Peritoneal macrophages attenuate retinal ganglion cell survival and neurite outgrowth

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

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.

1Joint Shantou International Eye Center of Shantou University and the Chinese University of Hong Kong, Shantou, Guangdong Province, China; 2Shantou University Medical College, Shantou, Guangdong Province, China; 3Department of Ophthalmology and Visual Sciences, the Chinese University of Hong Kong, Hong Kong Special Administrative Region, China
*Correspondence to: Ling-Ping Cen, PhD, cenlp@hotmail.com.
https://orcid.org/0000-0003-3876-0606 (Ling-Ping Cen)
#Both authors contributed equally to this work.

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Peritoneal macrophages have been also 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 with ketamine (100 mg/mL; Fujian Gutian Pharma Co., Ltd., 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, Burlington, 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/mm². 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 scarring. 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/mm² 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; Sigma-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...
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 3A and D). 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 = 0.007), and similarly, the activated peritoneal macrophages without zymosan pretreatment further reduced the number and length 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...
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-activated 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 < 0.05, vs. PM group (one-way analysis of variance with post hoc Tukey’s test). PM: peritoneal macrophages; PM-Zymosan: zymosan-treated peritoneal macrophages.

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. 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
Macrophages can release multiple pro-/anti-inflammatory cytokines, indicating that additional inflammation and oxidative stress might be harmful to the nervous system. A mild intraretinal 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 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 separate stress for RGCs, which might explain the further reduction in RGC survival and neurite outgrowth that is 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, intraretinal 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.
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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.
| Gene  | Primer sequence (5'→3')                  |
|-------|----------------------------------------|
| Cd86  | Forward: AAGACATGTGTAACCTGCACC          |
|       | Reverse: AACCGACTTTTTCCCGGTCCCT        |
| Nos2  | Forward: TGGTGAGGGGACTGGACTTT          |
|       | Reverse: TAGGAGATGACTGCTGGCG            |
| Cd16  | Forward: GCACCAGAGTCTAAGGGCAT          |
|       | Reverse: TAGGAGATGACTGCTGGCG            |
| Cd32  | Forward: CTTACGTTTCTCCTGGCGT           |
|       | Reverse: GAAGGCTGTCTGGCATCTGT          |
| Arg1  | Forward: CCGCAGCATTAAAGGAAGGC          |
|       | Reverse: ATTGCCATACTGTGCTCCCA          |
| Cd206 | Forward: GGACTAAGGCAAGGGGCAAC          |
|       | Reverse: AATTGCCGTGAGTCCAAGAG          |
| Cd68  | Forward: ACAAGCAGCAGCAGAGGACAT         |
|       | Reverse: GTTGATTGTGCTCTCGGCTGT         |
| Il-1b | Forward: CTTTGTCAGTGTCCTGCAAGC         |
|       | Reverse: AGTCAAGGGCTTGGAAGCAGA         |
| Il-6  | Forward: CTCTCCGCAAGAGACTTCCA          |
|       | Reverse: AGTCTCTCCTCAGGACTTGT          |
| Tnf-α | Forward: ATGGGCTCCCCCTCCTACGTA         |
|       | Reverse: GCTTGGCTGTTTGCTACGAC          |
| Nos1  | Forward: CCTCAGGCTTGGGTCTTGTTT         |
|       | Reverse: GTGTTGGGCTGCGGAATAG           |
| Cntf  | Forward: CTTCCTTCCAAGTCGCTG            |
|       | Reverse: TGTCCTGTTGAAGGCTCCTC          |
| Gapdh | Forward: ATCAAGAGGGTGGAAGCAGG          |
|       | 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.

| 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 |