Application of Intracellular Toll-Like Receptor Ligands


1Department of Optometry and Vision Sciences, University of Melbourne, Parkville, Victoria, Australia
2Department of Anatomy & Developmental Biology, School of Biomedical Sciences, Monash University, Victoria, Australia
3School of Molecular Sciences, Department of Biochemistry, La Trobe Institute for Molecular Science, La Trobe University, Bundoora, Australia
4Lions Eye Institute and the Centre for Ophthalmology and Visual Sciences, University of Western Australia, Perth, Western Australia, Australia
5Section of Immunology and Infection, Institute of Medical Sciences, University of Aberdeen, Aberdeen, United Kingdom

Retinal Microglial Activation Following Topical Application of Intracellular Toll-Like Receptor Ligands

Correspondence: Holly R. Chinnery, Department of Optometry and Vision Sciences, University of Melbourne, Parkville, VIC 3010, Australia; holly.chinnery@unimelb.edu.au.

Submitted: June 29, 2015
Accepted: September 22, 2015
Citation: Chinnery HR, Naranjo Golborne C, Leong CM, Chen W, Forrester JV, McMenamin PG. Retinal microglial activation following topical application of intracellular toll-like receptor ligands. Invest Ophthalmol Vis Sci. 2015;56:7377–7386. DOI:10.1167/iovs.15-17587

PURPOSE. We previously have reported that application of the intracellular toll-like receptor (TLR)9 ligand CpG-ODN onto the injured corneal surface induces widespread inflammation within the eye, including the retina. We tested the hypothesis that topical application of two other intracellular TLR agonists, Poly I:C and R848, would cause retinal microglial activation and migration into the subretinal space.

METHODS. C57BL/6J wild-type and CX3CR1^GFP/ mice were anesthetized and received central corneal abrasions followed by topical application of Poly I:C (TLR3 agonist), R848 (TLR7/8 agonist), or CpG-ODN (TLR9 agonist). Eyes were imaged in vivo by using spectral-domain optical coherence tomography to assess and quantify vitreous cells and retinal edema. Tissues were processed for whole-mount immunofluorescence staining or gene expression studies. Microglial activation was determined by morphologic changes, major histocompatibility complex (MHC) class II reactivity, and migration to the subretinal space. Expression of proinflammatory cytokine gene IL-6, IL-1β, IFN-γ, and MCP-1 in retinal tissues were analyzed.

RESULTS. At 24 hours, topical treatment with CpG-ODN and R848, but not Poly I:C, led to altered microglial morphology. One week after CpG-ODN and R848 treatment, eyes exhibited vitritis and mild inner retinal edema, increased number of subretinal Iba-1^+ cells, and an increase in MHC II^+ cells in the neural retina. Proinflammatory cytokine genes were upregulated after R848 treatment, whereas in the CpG-ODN group, only IL-1β and MCP-1 were significantly upregulated. Retinal microglial activation was not observed in the Poly I:C–treated group.

CONCLUSIONS. Topical application of CpG-ODN and R848, but not Poly I:C, to the damaged corneal surface can cause activation and migration of retinal microglia.

Keywords: microglia, toll-like receptors, macrophages, inflammation
motifs, which are abundant in the genomes of many bacteria and viruses but are not present in eukaryotic nuclear DNA. TLR7/8 detects single-stranded RNA (ssRNA) from the genomes of ssRNA viruses and TLR3 acts as sensor for viral double-stranded RNA. Toll-like receptor activation in the retina is predictably implicated in the host response to infectious microorganisms such as *Staphylococcus aureus* and *Bacillus cereus*. However, it is becoming increasingly apparent that TLR activation of innate inflammatory pathways is involved in a wide range of sterile retinal pathologies including photoreceptor degeneration and ischemia reperfusion injury. TLR3 ligands can directly induce apoptosis of retinal pigment epithelial cells in vitro, and genetic studies suggest a protective role for specific genetic variants of TLR3 in geographic atrophy. We previously have reported that topical application of oligonucleotide 1826 (type B, TLR9 ligand), R848 (Resiquimod, a synthetic analog of the viral TLR7/8 ligand), Poly IC (low-molecular-weight TLR3 ligand) or sterile saline was applied to each eye. In the absence of corneal epithelial debridement, 20 μg of either phosphorothioate CpG oligonucleotide 1826 (type B, TLR9 ligand), R848 (Resiquimod, TLR7/8 ligand), Poly IC (low-molecular-weight TLR3 ligand) or sterile saline was applied to each eye. In the absence of corneal epithelial damage or debridement there was no response to ligands. Similarly, in previous studies we have shown that scrambled CpG (i.e., GpC-ODN) does not elicit a corneal inflammatory response. All TLR ligands were purchased from Invivogen (San Diego, CA, USA). At 24 hours or 1 week after treatment, animals were euthanized and eyes placed into either 4% paraformaldehyde (PFA; Sigma-Aldrich, Castle Hill, New South Wales, Australia), RNAlater (Life Technologies, Carlsbad, CA, USA) or snap-frozen in optimal cutting temperature compound (Tissue-Tek; ProSciTech, Kirwan, Queensland, Australia) for tissue processing.

**Spectral-Domain Optical Coherence Tomography (SD-OCT)**

At 1 week post treatment, mice were anesthetized and dilating drops were applied to the cornea (Mydriacyl 0.5%; Alcon Laboratories, Frenchs Forest, NSW, Australia). Mice were placed on the animal imaging mount and the rodent alignment stage was attached to the Bioptigen SD-OCT imaging device (Bioptigen, Inc., Durham, NC, USA). Once the eye was aligned with the noncontact mouse retina lens, any excess mydriatic drops were absorbed by using a cotton tip and saline applied once to prevent corneal drying. Volumetric 1.4 mm × 1.4 mm scans containing optic nerve head were acquired at a rate of 1000 A-scans per 100 B-scans.

**Quantification of Vitreous Cells in SD-OCT Scans**

Vitreous opacities in SD-OCT images have been histologically verified in previous studies to be infiltrating inflammatory cells. Posterior segment images were acquired by using the Envisu R-2200 OCT (Bioptigen, Inc.) and analyzed by using FIJI, an open-sourced platform. To quantify the inflammatory response, a custom FIJI script was written to automate the following process. Firstly, all images underwent binary conversion by using Yen thresholding. A median filter was then applied to remove one pixel-wide speckle noise. Next, a 15 × 50 pixel-wide region of interest was created and counted by using the Analyze Particles function on FIJI.

**Quantification of the Retinal Nerve Fiber Layer (RNFL) Thickness**

Thicknesses of individual retinal layers at multiple cross-sectional images were determined by using the Bioptigen segmentation software InVivoVue Diver. Manual calipers were used to mark distinct boundaries of the retinal sublayers from the superior, nasal, inferior, and temporal quadrants approximately 225 μm from the optic nerve head. The thickness of the RNFL was defined by the distance between the inner limiting membrane and the ganglion cell layer (GCL).

**Real-Time Reverse Transcription PCR**

Total RNA was extracted from retinal tissue by using the PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and subjected to on-column digestion of DNA by using PureLink DNase Set (Invitrogen). DNase-treated RNA was reverse transcribed to cDNA, with each reaction containing 500 ng RNA per 10 μL reaction volume (Transcriptor First Strand cDNA Synthesis Kit; Roche Applied Sciences, Mannheim, Germany). Mock cDNA reactions, in which the enzyme reverse transcriptase was omitted (No-RT controls), were also generated in order to ensure the removal of genomic DNA had been successful. Synthesized cDNA served as template in real-time PCR reactions using Fast SYBR Green Master Mix (Applied
### Immunofluorescence Staining of Retinal and Choroidal Tissue

Retinas from Cx3cr1<sup>−/−</sup> mice, as well as retinas and choroids from C57BL/6 wild-type (WT) mice, were dissected and divided into radial portions to be used in whole-mount immunofluorescence staining, as previously described. 54 Tissue whole-mounts were incubated in 20 mM ethylenediaminetetraacetic acid for 30 minutes at 37°C and blocked in 0.3% Triton X-100, 5% bovine serum albumin in PBS for 30 minutes at room temperature before antibody incubation. Tissues were then incubated with rabbit anti-mouse ionized calcium-binding adaptor molecule 1 (Iba-1; 1:400; Wako Pure Chemical Industries, Osaka, Japan) and rat anti-mouse MHC class II (M5/114.15.2, 1:400; BD Pharmingen, San Diego, CA, USA). Incubations were carried out for four hours at room temperature, followed by overnight incubation at 4°C. The following day, whole-mounts were washed three times in PBS and incubated with biotinylated goat anti-rat (1:400; Amersham Biosciences, Buckinghamshire, UK) diluted in blocking buffer, for two hours at room temperature. Following an additional three washes in PBS, whole-mounts were incubated with AlexaFluor 647 goat anti-rabbit (1:300; Molecular Probes, Carlsbad, CA, USA) and Streptavidin Cy3 (1:300; Molecular Probes) diluted in PBS. In some experiments, whole-mounts were instead incubated with biotinylated goat anti-rabbit (1:300; Vector Laboratories, Burlingame, CA, USA) followed by Streptavidin Cy3, as described above for biotinylated goat anti-rat. Whole-mounts were finally incubated with Hoechst/PBS (1/500) for 10 minutes at room temperature and cover-slipped with the vitreous side up (Iba-1–stained Cx3cr1<sup>gfp/−</sup> retinas and Iba-1/MHC class II–stained C57BL/6 retinas) or with the photoreceptors side up (Iba-1–stained C57BL/6 retinas), the latter being referred to as photoreceptor layer (PRL) in the remainder of the article. Choroidal whole-mounts were mounted with the retinal pigment epithelium (RPE) side up, thus making the apical surface of the RPE visible for quantitation of subretinal macrophages. Negative controls were generated by omitting the primary antibody from the immunostaining protocol or substituting the primary antibody with an isotype control antibody.

Microglial stratification in retinal layers was analyzed by using cryosections of eyes from Cx3cr1<sup>−/−</sup> mice. In brief, 5-μm sections were fixed for 30 minutes in 4% PFA and incubated with Hoechst for 10 minutes.

### Quantitative Analysis of the Number and Characterization of Retinal Microglia

To quantify and characterize retinal microglia, Iba-1-immunostained Cx3cr1<sup>−/−</sup> retinas were analyzed by confocal microscopy. Previous studies have reported no difference in the number and phenotype of retinal microglia in Cx3cr1<sup>−/−</sup> mice compared with WT mice. Z-stacks of 1-μm optical sections were taken by using the 20× objective (SP5; Leica Microsystems, Mannheim, Germany). The total number of microglia was counted in z-series videos by using FIJI/ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) and numbers were converted to represent density/mm².

In retinas from each treatment group (n = 5), three representative fields encompassing the central, paracentral, and peripheral regions were selected for counting. In each field, cell bodies were counted separately in the inner plexiform layer (IPL) and outer plexiform layer (OPL). Imaging was performed and the lengths of processes were determined by measuring the distance from the centroid of a cell body to the tip along the processes.

### Table: Primer Sequences of Proinflammatory Genes Analyzed by Real-Time RT-PCR

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5'-3')</th>
<th>Reverse Primer (5'-3')</th>
<th>Amplicon Size, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>IFN-γ</td>
<td>AGCCAAAGCAGAGGCCGAAA</td>
<td>CTGGACCTGTGGTGTGTTGA</td>
<td>72</td>
</tr>
<tr>
<td>IL-1β</td>
<td>TGTCACCTACCTGGAAGGTGCT</td>
<td>TGGTTGCTCCCATACCGATG</td>
<td>241</td>
</tr>
<tr>
<td>MCP-1</td>
<td>GCTGAGAACCTTGGCAGA</td>
<td>210</td>
<td></td>
</tr>
<tr>
<td>IL-6</td>
<td>GCCAGGTGCTCTAGACAGA</td>
<td>155</td>
<td></td>
</tr>
<tr>
<td>Rpl32</td>
<td>TTAACGCGAACTTGCGGGAAAC</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>

- To quantify the number of cells expressing MHC class II in response to treatment, confocal z-series of Iba-1/MHC class II–stained retinas from C57BL/6 WT mice were acquired with a 20× objective (SP5; Leica Microsystems). Z-stacks consisted of 3- to 4-μm optical sections, and two random fields were captured for each retinal whole-mount (each field representing 0.6006 mm²). Cells expressing MHC class II were manually counted by using FIJI/ImageJ. For any given animal, the number of MHC class II–expressing cells in each field were divided with the total area imaged, hence represented as cells/mm².
surface of whole-mounts was imaged with a 20× objective (Olympus Provis AX70 microscope; Olympus, Tokyo, Japan) and captured with a Sis FVII digital camera. Three random fields were captured for each whole-mount (each field representing 0.1462 mm²). Cells expressing Iba-1 were manually counted by using FIJI/ImageJ and represented as cells/mm².

**Statistical Analyses**

Statistical analyses were carried out by using unpaired, two-tailed Mann-Whitney test or unpaired, two-tailed Student’s t-test (GraphPad Prism, v6; GraphPad Software, Inc., La Jolla, CA, USA). A P value < 0.05 was considered significant.

**RESULTS**

**Microglial Activation in Response to TLR3, TLR7/8, and TLR9 Ligands at 24 Hours Post Treatment**

To facilitate visualization of microglia morphology we used Cx3cr1gfp/+ mice in which most monocyte-derived cells are GFP⁺. Cryosections of saline, CpG, R848- and Poly I:C–treated eyes demonstrated the localization of microglia in the GCL and plexiform layers of the mouse retina (Fig. 1A).

---

**Figure 1.** GFP⁺ retinal microglial response to TLR3, TLR7/8, and TLR9 ligands 24 hours post application to the debrided cornea. (A) Confocal imaging of GFP⁺ microglia in retinal histologic sections (top panel) and whole-mounts (costained with Iba1; bottom panel) from Cx3cr1gfp/+ mice. Histologic cross-sections reveal normal microglial distribution following CpG, R848, and Poly I:C treatment. En face confocal images of the IPL show an increase in Iba1 expression in CpG- and R848-treated mice, with a subset of Iba2⁻¹ GFP⁺ cells in CpG-treated eyes (bottom panel, arrows). (B) Analysis of dendrite length in retinal GFP⁺ microglia from Cx3cr1gfp/+ mice, located in the IPL. Treatment with CpG and R848 induces morphologic changes in retinal microglia, with cells appearing more amoeboid as opposed to the ramified shape displayed by Poly I:C-treated mice and saline controls. (C) Quantification shows that CpG and R848 treatment significantly decreases the dendrite length of these microglia (n = 3–5 per treatment group). (D) Quantification of microglial density confirms that there is no difference between treatment groups (n = 5 per treatment group). P values were generated by two-tailed Mann-Whitney test, in which *P < 0.05 was considered significant. Error bars depict mean ± SEM. Scale bars: 100 μm. Hoechst was used as nuclear stain (blue; [A]).
Representative en face confocal images of Iba-1–stained retinal whole-mounts demonstrated the presence of Iba-1+ Cx3cr1GFP+ microglia in the IPL and outer plexiform layer (not shown). The presence of less ramified Iba-1+ Cx3cr1GFP+ macrophages (arrows), indicating infiltrating macrophages, was most apparent after Cpg-ODN treatment but not saline, R848, or Poly I:C treatment (Fig. 1A). Dendritic tracing performed on randomly selected microglia in the IPL in retinal whole-mounts from all experimental groups revealed that the average dendrite length, used as a measure of microglial activation, was significantly reduced in Cpg-ODN– and R848-treated eyes but unchanged in saline- and Poly I:C–treated eyes (Figs. 1B, 1C). Despite differences in the dendrite length of microglia in Cpg-ODN– and R848-treated eyes, the density of microglia was similar across treatment groups at 24 hours (Fig. 1D).

**Moderate Vitritis and Microglial Activation in Cpg-ODN–Treated Eyes at 1 Week**

We have previously reported that topical application of Cpg-ODN does indeed induce the infiltration of inflammatory cells into the vitreous at 24 hours.26 To further examine the posterior segment inflammatory response after 1 week, SD-OCT images of the posterior segment were collected. Representative SD-OCT B-scans of the optic nerve head and retina at 1 week post treatment demonstrated a clear vitreous in saline-treated eyes (Fig. 2A), compared with the presence of numerous round inflammatory cells after CpG-ODN treatment (Figs. 2A, 2B), which conforms with descriptions of vitritis in previous studies.30 As compared to saline-treated eyes, the RNFL thickness was increased by approximately 20% in Cpg-ODN–treated eyes (Fig. 2C). Proinflammatory cytokine mRNA (II-1β and MCP-1) was upregulated in Cpg-ODN–treated eyes (Fig. 2D). Microglia appeared more numerous and less ramified in the IPL than in saline-treated controls (Fig. 2E). Iba-1+ macrophages in the subretinal space were a distinctive characteristic of Cpg-ODN–treated eyes but were absent in saline-treated controls (Fig. 2F). Furthermore, in Cpg-ODN–treated eyes there was a statistically significant increase in microglial density in the IPL (Fig. 2G), and Iba-1+ cells in the subretinal space, with evidence of cellular processes extending through the nuclear layers, which is not a feature of the distinctive laminar arrangement of microglia in normal eyes (Fig. 2H). In addition, the numbers of MHC class II+ cells throughout the entire thickness of the neural retina increased significantly (Fig. 2I). Representative images of MHC II+ cells in the inner retina are shown in saline- and Cpg-ODN–treated eyes (Fig. 2J).

**Severe Vitritis and Microglial Activation in R848-Treated Eyes at 1 Week**

Analysis of the posterior segment of R848-treated eyes revealed a significant number of inflammatory cells in the vitreous, compared with saline-treated eyes (Figs. 3A, 3B), which was approximately 3-fold greater than with Cpg-ODN treatment (see Fig. 2B). The RNFL was significantly thicker after R848 treatment (Fig. 3C), and gene expression analysis revealed extensive and significant upregulation of all proinflammatory cytokines tested (Fig. 3D). Cx3cr1GFP+ microglia appeared to be less ramified and had larger cell bodies in R848-treated eyes than saline controls, which were visible on both cryosections and Iba-1–stained retinal whole-mounts (Fig. 3E). Large numbers of Iba-1+ macrophages were present in the subretinal space after R848 treatment (Figs. 3F, 3G). The total number of Cx3cr1GFP+ microglia (Fig. 3H) and MHC II+ cells within the neural retina was significantly increased, compared to control eyes (Figs. 3I, 3J), and an order of magnitude greater than in Cpg-treated eyes (see Fig. 2J). In TLR7−/− mice treated with R848, few microglia were observed in the subretinal space, and rare MHC II–expressing microglia were observed in the neural retina. Microglia exhibited typical ramified morphology and were distributed normally in the retinal layers (Supplementary Fig. S1).

**Absence of Vitritis and Microglial Activation Following Topically Applied TLR3 Agonist Poly I:C at 1 Week**

Having previously reported that topically applied Poly I:C leads to infiltration of MHC II+ macrophages to the injured mouse cornea after 1 week,36 we wished to assess whether this TLR3 agonist would elicit vitritis and microglial activation similar to the other intracellular TLR agonists used in the present study. In WT mice whose cornea had been exposed to TLR3 ligand 1 week earlier, SD-OCT imaging of the central retina demonstrated a normal vitreous that, similar to saline-treated controls, was devoid of inflammatory cells (Figs. 4A, 4B). There was no significant difference in the thickness of the RNFL (Fig. 4C) and mRNA expression levels of the cytokines IFN-γ, IL-1β, IL-6, and MCP-1 (Fig. 4D), compared to controls. Analysis of cryosections from treated Cx3cr1GFP+ mice revealed normal morphology of microglia (Fig. 4E) and the absence of Iba-1+ macrophages in the subretinal space (Figs. 4F, 4G). There was no quantitative difference in the density of Cx3cr1GFP+ microglia (Fig. 4H) or MHC II+ cells in the neural retina (Figs. 4I, 4J) between control and Poly I:C–treated eyes. In summary, the TLR3 ligand Poly I:C and saline-treated controls failed to elicit any inflammatory changes in the posterior segment as a sequelae to topical application to the debrided cornea.

**DISCUSSION**

Activation of retinal microglia has been implicated in the development and progression of retinal degenerative diseases such as diabetic retinopathy,37 retinitis pigmentosa,38 and age-related macular degeneration.39 We have previously demonstrated that toll-like receptor–induced anterior segment inflammation and systemic viral infection can cause retinal inflammation in mice.26,40 In the present study we sought to extend our previous report showing that topically applied TLR9 agonist Poly I:C can induce retinal inflammation in mice by elucidating whether other topically applied intracellular TLR ligands can cause activation of retinal microglia and induce their migration into the subretinal space. The SD-OCT imaging was valuable in quantifying vitreous infiltrates and measuring changes in retinal thickness; in addition, we took advantage of Cx3cr1GFP+ reporter mice to perform image analysis of microglia morphology as evidence of activation. Our data revealed that another intracellularly recognized TLR ligand, R848, leads to marked activation and migration of retinal microglia, inner retinal edema, and upregulation of proinflammatory genes after topical application to the injured cornea. No signs of innate inflammation were observed in the retina of eyes in which ligands were topically applied to the intact corneal surface (data not shown), confirming that breach of the corneal epithelium, a normally effective barrier to microbial products,41 is required for activation of the innate immune response.

R848, a synthetic mimic of naturally occurring single-stranded RNA, activates cells expressing TLR7 and TLR8 and is involved in mediating antiviral responses.42 Owing to its ability to modify immune responses, it has been used as a topical agent to treat precancerous skin lesions (actinic keratoses) and herpes simplex virus–2–associated lesions, and as an adjuvant
in vaccines. However, to date we are unaware of any use in the context of ocular infections. Murine TLR8 has been previously suggested to be nonfunctional, which is evidenced by a lack of responsiveness to R848 treatment in TLR7-deficient mice, even though TLR8 is present. Our data demonstrating a lack of microglial activation in TLR7/− mice in response to topical R848 support these previous studies and confirm the specificity of R848 as a TLR7 ligand.

**Figure 2.** Stimulation of corneal TLR9 by CpG induces a retinal microglial activation at 1 week post treatment. Retinal microglial activation and vitritis in WT C57BL/6 mice (A–D, F, I, J) or Cx3cr1GFP/− mice (E, H) in response to topical CpG application. (A, B) The OCT imaging and quantification reveal an increased number of infiltrating vitreous cells (arrows) in the eyes of CpG-treated mice compared with saline treatment. Additional effects of CpG treatment include thickening of the RNFL (C) and upregulation of mRNA of proinflammatory genes IL-1β and MCP-1 (D). (E, G) Confocal imaging of GFP+ microglia in retinal histologic sections (top panel) and whole-mounts (costained with Iba1; bottom panel) from Cx3cr1GFP/− mice. Histologic cross-sections (E) reveal occasional vertical orientation of GFP+ cells, indicating microglial migration. Optical sections of the IPL show an increase in Iba1 expression in CpG-treated mice. (F, H) Confocal imaging of the subretinal space—consisting of the PRL and the RPE on the choroidal surface—reveals the presence of Iba1+ macrophages in CpG-treated eyes, cells that are virtually absent in saline controls. (H) Quantification of GFP+ microglial density in the retina reveals a significant increase after CpG treatment. (I, J) Confocal imaging and quantitative analysis of MHC class II+ cells in retinal whole-mounts. The CpG treatment induces increased MHC class II expression in the retina. Each treatment group consisted of n = 6 unless stated otherwise. P values were generated by two-tailed Mann-Whitney test (B, C, G–I) or two-tailed Student’s t-test (D). P values of <0.05 were considered significant, with asterisks defined as follows: *P < 0.05 and **P < 0.01. Bars depicted represent mean ± SEM. Scale bars: 100 μm. Hoechst was used as nuclear stain (blue; E, F, J).
TLR3 is another nucleic acid–sensing TLR, which recognizes double stranded RNA of viral origin and Poly I:C is a synthetic analog of viral dsRNA. Addressing whether topically applied TLR3 ligand can induce intraocular inflammation is of particular interest in light of recent controversial studies implicating a role for nucleic acid–sensing TLR3 in the development of geographic atrophy in age-related macular degeneration. Furthermore, TLR3 is activated by endogenous products of damaged RPE cells and is involved in retinal degenerative processes in mice with defective clearance of all-

**Figure 3.** Stimulation of corneal TLR7/8 by R848 induces retinal microglial activation at 1 week post treatment. Microglial activation and vitritis in the retina of WT C57BL/6 mice (A–D, F, G, I, J) or Cx3cr1GFP/+ mice (E, H) in response to topical R848 application. (A, B) The OCT imaging reveals the presence of significant number of vitreous cells (arrows) infiltrating the eyes of R848-treated mice compared with saline-treated mice. Additional effects of R848 treatment include thickening of the RNFL (C; n = 5–6) and a significant upregulation of the proinflammatory genes IFN-γ, IL-1β, MCP-1, and IL-6 (D). (E, G) Confocal imaging of GFP+ microglia in retinal histologic sections (top panel) and whole-mounts (costained with Iba1; bottom panel) from Cx3cr1GFP/+ mice. Histologic cross-sections reveal the presence of occasional, vertically arranged microglia, indicating migration. Optical sections of the IPL show an increase in Iba1 expression in R848-treated mice compared to saline-treated mice (n = 3 per treatment group). (E, H) Large numbers of Iba1+ cells in R848-treated mice, cells that are virtually absent in saline controls. (I, J) Confocal imaging of retinal whole-mounts after R848 application to the debrided cornea, which induces strong MHC class II expression in the retina. Each treatment group consisted of n = 6 unless stated otherwise. P values were generated by two-tailed Mann-Whitney test (B, C, G–I) or two-tailed Student’s t-test (D). P values of <0.05 were considered significant, with asterisks defined as follows: *P < 0.05, **P < 0.01, and ***P < 0.001. Bars depicted represent mean ± SEM. Scale bars: 100 μm. Hoechst was used as nuclear stain (blue; [E, F, J]).
trans-retinal in the photoreceptors. Exogenous delivery of 2 μg Poly I:C into the subretinal space also causes severe retinal inflammation and photoreceptor necrosis in mice. Thus, while it is clear that direct exposure to TLR3 ligands in the mouse retina can trigger innate immune responses, our data demonstrated that this ligand does not cause retinal inflammation after topical corneal application. It is possible that, unlike the highly stable and low-molecular-weight CpG-ODN and R848, the stability and relatively larger size of Poly I:C limit its diffusion through the ocular media. However, in a recent study...
Retinal Microglial Activation by TLRs

of ocular pharmacokinetics of fluorochrome-labeled nanoparticles, RNA nanoparticles, but not dsRNA, were easily detectable in the posterior segment of the eye after subconjunctival delivery. The inability of dsRNA to traverse the ocular media, despite being of similar size to the RNA nanoparticles, may explain why this TLR ligand failed to induce intraocular inflammation in our model.

Time-lapse intravital imaging studies of brain microglia have elegantly shown that upon activation by diverse stimuli, such as local laser lesions or injection of picogram quantities of LPS, ramified microglia respond by rapidly changing cell motility, polarity, and activity in an adenosine triphosphate–dependent manner. As morphologic alterations in microglia are well recognized and widely accepted as a means of assessing functional status, we investigated microglial dendrite length as an indicator of their response to topical intracellular TLR ligands. We observed retraction of retinal microglial dendrites 24 hours after topical application of TLR7/8 and TLR9 ligands, but not the TLR3 ligand Poly IC, to the injured cornea. In addition to alterations in microglial morphology, loss of microglial stratification and the appearance of MHC II+ cells in the retina and Iba-1+ cells in the subretinal space are other indications of microglial activation in response to a proinflammatory milieu in the retina. The presence of subretinal macrophages in particular has been reported in a range of rodent models of retinal conditions including acute elevated intraocular pressure, light-induced degeneration, and aging. The presence of subretinal macrophages 1 week following TLR7/8 and TLR9 activation in the cornea parallels reports of macrophage migration to the subretinal space in rodent models of systemic viral and fungal infections, and highlights the potential significance of these cellular responses in the development of retinal diseases.

This study provided further evidence that anterior segment inflammation following topical application of TLR ligands to the injured ocular surface can lead to altered microglia homeostasis deeper in the retina. This is potentially significant when considering the well-described age-associated priming of microglia and the subsequent exaggerated and prolonged inflammatory responses following innate immune activation in aged animals. Thus, activation of retinal microglia during corneal microbial infection may play a role in early-onset retinal degenerative diseases, where chronic inflammation is implicated. These data suggest that TLR7/8 and TLR9 receptor activation accompanying viral and bacterial infections of the cornea may have unsuspected consequences for the normally quiet immunologic environment of the neural retina.

Acknowledgments

The authors thank Janet Choi (University of Melbourne) who provided technical help with animal studies, tissue processing, and image analysis and Jeremiah Lim (University of Melbourne) who helped with image analysis of vitreous cells. Supported by funding from the National Health and Medical Research Council of Australia (Project Grants 1026301 and 1042612) (HRC, JVE, and PGM).

Disclosure: H.R. Chinnery, None; C. Naranjo Golborne, None; C.M. Leong, None; W. Chen, None; J.V. Forrester, None; P.G. McMenamin, None

References

Retinal Microglial Activation by TLRs


