Research Article

Peritoneal macrophages attenuate retinal ganglion cell survival and neurite outgrowth

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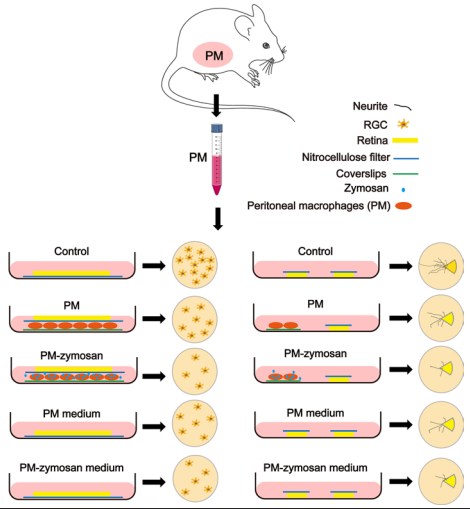
Graphical Abstract *Rat primary peritoneal macrophages attenuates retinal ganglion cell (RGC) survival and neurite outgrowth in the retinal explant co-culture system through the macrophage-derived factors*



Abstract

Inflammation is a critical pathophysiological process that modulates neuronal survival in the central nervous system after disease or injury. However, the effects and mechanisms of macrophage activation on neuronal survival remain unclear. In the present study, we co-cultured adult Fischer rat retinas with primary peritoneal macrophages or zymosan-treated peritoneal macrophages for 7 days. Immunofluorescence analysis revealed that peritoneal macrophages reduced retinal ganglion cell survival and neurite outgrowth in the retinal explant compared with the control group. The addition of zymosan to peritoneal macrophages attenuated the survival and neurite outgrowth of retinal ganglion cells. Conditioned media from peritoneal macrophages also reduced retinal ganglion cell survival and neurite outgrowth. This result suggests that secretions from peritoneal macrophages mediate the inhibitory effects of these macrophages. In addition, increased inflammation- and oxidation-related gene expression may be related to the enhanced retinal ganglion cell degeneration caused by zymosan activation. In summary, this study revealed that primary rat peritoneal macrophages attenuated retinal ganglion cell survival and neurite outgrowth, and that macrophage activation further aggravated retinal ganglion cell degeneration. This study was approved by the Animal Ethics Committee of the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong, Shantou, Guangdong Province, China, on March 11, 2014 (approval No. EC20140311(2)-P01).

Key Words: *in vitro*; inflammation; macrophages; model; neurite outgrowth; optic nerve; retinal ganglion cells; survival

Chinese Library Classification No. R456; R774; R364.5

Introduction

Central nervous system neurons, including retinal ganglion cells (RGCs), lack regenerative power to maintain their survival and axonal regeneration after disease or injury (Berkelaar et al., 1994). Despite a shortage of effective clinical treatments, the degenerative processes can be partially ameliorated by modulating the microenvironment (Laha et al., 2017). Multiple preclinical strategies have been studied to enhance RGC survival and promote axonal regeneration, including the exogenous supplementation of neurotrophic factors

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(Carmignoto et al., 1989; Di Polo et al., 1998; Koeberle and Ball, 1998; Cui et al., 1999), peripheral nerve grafting to the optic nerve (ON) stump (Richardson et al., 1980; David and Aguayo, 1981, 1985), counteracting the inhibitory signals associated with myelin and glial scar formation (Fischer et al., 2004; Yu et al., 2008), and stem cell therapy (Cen et al., 2018). Understanding treatment responses and mechanisms may facilitate the development of effective treatments for neuronal protection.

Mild intraocular inflammation induced by lens injury or macrophage activation has been demonstrated to promote RGC survival and axonal regeneration (Leon et al., 2000; Yin et al., 2003). Oncomodulin, a small calcium-binding protein, has been suggested as the most effective macrophage-derived neurite outgrowth stimulatory factor *in vitro* (Yin et al., 2006). However, macrophages have also been reported to aggravate neuronal damage in experimental autoimmune encephalomyelitis (Alrashdi et al., 2019), acute intraocular pressure elevation injury, and spinal cord traumatic injury models (Leibovitch et al., 1991), thus indicating the complex effects of macrophages on the regulation of neuronal survival. Different varieties of macrophages and different modes of macrophage stimulation can result in neuroprotective or neurotoxic effects (Kroner et al., 2014; Liu et al., 2019). Blood-borne monocytes have been reported to positively contribute to RGC axon regeneration (Yin et al., 2003). Peritoneal macrophages consist of embryonically established local macrophages and blood-borne monocytes that migrate into the peritoneal cavity in response to infectious or inflammatory stimuli (Epelman et al., 2014; Cassado Ados et al., 2015). It remains unknown whether peritoneal macrophages can enhance RGC survival and axon regeneration after ON injury. We postulate that peritoneal macrophages, similar to blood monocytes, can contribute to neural repair upon injury. In the present study, we aimed to determine the biological effects of peritoneal macrophages on RGC survival and neurite outgrowth in a retinal explant co-culture system. In addition, the effects and mechanisms of peritoneal macrophage activation on RGC survival and neurite outgrowth were also investigated.

Materials and Methods

Animals

Adult female Fischer 344 (F344) rats (8–10 weeks old) were purchased from Vital River (Beijing, China; license No. SCXK (Jing) 2016-0006), and were housed under standard specific-pathogen-free conditions with a 12-hour dark/light cycle, with food and water available *ad libitum*. All experimental procedures were conducted according to the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research, and were approved by the Animal Ethics Committee of the Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong, China, on March 11, 2014 (approval No. EC 20140311(2)-P01). All surgeries were performed under general anesthesia of a 1:1 mixture (1.5 mL/kg) of ketamine (100 mg/mL; Fujian Gutian Pharma Co., Ltd., Ningde, Fujian Province, China) and xylazine (20 mg/mL; Sigma-Aldrich, Bulington, MA, USA).

Primary peritoneal macrophage isolation and culture

Peritoneal macrophages were isolated according to previously described procedures (Malorni et al., 1991). Briefly, after animals were euthanized, the peritoneal cavity was filled with Hank's balanced salt solution for 5 minutes, and the peritoneal cavity fluid was subsequently collected. The collected cells were centrifuged at 300–400 × *g* for 10 minutes. The cells were then allowed to adhere for 30 minutes at 37°C. Peritoneal macrophages were retained after removing the loosely adhered and suspended cells. The macrophages were pre-labeled with 0.2% 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Cat# MAN0001776; Invitrogen, Rockville, MD, USA) and cultured in Neurobasal-A medium (Gibco BRL, Rockville, MD, USA) supplemented with 1× B27 supplement (Gibco BRL), glutamine (Gibco BRL) and 1× penicillin/streptomycin (Gibco BRL). Before the co-culture experiments, the peritoneal macrophages were cultured on coverslips for 8 hours with a density of 60 cells/mm^2. For macrophage activation, peritoneal macrophages were treated with zymosan (a macrophage activator (Yin et al., 2003, 2006); 1.25 mg/mL; Cat# Z4250; Sigma-Aldrich). The zymosan was washed off before the co-culture experiments. In addition, the peritoneal macrophage-conditioned media (both with and without zymosan treatment) was also collected to examine the effects of macrophage secretion on RGCs. To confirm the identification of the macrophages, immunofluorescence analysis was performed using the macrophage markers CD68 and Iba-1.

For the isolation of white blood cells, each blood sample was centrifuged at 800 × *g* for 5 minutes. Next, 5 mL of sterile water was added and mixed completely for 30 seconds to rupture the red blood cells. The white blood cells were purified and collected with the addition of 5 mL of 1.8% NaCl solution, and centrifuged at 400 × *g* for 10 minutes.

Retinal explant culture for RGC survival

For the RGC survival analysis (Cen et al., 2018), rat eyes were directly dissected without pre-ON crush after scarification. Each retina was dissected by four cuts and mounted onto a nitrocellulose filter paper with the RGC layer facing upward, and the whole retina was cultured in B27-supplemented Neurobasal-A media with peritoneal macrophages attached to the coverslips for 7 days at 37°C (**Figure 1A**). For the macrophage secretion analysis, the macrophages were replaced by their conditioned media in the co-culture system. The retinas that were cultured in B27-supplemented Neurobasal-A medium alone served as the control group.

Retinal explant culture for neurite outgrowth

Because previous studies have demonstrated that retinas without pre-ON crush injuries possess low neurite outgrowth ability (Bähr, 1991; Dotan et al., 2001; Fischer et al., 2004), a pre-conditioning ON crush injury was performed before collecting the retina for the neurite outgrowth experiments (Yin et al., 2003, 2006). Briefly, under general anesthesia, the left ON was exposed after separating the superior conjunctiva and the eyeball under an operating microscope. Using angled jeweler's forceps (Dumont #5; Roboz, Rockville, MD, USA), the ON was crushed at 1.5 mm behind the ON head for 5 seconds, without injuring the ophthalmic artery. The ON injury was verified by a clear appearance at the crush site.

Seven days after the ON crush injury, the retina was collected and co-cultured with the peritoneal macrophages (60 cells/mm^2 on coverslips) according to a previously described protocol (Malorni et al., 1991). Briefly, the retina was dissected with four cuts and mounted onto a nitrocellulose filter paper with the RGCs facing upward, and was then cut into eight equal pieces and divided into the different treatment groups. The RGC layer was placed on a culture plate coated with poly-lysine (200 μg/mL; Sigra-Aldrich) and laminin (20 μg/mL; Gibco BRL), and the retinal explant was co-cultured with peritoneal macrophages in B27-supplemented Neurobasal-A media for 7 days at 37°C (**Figure 1B**). For the macrophage secretion analysis, the macrophages were replaced by their conditioned media in the co-culture system. The retinas that were cultured without the addition of macrophages or conditioned media served as the control group.

RGC survival and neurite outgrowth analyses

Immunofluorescence analysis was applied to visualize

RGCs in the retinal explant cultures. Following the co-culture experiments, the retinal explants were fixed in 4% paraformaldehyde for 2 hours and blocked with 10% normal goat serum and 0.2% Triton® X-100 for 1 hour. Next, the explants were incubated with anti-βIII-tubulin (TuJ1) antibody (1:400; rabbit; Cat# 5568S; Cell Signaling Technology, Inc., Danvers, MA, USA) overnight at 4°C, followed by fluorescein isothiocyanate-conjugated secondary antibody (1:400; goat anti-rabbit; Sigma-Aldrich) for 2 hours at room temperature. The retinal explants were then mounted with the nitrocellulose filters and examined under a confocal microscope (Leica TCS SP5 II; Leica Microsystems, Wetzlar, Germany). The number of RGCs was counted in each field (0.443 × 0.334 mm) on a grid in the microscope. At least 50 fields were counted for each retina, and the average density of the labeled RGCs in each retina was determined. To analyze cell apoptosis in the retinal explant after 7 days of culture, retinal explants were fixed overnight in 4% paraformaldehyde. After fixation, each retina was cryoprotected with graded 10–30% sucrose in phosphate-buffered saline, sectioned at 10 μm using a cryostat (Leica), and permeabilized with 0.2% Triton® X-100 solution (Sigma-Aldrich) in phosphate-buffered saline for 5 minutes at room temperature. Next, the retinas were stained with anti-TuJ1 antibody and the respective secondary antibody, before being incubated in terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) reaction mixture at 37°C for 1 hour. The TuJ1 and TUNEL double-labeled cells were imaged using a confocal microscope.

To visualize neurite outgrowth, the explants attached to the surface coating were imaged under an inverted fluorescent microscope (Leica). The numbers of neurites growing out from the explant were counted, and the mean number of neurites per explant was compared between each group. The average neurite length was calculated as the average length of the five longest neurite outgrowths measured from each retinal explant. This analysis was performed using ImageJ software (version 1.47; National Institutes of Health, Bethesda, MD, USA).

Gene expression analysis

To verify the identification of the isolated peritoneal macrophages and to delineate the mechanisms of macrophage activation on RGC survival, the expressions of macrophage (Cd32, Cd16, Cd68, Cd86, and Cd206), inflammatory (tumor necrosis factor (Tnf), interleukin (Il)-1b, and Il-6), oxidative (nitric oxide synthase (Nos)1, Nos2, and arginase-1 (Arg1)), and neurotrophic factor (ciliary-derived neurotrophic factor, (Cntf)) genes were examined by semi-quantitative polymerase chain reaction (PCR) or SYBR green PCR. Briefly, total RNA was extracted from the cultured peritoneal macrophages using TRIzol (Invitrogen), and 1 μg of total RNA was reverse transcribed into cDNA using SuperScript III reverse transcriptase (Invitrogen). The relative gene expression was determined by semi-quantitative PCR (35 cycles) with LightCycler 480 real-time PCR system (Roche, Basel, Switzerland) with specific primers (**Additional Table 1**). A housekeeping gene (glyceraldehyde 3-phosphate dehydrogenase, *Gapdh*) was used for normalization. The relative gene expression of each sample was calculated using the $2^{-\Delta\Delta Ct}$ method and expressed as the relative fold change.

Statistical analysis

Five retinas were analyzed for each treatment group. The data are expressed as the mean ± standard deviation (SD) of five samples. The independent samples *t*-test or one-way analysis of variance with *post hoc* Tukey's test (to correct for multiple testing) was used to compare the means among the different treatment groups. All statistical analyses were performed using SPSS Statistics v21 software (IBM SPSS Inc., Chicago, IL, USA). Significance was defined as $P < 0.05$.

Results

Characterization of the isolated primary peritoneal macrophages

The identity of the isolated primary peritoneal macrophages was verified by gene expression analysis. The PCR analysis showed that, similar to the white blood cells collected from the peripheral blood, the isolated peritoneal cells expressed the macrophage marker genes Cd32, Cd16, Cd68, Cd86, and Cd206 (**Figure 2A**). Immunofluorescence analysis confirmed that the isolated peritoneal macrophages were round in shape, and 96.2 ± 2.6% and 93.7 ± 2.7% of the isolated cells expressed the macrophage markers Iba-1 and CD68, respectively (**Figure 2B**). With zymosan treatment, the activated peritoneal macrophages became larger in size and vacuoles were observed in the cytoplasm. Our results indicated that the primary cells isolated from the rat peritoneal cavity were peritoneal macrophages, and these cells were therefore subjected to further RGC survival and neurite outgrowth analyses.

Peritoneal macrophages attenuate RGC survival and inhibit neurite outgrowth

After 7 days of co-culture with primary peritoneal macrophages, the RGCs underwent cell apoptosis in the retinal explants (**Additional Figure 1**). Compared with the control group, a significant reduction in the number of RGCs was observed after co-culture ($P < 0.001$; **Figure 3A**, **B**, and **D**). Additionally, the number of neurites regenerated from the retinal explants was also significantly decreased in the co-culture with primary peritoneal macrophages, decreasing by 73.21% compared with the control group ($P < 0.001$; **Figure 4A** and **B**). Furthermore, in the peritoneal macrophage co-culture group, the neurite outgrowth lengths were 71.01% shorter than those in the control group ($P < 0.001$; **Figure 4C**). These results indicate that the primary peritoneal macrophages might attenuate RGC survival and inhibit neurite outgrowth in the retinal explant culture model.

Because we have previously demonstrated that macrophage activation by zymosan enhances RGC survival and axonal regeneration in rats (Kurimoto et al., 2010), we next evaluated the effect of peritoneal macrophage activation by zymosan on RGC survival and neurite outgrowth in the retinal explant culture model. With zymosan pretreatment, the activated peritoneal macrophages significantly reduced the number of RGCs by 57.27% compared with the control group ($P < 0.001$; **Figure 3A**, **C**, and **D**). Macrophage activation further reduced the survival of RGCs by 10.01% ($P = 0.007$) compared with peritoneal macrophages without zymosan pretreatment. Similarly, the activated peritoneal macrophages significantly reduced the number and length of neurite outgrowths by 83.04% ($P < 0.001$) and 79.71% ($P < 0.001$), respectively, compared with the control group (**Figure 4A–C**). Macrophage activation further reduced the number of neurite outgrowths by 9.82% ($P = 0.042$), but had no effect on neurite length ($P = 0.161$), compared with peritoneal macrophages without zymosan pretreatment. Our results suggest that macrophage activation aggravates the attenuation of RGC survival and neurite outgrowth that is induced by peritoneal macrophages.

Macrophage-secreted factors attenuate RGC survival and inhibit neurite outgrowth

The effect of macrophages on RGC survival and axonal regeneration has been reported to be mediated by macrophage-derived factors, including inflammatory cytokines and neurotrophic factors (Yin et al., 2003). To validate this previous finding, we evaluated the effects of macrophage-derived conditioned media on RGC survival and neurite outgrowth. Similar to the effects of the primary peritoneal macrophages, the conditioned media derived from these macrophages reduced the number of RGCs and neurite

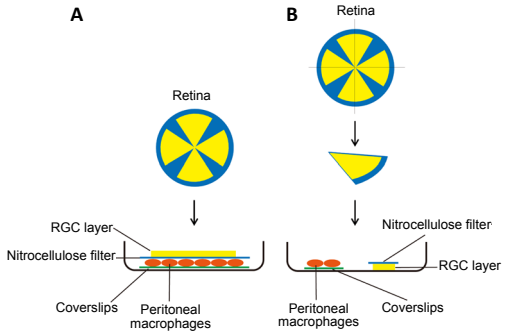

Figure 1 | Schematic diagram of retinal ganglion cell (RGC) survival and neurite outgrowth analyses in the retinal explant culture system.
(A) For the RGC survival analysis, the retina was dissected by four cuts and mounted onto a nitrocellulose filter paper, with the RGC layer facing upward. The whole retina was cultured in media with the peritoneal macrophages attached to coverslips for 7 days. (B) For the neurite outgrowth analysis, the retina was dissected with four cuts and mounted onto a nitrocellulose filter paper, with the RGCs facing upward. The mounted retina was then cut into eight equal pieces and divided into the different treatment groups. The RGC layer was placed on a culture plate coated with poly-lysine (200 μg/mL) and laminin (20 μg/mL), and was cultured with peritoneal macrophages in the media for 7 days.

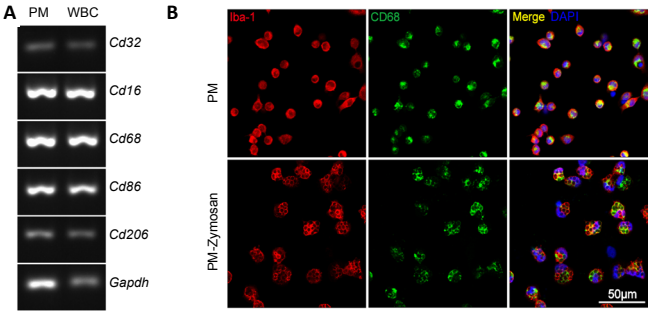

Figure 2 | Characterization of the isolated primary peritoneal macrophages.
(A) Semi-quantitative polymerase chain reaction analysis showed that, similar to white blood cells collected from peripheral blood, the isolated peritoneal macrophages expressed the macrophage marker genes *Cd32*, *Cd16*, *Cd68*, *Cd86*, and *Cd206*. *Gapdh* was used as the housekeeping gene. (B) Immunofluorescence analysis of the macrophage markers Iba-1 (red, Alexa Fluor Plus 555) and CD68 (green, Alexa Fluor Plus 488) in the isolated peritoneal macrophages and zymosan-activated peritoneal macrophages. The activated macrophages became larger, with vacuoles in the cytoplasm. Blue: DAPI, a nuclear counter-stain. Scale bar: 50 μm. DAPI: 4′,6-Diamidino-2-phenylindole; Gapdh: glyceraldehyde 3-phosphate dehydrogenase; PM: peritoneal macrophage; PM-Zymosan: zymosan-treated peritoneal macrophages; WBC: white blood cell.

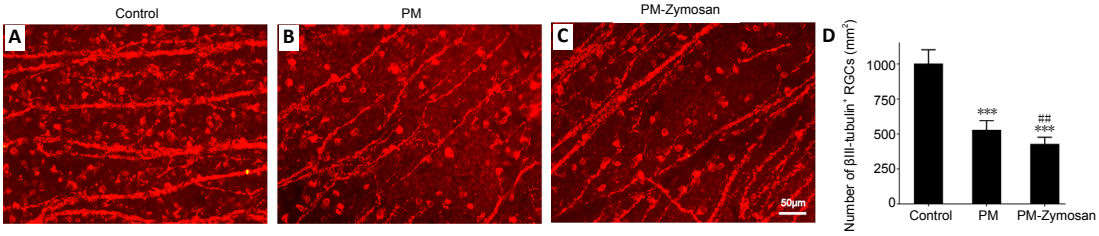

Figure 3 | Effects of peritoneal macrophages and zymosan activation on retinal ganglion cell (RGC) survival.
Primary peritoneal macrophages were treated with or without zymosan for 8 hours, and retinal explants were co-cultured with the primary peritoneal macrophages for 7 days. (A–C) Immunofluorescence analysis of RGCs in the retinal wholemounts. Both primary peritoneal macrophages and zymosan-treated peritoneal macrophages significantly reduced the number of RGCs compared with the control group. Red: βIII-tubulin-positive, stained by fluorescein isothiocyanate. Scale bar: 50 μm. (D) Cell count analysis of RGCs. Data are expressed as the mean ± SD (n = 5). ***P < 0.001, *vs*. control group; ##P < 0.01, *vs*. PM group (one-way analysis of variance with *post hoc* Tukey's test). PM: Peritoneal macrophages; PM-Zymosan: zymosan-treated peritoneal macrophages.

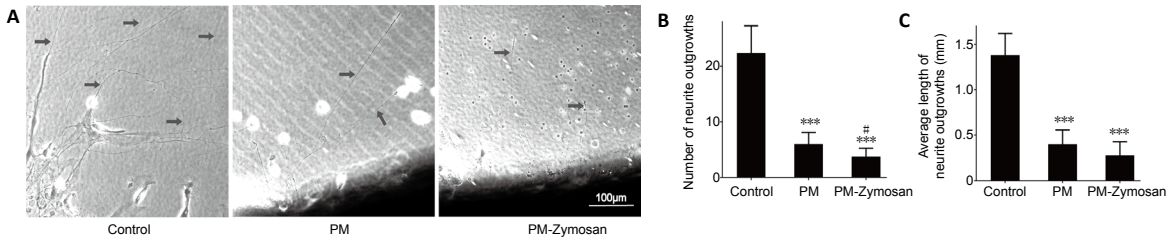

Figure 4 | Effects of peritoneal macrophages and zymosan activation on retinal ganglion cell neurite outgrowth.
Primary peritoneal macrophages were treated with or without zymosan for 8 hours, and retinal explants were co-cultured with the primary peritoneal macrophages for 7 days. (A) Cell culture photos of neurite outgrowth from the retinal explants. The number of neurite outgrowths decreased in both the primary peritoneal macrophage group and the zymosan-treated peritoneal macrophage group. Arrows indicate regenerating neurites. Scale bar: 100 μm. (B) Neurite counts from the explant culture. Primary peritoneal macrophages and zymosan-treated peritoneal macrophages significantly reduced the number of neurite outgrowths compared with the control group. (C) Length measurements of the neurite outgrowths. Primary peritoneal macrophages and zymosan-treated peritoneal macrophages significantly reduced the length of neurite outgrowths compared with the control group. Data are expressed as the mean ± SD (n = 5). ***P < 0.001, *vs*. control group; #P < 0.05, *vs*. PM group (one-way analysis of variance with *post hoc* Tukey's test). PM: Peritoneal macrophages; PM-Zymosan: zymosan-treated peritoneal macrophages.

outgrowths by 34.94% (P < 0.001; **Figure 5A**) and 64.29% (P < 0.001; **Figure 5B**), respectively, compared with the control group. The macrophage-derived conditioned media also reduced the length of neurite outgrowths by 57.97% (0.58 ± 0.16 mm, P < 0.001; **Figure 5C**) compared with the control group. Moreover, with the conditioned media derived from the zymosan-activated peritoneal macrophages, the number of RGCs and the number and length of neurite outgrowths were also significantly decreased by 45.16% (P < 0.001; **Figure 5A**), 74.11% (P < 0.001; **Figure 5B**), and 69.57% (P < 0.001; **Figure 5C**), respectively, compared with the control group. Additionally, the conditioned media derived from the zymosan-activated peritoneal macrophages further reduced the number of RGCs and neurite outgrowths, by 10.23% (P = 0.017) and 9.82% (P = 0.036), but had no effect on neurite length (P = 0.126) compared with the conditioned media from peritoneal macrophages without zymosan pretreatment. These results indicate that macrophage-secreted factors may be responsible for the attenuation of RGC survival and neurite outgrowth induced by peritoneal macrophages.

Increased inflammatory and oxidative stress gene expression but reduced neurotrophic factor gene expression in zymosan-treated peritoneal macrophages

To explore the mechanisms of macrophage activation on RGC survival and neurite outgrowth, we determined the

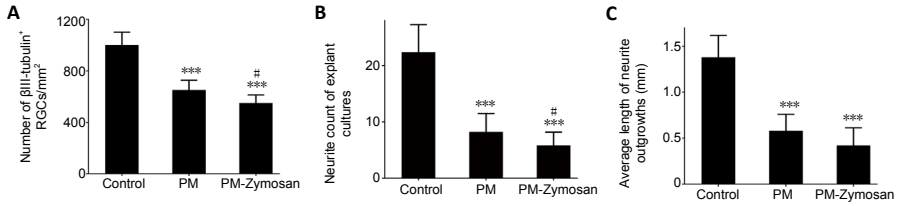

Figure 5 | **Effects of peritoneal macrophage-derived conditioned media and zymosan activation on retinal ganglion cell (RGC) survival and neurite outgrowth.**

Primary peritoneal macrophages were treated with or without zymosan for 8 hours, and retinal explants were cultured with the peritoneal macrophage-derived conditioned media for 7 days. (A) Cell count analysis of the RGCs. The conditioned media derived from both primary peritoneal macrophages and zymosan-treated peritoneal macrophages significantly reduced the number of RGCs compared with the control group. (B) Neurite count of the explant cultures. The conditioned media derived from both primary peritoneal macrophages and zymosan-treated peritoneal macrophages significantly reduced the number of neurite outgrowths compared with the control group. (C) Length measurements of the neurite outgrowths. The conditioned media derived from both primary peritoneal macrophages and zymosan-treated peritoneal macrophages significantly reduced the length of the neurite outgrowths compared with the control group. Data are expressed as the mean ± SD (n = 5). ***P < 0.001, *vs*. control group; #P < 0.05, *vs*. PM group (one-way analysis of variance with *post hoc* Tukey's test). PM: Peritoneal macrophages; PM-Zymosan: zymosan-treated peritoneal macrophages.

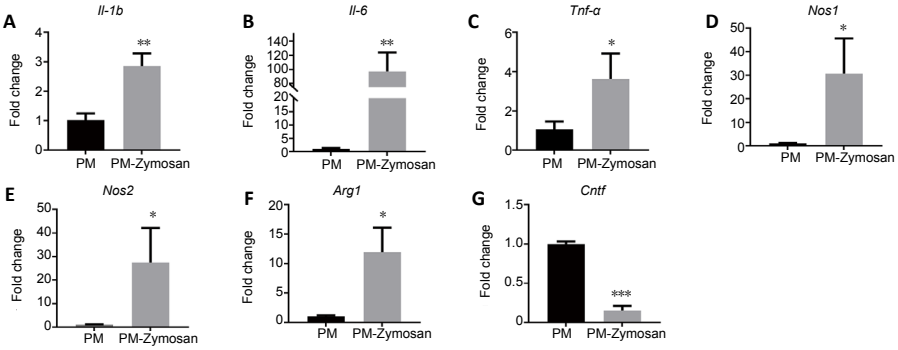

Figure 6 | **Inflammatory, oxidative, and neurotrophic factor gene expression in peritoneal macrophage activation.**

Primary peritoneal macrophages were treated with or without zymosan for 8 hours, and the expressions of inflammatory (*Tnf-α*, *Il-1b*, and *Il-6*), oxidative (*Nos1*, *Nos2*, and *Arg1*), and neurotrophic factor (*Cntf*) genes were determined using Sybr green PCR. The expressions of inflammation- (*Tnf-α*, *Il-1b*, and *Il-6*) and oxidation-related (*Nos1*, *Nos2*, and *Arg1*) genes were upregulated in the zymosan-treated peritoneal macrophages compared with the untreated peritoneal macrophages, and *Cntf* was downregulated. Data are expressed as the mean ± SD (n = 5). *P < 0.05, **P < 0.01, ***P < 0.001, *vs*. PM group (independent samples t-test). Arg1: Arginase 1; Cntf: ciliary-derived neurotrophic factor; Il: interleukin; Nos: nitric oxide synthase; PM: peritoneal macrophages; PM-Zymosan: zymosan-treated peritoneal macrophages; Tnf-α: tumor necrosis factor alpha.

expressions of inflammatory, oxidative, and neurotrophic factor genes. SYBR green PCR analysis revealed that, compared with the untreated peritoneal macrophages, expressions of the inflammation-related genes *Il1b*, *Il6*, and *Tnf* were significantly upregulated in the zymosan-treated peritoneal macrophages, by 2.84-, 94.68-, and 3.45-fold, respectively (P < 0.05; **Figure 6A–C**). Moreover, compared with the untreated peritoneal macrophages, expressions of the oxidation-related genes *Nos1*, *Nos2*, and *Arg1* were also significantly upregulated in the zymosan-treated peritoneal macrophages, by 27.68-, 24.44-, and 11.45-fold, respectively (P < 0.05; **Figure 6D–F**). In contrast, the neurotrophic factor gene *Cntf* was significantly downregulated, by 7.14-fold, in the zymosan-treated peritoneal macrophages compared with the untreated peritoneal macrophages (P < 0.05; **Figure 6G**). Our results suggest that increased inflammation and oxidative stress, but reduced neurotrophic factor expression, might be associated with the reduction in RGC survival and neurite outgrowth that was induced by activated peritoneal macrophages.

Discussion

Inflammation is a complex pathophysiological process in the host defense response for maintaining homeostasis after tissue injury (Mo et al., 2020). Protective or detrimental effects are regulated in different circumstances by the mode of stimulation. A mild intraocular inflammatory response has been demonstrated to contribute to RGC survival and axonal regeneration after ON injury (Leon et al., 2000; Yin et al., 2003; Luo et al., 2007). Macrophages can release multiple pro-/anti-inflammatory cytokines for RGC regeneration. Of these, brain-derived neurotrophic factor, platelet-derived growth factor, and glial cell line-derived neurotrophic factor can all promote RGC survival (Yan et al., 1999; Osborne et al., 2018), whereas oncomodulin is effective for axonal regeneration (Yin et al., 2006). However, macrophages can also secrete neurotoxic factors, including nitric oxide, TNF-α, IL-6, and IL-1β (Gunawardena et al., 2019). In the present study, peritoneal macrophage activation by zymosan upregulated the expression of inflammation- (*Tnf*, *Il-1b*, and *Il-6*) and oxidation-related (*Nos1*, *Nos2*, and *Arg1*) genes, implying that inflammation and oxidative stress are enhanced after zymosan activation. The enhanced inflammation and oxidation may induce extra stress for RGCs, which might explain the further reduction in RGC survival and neurite outgrowth that was induced by the zymosan-treated peritoneal macrophages. Similar to our results, it has been reported that *Nos2* expression increases in rodent peritoneal macrophages with the treatment of lipopolysaccharide (Szabó et al., 2004) or S-antigen (Bae, 2000). In addition, increased expression of *Nos2* and *Arg1* has also been reported in rodent bone marrow-derived macrophages (Kroner et al., 2014). However, further investigations are required to understand how the inflammation and oxidation induced by peritoneal macrophages can promote RGC degeneration, as well as to clarify the responses of different types of macrophages, intravitreal macrophages, and blood-borne monocytes after ON injury.

The balance of beneficial and detrimental effects of macrophages depends on different pathological conditions, the macrophage subpopulations, and the timing of activation (Yin et al., 2003; Cui et al., 2009). We have previously demonstrated that blood-borne monocytes can be attracted to the retina by the intravitreal injection of CNTF, and can then promote RGC survival and axonal regeneration (Cen et al., 2007). Blood-borne monocytes, but not peritoneal macrophages, can chemotactically respond to CNTF. In the present study, we demonstrated for the first time that peritoneal macrophages are detrimental to RGC survival and neurite outgrowth in the retinal explant co-culture system. Macrophage-secreted factors may be responsible for the reduction in RGC survival and neurite outgrowth that is induced by peritoneal macrophages. Critically, with zymosan pretreatment, activated peritoneal macrophages further attenuated RGC survival and neurite outgrowth. Macrophage activation enhanced the expression of inflammation- (*Tnf*, *Il1b*, and *Il6*) and oxidation-related (*Nos1*, *Nos2*, and *Arg1*) genes, indicating that additional inflammation and oxidative stress by peritoneal macrophage-derived cytokines and nitric oxide might be harmful to the nervous system. A

previous study has reported that the intravitreal application of peritoneal macrophages contributes to neovascularization in an oxygen-induced retinopathy mouse model (Kataoka et al., 2011). Macrophages have remarkable plasticity and can modify their physiological responses to different stimuli, giving rise to different macrophage subpopulations with distinct functions (García Talavera et al., 1991). Classical activated macrophages can respond to interferon-γ and TNF (Jorens et al., 1991), whereas wound-healing alternatively activated macrophages respond to IL-4 (Cenciarelli et al., 1991). After migrating from the blood to the peritoneal cavity, macrophages acquire specific physiological features in response to the peritoneal environment, and serve as the first defense against micropathogens or tumors in the immune reaction via phagocytosis and antigen presentation (García Talavera et al., 1991). However, these peritoneal macrophages are detrimental to RGC survival and neurite outgrowth, as we have demonstrated in the present study. The macrophage-secreted factors that lead to this detrimental effect on RGCs need to be determined, to further illustrate the mechanisms of peritoneal macrophage-induced RGC degeneration.

In summary, our results revealed that rat primary peritoneal macrophages attenuated RGC survival and neurite outgrowth in the retinal explant co-culture system, via the secretion of macrophage-derived factors. Macrophage activation further aggravated the attenuation of RGC survival and neurite outgrowth, through the upregulation of inflammation- and oxidation-related genes. The macrophage-secreted factors that have detrimental effects on RGCs may be potential treatment targets to prevent RGC degeneration.

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Additional files:
Additional Table 1: *Gene primer sequence.*
Additional Figure 1: *Apoptosis analysis on RGCs in the retinal explant after 7-day culture.*
Additional file 1: *Original data of the experiment.*

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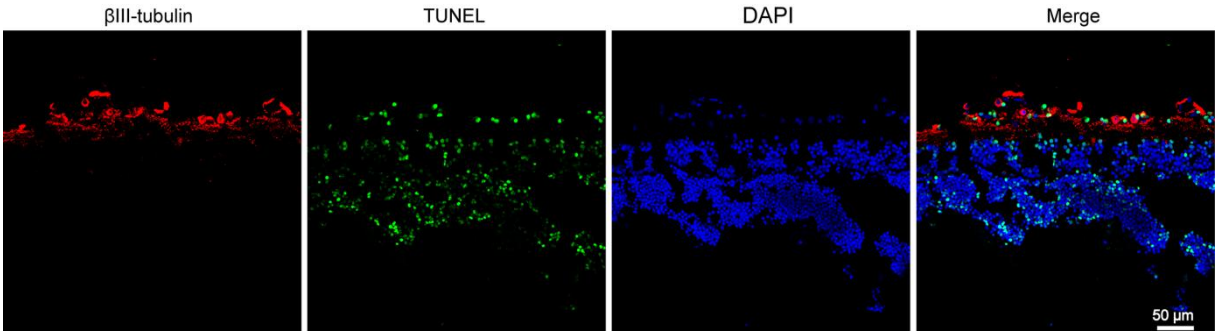

Additional Figure 1 Apoptosis analysis on RGCs in the retinal explant after 7-day culture.

The retinal explants were fixed after 7 days culture, and the explants were sectioned and stained with the anti-βIII-tubulin antibody (red, Alexa Fluor Plus 555) as well as the TUNEL reaction mixture (green) and DAPI (blue). Scale bar: 50μm. DAPI: 4′,6-Diamidino-2-phenylindole; RGC: retinal ganglion cell; TUNEL: terminal deoxynucleotidyl transferase dUTP nick end labeling.

Additional Table 1 Gene primer sequence

Gene	Primer sequence (5'-3')
Cd86	Forward: AAGACATGTGTAACCTGCACC
	Reverse: AACCGACTTTTTCCGGTCCT
Nos2	Forward: TGGTGAGGGGACTGGACTTT
	Reverse: ATCCTGTGTTGTTGGGCTGG
Cd16	Forward: GCACCAGAGTCTAAGGGCAT
	Reverse: TAGGAGATGAACTGCTGGCG
Cd32	Forward: CTTACGGTTTCCTCTGGCGT
	Reverse: GAAGGCTGTCTGGCATCTGT
Arg1	Forward: CCCGCAGCATTAAGGAAAGC
	Reverse: ATTGCCATACTGTGGTCTCCA
Cd206	Forward: GGACTAAGCCAAGGGGCAAC
	Reverse: AATTGCCGTGAGTCCAAGAG
Cd68	Forward: ACAAGCAGCACAGTGGACAT
	Reverse: GTTGATTGTCGTCTCCGGGT
Il-1b	Forward: CCTTGTGCAAGTGTCTGAAGC
	Reverse: AGTCAAGGGCTTGGAAGCAA
Il-6	Forward: CTCTCCGCAAGAGACTTCCA
	Reverse: AGTCTCCTCTCCGGACTTGT
Tnf-α	Forward: ATGGGCTCCCTCTCATCAGT
	Reverse: GCTTGGTGGTTTGCTACGAC
Nos1	Forward: CCTCAGGCTTGGGTCTTGTTA
	Reverse: GTGTTGTTGGGCTGGGAATAG
Cntf	Forward: CTTCTTCTCCAAGTCGCTGC
	Reverse: TGTCCACTGTGAAAGCTCCT
Gapdh	Forward: ATCAAGAAGGTGGTGAAGCAGG
	Reverse: AGGTGGAAGAATGGGAGTTGCT

Arg1: Arginase 1; Cntf: ciliary-derived neurotrophic factor; Gapdh: glyceraldehyde 3-phosphate dehydrogenase; Il: interleukin; Nos1: nitric oxide synthase 1; Nos: nitric oxide synthase; Tnf-α: tumor necrosis factor alpha.

Effects of peritoneal macrophages and the zymosan activation on retinal ganglion cell (RGC) survival.					
Groups	RGC numbers in different samples				
Control	1129	966	894	1082	944
PM	600	467	449	569	560
PM-Zymosan	456	389	499	405	394

Effects of peritoneal macrophages and the zymosan activation on retinal ganglion cell neurite outgrowth.					
Numbers of Numbers of outgrowth neurites/explant					
Groups	Neurite numbers in different samples				
Control	25	16	21	29	21
PM	4	7	4	9	6
PM-Zymosan	4	6	3	2	4

Average length of outgrowth neurites (mm)					
Groups	Average length in different samples				
Control	1.7	1.5	1.4	1.1	1.2
PM	0.3	0.6	0.4	0.2	0.5
PM-Zymosan	0.3	0.2	0.5	0.1	0.3

Effects of peritoneal macrophage-derived conditional media and the zymosan activation on retinal ganglion cell (RGC) survival and neurite outgrowth.					
Groups	RGC numbers in different samples				
Control	1129	966	894	1082	944
PM	721	730	550	642	620
PM-Zymosan	623	532	612	485	498

Groups	Neurite numbers in different samples				
Control	25	16	21	29	21
PM	9	4	8	7	13
PM-Zymosan	3	6	4	7	9

Average length of outgrowth neurites in conditional media (mm)					
Groups	Average length in different samples				
Control	1.7	1.5	1.4	1.1	1.2
PM	0.4	0.7	0.4	0.8	0.6
PM-Zymosan	0.4	0.7	0.2	0.5	0.3

Inflammation, oxidation and neurotrophic factor gene expression in peritoneal macrophage activation.
Relative expression (ΔCt) in different groups

Genes	PM			PM-Zymosan		
Il-1b	2.708	2.082	2.502	0.703	0.948	1.131
Il-6	8.779	8.255	9.023	1.759	2.027	2.576
Tnf-a	5.037	3.956	4.641	2.517	2.330	3.420
Nos1	7.045	6.919	7.505	1.716	2.051	3.329
Nos2	5.827	5.651	6.243	0.537	1.056	2.294
Arg1	8.795	8.245	8.295	4.394	5.030	5.361
Cntf	10.438	10.440	10.360	13.174	12.655	13.857