLRP3 inflammasome pathway by upregulating the activity and expression of SIRT1, thereby alleviating the occurrence of retinal cell scorch death.

Müller cells are a kind of macroglia unique to the retina. We verified the significant activation state of Müller cells after I/R, while resveratrol attenuated the activation of glial cells (Fig. 4). Recently, the proinflammatory role of Müller cells in retinal damage has been progressively explored. Müller cells in chronically hypertensive mice are activated and subsequently induce microglial activation via the ATP/P2 × 7 receptor pathway, and the two interact to secrete large amounts of proinflammatory factors.[30] In addition, Zhang et al.[31] detected elevated secretion of proinflammatory cytokines including IL-1β, TNF-α, IL-6, IL-18 and the NLRP3 inflammasome by high glucose-treated Müller cells. In the present study, we observed that Müller cell lines cultured in vitro could undergo pyroptosis and secrete the inflammatory factor IL-1β via the NLRP3/Caspase-1 inflammasome pathway in response to LPS induction. Consistent with our results, Pronin et al. also visualized the pyroptosis of Müller cells after retinal I/R injury in mice, as manifested by colocalization of GS with Caspase-1 and ASC proteins.[11] Our further observations revealed that the addition of resveratrol significantly curtailed the NLRP3/Caspase-1 inflammasome pathway activated by Müller cells, alleviat-

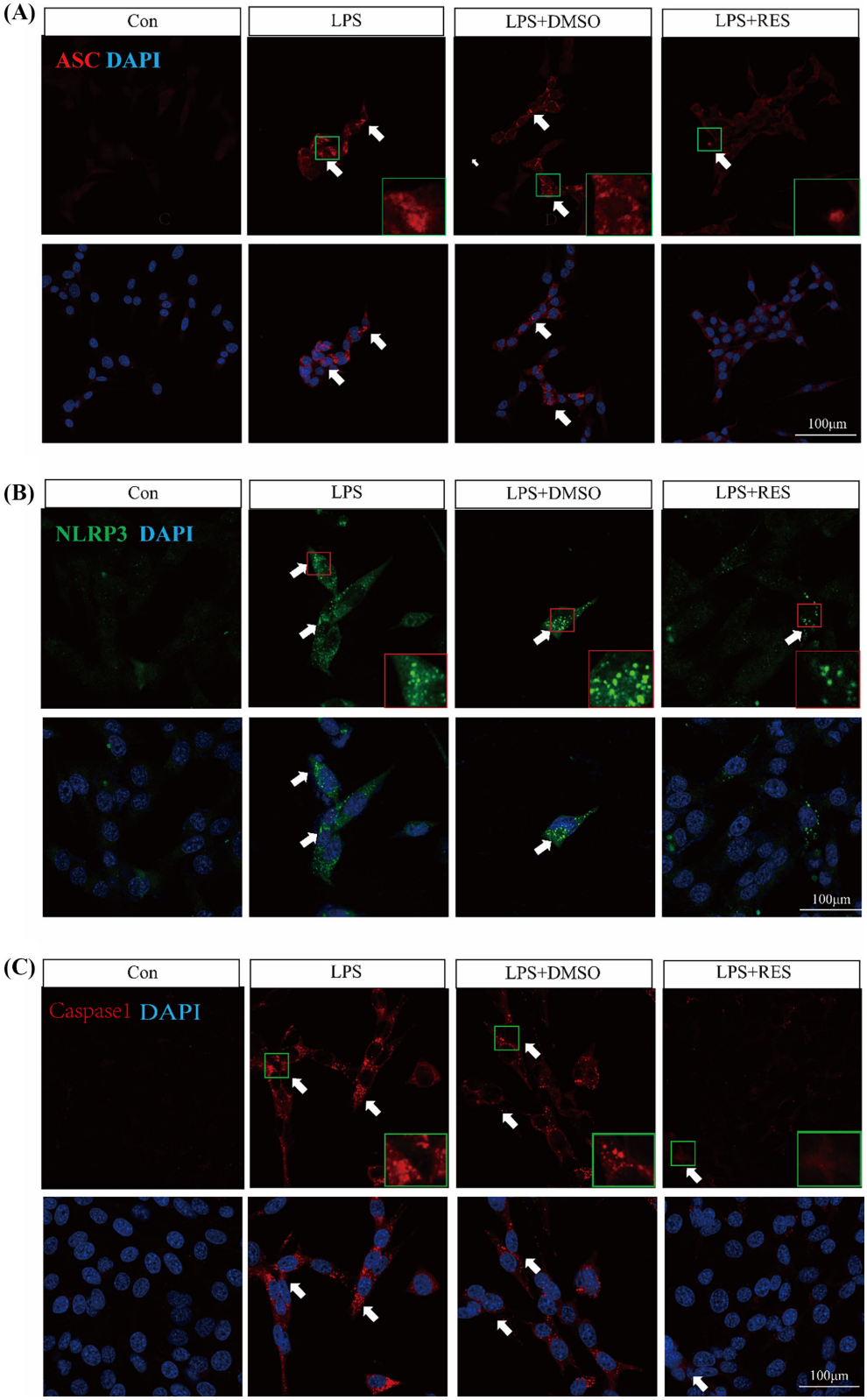

Figure 8. Resveratrol reduces LPS-induced inflammasome in retinal Müller cells. (**A–C**) Immunofluorescent staining of TMS1/ASC (*red*), NLRP3 (*green*), Caspase-1p20 (*red*), and DAPI (blue) in r-MC in different groups. *Scale bar:* 200×. Con, control; RES, resveratrol.

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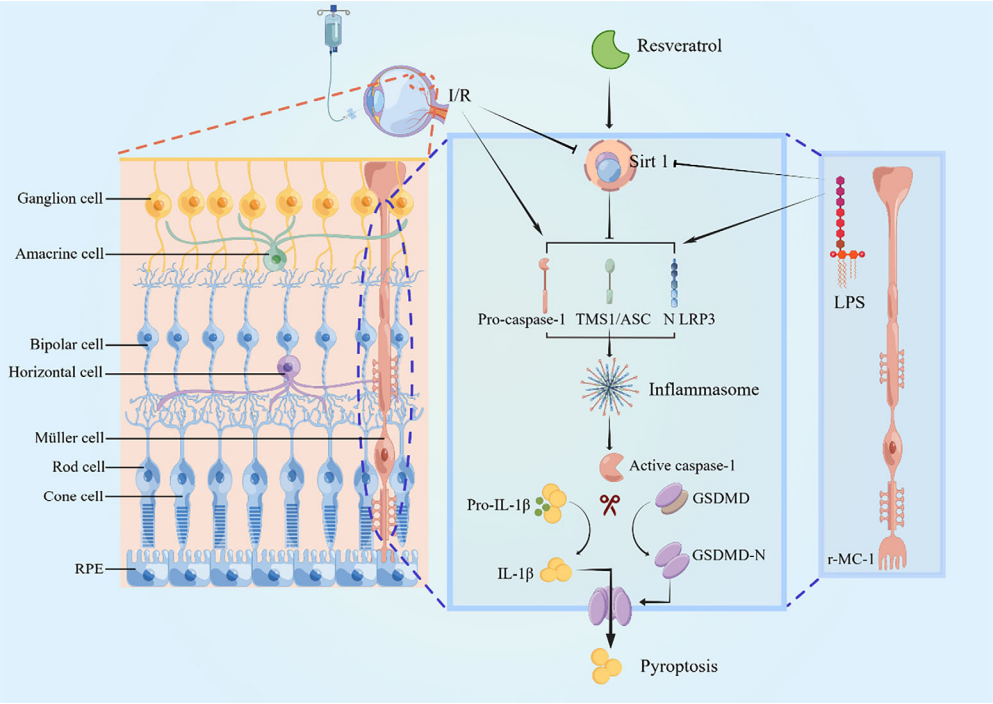

Figure 9. Schematic diagram of resveratrol inhibiting the pyroptotic pathway in retinal Müller cells. Resveratrol upregulated SIRT 1 expression and attenuated the activation of r-MC induced by retinal ischemia/reperfusion injury, as well as inhibited the activation of NLRP3 inflammasome, thereby suppressing the onset of pyroptosis.

ing cell pyroptosis as well as the secretion of inflammatory factors.

Inflammasomes existed in distinct cell types, and in addition to NLRP3, NLRP1 inflammasomes have also been identified in mouse and rat models of acute glaucoma and play a crucial role in the pathogenesis of glaucoma.[32] Research conducted in human glaucoma reported increasing levels of the NLRP3 inflammasome, Caspase-1, and Caspase-8,[33] whereas Caspase-8 also appears to be capable of activating the HIF-1α-NLRP12/NLRP3/NLRC4 inflammasome pyroptosis pathway in mouse models of acute glaucoma.[34] Additionally, activation of the AIM2 inflammasome was detected in Müller cells.[11] Whether there is a unified regulatory mechanism upstream of diverse inflammasome assemblies in the pathogenesis of glaucoma remains unknown. In addition, whether SIRT1 has an inhibitory impact on pyroptosis induced by other inflammasome pathways awaits further exploration.

It is widely accepted that retinal cells undergo death in more than one way.[7] Resveratrol not only minimizes apoptosis by inhibiting the HIF-1a/VEGF pathway, blocking the p38/p53 pathway and enhancing the potency of the PI3K/Akt pathway[26,35] but also activates autophagy to preserve the mitochondrial function of the retina by restricting the Akt/mTOR pathway[36] in zebrafish, which clearly indicates that the function of resveratrol in retaining retinal function is multifaceted. While pyroptosis occurs in the early stage of retinal degeneration,[25] our results complement its inhibition of pyroptosis, which is highly innovative and significant. In addition, several randomized controlled trials have illustrated that resveratrol supplementation is effective in increasing SIRT1 expression in humans,[37–39] although its role in human eyes still needs to be further examined.

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