A possible mechanism of microglia-photoreceptor crosstalk

Li-ping Yang,‡ Xiu-an Zhu,‡ Mark O.M.Tso

1Peking University Eye Center, Peking University Third Hospital, Peking University, Beijing, China; 2 Wilmer Eye Institute, Johns Hopkins University School of Medicine, Baltimore, MD

Purpose: The goal of this study was to explore the relationship between photoreceptor apoptosis and retinal microglial activation.

Methods: A murine photoreceptor cell line (661W cells) was exposed to LPS-treated microglial cell conditioned medium (MGCM), and cell viability was assessed by terminal dUTP transferase nick end labeling (TUNEL) and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, microglia were exposed to culture media from light-damaged 661W photoreceptor cells (PRCM), and microglial activation was assessed morphologically by phase contrast microscopy. Reverse transcription polymerase chain reaction was used to examine mRNA levels of several chemokines and noxious factors in the MGCM-treated photoreceptor cells and the PRCM-treated microglial. Western blotting was used to analyze NF-κB p65 subunit, phosphorylated MAPKs p38, p44/42 (Erk1/2), and c-Jun N-terminal kinase (JNK).

Results: Our results showed 37% of 661W cells underwent apoptosis following exposure to MGCM for 24 h. MGCM-induced death was associated with down-regulation of chemokine expression (i.e., eotaxin and RANTES), up-regulation of inflammatory mediators (i.e., MIP-1α, MIP-1β, IL-10, iNOS, and TNF-α), and increased phosphorylation of p38, p44/p42, and JNK. Retinal microglia acquired an activated phenotype after exposure to PRCM for 24 h. Microglial activation was accompanied by increased NF-κB p65 expression, increased phosphorylation of p38 and JNK, and up-regulation of chemokines (i.e., eotaxin and RANTES) and inflammatory mediators (i.e., iNOS and IL-10).

Conclusions: Light-damaged photoreceptors release immunological signaling molecules that attract microglia, resulting in microglial activation and subsequent further degeneration of remaining photoreceptors. These results also suggest that p38, p44/42, and JNK may regulate glial-induced photoreceptor death and that p38, JNK, and NF-κB may regulate photoreceptor-induced microglial activation.

In retinal diseases, such as inherited retinal degeneration [1-3], light-induced retinal degeneration [4,5], retinal detachment [6], laser photocoagulation [7], and age-related macular degeneration [8], photoreceptor cell death is often accompanied by microglial activation and migration from the inner retinal layer (IRL) to the outer nuclear layer (ONL) or subretinal space. Microglial activation involves the production of growth factors and the phagocytosis of potentially toxic cellular debris, and both of these cellular activities are believed to be beneficial to the surviving neurons [9]. Minghetti and colleagues demonstrated microglial cells released immunoregulatory and neuroprotective agents in response to chronic neurodegenerative diseases and in interaction with phosphatidyl serine-expressing apoptotic cells [10]. It has been shown that, in response to oxygen-glucose deprivation, microglia come into close physical contact with neurons in the ischemic brain regions and help to protect against neuronal damage [11]. However, a close spatial and temporal relationship has been observed between invading microglial cells and degenerating photoreceptor cells, suggesting that microglial cells may be involved in photoreceptor cell death [3,4]. Furthermore, microglia-derived factors have been shown to promote photoreceptor cell death in vitro [12,13]. In addition, inhibition of microglial activation slowed photoreceptor death in animal models of inherited [14] and light-induced retinal degeneration [15]. These contradictory observations prompted us to further investigate the relationship between microglial activation and photoreceptor apoptosis.

Chemokines are chemotactic cytokines that act through G-protein coupled receptors. These immune-signaling molecules are known to cause secondary damage in inflammation and aggravate neuro-degeneration [16-18]. It has been suggested that, in inherited and light-induced retinal degeneration, degenerating photoreceptors release chemokines, which attract microglia to the retinal lesion site [3,4]. Following recruitment, activated microglia secrete pro-inflammatory cytokines and other cytotoxic factors that induced neuronal degeneration [19-21]. The immunological signaling molecules produced by activated microglia and by damaged photoreceptors remain to be fully defined.

In the present study, a murine photoreceptor cell line (661W cells) was exposed to culture media from lipopolysaccharide (LPS)-activated retinal microglia (microglial conditioned medium; MGCM), and retinal microglia were exposed to culture media from light-damaged 661W cells (photoreceptor conditioned medium; PRCM). The inflammatory mediators, chemokines, and signaling pathways regulating MGCM-induced photoreceptor apoptosis and PRCM-induced photoreceptor apoptosis were then explored.

Correspondence to: Li-ping Yang, Peking University Eye Center, Peking University Third Hospital, 49 North Garden Road, Haidian District, Beijing, 100083, P.R.China Phone: +86 10 8208 9946; FAX: +86 10 8208 9951; email: alexlipingyang@gmail.com
METHODS

661W cell culture: The 661W photoreceptor cell line was a generous gift of Dr. Al-Ubaidi, University of Oklahoma. Cultures were grown in DMEM/F-12 (1:1; Invitrogen Corporation, Grand Island, NY) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (basal medium) and were maintained at 37 °C in a humidified, 5% CO₂ atmosphere. For experiments involving exposure to MGCM, 661W cells were plated in 35 mm culture dishes and allowed to attach overnight. Next, 661W cells were washed twice with basal media and incubated with MGCM. As a control, we used 661W cells cultured in basal medium with or without LPS for 48 h.

At this point the cells were stable, and had undergone nutrient and glucose depletion similar to those exposed to MGCM.

A 661W photoreceptor cell conditioned medium with photic injury (PRCM) was generated as follows. After being cultured in basal media for 24 h, the cells were exposed to green light at 4.5 mWatt/cm² for 4 h. Cells were cultured for an additional 48 h at 37 °C. Culture media were collected and centrifuged to remove all cellular components. The conditioned medium was then supplemented with 0.1% BSA, which served as a carrier protein. As a control, we used 661W photoreceptor cell conditioned medium without photic injury. The control cells were shielded from light and kept under similar conditions as the cells in the light exposure paradigm.

Primary retinal microglial culture: Retinas were isolated from newborn Sprague-Dawley rats and incubated at 37 °C for 20 min in Ca²⁺/Mg²⁺-free Hank’s balanced salt solution containing 0.25 mg/ml trypsin and 0.4 mg/ml EDTA. The enzyme-treated tissues were then dissociated into single cells by gentle pipetting. Cells were collected by centrifugation and re-suspended in DMEM/F-12 (1:1) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (basal medium). Cells were seeded at a density of 10⁶ cells per ml in T75 culture flasks (Corning Incorporation, NY) and incubated at 37 °C in a humidified, 5% CO₂ atmosphere.

RESULTS

For experiments involving exposure to MGCM, 661W cells were plated in 35 mm culture dishes and allowed to attach overnight. They were then exposed to MGCM for an additional 24 h. Cells were then fixed with 4% paraformaldehyde and processed for immunofluorescent staining. No GFAP and cellular retinaldehyde-binding protein labeling was present, indicating an absence of astrocytes and Müller cells. After harvesting, microglial cells were re-seeded in poly-L-lysine coated 35 mm culture dishes and either treated with LPS to generate conditioned media (see below) or exposed to conditioned media. For the latter, microglial cells were allowed to attach overnight. They were then washed twice with basal medium and incubated with PRCM. Microglial cells cultured in basal medium for 72 h served as a control.

MGCM were collected from microglia cultured for 48 h in basal medium containing 250 ng/ml LPS (Sigma-Aldrich, St. Louis, MI). Cell-free culture media obtained by centrifugation were supplemented with 0.1% BSA.

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay: Cell viability was determined by spectroscopic measurement of the reduction of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, obtained from Sigma-Aldrich. MTT assay is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form a dark blue formazan crystal, which is largely impermeable to cell membranes, thus resulting in its accumulation within healthy cells. Solubilization of the cells by the addition of a detergent resulted in the liberation of the crystals which are solubilized. Cell viability is directly proportional to the level of the formazan product created. After 661W cells were exposed to MGCM for 24 h, media were discarded, and cells were washed three times with PBS. Each dish was then incubated in 1 ml of 0.5 mg/ml MTT solution for 2 h at 37 °C. The MTT solution was then discarded, and formazan precipitates were dissolved in 500 µl of DMSO by agitating dishes for five min at 200 rpm on an orbital shaker. The absorbance at 570 nm was determined with a micro-plate reader (Bio-Rad). Each experiment was performed in triplicate.

3' end labeling of fragmented DNA by terminal deoxynucleotidyl transferase-mediated fluorescent dUTP nick end labeling: We cultured 661W cells for 24 h and subsequently exposed them to MGCM for an additional 24 h. Cells were then fixed with 4% paraformaldehyde and processed for transferase-mediated fluorescent dUTP nick end labeling (TUNEL) using a commercially available apoptosis kit (DeadEnd™ Fluorometric TUNEL System, Promega Corporation, Madison, WI). TUNEL was performed according to the manufacturer’s instructions and as described by Gavrieli.

Primer sequences were designed using the Primer Express Program and purchased from AuGCT Biotechnology, Beijing, P. R. China.

Primer sequences were designed using the Primer Express Program and purchased from AuGCT Biotechnology, Beijing, P. R. China.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequences (5'-3')</th>
<th>Location (nt)</th>
<th>Annealing temperature (°C)</th>
<th>Product size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>iNOS</td>
<td>F: GCTCCATGACTCTCAGCACAG</td>
<td>NT 3164-3509</td>
<td>63</td>
<td>345</td>
</tr>
<tr>
<td></td>
<td>R: TGGAGAGAGGTACAAACGAGGT</td>
<td>NT 3155-3509</td>
<td>63</td>
<td>345</td>
</tr>
<tr>
<td>Rantes</td>
<td>F: CTGCATCCCTCACCGTCATC</td>
<td>NT 17-242</td>
<td>63</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>R: CATCTCCTTCCATGCTCTCTCCT</td>
<td>NT 242-207</td>
<td>63</td>
<td>320</td>
</tr>
<tr>
<td>Eotaxin</td>
<td>F: GCACGCTGAAAGCCATAGTCT</td>
<td>NT 204-557</td>
<td>63</td>
<td>353</td>
</tr>
<tr>
<td></td>
<td>R: CACTTCTTCTCTGGGTTGGCA</td>
<td>NT 542-505</td>
<td>63</td>
<td>353</td>
</tr>
<tr>
<td>MIP-1</td>
<td>F: CCAAGAGAGCTGCTGCGAACG</td>
<td>NT 307-327</td>
<td>63</td>
<td>320</td>
</tr>
<tr>
<td></td>
<td>R: GGGCAGGAAATCTGAACGTG</td>
<td>NT 307-327</td>
<td>63</td>
<td>320</td>
</tr>
<tr>
<td>Rantes</td>
<td>F: CCACTCCTGCTGCTGCTGCTG</td>
<td>NT 139-419</td>
<td>63</td>
<td>280</td>
</tr>
<tr>
<td></td>
<td>R: ACCCATTCCCTTCACAGAGCA</td>
<td>NT 786-1031</td>
<td>63</td>
<td>280</td>
</tr>
<tr>
<td>α-Actin</td>
<td>F: CATCCTGCGTCTGGACCT</td>
<td>NT 603-1082</td>
<td>63</td>
<td>479</td>
</tr>
<tr>
<td></td>
<td>R: ACCCATTCCCTTCACAGAGCA</td>
<td>NT 786-1031</td>
<td>63</td>
<td>479</td>
</tr>
<tr>
<td>β-Actin</td>
<td>F: CATCCTGCGTCTGGACCT</td>
<td>NT 603-1082</td>
<td>63</td>
<td>479</td>
</tr>
<tr>
<td></td>
<td>R: ACCCATTCCCTTCACAGAGCA</td>
<td>NT 786-1031</td>
<td>63</td>
<td>479</td>
</tr>
</tbody>
</table>

Table 1. Oligonucleotides used for reverse transcription-polymerase chain reaction in murine photoreceptor (661W) cells in 661W cells

Table 2. Oligonucleotides used for reverse transcription-polymerase chain reaction in retinal microglial cells
et al. [22]. Briefly, the slides were permeabilized in 0.1% Triton X-100 in PBS for 1 h. Following washes in PBS the fluorescent TUNEL mixture was added for 1 h at 37 °C in the dark. After further washes, the slides were counterstained with Hoechst to enable an accurate determination of the number of apoptotic cells. Counting was performed by a single masked individual. The field was chosen randomly, and a 1000X magnification was used for counting. Six fields were chosen for every specimen.

Total RNA extraction and semi-quantitative reverse transcription polymerase chain reaction: Total RNA was extracted using the Trizol reagent (Invitrogen). Reverse transcription was performed with oligonucleotide primers using Superscript II reverse transcriptase (Invitrogen) according to manufacturer’s protocol. PCR primers and annealing temperatures for each of the target genes examined in 661W cells and microglia are given in Table 1 and Table 2, respectively. Based on our experiments with series diluted template, amplification for 30-35 cycles were in the linear range of detection for the examined genes. Because of this, we chose to use 35 cycles of amplification for all the genes in this study. PCR products were separated on 2% agarose gels and analyzed with Quantity One 1-D Analysis software (Bio-Rad, Richmond, CA). Target gene expression was normalized to β-actin expression. All PCR experiments were repeated four times.
Western blotting: Cells were lysed in buffer containing 50 mM Tris-Cl (pH 8.0), 0.02% sodium azide, 1 µg/ml aprotinin, 1% NP-40, and 100 µg/ml phenylmethylsulfonyl fluoride (PMSF). Final protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Rockford, IL) according to the manufacturer’s specifications. Western blotting was performed as previously described [23]. Briefly, for each sample the same amount of total protein was added to a well of a 10% acrylamide gel and resolved by SDS-PAGE. The separate proteins were transferred to a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). Non-specific binding was blocked by incubation with 5% nonfat milk at room temperature for 2 h before overnight incubation with primary antibodies directly against NFκB p65, phospho-p38, phospho-p44/42 (ERK), or phospho-c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs; Promega) at 4 °C. Where indicated, anti-ERK2 MAPK polyclonal antibody was used to assay total ERK levels, and it served as a loading control. To evaluate other proteins, we stripped membranes in stripping buffer (62.5 mM Tris-Cl, pH 6.8; 2% SDS; 100 mM β-mercaptoethanol) for 30 min at 50 °C and re-probed. Western blotting experiments were repeated four times.

Statistical analysis: Comparisons of group means in RTPCR and Western blotting were performed by single-factor ANOVA. Comparisons of group means in MTT assay were performed by student’s t-test. A p<0.01 was accepted as significant. All values are expressed as mean±SD.

RESULTS

Microglial cell conditioned medium induced 661W photoreceptor cell death: When grown in basal medium, 661W cells had a flattened and plump appearance and only a few small intercellular spaces (Figure 1A). These cultures did not exhibit any TUNEL-positive labeling (Figure 2A). Lipopolysaccharide (LPS) treatment for 24 h did not alter the morphology of cells nor exhibit TUNEL-positive labeling. In contrast, exposure to MGCM for 24 h led the cells to become spindle-shaped, and large intercellular spaces were observed (Figure 1B). As determined by nuclear incorporation of fluoresceinated dUTP (Figure 2B), 37% of the cells underwent apoptosis. The MTT cell viability assay did not detect a significant difference between viability in cultures grown in basal media in the presence or absence of LPS (Table 3). However, cell viability decreased at least 1.6 fold after exposure to MGCM (p<0.01 versus cultures grown in basal media-LPS; Table 3).

Photoreceptor cells induced an activated phenotype in microglia: The effect of PRCM on microglial morphology was examined by phase-contrast microscopy. Microglial cells grown in the basal medium had features typical of quiescent microglia. These included ramified shapes, long single processes, and a small cell soma (Figure 3A). Following exposure to 661W cell conditioned medium without photic injury, some cells became larger and rounder, and some remained unchanged (Figure 3B). Following exposure to PRCM, the cells grew the characteristic ameboid shape of activated microglia (Figure 3C).

Microglial cell conditioned medium elicited pro-inflammatory gene expression in 661W cells: To understand the expression of chemokines and noxious factors in MGCM-induced 661W cell death, we collected total mRNA at previously determined times after MGCM exposure and analyzed for mRNA levels of seven selected target genes. Seven genes included MIP-1α, MIP-1β, eotaxin, RANTES, IL-10, iNOS, and TNF-α (Figure 4). No MIP-1α, MIP-1β, or IL-10 transcripts could be detected in 661W cells grown in the basal

<table>
<thead>
<tr>
<th>Treatment</th>
<th>MTT cell viability assay</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultures grown in basal medium</td>
<td>0.96±0.103</td>
</tr>
<tr>
<td>Cultures grown in basal medium with LPS</td>
<td>0.86±0.023</td>
</tr>
<tr>
<td>Cultures grown in MGCM</td>
<td>0.59±0.034 *</td>
</tr>
</tbody>
</table>

The values are expressed as mean±SD of three individual experiments. Statistical comparisons were made by compared t test. Asterisk indicates p<0.01 was considered statistically significant when compared with cultures grown in basal medium with or without LPS treatment.

Figure 3. The effect of photoreceptor cell conditioned medium on retinal microglial morphology. A: Phase contrast images of primary retinal microglial cells cultured in basal media for 48 h. B: Following exposure to 661W cell conditioned medium without photic injury, some cells became larger and rounder (arrow), some cells remain unchanged. C: Following exposure to 661W cell conditioned medium with photic injury (PRCM), the cells grew notably the characteristic ameboid shape of activated microglia (arrowhead).
media in the presence or absence of LPS. However, exposure to MGCM elicited an up-regulation of all of these genes (Figure 4). MIP-1α expression peaked at 24 h, whereas, MIP-1β and IL-10 peaked at 12 h after exposure. In addition, 661W cells grown in the basal medium constitutively expressed modest quantities of eotaxin and RANTES mRNA. Their expression was not altered after LPS treatment. On the other hand, MGCM exposure markedly decreased the levels of both transcripts (Figure 4). Eotaxin and RANTES mRNA levels reached a minimum at 12 h and 4 h after MGCM exposure, respectively. Both iNOS and TNFα mRNA levels were readily detectable in 661W cells grown in the basal media and were slightly up-regulated by LPS treatment. Following exposure to MGCM, their expression was markedly increased and reached a peak at 24 h.

Photoreceptor cells elicited pro-inflammatory gene expression in retinal microglia: The expression of pro-inflammatory factors in PRCM-induced microglial activation, were

![Figure 4](http://www.molvis.org/molvis/v13/a232/) Figure 4. The effect of microglia cell conditioned medium on 661W photoreceptor expression of chemokines and noxious factors. A: Representative gel images showed the indicated RT-PCR products at various times after treatment with microglial cell conditioned medium (MGCM). B: Quantification of MGCM-induced changes in mRNA expression. In all cases, relative mRNA levels were normalized to β-actin. *p<0.01 versus cells grown in basal medium (NC).

![Figure 5](http://www.molvis.org/molvis/v13/a232/) Figure 5. The effect of photoreceptor cell conditioned medium on microglial expression of chemokines and noxious factors. A: Representative gel images showed the indicated RT-PCR products at 12 h after treatment with 661W cell conditioned medium with or without photic injury. B: Quantification of changes in mRNA expression. The relative mRNA levels were normalized to β-actin. *p<0.01 versus cells grown in basal medium (NC). Hash mark p<0.01 versus cells grown in 661W cell conditioned medium without photic injury.
examined by measuring the mRNA levels of eotaxin, RANTES, IL-10, and iNOS at 12 h after PRCM exposure (Figure 5). Retinal microglial cells grown in the basal medium constitutively expressed moderate levels of eotaxin and RANTES mRNA. IL-10 and iNOS mRNA could also be detected under these conditions. Following exposure to 661W cell conditioned medium without photic injury, the mRNA level for IL-10 and iNOS were not changed much, and the mRNA level for eotaxin and RANTES were up-regulated. One important finding was, the mRNA levels for all these genes were markedly up-regulated in response to PRCM.

The microglial cell conditioned medium-induced 661W cell death was associated with MAPK activation: Unpublished results from our laboratory suggest that the MAPKs and NF-κB may be involved in light-induced photoreceptor apoptosis and LPS-induced microglial activation. MAPKs are serine/threonine kinases that play an instrumental role in signal transduction from the cell surface to the nucleus, and they include p38 MAPK (p38), extra-cellular signal-regulated kinase (p44/42), and JNK [24]. NF-κB, a ubiquitous transcriptional factor that regulates a broad range of genes, plays a pivotal role in cell death and survival [25-27]. The role of NF-κB or MAPK

Figure 6. The effect of microglial cell conditioned medium on NF-κB p65 levels and MAPK phosphorylation in 661W cells. A: Representative western blots images showed the time course of changes in levels of NF-κB p65, phosphorylated p38 (p-p38), phosphorylated p44/42 (p-p44/42), and phosphorylated JNK (p-JNK) in response to microglial cell conditioned medium (MGCM). B: Quantified levels of the indicated protein. The relative levels of each protein were normalized to ERK2. *p<0.01 versus cells grown in basal medium (NC).
signaling pathways in MGCM-induced 661W cell death were studied by examining levels of the p65 subunit of NF-κB and levels of phosphorylated p38 (p-p38), p44/42 (p-p44/42), and JNK (p-JNK) using Western blot analysis (Figure 6). We found 661W cells expressed high levels of NF-κB p65 when grown in the basal medium. However, NF-κB p65 levels were not altered in response to LPS or MGCM exposure. When cultured in basal media, 661W cells contained minute levels of p-p38, p-p44/42, and p-JNK. Phosphorylation of these MAPKs was not altered by LPS treatment but was transiently increased by MGCM. Phosphorylation of p38 was increased following 20 min exposure to MGCM and peaked at 30 min, then slowly declined thereafter, returning to baseline at 2 h. Phosphorylation of p44/42 MAPK also increased following 20 min exposure and peaked at 30 min, but it persisted until 2 h, after which time the expression returned to basal level. Phosphorylation of JNK was increased following 20 min exposure of MGCM and peaked at 1 h. High levels of phosphorylated JNK persisted until 4 h and returned to baseline thereafter.

**PRCM-induced microglial activation was associated with NF-κB upregulation and MAPK activation:** The role of NF-κB or MAPK signaling pathways in PRCM-induced microglial activation was determined by performing Western blot analysis of NF-κB p65, p-p38, p-p44/42, and p-JNK on microglial lysates (Figure 7). Retinal microglia expressed low levels of the p65 subunit of NF-κB in basal medium. After exposure to 661W cell conditioned medium without photic injury for 30 min, NF-κB p65 was up-regulated. After exposure to PRCM for 30 min, NF-κB p65 was markedly up-regulated. Retinal microglia expressed high levels of p-p44/42 and minute levels of p-p38 and p-JNK in basal media. Following exposure to 661W cell conditioned medium without photic injury for 30 min, all examined proteins barely changed. Following exposure to PRCM for 30 min, p-p38 and p-JNK MAPKs were markedly up-regulated, but the level of p-p44/42 MAPK remained unchanged.

**DISCUSSION**

In the present study, we employed light-damaged 661W cells to study the effects of soluble factors secreted by these cells on microglial activation. We also investigated the reciprocal effects of factors secreted by LPS-activated retinal microglia on 661W photoreceptor cells. The photoreceptor cell line 661W was originally isolated from a transgenic mouse line expressing the H1T1 construct, which was comprised of SV40 T-antigen driven by the human inter-photoreceptor retinol binding protein promoter [28]. In spite of their proliferative state, the 661W cells appeared to express several photoreceptor cell markers [29]. In addition, 661W cells also possessed light-induced cell death pathways similar to those observed in photoreceptor cells in vivo [30]. Thus, this cell line is a useful model for the study of photoreceptor cell death mechanisms.

Our results demonstrated that MGCM induced photoreceptor apoptosis, and PRCM induced microglial activation. Together, these findings were consistent with the hypothesis that, in retinal disease, the primary apoptotic photoreceptor cells release immunological signaling molecules that activate and recruit microglial cells to the lesioned site. In turn, these activated microglia not only acted as phagocytic cells to clear the debris [1], but also released a variety of potentially harmful substances, which triggered death of the remaining photoreceptors. It was noteworthy that IL-10 mRNA was up-regulated in 661W photoreceptor cells after treatment with MGCM and in microglial cells after treatment with PRCM. IL-10 is an important anti-inflammatory cytokine that has been shown to suppress the harmful effects of activated microglia [21,31]. Ledeboer and colleagues demonstrated that IL-10 inhibited LPS-induced production of several inflammatory mediators.
and toxic factors [31]. The production of pro-inflammatory factors in the retina was under strict control to maintain homeostasis. This might explain why, after retinal reattachment, the activated microglial cells returned to a resting state, and the remaining photoreceptors survived for a relatively long period [6].

It has been shown that NF-κB played an important role in photo-oxidative stress induced photoreceptor apoptosis. Exposure of photoreceptor cells to light generated reactive oxygen intermediates (ROI) that activated caspase-1, resulting in proteolytic cleavage of NF-κB [30]. However, decreased levels of NF-κB p65 were not observed during photoreceptor apoptosis induced by MGCM. MGCM has previously been shown to contain pro-inflammatory factors and other toxic factors such as MIP-1α, NO, and TNFα [21,32,33]. The toxic effects of NO on photoreceptors has been well-studied and has been attributed to the ability of NO to do the following: (1) increase cyclic guanosine monophosphate (cGMP), leading to excess calcium influx [34]; (2) trigger adenosine diphosphate (ADP)-ribosylation of photoreceptor proteins, which modulated their activity [35]; and (3) contribute to the production of the peroxytirute anion (peroxytirute-ONO₂), which is highly cytotoxic [36]. Also, TNFα has been shown to enhance the excitotoxic effects of glutamate [37]. The observation that there was no down-regulation of NF-κB during MGCM-induced photoreceptor apoptosis suggested that, under these experimental conditions, there was no crosstalk between these pro-inflammatory pathways and IL-1β converting enzyme (or caspase-1).

Both p38 and JNK are prototypical stress-response kinases, which are activated by diverse stress such as inflammatory cytokines [38]. Phosphorylation of p38 resulted in activation of MAPK-activated protein 2 and activating transcription factor 2 (ATF-2); whereas, phosphorylation of JNK resulted in c-Jun induction [39]. On the other hand, the p44/42 was activated in response to growth factors, oxidative stress, or glutamate receptor stimulation [40-42]. Phosphorylation of p44/42 was involved in immediate early gene induction and hyperphosphorylation of EIK-1 and cAMP/calcium-responsive element binding proteins [43]. In the eye, MAPKs have been shown to be activated and involved in neuronal cell death during retinal degeneration [44] and in retinal ischemia [45,46], light damage [47], axotomy of the optic nerve [48], and NMDA-induced retinal cell death [49]. Moreover, p38 and JNK inhibitors have been found to partially rescue neurons from glia-induced death [50]. Our findings p38, JNK, and p44/42 were transiently activated by MGCM were consistent with these findings, and might be indicative of a role for the MAPKs in MGCM-induced photoreceptor death.

Both the MAPK and NF-κB signaling pathways have been implicated in microglial/macrophage activation [51,52]. Evidence suggested that expression of activated p38 by microglia plays a key role in the production of inflammatory cytokines and free radicals such as NO in the central nervous system [53]. Furthermore, inhibition of p38 activation in microglia after transient middle cerebral artery occlusion in rats significantly reduced the infarct volume and the induction of various pro-inflammatory factors such as iNOS, TNF-α, IL-1β, and COX-2 [54]. NF-κB activation has been shown to play a dominant role in iNOS expression and NO production in microglial cells [55]. In the present study, activation of p38 and JNK MAPK as well as upregulation of NF-κB p65 occurred in response to PRCM, suggesting that they may play a role in PRCM-induced microglial activation. The relationship between MAPK and NF-κB signaling on cytokine responses was not fully defined and may depend on the particular cell systems examined. Some investigations in peripheral cells have provided evidence that NF-κB was downstream of the MAPKs. For example, p38 has been demonstrated to phosphorylate p65, which enhanced its binding properties [56-58]. Also, JNK and p38 have been shown to indirectly up-regulate NF-κB binding activity by enhancing rapid degradation of I-κB components [59,60]. Other studies suggested that the NF-κB and MAPK pathways are two independent signaling pathways [61,62]. Additional studies will be necessary to clarify the relationship between MAPK and NF-κB in PRCM-induced microglial activation.

It was noteworthy that exposure of microglial cells to 661W cell conditioned medium without photic injury, the cells became larger and rounder. The mRNA expression of eotaxin and RANTES, and the protein expression of NF-κB p65 were up-regulated. Our studies demonstrated (data not shown) that the 661W photoreceptor cells constitutively expressed modest quantities of MCP-1 and MCP-3, which was undetectable in the normal retina [4]. MCP-1 and MCP-3 are members of the CC chemokine family, which was considered to be the principal chemokines involved in recruitment of monocytes and macrophages to the site of inflammation. Previous studies suggested that they might play a role in activating and recruiting microglia to the outer nuclear layer and subretinal space in light-induced [4] and inherited retinal degeneration [63]. The 661W cell conditioned medium without photic injury-induced microglial reaction might be due to the chemokines (such as MCP-1, MCP-3) in the culture medium.

In summary, the interplay between immunological signaling molecules released by photoreceptors and microglia and the downstream targets of these molecules was complex. Nevertheless, our data pointed to a role for NF-κB and MAPK signaling pathways in photoreceptor apoptosis and microglial activation, and will be valuable in unraveling the mechanisms underlying microglia-photoreceptor crosstalk.

ACKNOWLEDGEMENTS
This work was supported in part by the National Natural Science Foundation of China (30600689), Michael Panitch Research Fund, RPB Foundation, the Oliver Birckhead Research Fund, and Research Fund from Peking University Third Hospital. The authors are grateful to Professor Al-Ubaidi for providing the 661W photoreceptor cells.

REFERENCES
1. Thanos S. Sick photoreceptors attract activated microglia from the ganglion cell layer: a model to study the inflammatory cascades in rats with inherited retinal dystrophy. Brain Res 1992;
588:21-8.
51. Uesugi M, Nakajima K, Tohyama Y, Kobasa S, Kurihara T. Nonparticipation of nuclear factor kappa B (NFkappaB) in the signaling cascade of c-Jun N-terminal kinase (JNK)-and p38 mitogen-activated protein kinase (p38MAPK)-dependent tumor necrosis factor alpha (TNFalpha) induction in lipopolysaccha-