Innate Immune Interference Attenuates Inflammation In Bacillus Endophthalmitis

Md Huzzatul Mursalin,1,2 Phillip S. Coburn,2,3 Frederick C. Miller,4 Erin T. Livingston,1 Roger Astley,2,3 and Michelle C. Callegan1–3

1Department of Microbiology and Immunology, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States
2Department of Ophthalmology, Dean McGee Eye Institute, Oklahoma City, Oklahoma, United States
3Dean McGee Eye Institute, Oklahoma City, Oklahoma, United States
4Department of Cell Biology and Department of Family and Preventive Medicine, University of Oklahoma Health Sciences Center, Oklahoma City, Oklahoma, United States

Keywords: endophthalmitis, inflammatory response, TLR, Bacillus, inflammation, retina, blindness

Bacterial endophthalmitis is a dangerous ocular infection that is considered a medical emergency. This intraocular infection is often caused by Gram-positive bacteria.1–4 The infecting organism can be introduced into the immune-privileged posterior chamber of the eye by an injury (post-traumatic), after a surgical procedure (post-operative), or by migration of organisms into the eye from an extraocular site of infection (endogenous).5–8 Regardless of the route of infection, the signs and symptoms are quite similar, ranging from red eyes and swollen eyelids to severe intraocular pain and vision loss.1,14 The severity of the disease can range from benign inflammation that responds to treatment, to a fulminant, rapidly progressing, intractable infection. From a treatment standpoint, Bacillus endophthalmitis cases are difficult and are often associated with worse patient outcomes than infections caused by other pathogenic species.6,8–11 The pathogenicity of Bacillus endophthalmitis is linked to the inflammmogenic potential of the bacterial cell wall and the secretion of toxins and enzymes.12–18 The severe nature of Bacillus endophthalmitis requires prompt and rapid therapy to halt disease progression. However, Bacillus endophthalmitis may result in vision loss within 12 to 24 hours of infection, despite treatment that might otherwise effectively attenuate endophthalmitis caused by less virulent ocular pathogens.9

Bacillus is a Gram-positive, motile, spore-forming rod, and is found in numerous environments, especially in soil.19–21 Metabolically-inactive Bacillus cereus triggered intraocular inflammation in a rabbit experimental endophthalmitis model, suggesting a significant role of cell wall components in inciting inflammation.11 In addition to the thick outer layer containing peptidoglycan, lipoteichoic...
Acid, and lipoproteins, the unique architecture of the Bacillus envelope also includes flagella, pili, and S-layer, which is comprised of proteinaceous subunits arranged in a monocellular crystalline array.22–25 If present, S-layer protein (SLP) is one of the most plentiful proteins in the bacterial cell envelope and provides the organism with a selective advantage in diverse habitats.26 SLP contributes to colonization by promoting bacterial adherence and biofilm formation,27,28 as well as protecting the bacteria against complement killing and phagocytosis.29–32 We recently reported that the absence of SlpA resulted in protection of retinal function, reduced inflammatory cell influx, and muted disease severity in mouse experimental Bacillus endophthalmitis.33 We also reported Bacillus SlpA as a stimulator of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in human retinal Muller cells in vitro.34 SLP also protected Bacillus from being phagocytized by neutrophils and retinal cells and also impacted bacterial adherence to retinal cells.35 SLP appears to be a significant virulence factor in the pathogenesis of Bacillus endophthalmitis.

During infection, ocular immune privilege is compromised by an overwhelming, acute inflammation resulting from interactions between innate receptors and microbial ligands. We reported that Toll-like receptors (TLR) 2 and 4, but not TLR5, were necessary for robust intraocular inflammation during Bacillus infection.36–38 Being a Gram-positive pathogen, Bacillus does not produce lipopolysaccharide (LPS), so the specific ligand on Bacillus that interacts with TLR4 remained unknown. We reported that Bacillus SLP activated both TLR2 and TLR4.34 When activation of these TLRs was blocked by injection of oxidized phospholipid (OxPAPC) during experimental endophthalmitis, disease severity and overall inflammation were reduced and retinal function was retained compared to that in untreated control mice.34

Reactive oxygen species (ROS) are important effectors produced by inflammatory cells and are essential for innate defense. A slight elevation of ROS level is critical for diverse cellular mechanisms. Phospholipids, such as 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphorylcholine (PAPC), are located in cell membranes and lipoproteins. During inflammation, the interaction of ROS with cellular components leads to the modification of membrane phospholipids. Oxidation of PAPC leads to the production of OxPAPC that elicits distinct biological responses and contribute to the amplification, initiation, and resolution of inflammation. OxPAPC inhibits bacterial lipopeptide- and LPS-induced signaling via TLR2 and TLR4 pathways. OxPAPC blocks signaling in TLR2 and TLR4 pathways by competing with CD14, lipid binding protein (LBP), and MD2.39,40

TLR-ligand interactions during intraocular infection generates inflammatory mediators that recruit neutrophils into the eye.41–43 When TLRs recognize surface molecules on pathogenic bacteria, signals are transmitted via the adaptor molecules to the signaling molecules which are located in the cytoplasm. Signals from cytoplasm reach the nucleus and activate inflammatory mediator transcription. We reported that both myeloid differentiation primary response gene-88 (MyD88) and Toll/interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon-β (TRIF) regulate this inflammation signaling cascade during murine experimental endophthalmitis.38 Neutrophils are the primary innate responders and may also cause damage to the retina during an intraocular infection.44–45 Therefore inflammatory mediators that recruit neutrophils are crucial for disease outcome. During experimental Bacillus endophthalmitis, the absence of tumor necrosis factor (TNF) α resulted in reduced neutrophil infiltration into the eye, which resulted in an elevated bacterial load and damage to ocular tissues.44 The absence of TNFα was compensated for by elevated expression of other mediators such as chemokine (C-X-C motif) ligand (CXCL) 1, CXCL2/MIP1α, and IL-6, which might have contributed to delayed recruitment of neutrophils and overall pathogenesis.45 We reported that the absence of CXCL1 or anti-CXCL1 treatment led to a blunted inflammatory response but improved clinical outcome, suggesting a potential benefit for targeting neutrophil chemoattractants to curb inflammation-mediated damage.35

In endophthalmitis, the use of anti-inflammatory therapies with antibiotics has not proven completely effective at improving disease outcomes.3–8 The primary purpose of the innate inflammatory response is to sense and eliminate invading pathogens as quickly as possible. However, the highly inflammogenic Bacillus-induced ocular inflammatory response is often so robust that it is difficult to control and ultimately results in vision loss.3,46 At present, a universal therapeutic regimen to prevent vision loss in Bacillus endophthalmitis is not available. Since the absence of single proinflammatory mediators could be offset by the synthesis of others, identifying anti-inflammatory agents with the potential to block multiple inflammatory pathways might have potential as future anti-inflammatory therapeutics.

An acute and potentially damaging intraocular inflammation is one of the major problems of bacterial endophthalmitis. We reported that an absence of TLR2 or TLR4 in knockout mice or inhibition of TLR2 and TLR4 activation by OxPAPC improved the clinical outcome of experimental Bacillus endophthalmitis.33,36,37 OxPAPC-mediated interference of innate activation is well documented.39,40,47,48 In this study, we investigated how interfering with innate immune pathways during the early stage of Bacillus infection would impact inflammation during the later stage of infection. Because inhibition of TLR signaling serves as a promising treatment option for inflammatory diseases,49 we hypothesized that innate interference could be protective for a rapidly blinding disease like Bacillus endophthalmitis. Intraocular pathway interference was achieved by injection with SLP-deficient Bacillus (ΔslpA) or treatment of experimental Bacillus endophthalmitis with OxPAPC. NanoString analysis of a mouse inflammation panel revealed that innate interference considerably reduced inflammatory gene expression in the whole infected eye. Our findings suggest that the attenuation of innate activation may be a favorable anti-inflammatory option for the treatment of Bacillus endophthalmitis.

**Materials and Methods**

**Ethical Statement**

All mouse experiments were performed in accordance with the guidelines in the Guide for the Care and Use of Laboratory Animals and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences Center (protocol numbers 15-103, 18-043, and 18-087).
Innate Immune Interference and Endophthalmitis

**FIGURE 1.** Interfering with TLR2 and TLR4 activation affected inflammatory gene expression in the eye. (A) Experimental outline of the NanoString experiment. (B) PCA analysis of mouse inflammatory gene. All WT-infected eyes cluster together. Except for one WT+OxPAPC eye, all uninfected, ∆slpA-infected, and two WT+OxPAPC eyes clustered together. (C) Variations in the diversity of gene expression profiles across different groups. Shannon index was used to calculate the diversity. Statistical significance was calculated using unpaired t-test. *P = 0.0152, **P = 0.0043, and #P = 0.0198. (D) Heatmap for the visualization of gene expression. The red and green color gradient represents the upregulation and downregulation of genes. Red indicates increased, whereas green represents reduced gene expression. Sample groups were color-coded by gray (uninfected), black (WT-infected), light blue (WT+OxPAPC), and deep blue (∆slpA-infected). Functional groups were color-coded in the Y axis as follows: chemoattractants (light pink), complement (light blue), cytokines (yellow), innate elements (purple), kinases (deep pink), and peptidases (green).

**Bacterial Strains**

*Bacillus thuringiensis* subsp. Galleriae NRRL 4045 (WT) and its isogenic S-layer protein-deficient mutant (∆slpA) were used to initiate experimental endophthalmitis, as previously described. These strains were grown in brain heart infusion (BHI; VWR, Radnor PA) broth for 18 hours to early stationary phase and diluted to 100 CFU/0.5 μL for intravitreal injections.

**Mice and Intraocular Infection**

C57BL/6j mice were purchased from Jackson Laboratories (Bar Harbor, ME, USA; stock no. 000664). Upon arrival, mice were housed on a 12 hour on/12 hour off light cycle in biohazard level 2 conditions, and acclimatized for at least two weeks to equilibrate their microbiota. A ketamine (85 mg/kg body weight; Ketasthesia, Henry Schein Animal Health, Dublin, OH, USA) and xylazine (14 mg/kg body weight; AnaSed; Akorn Inc., Decatur, IL, USA) cocktail was used to sedate the mice. Sample size was determined based on our previous gene expression study. Four groups of 8- to 10-week-old mice with three mice in each group were used in these experiments (Fig. 1A). Groups 1 and 2 were infected with 100 CFU WT *B. thuringiensis*/0.5 μL BHI, and Group 3 was infected with 100 CFU ∆slpA *B. thuringiensis*/0.5 μL BHI, as previously described. At four hours postinfection,
Group 2 was intravitreally treated with the synthetic TLR2/4 inhibitor OxPAPC (Invivogen, Carlsbad, CA, USA; 30 ng/μL) (WT+OxPAPC).34 Uninfected mice (Group 4) were used as control.

Harvesting Mouse Eyes and RNA Preparation

At 10 hours postinfection, all mice were euthanized by CO2 inhalation. Experimental and control eyes were harvested and transferred to individual 1.5 mL screw-cap tubes containing sterile 1 mm glass beads (BioSpec Products, Inc., Bartlesville OK, USA) and 400 μL lysis buffer (RLT) from an RNeasy kit (Qiagen, Germantown, MD, USA). Eyes were homogenized using a Mini-BeadBeater (BioSpec Products Inc.) for 120 seconds (two pulses of 60 seconds each). Eye homogenates were spun in a centrifuge and transferred into another screw-capped tube containing 0.1 mm glass beads (BioSpec Products Inc.) and homogenized for 60 seconds in the Mini-BeadBeater. Tissue lysates were recovered by centrifugation and processed for total RNA purification using the RNeasy kit according to the manufacturer’s instructions. Genomic DNA was removed (TURBO DNA-free kit; ThermoFisher Scientific, Inc., Waltham, MA, USA), and eluted RNA was cleaned and concentrated (RNA Clean & Concentrator; Zymo Research, CA, USA). RNA purity and concentrations were confirmed via Nanodrop.

NanoString Analysis

In this technique, biotin-labeled capture and fluorescent-labeled reporter probes hybridize to specific target transcripts. The nCounter NanoString analysis system counts the immobilized RNAs using their barcodes. The NanoString assay was performed using total RNA samples from whole mouse eye. From each sample, 100 ng of RNA was hybridized with NanoString’s XT PGX Mnv2 Inflammation code set containing 248 mouse inflammatory genes and six housekeeping genes using NanoString’s nCounter XT Code-Set Gene Expression Assay protocol. After hybridization for 16 hours at 65°C, the samples were loaded onto an nCounter Cartridge and run on the NanoString Sprint platform. Data was normalized using NanoString’s nSolver Analysis Software v 4.0. Normalized data of 248 inflammatory genes was then used to analyze the expression profile in WT-infected, WT-infected and OxPAPC-treated (WT+OxPAPC), and ΔslpA-infected eyes compared to uninfected eyes.

Statistics

NanoString analysis was performed in NanoString’s nSolver Analysis Software v 4.0. After data normalization, GraphPad Prism 7 (Graph-Pad Software, Inc., La Jolla, CA, USA) was used for the comparative analysis. Multiple t-tests were performed to compare the means of individual genes from two different groups. Gene expression in uninfected eyes was compared with that of WT-infected, WT+OxPAPC, and ΔslpA-infected eyes. We also compared WT-infected eyes with WT+OxPAPC and ΔslpA-infected eyes. P values less than 0.05 were significant.

RESULTS

Interfering with TLR2 and TLR4 Activation Affected Inflammatory Gene Expression in the Eye

*Bacillus* induces a robust intraocular inflammatory response that irreversibly damages nonregenerative retinal tissues and results in a devastating clinical outcome. *Bacillus* SLP likely triggers the host inflammatory pathway by activating both TLR2 and TLR4.33,34 To explore the interference of innate activation on intraocular inflammatory responses during the later stages of *Bacillus* endophthalmitis, we performed NanoString analysis on total RNA isolated from our experimental groups. Figure 1A represents the schematics of our experimental approach. In Figure 1B, we performed principal component analysis (PCA) to understand the variability of gene expression in our experimental groups. The normalized mouse inflammatory genes were plotted in ClustVis, an internet-based tool for imaging multivariate data clustering. All replicates of WT-infected eyes clustered together. Except for one eye in the WT+OxPAPC group, replicates of three uninfected, three ΔslpA-infected, and two WT+OxPAPC eyes were clustered together, indicating transcriptional similarities among these groups. In Figure 1C, we calculated the Shannon diversity index to understand the diversities of expressed genes between groups. Compared to uninfected eyes, gene expression was more diverse in WT-infected eyes. However, compared to WT-infected eyes, gene expression was less diverse in WT+OxPAPC or ΔslpA-infected eyes. In Figure 1D, we used the R package “pheatmap” for visualization of gene expression in our groups.33 Most of the inflammatory genes in three uninfected, three ΔslpA-infected, and two WT+OxPAPC eyes had low levels of expression. In contrast, most of the genes in three WT-infected eyes and one WT+OxPAPC eye had high expression levels. Branch lengths at the top of the heat map represent correlations of gene expression, with longer branches indicating a lower correlation. All uninfected, two WT+OxPAPC, and ΔslpA-infected eyes were highly correlated with each other, whereas all WT-infected and one WT+OxPAPC eye were highly correlated with one another. On the Y-axis, we grouped and color-coded these inflammatory genes based on their function: chemoattractants, complement, innate immune genes, cytokines, kinases, and peptidases. Together, the distinct gene expression clusters and expression patterns demonstrate that mouse ocular inflammatory gene expression was muted if SLP was absent in the infecting strain or if infected eyes were treated with the TLR inhibitor OxPAPC.

Interfering with TLR2 and TLR4 Activation Reduced Ocular Inflammatory Responses

We further probed our NanoString data to quantify genes that were altered in our experimental groups. Figures 2A through 2C and 2Gi and 2Gii demonstrate that among the 248 mouse inflammatory genes, 137 genes were significantly upregulated in WT-infected eyes compared to uninfected eyes. However, the number of significantly upregulated genes in WT+OxPAPC eyes and ΔslpA-infected eyes was only 44 and 34, respectively (Fig 2Gii) compared to that of uninfected eyes. Compared to uninfected eyes, expression of nine genes, which were common to all noncontrol groups, was significantly increased (Fig 2Gi). Similarly, expression of 91 genes which were common to all noncon-
Interfering with TLR2 and TLR4 activation reduced ocular inflammatory responses. Volcano plot analysis of NanoString data derived from WT-infected, WT+OxPAPC, and ΔslpA-infected eyes compared with A–C uninfected eyes and D, E WT-infected eyes. For A to C and D to E the x-axis indicates the log fold change relative to uninfected eyes and WT-infected eyes, respectively. The y-axis shows the negative log10 P-value. Each dot represents an individual gene. TLR2 and TLR4 are indicated by a blue and red dot, respectively. Significance was assessed using multiple t-tests, and P values of < 0.05 (dotted line) were considered to be significant. Any gene above the dotted line was either significantly (A–C) increased or (D, E) decreased. F Percent of differentially expressed mouse inflammatory genes in WT+OxPAPC and ΔslpA-infected eyes relative to uninfected eyes or WT-infected eyes. G Venn diagram (i–iv) showing the number of differentially expressed genes in WT+OxPAPC and ΔslpA-infected eyes compared to uninfected or WT-infected eyes. Shading indicates no change in expression (dark gray), increased expression (black), and reduced expression (light gray).

trol groups remained unchanged compared to that of uninfected eyes (Fig. 2Gii). We found 66 and 123 genes significantly decreased in WT+OxPAPC and ΔslpA-infected eyes, respectively, compared to WT-infected eyes (Figs. 2D, 2E, and 2Gii). Compared to TLR2 and TLR4 expression in WT-infected eyes, the expression of TLR2 (blue) and TLR4 (red) in WT+OxPAPC and ΔslpA-infected eyes was decreased, suggesting that OxPAPC indeed interfered with the innate activation of those two pathways. In Figure 2Giii, the expression of 61 significantly upregulated genes in WT-infected eyes was blunted in WT+OxPAPC and ΔslpA-infected eyes. One gene, protein kinase C alpha (PRKCA), was significantly downregulated in WT-infected eyes but upregulated in WT+OxPAPC and ΔslpA-infected eyes (Supplementary Table S1). There were no differences in expression of 103 genes in the four groups (Fig. 2Giv). Compared to uninfected eyes, 55% of total genes were differentially expressed in WT-infected eyes. In contrast, only 18% and 14% of genes were differentially expressed in WT+OxPAPC and ΔslpA-infected eyes compared to uninfected eyes, respectively. Compared to WT-infected eyes, expression of 27% and 50% genes was decreased in WT+OxPAPC and ΔslpA-infected eyes, respectively (Fig. 2F). Taken together, these findings further suggested that inflammation was not as robust when SlpA was absent in Bacillus or when TLR2 and TLR4 was inhibited by OxPAPC.

Interfering with TLR2 and TLR4 Activation Blunted Complement Gene Expression in the Eye

Activation of the complement cascade is an initial and important line of defense in bacterial infection. Studies about the impact of complement factors in the pathogen-
Innate Immune Interference and Endophthalmitis

FIGURE 3. Interfering with TLR2 and TLR4 activation blunted complement gene expression in the eye. Analysis of complement factor expression in WT-infected, WT+OxPAPC, and ΔslpA-infected eyes. (A) Fold changes of complement factor expression in WT-infected, WT+OxPAPC, and ΔslpA-infected eyes compared to uninfected eyes. (B) Compared to WT-infected eyes, decreased expression of C3, Cfb, C6, C7, C1qa, C1qb, C1ra, C8b, and C1s transcripts were observed in WT+OxPAPC or ΔslpA-infected eyes. Values represent the mean ± SEM of complement counts at 10 hours after infection for three different mouse eyes and a P value of < 0.05 was considered significant.

esis of intraocular diseases is limited. Cobra venom factor-decomplemented Guinea pigs had impaired host defense during intraocular infection with *Staphylococcus epidermidis* and *Pseudomonas aeruginosa*. When complement levels returned to baseline in those guinea pigs, their host defenses recovered. In contrast, the absence of complement component C3 did not alter inflammation in a mouse model of experimental *S. aureus* endophthalmitis. These findings suggest diversity in the contribution of the complement cascade in different endophthalmitis models. Figure 3A depicts the log fold change of complement pathway components in WT-infected, WT+OxPAPC, and ΔslpA-infected eyes compared to uninfected eyes. Expression of complement factors b (Cfb), C3, C6, and C7 in WT-infected eyes was 19.6-, 9.9-, 2.9-, and 2.6-fold higher, respectively, compared to uninfected eyes. In contrast, compared to WT-infected eyes, the expression of these genes was decreased in both WT+OxPAPC and ΔslpA-infected eyes (Supplemen-
Interfering with TLR2 and TLR4 Activation

Reduced Chemoattractant Gene Expression in the Eye

During endophthalmitis, cytokines and chemokines drive ocular inflammation. In TNFα−/− and CXCL1−/− mice, neutrophil recruitment was suppressed. However, this resulted in an elevated bacterial load and disease severity in TNFα−/− mice,45 but no change in bacterial load and a reduction in disease severity in CXCL1−/− mice.45 These contrasting outcomes suggest a potential role of additional inflammatory cytokines during infection. Here, we focused on the expression of 51 mouse inflammatory cytokines during Bacillus endophthalmitis. Figure 5A depicts the log-fold changes of these genes in our experimental groups. Eighteen inflammatory cytokine genes were significantly increased in WT-infected eyes compared to uninfected eyes. These genes were decreased in WT+OxPAPC or ∆slpA-infected eyes compared to WT-infected eyes (Fig. 5B, Supplementary Table S4). Figure 5C depicts the expression of IL-1β, IL-6, and TNFα, and several other proinflammatory cytokines in our infection and treatment groups. In addition to IL-1β, IL-6, and TNFα, the expression of colony-stimulating factor (CSF)3, CSF2, IL-1α, IL-23α, transforming growth factor (TGF)β1, and IL-12β were significantly increased in WT-infected eyes compared to uninfected eyes. Expression of CSF3, IL-6, IL-1β, CSF2, IL-1α, TNFα, and IL-23α was significantly decreased in both WT+OxPAPC and ∆slpA-infected eyes compared to WT-infected eyes. Expression of TGFβ1 and IL-12β was reduced only in ∆slpA-infected eyes compared to WT-infected eyes.

Proinflammatory cytokines function in association with cytokine inhibitors to regulate inflammation. The expression of anti-inflammatory cytokines IL-1 receptor accessory protein (rap), IL-10, and IL-1 receptor antagonist (rn) was significantly increased in WT-infected eyes compared to uninfected eyes, but significantly reduced in ∆slpA-infected eyes compared to WT-infected eyes. The expression of IL-10 was reduced only in WT+OxPAPC eyes compared to WT-infected eyes (Fig. 5D). The expression of anti-inflammatory cytokines IL-13 and IL-4 were not changed in all groups. Relative to uninfected eyes, expression of CSF3, IL-6, IL-1β, CSF2, IL-1α, and TNFα was 50-fold or higher (Supplementary Table S4). These findings not only identified several inflammatory cytokines that were not expressed at four hours postinfection in Bacillus endophthalmitis but also identified blunted expression of others when TLR2 and TLR4 activation was blocked. Collectively, these findings suggested that the innate inhibition could be a viable strategy to avert inflammatory cytokine production during Bacillus endophthalmitis.

Interfering with TLR2 and TLR4 Activation

Reduced Cytokine Gene Expression in the Eye

During endophthalmitis, cytokines and chemokines drive ocular inflammation. In TNFα−/− and CXCL1−/− mice, neutrophil recruitment was suppressed. However, this resulted in an elevated bacterial load and disease severity in TNFα−/− mice,45 but no change in bacterial load and a reduction in disease severity in CXCL1−/− mice.45 These contrasting outcomes suggest a potential role of additional inflammatory cytokines during infection. Here, we focused on the expression of 51 mouse inflammatory cytokines during Bacillus endophthalmitis. Figure 5A depicts the log-fold changes of these genes in our experimental groups. Eighteen inflammatory cytokine genes were significantly increased in WT-infected eyes compared to uninfected eyes. These genes were decreased in WT+OxPAPC or ∆slpA-infected eyes compared to WT-infected eyes (Fig. 5B, Supplementary Table S4). Figure 5C depicts the expression of IL-1β, IL-6, and TNFα, and several other proinflammatory cytokines in our infection and treatment groups. In addition to IL-1β, IL-6, and TNFα, the expression of colony-stimulating factor (CSF)3, CSF2, IL-1α, IL-23α, transforming growth factor (TGF)β1, and IL-12β were significantly increased in WT-infected eyes compared to uninfected eyes. Expression of CSF3, IL-6, IL-1β, CSF2, IL-1α, TNFα, and IL-23α was significantly decreased in both WT+OxPAPC and ∆slpA-infected eyes compared to WT-infected eyes. Expression of TGFβ1 and IL-12β was reduced only in ∆slpA-infected eyes compared to WT-infected eyes.

Proinflammatory cytokines function in association with cytokine inhibitors to regulate inflammation. The expression of anti-inflammatory cytokines IL-1 receptor accessory protein (rap), IL-10, and IL-1 receptor antagonist (rn) was significantly increased in WT-infected eyes compared to uninfected eyes, but significantly reduced in ∆slpA-infected eyes compared to WT-infected eyes. The expression of IL-10 was reduced only in WT+OxPAPC eyes compared to WT-infected eyes (Fig. 5D). The expression of anti-inflammatory cytokines IL-13 and IL-4 were not changed in all groups. Relative to uninfected eyes, expression of CSF3, IL-6, IL-1β, CSF2, IL-1α, and TNFα was 50-fold or higher (Supplementary Table S4). These findings not only identified several inflammatory cytokines that were not expressed at four hours postinfection in Bacillus endophthalmitis but also identified blunted expression of others when TLR2 and TLR4 activation was blocked. Collectively, these findings suggested that the innate inhibition could be a viable strategy to avert inflammatory cytokine production during Bacillus endophthalmitis.
Interfering with TLR2 and TLR4 activation impacted innate immune gene expression in the eye. Expression of innate immune genes in mouse eyes. (A) Venn diagram showing the number of innate immune genes differentially upregulated between the groups. (B) Heatmap showing the expression of extracellular and intracellular innate receptors. TLR2, TLR4, TLR6, TLR8, intracellular receptor Nod2, and Nlrp3 were significantly upregulated in WT untreated eyes compared to uninfected eyes. (C) Innate immune genes such as MyD88, NF-κB, RelA, RelB, Ptsg2, Hspb1, STAT2, STAT3, and Hspb1 were significantly upregulated in WT-infected eyes compared to uninfected eyes. Values represent the mean ± SEM of transcript counts at 10 hours after infection for three different mouse eyes, and *P < 0.05 was considered significant.

Figure 6A depicts the log fold changes of 27 inflammatory chemokines and receptors that belong to CXC and CC groups. Fifteen chemokines were significantly increased in WT-infected eyes compared to uninfected eyes. Compared to WT-infected eyes, expression of these 15 chemokines was significantly reduced in WT+OxPAPC or ΔS/pA-infected eyes (Fig. 6B, Supplementary Table S5). Figure 6C depicts the expression of chemoattractants in our infection and treatment groups. Expression of CXCL1, CXCL2, CXCL10, CCL2, CCL3, CXCL3, CCL20, and CCL4 was elevated in WT-infected eyes compared to uninfected eyes. Expression of these genes was reduced 10-fold or greater in WT+OxPAPC or ΔS/pA-infected eyes compared to WT-infected eyes. Figure 6D depicts analysis of the expression of chemokine receptors. Expression of chemokine (C-C motif) receptor (CCR)1, chemokine (C-X-C motif) receptor (CXCR)2, CXCR4, and CCR7 was greater in WT-infected eyes compared to uninfected eyes, but was significantly reduced...
FIGURE 5. Interfering with TLR2 and TLR4 activation reduced cytokine gene expression in the eye. Expression of inflammatory cytokines from WT-infected, WT+OxPAPC, Δsll4-infected eyes were analyzed. (A) Log fold change of 51 mouse inflammatory cytokines from WT-infected, WT+OxPAPC, and Δsll4-infected eyes, compared to uninfected eyes. The darkest shading corresponds to the greatest fold changes. (B) Venn diagram showing the number of cytokine genes differentially expressed between the groups. (C) Elevated expression of proinflammatory cytokines CSF3, IL-6, IL-1β, CSF2, IL-1α, TNFα, IL-23α, TGFβ1, and IL-12β in WT-infected eyes compared to uninfected eyes. Compared to WT-infected eyes, expression of these pro-inflammatory cytokines was significantly reduced in WT+OxPAPC or Δsll4-infected eyes. (D) Compared to uninfected eyes, expression of anti-inflammatory cytokines IL-1rap, IL-10, and IL-1rn was elevated in WT-infected eyes, but was reduced significantly in WT+OxPAPC or Δsll4-infected eyes. Values represent the mean ± SEM of transcript counts at 10 hours after infection (N = 3). *P < 0.05 was considered significant.
FIGURE 6. Interfering with TLR2 and TLR4 activation reduced chemoattractant gene expression in the eye. NanoString analysis of CXC and CC chemokine groups analyzed in WT-infected, WT+OxPAPC, and ΔslpA-infected mouse eyes. (A) Log fold changes of inflammatory chemokines from WT-infected, WT+OxPAPC, and ΔslpA-infected eyes, compared to uninfected eyes. The darker shading corresponds to the greatest log-fold changes. (B) Venn diagram showing the number of chemoattractants differentially expressed between the groups. (C) At 10 hours after infection, expression of CXCL2, CXCL1, CXCL3, CCL20, CCL4, CCL3, CCL2, CCL7, CXCL10, and CXCL5 were upregulated in WT-infected, and significantly reduced in WT+OxPAPC and ΔslpA-infected eyes. (D) Expression of chemokine receptor CCR1, CXCR2, CXCR4, and CCR7 was also significantly upregulated in WT-infected eyes and reduced in WT+OxPAPC and ΔslpA-infected eyes. Values represent the mean ± SEM of transcript counts at 10 hours after infection for three individual mouse eyes. *P < 0.05 was considered significant.
In WT+OxPAPC or ΔslpA-infected eyes compared to WT-infected eyes. CCR7 expression was significantly increased in WT-infected eyes compared to uninfected eyes, and significantly reduced in ΔslpA-infected eyes compared to WT-infected eyes. There was no change in CCR7 expression between WT-infected and WT+OxPAPC eyes. Expression of CXCL2, CXCL1, CXCL3, CCL20, CCL4, CCL3, and CCL2 was 500-fold or higher relative to that of uninfected eyes (Supplementary Table S5). In addition to confirming our retina-specific transcriptome data at 4 hours postinfection with Bacillus, these findings identified the expression of additional chemoattractants in the whole eye during the later stages of infection. Altogether, these results demonstrated that the chemokine response is blunted when SlpA is absent in Bacillus and when TLR2 and TLR4 are inhibited by OxPAPC. Collectively, these results suggested the inhibition of TLR2 and TLR4 pathways might be a viable strategy to block the chemoattractant synthesis during Bacillus endophthalmitis, arresting the recruitment of inflammatory cells into the eye.

**DISCUSSION**

The inflammatory response is generally defined as a protective reaction to stimulation of the host defense system to invading pathogens or endogenous signals. When this protective response goes unchecked, dysregulated inflammatory responses occur and can worsen the disease severity. Cells involved in immune responses produce plethora of mediators including cytokines, chemokines, antibodies, and complement to aid in the war against invading pathogens. Although inflammatory responses protect tissue against these insults, the inflammation itself could cause damage. Immune privileged tissues resist immunogenic inflammation through multiple mechanisms. A well-controlled system to regulate the inflammatory response in the eye is necessary. We reported that Bacillus reach their maximum growth faster than most other Gram-positive endophthalmitis pathogen in the eye. Bacillus endophthalmitis is well recognized for creating an aggressive acute inflammatory response that results in retinal damage with rapid loss of vision within 12 to 24 hours, even as antibiotic and anti-inflammatory treatment is given. Avirulent *S. epidermidis* is generally cleared by an active but relatively tame inflammatory response. Current therapies for this severe blinding infection include intravitreal antibiotics that can sterilize the eye if given at an early phase of infection. Corticosteroids use in endophthalmitis is routine, but their usefulness has not been without controversy. Therefore identifying critical elements of inflammatory pathways that can be targeted to inhibit the intraocular inflammation are viable prospects for future therapeutics in Bacillus endophthalmitis. One of the earliest and most important events in inflammation is the recognition of the invading organisms by the host innate defense system. The retina is comprised of different types of cells necessary for the visual cycle, maintaining retinal integrity, and homeostasis. These cells also act as a defensive barrier to protect against invading pathogens by expressing innate receptors. We reported that during infection, Bacillus migrates in close proximity to the inner limiting membrane (ILM) of the retina, facilitating potential interactions between the retina and invading microbes. As such, the outer most layer of the bacterium may be the first to interact with innate receptors on cells lining the ILM.

S-layer protein (SLP), the outer most layer of the *Bacillus* envelope, serves as a protective barrier for the pathogen. Besides maintaining cell wall integrity, SLP provides additional benefits for the microbes to survive in diverse host environments, but can also activate the immune system. We recently reported that an absence of Bacillus SLP reduced disease severity and damage to the eye in murine experimental endophthalmitis. Activation of innate immunity and its effects on host tissue are significant events which contribute to the severity and poor visual outcomes of Bacillus endophthalmitis. In a follow-up study, we reported Bacillus SLP as an activator of the TLR2 and TLR4 pathways. In a recent transcriptome study of retinas of eyes infected with Bacillus, we reported elevated expression of 72 genes at four hours postinfection and decreased expression of these 72 genes in the absence of TLR4. The reduced inflammation and improved clinical outcomes in the absence of TLR2 or TLR4 or after TLR2 and TLR4 inhibition suggests innate immune pathway inhibition as a prospective option for the anti-inflammatory arm of treatment of Bacillus intraocular infection.

The nature of ocular tissue damage arising from an excessive inflammatory response in bacterial endophthalmitis is not completely understood. Based on our recent studies demonstrating positive clinical outcomes when TLR2 and TLR4 pathways are blocked, and building upon findings that treatment of experimental Bacillus endophthalmitis at four hours or earlier resulted in an improved clinical outcome, we further probed this treatment strategy by using NanoString to compare gene expression in whole eyes in our experimental groups. NanoString is better for targeted transcriptomics since there is no need for preparing gene libraries, enzymes, and processing as in other next generation sequencing (NGS) techniques. Unlike other alternatives, NanoString does not require polymerase activity, and therefore the chance of introducing bias is lower. NanoString can identify RNAs in a heterogeneous sample, even one that contains cells from different species. In this study, we focused on 248 mouse genes potentially associated with the inflammatory response in experimental murine Bacillus endophthalmitis. Distinct clusters of infection groups in our principal component analysis suggested a similar pattern of gene expression within each group. Except for one eye in the WT+OxPAPC group, we observed specific clusters in our groups. One explanation for this outlier could be a microinjection error during intraocular OxPAPC delivery. Overall, however, the clustering of uninfected, ΔslpA-infected, and WT+OxPAPC eyes suggested transcriptional similarities among these groups.

Most of the genes analyzed were differentially expressed in WT-infected eyes compared to uninfected eyes. Expression of most of these genes were reduced in WT+OxPAPC, and ΔslpA-infected eyes compared to WT-infected eyes. Expression of 61 genes which were significantly increased in WT-infected eyes compared to uninfected eyes were decreased in WT+OxPAPC or ΔslpA-infected eyes compared to WT-infected eyes (Supplementary Table S1). Only one gene, protein kinase C alpha (PRKCa), was decreased in WT-infected eyes compared to uninfected eyes but significantly increased in ΔslpA-infected and WT+OxPAPC eyes. In the retina, PRKCa modulates rod photoreceptor and bipolar cell function. PRKCa and few other isoforms of PRKCa are reported to be present in ganglion, amacrine, and RPE cells. PRKCa contributes to cellular proliferation, differentiation, motility, apoptosis, and inflammation. It has been
reported that TLR1/2-driven activation of AP-1, MAPK, and NF-κB and secretion of IL-6, IL-10, and TNF-α by dendritic cells requires the activation of PRKCa. Overexpression of PRKCa resulted in loss of tight junctions, which reduced the transepithelial permeability in an epithelial cell line LLC-PK1. PRKCa has been linked to the regulation of the production of nitric oxide, an innate effector molecule and inflammation modulator in vascular smooth muscle, murine macrophages, and murine microglia. PKC has also been shown to induce the production of various inflammatory cytokines from human bronchial epithelia cells, suggesting that PKC activation might be essential for the initial defense mechanism in the airway against pathogens. The absence of PRKC isoform δ in mice resulted in reduced inflammatory mediator production and neutrophil infiltration in an asbestos-associated disease model. We observed decreased expression of PRKCa in WT-infected eyes compared to uninfected eyes, which suggested a potential anti-inflammatory property of PRKCa. However, this was not reflected in the pathogenesis in vivo, since we found increased PMN influx in WT-infected eyes. PKC is expressed differentially in various mammalian tissues, and is highly enriched in brain and lymphoid organs. Together, this suggests that PRKC function and contributions to inflammation may be tissue and organ-specific.

Improving treatment options is a main focus of our research in endophthalmitis, which is highly critical for Bacillus endophthalmitis, where the majority of infected eyes lose vision. The contribution of the host immune response in mediating bystander damage and visual function loss is still not completely understood. Complement is an integral component of the innate defense against infection and a prime mediator of tissue inflammation. We recently reported that pentraxin 3 (PTX3), which activates the classical complement pathway via C1q to expedite pathogen recognition and clearance, was expressed as early as 4 hours in retinas of mouse eyes infected with Bacillus. In this study, TLR2 and TLR4 interference resulted in decreased expression of PTX3 and other complement pathway-related genes. In endophthalmitis caused by S. epidermidis and P. aeruginosa, complement exhibited a beneficial effect in ocular defense. However, for S. aureus endophthalmitis, complement was not protective. Because the interplay between complement and bacteria is relevant in inflammation, studying the role of complement pathway-associated genes in the pathogenesis of a rapidly blinding infection like Bacillus endophthalmitis may be valuable.

Innate recognition activation of associated pathways are complex events requiring coordinate regulation of multiple cellular signaling components. We previously reported the role of TLR2 and TLR4 in the pathogenesis of Bacillus endophthalmitis. TLR2 is also important for inflammatory responses in S. aureus endophthalmitis. TLR6 forms a heterodimer with TLR2 and recognizes diacylated lipoproteins such as lipoteichoic acid on the cell wall of Gram-positive bacteria. Synergistic interactions of TLR2/6 and TLR9 provide resistance against lung infection. However, TLR8, which is an innate endosomal receptor, usually senses viral infection. However, TLR8 may also detect RNA from S. aureus, which occurs when the bacterium is internalized and degraded inside phagocytic cells. Activation of TLR2 could serve as an inhibitor of TLR8 activation inside the cell. This counter-inhibition might serve as a safety mechanism to avoid exaggerated immune activation. Because TLR2 and TLR8 sense different PAMPs, this cross-regulation represents a fine-tuning to the excessive immune response toward different classes of pathogens. Neither TLR6 nor TLR8 expression was detected at four hours postinfection in retinas of B. cereus-infected mice. However, TLR6 and TLR8 were expressed, but not to a great degree at 10 hours in B. cereus-infected mouse eyes in this study, so this expression may emanate from tissues or cells other than in the retina. Inflammomasomes are important because they aid in maintaining homeostasis with commensals and protecting tissues against pathogens. Although we reported that the absence of Nrpl3 in Nrpl3−/− global knockout mice did not affect the clinical outcomes in Bacillus endophthalmitis at eight hours postinfection, a recent report suggested that B. cereus non-hemolytic enterotoxin (NHE) activated the NLRP3 inflammasome and caused pyroptosis in primary BMDMs and that NHE and hemolysin BL functioned synergistically to induce inflammation in mice. Here, we observed significant increased expression of TLR2, TLR4, and Nrpl3 in WT-infected eyes compared to uninfected eyes. Expression of these innate receptors were reduced after TLR2 and TLR4 inhibition or infection with the ΔslpA mutant. These results suggested that interference of innate pathways affected the expression of other innate receptors in the eye, perhaps further limiting inflammation.

An absence of MyD88 or TRIF in mice resulted in improved clinical outcomes of Bacillus endophthalmitis. The importance of MyD88 has also been reported for S. aureus endophthalmitis. Arachidonic acid–derived lipid mediators control cell metabolism, proliferation, migration, and apoptosis. Prostaglandin-endoperoxide synthase (Ptgs) 2 converts arachidonic acid to prostaglandin endoperoxide H2 (PGE2), which immunomodulates neutrophil activation. Ptgs2 was upregulated in a TLR4-dependent manner in the retinas of mice infected with Bacillus. Activation of TLRs and their adaptors transfer the activation signal via a series of factors that ultimately activate inflammatory transcription factor NF-κB in the nucleus. We recently reported that Bacillus SLP activated NF-κB in human retinal Muller cells. Here, we observed increased expression of MyD88, Ptgs2, RelA, RelB, STAT2, STAT3, and Hspb1 in WT-infected eyes compared to that of uninfected eyes. We also observed blunted expression of these innate immune genes when TLR2 and TLR4 pathways were interfered with. Unfortunately, TRIF expression was not included in the NanoString panel, so this was not tested. Phosphorylation of STAT3 and Hspb1 influences NF-κB activation and promotes the inflammatory response. Retinal transcriptome analysis of S. aureus endophthalmitis showed elevated expression of STAT3 in a TLR2-dependent manner. SOCS3, which is a negative regulator of cytokine signaling, inhibits STAT3 phosphorylation and prevents over-activation of proinflammatory genes. The enhanced expression of STAT3 in our study may help to explain why increased TLR4-dependent expression of SOCS3 failed to negatively regulate inflammatory gene expression.

The Bacillus cell wall incites robust intraocular inflammation. Bacillus SLP triggers the expression of inflammatory mediators CXCL1, TNFα, CCL2, and IL-6 in human retinal Muller cells. Inflammatory cytokines, which are polypeptides, can act as intercellular messengers and mediate the process of inflammation and repair. In the eye, in response to an infection or injury, inflammatory cytokines can be produced by corneal epithelium, RPE, microglia, macrophages, endothelial cells, and other immune cells. Here, we analyzed the expression of 51 inflammatory
cytokines and found 50-fold or greater expression of IL-1α, IL-1β, IL-6, CSF2, CSF3, and TNFα in WT-infected eyes (Supplementary Table S4). CSF is a glycosylated protein that stimulates neutrophil production and release into the blood, and enhances their survival, differentiation, proliferation, and functions.31,52 Quite a bit is known about CSF's role in treating neutropenia and hematopoietic mobilization, but information about its role in ocular infection is limited. Neutrophils are recruited into the eye as early as four hours postinfection and are the primary immune cells that migrate into the vitreous after infection.53 Because of its role in neutrophil migration, the increased expression of CSF in the eye may contribute to the rapid migration of neutrophils into the eye in experimental Bacillus endophthalmitis. Because we did not observe an increase in the expression of CSF at four hours postinfection in retinas of infected mouse eyes,56 the source of CSF may not be the retinal cells at this early stage of infection.

IL-6 is both a pro- and anti-inflammatory cytokine, depending upon the environment.95 IL-6 expression increases during the course of bacterial endophthalmitis53,77 in a TLR2- and TLR4-dependent manner.36,57 Expression of IL-6 and IL-1β was also increased with time in retinas of S. aureus-infected mice; however, compared to wild type, expression of these mediators was reduced when TLR2 was absent.98 Surprisingly, the absence of IL-6 in IL-6−/− mice did not affect the overall outcome of Bacillus endophthalmitis.95 Here, interference with TLR 2 and TLR4 also resulted in reduced expression of the anti-inflammatory cytokine IL-10 at 10 hours postinfection. Intravenous administration of IL-10, a potent inhibitor of cytokine production, reduced neutrophil chemotaxis in LPS-induced uveitis.94,95 We also observed increased expression of the homologous and are powerful neutrophil chemoattractants.96–98 We reported TLR4-dependent expression of CCL2 and CCL3 in the retinas of Bacillus-infected mouse eyes at four hours postinfection.50 Here, we demonstrated blunted expression of CCL2, CCL3, CCL4, CCL14, and CCL20 when TLR2 and TLR4 activation was interfered with. CXCR2, CXCR4, CCR1 are the major CXC and CC chemokine receptors that bind and respond to chemokines to mobilize immune cells. CXCR1 and CXCR2 interact with CXCL1-8 and are expressed on the neutrophil surface.109 Therefore the increased expression of CXC and CC receptors and chemoattractants in WT-infected eyes may be related to the presence of neutrophils in the intraocular environment at this stage of infection. We reported that the absence of CXCL1 in CXCL1−/− mice or intraocular injection of anti-CXCL1 improved the clinical outcome of Bacillus endophthalmitis, resulting in minimal inflammation and retained retinal function.15 The absence of TLR2 resulted in reduced expression of CXCL2 in mouse retinas during experimental S. aureus endophthalmitis.88 Although CCL2 is not known to recruit neutrophils, treatment with anti-CCL2 or anti-CCL3 significantly blunted neutrophil infiltration, resulting in attenuated corneal damage in a mouse model of P. aeruginosa keratitis.109 The expression of more than half of the chemokines that we analyzed was blunted when TLR2 and TLR4 activation was interfered with (Supplementary Table S5). Together, these findings suggested a therapeutic potential for targeting CC and CXC chemokines in controlling ocular inflammation during Bacillus endophthalmitis and possibly other forms of endophthalmitis.

During ocular infection with an avirulent organism, the innate immune response is usually sufficient to clear the infection. However, ocular infections caused by a more virulent pathogen such as Bacillus are not easily cleared, and the robust innate response induced by Bacillus cell wall components can lead to significant host-mediated damage. Therapeutics designed to counteract the activities of individual bacterial products might not prevent the damage caused by numerous other toxic microbial factors, nor reduce inflammation-induced injury. The fact that the absence of Bacillus S-layer had such a profound effect on intraocular inflammation and infection highlights S-layer as an important virulence factor in this disease. The capacity of Bacillus S-layer to activate both TLR2 and TLR4 may partially explain why Bacillus endophthalmitis results in such an explosive inflammatory response.

Innate inhibition by interfering the TLR pathways has been promising in several inflammation-associated diseases such as Gram-negative bacterial sepsis, acute lung inflammation injury, atherosclerosis, acute and chronic inflammatory pain.37,49,105–109 The negative regulation of inflammation by oxidized phospholipid has been effective for the treatment of these diseases. Oxidized phospholipids have been shown to inhibit LPS-induced pyroptosis, IL-1β release, and septic shock, providing a basis for therapeutic innate immune interference in Gram-negative bacterial sepsis.87 Oxidized phospholipids also significantly reduced LPS-induced cytokine production, barrier disruption, and tissue inflammation in rats.110 Because the inflammatory response triggered by the innate immune response to Bacillus endophthalmitis may cause severe damage to ocular tissues, targeting these pathways could be a viable anti-inflammatory approach which also preserves retinal function. We identified 25 inflammatory genes (Table) which were upregulated 50-fold or higher after infection.
with WT Bacillus, and demonstrated reduced expression of these 25 inflammatory genes after OxPAPC-mediated TLR2 and TLR4 inhibition or infection with a SLP-deficient Bacillus. The expression of 12 chemotaxants among these 25 genes was likely the driver of excessive neutrophil infiltration during Bacillus infection. Together, these results suggest that Bacillus SLP potentially triggered the expression of these inflammatory genes, which could be prevented by inhibiting the activation of TLR2 and TLR4 pathways. Although we demonstrated improved clinical outcomes in Bacillus endophthalmitis when infected eyes were treated with OxPAPC, we did not test coadministration with antibiotics. The administration of anti-inflammatory drugs alone is not an acceptable standard of care for endophthalmitis.

Present treatment options, including intravitreal therapeutics and vitrectomy, are often unsuccessful at preventing irreversible damage to the retina that can result in total loss of vision. A complete understanding of the role of the host response in the disease process is required to design rational treatment strategies to improve the visual outcome of this disease. Infection with an SLP-deficient pathogen,\textsuperscript{33} chemical inhibition of TLR2 and TLR4 after infection,\textsuperscript{34} mice deficient in TLR2,\textsuperscript{36} TLR4,\textsuperscript{37} MyD88,\textsuperscript{38,85} and TRIF,\textsuperscript{38} mice pre-treated with TLR2 agonists,\textsuperscript{77,99} mice deficient in CXCL1,\textsuperscript{45} and anti-CXCL1 administration\textsuperscript{45} each resulted in reduced inflammation and better clinical outcomes. Collectively, these findings suggest targeting innate immune activation and their response elements as potential therapeutic strategies. Here, we identified several innate inflammatory genes which could be investigated further as potential anti-inflammatory targets in endophthalmitis.

Further studies are necessary to determine whether expression of these genes contributes directly to the pathogenesis of Bacillus endophthalmitis. All things considered, this study demonstrated a viable strategy to minimize the otherwise robust and sight-threatening inflammation in Bacillus endophthalmitis and perhaps this infection caused by other pathogens.

**Acknowledgments**

The authors thank Agnes Fouet (Institut Cochin, INSERM U1016, CNRS UMR8104, University Paris Descartes, Paris, France) for providing the bacterial strains, Md Abdul Wadud Khan (Department of Surgical Oncology, The University of Texas MD Anderson Cancer Center) for generating the diversity index and heatmap, Feng Li and Mark Dittmar (OUHSC P30 Live Animal Research Support Grant Award (to MCC and MHM), a Presbyterian Health Foundation Equipment Grant (to Robert

---

**Table.** Top 25 Differentially Expressed Mouse Inflammatory Genes in Bacillus-Infected Eyes at 10 Hours Postinfection

| Gene Symbol | Gene Title | Accession   | Un/WT | WT/WT+OxPAPC | WT/ΔslpA |
|-------------|------------|-------------|-------|--------------|----------|
| CXCL2       | Chemokine (C-X-C motif) ligand 2 | NM_009140.2 | 7605  | −87          | −277     |
| CXCL1       | Chemokine (C-X-C motif) ligand 1 | NM_008176.2 | 4403  | −98          | −169     |
| CXCL3       | Chemokine (C-X-C motif) ligand 3 | NM_020320.2 | 3091  | −153         | −336     |
| CSF3        | Colony-stimulating factor 3 (granulocyte) | NM_009971.1 | 2639  | −67          | −136     |
| CCL20       | Chemokine (C-C motif) ligand 20 | NM_016960.1 | 2086  | −157         | −118     |
| CCL4        | Chemokine (C-C motif) ligand 4 | NM_013652.1 | 1454  | −13          | −218     |
| IL6         | Interleukin 6 | NM_031168.1 | 1361  | −21          | −30      |
| CCL3        | Chemokine (C-C motif) ligand 3 | NM_011337.1 | 1269  | −39          | −151     |
| IL1f        | Interleukin 1 beta | NM_008361.3 | 848   | −18          | −53      |
| CCL2        | Chemokine (C-C motif) ligand 2 | NM_013652.3 | 824   | −10          | −14      |
| CHI3L3      | Chitinase 3-like 3 | NM_009892.1 | 434   | −200         | −185     |
| CCR1        | Chemokine (C-C motif) receptor 1 | NM_009912.4 | 272   | −5           | −107     |
| CSF2        | Colony stimulating factor 2 (granulocyte-macrophage) | NM_009969.4 | 268   | −78          | −223     |
| CCL7        | Chemokine (C-C motif) ligand 7 | NM_013654.2 | 243   | −8           | −16      |
| CXCR2       | Chemokine (C-C motif) receptor 2 | NM_009090.3 | 231   | −43          | −82      |
| PTGS2       | Prostaglandin-endoperoxide synthase 2 | NM_011198.3 | 218   | −11          | −31      |
| IL-1α       | Interleukin 1 alpha | NM_010554.4 | 166   | −24          | −67      |
| NLRP3       | NLR family, pyrin domain containing 3 | NM_145827.3 | 140   | −18          | −37      |
| AREG        | Amphiregulin | NM_009704.3 | 131   | −12          | −55      |
| CXCL5       | Chemokine (C-X-C motif) ligand 5 | NM_009141.2 | 119   | −53          | −59      |
| CXCL10      | Chemokine (C-X-C motif) ligand 10 | NM_021274.1 | 104   | −7           | −21      |
| TNFα        | Tumor necrosis factor | NM_013693.1 | 91    | −18          | −38      |
| FOS         | FBβ osteosarcoma oncogene | NM_010234.2 | 82    | −13          | −13      |
| IL2α        | Interleukin 23, alpha subunit p19 | NM_031252.1 | 66    | −15          | −39      |
| IL1RN       | Interleukin 1 receptor antagonist | NM_031167.4 | 56    | 0            | −35      |

The fold changes of mouse inflammatory genes expression in WT-infected, WT+OxPAPC, and ΔslpA-infected eyes relative to uninfected and WT-infected eyes are shown. A negative fold change represents reduced expression relative to WT-infected eyes.
Innate Immune Interference and Endophthalmitis

E. Anderson, OUHSC, and an unrestricted grant to the Dean A. McGee Eye Institute from Research to Prevent Blindness.

Disclosure: M.H. Mursalin, None; P.S. Coburn, None; E.C. Miller, None; E.T. Livingston, None; R. Astley, None; M.C. Callegan, None

References

1. Nes T. Endophthalmitis. Ophthalmologist. 2018;115:697–706.
2. Durand ML. Endophthalmitis. Clin Microbiol Infect. 2013;19:227–234.
3. Durand ML. Endophthalmitis: An Overview. Cham, Switzerland: Springer International; 2016.
4. Relhan N, Forster RK, Flynn HW, Jr. Endophthalmitis: then and now. Am J Ophthalmol. 2018;178:xx–xxvii.
5. Callegan MC, Engelbert M, Parke DW, 2nd, Jett BD, Gilmore MS. Bacterial endophthalmitis: epidemiology, therapeutics, and bacterium-host interactions. Clin Microbiol Rev. 2002;15:111–124.
6. Callegan MC, Gilmore MS, Gregory M, et al. Bacterial endophthalmitis: therapeutic challenges and host-pathogen interactions. Prog Retin Eye Res. 2007;26:189–203.
7. Coburn PS, Callegan MC. Endophthalmitis. In: Rumelt ML. Endophthalmitis. In: Rumelt R, ed. Advances in ophthalmology. Rijeka, Croatia: IntechOpen; 2012:319–340.
8. Parkunan SM, Callegan MC. The pathogenesis of bacterial endophthalmitis. In: Durand MJ, Young LHY (ed), Endophthalmitis. Cham, Switzerland: Springer International Publishing; 2016:17–47.
9. Mursalin MH, Livingston ET, Callegan MC. The cereus matter of Bacillus endophthalmitis. Exp Eye Res. 2020;193:107959.
10. Coburn PS, Miller FC, LaGrow AL, et al. Disarming pore-forming toxins with biomimetic nanosponges in intraocular infections. mSphere. 2019;4:e00262–19.
11. Callegan MC, Booth MC, Jett BD, Gilmore MS. Pathogenesis of Gram-positive bacterial endophthalmitis. Infect Immun. 1999;67:3348–3356.
12. Callegan MC, Cochrane DC, Kane ST, Gilmore MS, Gominet M, Lereclus D. Contribution of membrane-damaging toxins to Bacillus endophthalmitis pathogenesis. Infect Immun. 2002;70:5381–5389.
13. Callegan MC, Cochrane DC, Kane ST, et al. Virulence factor profiles and antimicrobial susceptibilities of ocular Bacillus isolates. Curr Eye Res. 2006;31:693–702.
14. Callegan MC, Kane ST, Cochrane DC, Gilmore MS, Gominet M, Lereclus D. Relationship of plcR-regulated factors to Bacillus endophthalmitis virulence. Infect Immun. 2003;71:3116–3124.
15. Callegan MC, Kane ST, Cochrane DC, et al. Bacillus endophthalmitis: Roles of bacterial toxins and motility during infection. Invest Ophthalmol Vis Sci. 2005;46:3233–3238.
16. Moyer AI, Ramadan RT, Novosad BD, Astley R, Callegan MC. Bacillus cereus-induced permeability of the blood-ocular barrier during experimental endophthalmitis. Invest Ophthalmol Vis Sci. 2009;50:3783–3793.
17. Moyer AI, Ramadan RT, Thurman J, Burroughs A, Callegan MC. Bacillus cereus induces permeability of an in vitro blood-retina barrier. Infect Immun. 2008;76:1558–1567.
18. Beecher DJ, Olsen TW, Somers EB, Wong AC. Evidence for contribution of tripartite hemolysin BC, phosphatidylcholine-prefering phospholipase C, and collagenase to virulence of Bacillus cereus endophthalmitis. Infect Immun. 2000;68:5209–70.
19. Sankararaman S, Velauthum S. Bacillus cereus. Pediatr Rev. 2013;34:196–197.
20. Drobniewski FA. Bacillus cereus and related species. Clin Microbiol Rev. 1993;6:324–338.
21. Bottone EJ. Bacillus cereus, a volatile human pathogen. Clin Microbiol Rev. 2010;23:382–398.
22. Fouet A, Mesnage S. Bacillus anthracis cell envelope components. Curr Top Microbiol Immunol. 2002;271:87–113.
23. Siegel SD, Liu J, Ton-That H. Biogenesis of the Gram-positive bacterial cell envelope. Curr Opin Microbiol. 2016;34:31–37.
24. Dufresne K, Paradis-Bleau C. Biology and assembly of the bacterial envelope. Adv Exp Med Biol. 2015;883:1–47.
25. Rajagopal M, Walker S. Envelope structures of Gram-positive bacteria. Curr Top Microbiol Immunol. 2017;404:1–44.
26. Sleytr UB, Beveridge TJ. Bacterial S-layers. Trends Microbiol. 1999;7:253–260.
27. Sychanta D, Chapman RN, Bamford NC, Boons G-J, Howell PL, Clarke AJ. Molecular basis for the attachment of s-layer proteins to the cell wall of Bacillus anthracis. Biotechnology. 2018;57:1949–1953.
28. Sakakibara J, Nagano K, Murakami Y, et al. Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of Tannerella forsythiensis. Microbiology. 2007;153:866–876.
29. Thompson SA. Campylobacter surface-layers (S-layers) and immune evasion. Ann Periodontol. 2002;7:43–53.
30. Beveridge TJ, Pouwels PH, Sara M, et al. Functions of S-layers. FEMS Microbiol Rev. 1997;20:99–149.
31. Wang Y, Wei Y, Yuan S, et al. Bacillus anthracis S-layer protein BsaA binds to extracellular matrix by interacting with laminin. BMC microbiology. 2016;16:183–183.
32. Kotiranta A, Haapasalo M, Kari K, et al. Surface structure, hydrophobicity, phagocytosis, and adherence to matrix proteins of Bacillus cereus cells with and without the crystalline surface protein layer. Infect Immun. 1998;66:4895–4902.
33. Mursalin MH, Coburn PS, Livingston E, et al. S-layer impacts the virulence of Bacillus in endophthalmitis. Invest Ophthalmol Vis Sci. 2015;50:8683–8691.
34. Mursalin MH, Coburn PS, Livingston E, et al. Bacillus S-layer-mediated innate interactions during endophthalmitis. Front Immunol. 2020;11:00115.
35. Taylor AW, Ng TF. Negative regulators that mediate ocular immune privilege [published online ahead of print February 12, 2018]. J Leukoc Biol. https://doi.org/10.1002/JLB.3MR0817-337R.
36. Novosad BD, Astley RA, Callegan MC. Role of Toll-like receptor (TLR) 2 in experimental Bacillus cereus endophthalmitis. PLoS One. 2011;6:e28619.
37. Parkunan SM, Astley R, Callegan MC. Role of TLR5 and flagella in Bacillus intraocular infection. PLoS One. 2014;9:e100543.
38. Parkunan SM, Randall CB, Coburn PS, Astley RA, Staats RL, Callegan MC. Unexpected roles for toll-like receptor 4 and TRIF in intraocular infection with Gram-positive bacteria. Infect Immun. 2015;83:3926–3936.
39. Bochkov VN, Osokolkova OV, Birukov KG, Levonen AL, Binder CJ, Stockl J. Generation and biological activities of oxidized phospholipids. Antioxid Redox Signal. 2010;12:1009–1059.
40. Erridge C, Kennedy S, Spickett CM, Webb DJ. Oxidized phospholipid inhibition of toll-like receptor (TLR) signaling is restricted to TLR2 and TLR4: roles for CD14, LPS-binding protein, and MD2 as targets for specificity of inhibition. J Biol Chem. 2008;283:24748–24759.
41. Pearlman E, Johnson A, Adhikary G, et al. Toll-like receptors at the ocular surface. Ocul Surf. 2008;6:108–116.
Innate Immune Interference and Endophthalmitis

42. Yu F-SX, Hazlett LD. Toll-like receptors and the eye. Invest Ophthal Vis Sci. 2006;47:1255–1263.
43. Ramdani RT, Ramirez R, Novosad BD, Callegan MC. Acute inflammation and loss of retinal architecture and function during experimental Bacillus endophthalmitis. Curr Eye Res. 2006;31:955–965.
44. Ramdani RT, Moyer AL, Callegan MC. A role for tumor necrosis factor-alpha in experimental Bacillus cereus endophthalmitis pathogenesis. Invest Ophthalmol Vis Sci. 2008;49:4482–4489.
45. Parkunan SM, Randall CB, Astley RA, Furtado GC, Lira SA, Callegan MC. CXCL1, but not IL-6, significantly impacts intraocular inflammation during infection. J Leukoc Biol. 2016;100:1125–1134.
46. Miller FC, Coburn PS, Huzzatul MM, LaGrow AL, et al. TLR4 modulates inflammatory gene targets in the retina during Bacillus cereus endophthalmitis. BMC Ophthalmol. 2018;18:96.
47. Chu LH, Indramohan M, Ratsimandresy RA, et al. The oxidized phospholipid oxPAPC protects from septic shock by targeting the non-canonical inflammasome in macrophages. Nat Commun. 2018;9:996.
48. Ke Y, Zebda N, Oskolkova O, et al. Anti-Inflammatory Effects of OxPAPC Involve Endothelial Cell-Mediated Generation of LXA4. Front Physiol. 2017;8:508–508.
49. Gao W, Xiong Y, Li Q, Yang H. Inhibition of Toll-like receptor signaling as a promising therapy for inflammatory diseases: a journey from molecular to nano therapeutics. Front Physiol. 2017;8:508–508.
50. Coburn PS, Miller FC, LaGrow AL, et al. TLR4 modulates inflammatory gene targets in the retina during Bacillus cereus endophthalmitis. Ocul Immunol Inflamm. 2018;26:1262–1266.
51. Engelbert M, Gilmore MS. Fas ligand but not complement is critical for control of experimental Staphylococcus aureus Endophthalmitis. Invest Ophthalmol Vis Sci. 2005;46:2479–2486.
52. Bui DK, Carvounis PE. Evidence for and against intravitreal corticosteroids in addition to intravitreal antibiotics for acute endophthalmitis. Int Ophthalmol Clin. 2014;54:215–224.
53. Meredith TA, Aguilar HE, Drews C, et al. Intravitreal dexamethasone produces a harmful effect on treatment of experimental Staphylococcus aureus endophthalmitis. Trans Am Ophthalmol Soc. 1996;94:241–252; discussion 252–247.
54. Ching Wen Ho D, Agarwal A, Lee CS, et al. A review of the role of intravitreal corticosteroids as an adjuvant to antibiotics in infectious endophthalmitis. Ocul Immunol Inflamm. 2018;26:461–468.
55. Wiskur BJ, Robinson MI, Farrand AJ, Novosad BD, Callegan MC. Toward improving therapeutic regimens for Bacillus endophthalmitis. Invest Ophthalmol Vis Sci. 2008;49:1480–1487.
56. Sakalar YB, Ozekinci S, Celen MK. Treatment of experimental Bacillus cereus endophthalmitis using intravitreal moxifloxacin with or without dexamethasone. J Ocular Pharmacol Ther. 2011;27:593–598.
57. Shah GK, Stein JD, Sharma S, et al. Visual outcomes following the use of intravitreal steroids in the treatment of postoperative endophthalmitis. Ophthalmonol. 2009;107:486–489.
58. Astley RA, Coburn PS, Parkunan SM, Callegan MC. Modeling intraocular bacterial infections. Prog Retin Eye Res. 2016;54:30–48.
59. Fagan RP, Fairweather NE. Biogenesis and functions of bacterial S-layers. Nat Rev Microbiol. 2014;12:211–222.
60. Gerbino E, Carasi P, Mobili P, Serradell MA, Gómez-Zavaglia A. Role of S-layer proteins in bacteria. World J Microb Biot. 2015;31:1877–1887.
61. Goytain A, Ng T. NanoString nCounter technology: high-throughput RNA validation. Methods Mol Biol. 2020;2079:125–139.
62. Foye C, Yan IK, David W, et al. Comparison of miRNA quantitation by Nanostring in serum and plasma samples. PLoS One. 2017;12:e0189165–e0189165.
63. Tsang H-F, Xue VW, Koh S-P, Chiu Y-M, Ng LP-W, Wong S-CC. NanoString, a novel digital color-coded barcode technology: current and future applications in molecular diagnostics. Expert Rev Mol Diagn. 2017;17:95–103.
64. Xu W, Solis NV, Filler SG, Mitchell AP. Pathogen gene expression profiling during infection using a Nanostring nCounter platform. Methods Mol Biol. 2016;1361:57–65.
65. Wood JP, McCord RJ, Osborne NN. Retinal protein kinase C. Neurochem Int. 1997;30:119–136.
66. Xiong W-H, Pang J-J, Pennesi ME, Duvoisin RM, Wu SM, Morgans CW. The effect of PKCo on the light response of rod bipolar cells in the mouse retina. Invest Ophthalmol Vis Sci. 2015;56;4961–4974.
67. Loebering DJ, Lennartz MR. Protein kinase C and toll-like receptor signaling. Enzyme Res. 2011;2011:537821–537821.
68. Nakashima S. Protein kinase C alpha (PKC alpha): regulation and biological function. J Biochem. 2002;132:669–675.
69. Rosson D, O’Brien TG, Kampherstein JA, et al. Protein kinase C-α activity modulates transepithelial permeability and cell junctions in the LLC-PK1 epithelial cell line. J Biol Chem. 1997;272:14950–14953.
70. Leppänen T, Tuominen RK, Moilanen E. Protein kinase C and its inhibitors in the regulation of inflammation: inducible nitric oxide synthase as an example. Basic & Clin Pharmacol Toxicol. 2014;114:37–43.
71. Kim H, Zamel R, Bai X-H, Liu M. PKC activation induces toll-like receptor signaling. PLoS One. 2013;8:e64182.
72. Shukla A, Lounsbury KM, Barrett TF, et al. Asbestos-induced peribronchiolar cell proliferation and cytokine production are attenuated in lungs of protein kinase C knockout mice. Am J Patol. 2007;170:140–151.
73. Yoshida Y, Huang FL, Nakabayashi H, Huang KP. Tissue distribution and developmental expression of protein kinase C isoforms. J Biol Chem. 1988;263:9868–9873.
74. Kumar A, Singh CN, Glybina IV, Mahmoud TH, Yu F-SX. TLR2 ligand-induced protection against bacterial endophthalmitis. J Infect Dis. 2010;201:255–263.
75. Duggan JM, You D, Cleaver JO, et al. Synergistic interactions of TLR2/6 and TLR9 induce a high level of resistance to changing infection in mice. J Immunol. 2011;186:5916–5926.
76. Tuvim MJ, Gilbert BE, Dickey BF, Evans SE. Synergistic interactions of TLR2/6 and TLR9 induce a high level of resistance to changing infection in mice. J Immunol. 2011;186:5916–5926.
77. Lester SN, Li K. Toll-like receptors in antiviral innate immunity. J Mol Biol. 2014;426:1246–1264.
78. Moen SH, Ehrnström B, Kojen JF, et al. Human Toll-like receptor 8 (TLR8) is an important sensor of pyogenic bacte-
Innate Immune Interference and Endophthalmitis

ria, and is attenuated by cell surface TLR signaling. *Front Immunol.* 2019;10:1029.

82. Yerramothu P, Vijay AK, Willcox MDP. Inflammasomes, the eye and anti-inflammasome therapy. *Eve.* 2018;32:491–505.

83. Man SM, Kanneganti T-D. Regulation of inflammasome activation. *Immunol Rev.* 2015;265:6–21.

84. Fox D, Mathur A, Xue Y, et al. *Bacillus cereus* non-haemolytic enterotoxin activates the NLRP3 inflamma-

some. *Nature communications.* 2020;11:760–760.

85. Talreja D, Singh PK, Kumar A. In vivo role of TLR2 and MyD88 signaling in eliciting innate immune responses in *Staphylococcus endophthalmitis.* *Invest Ophthalmol Vis Sci.* 2015;56:1719–1732.

86. Tripathi T, Alizadeh H. Significance of arachidonic acid in ocular infections and inflammation. *Inflamm Cell Signal.* 2014;1:e301.

87. Herschman HR. Prostaglandin synthase 2. *Biochim Biophys Acta.* 1996;1299:125–140.

88. Rajamani D, Singh PK, Rottmann BG, Singh N, Bhasin MK, Loureiro B, Bonilla L, Block J, Fear JM, Bonilla AQS, Molineux G. Granulocyte colony-stimulating factors. *J Immunol Cell Biol.* 2014;70:856–859.

89. Fox D, Mathur A, Xue Y, et al. *Bacillus cereus* non-haemolytic enterotoxin activates the NLRP3 inflamma-

some. *Nature communications.* 2020;11:760–760.

90. Da Cunha AP, Zhang Q, Prentiss M, et al. The hierarchy of chemokines and cytokines. *J Immunol.* 2003;111:S460–S475.

91. Legler DF, Thelen M. Chemokines: chemistry, biochemistry and biological function. *Chimia (Aarau).* 2016;70:856–859.

92. Baggiolini M. Chemokines and leukocyte traffic. *Nature.* 1998;392:565–568.

93. Tanaka T, Narazaki M, Kishimoto T. IL-6 in inflammation, immunity, and disease. *Cold Spring Harb Perspect Biol.* 2014;6:a016295–a016295.

94. Molineux G. Granulocyte colony-stimulating factors. *Cancer Treat Res.* 2011;157:33–53.

95. Hayashi S, Guex-Crosier Y, Delvaux A, Velu T, Roberge FD. Interleukin 10 inhibits inflammatory cells infiltration in endotoxin-induced uveitis. *Graefes Arch Clin Exp.* 1996;234:633–636.

96. Nonas S, Birukova AA, Fu P, et al. A critical role for CCL2 and CCL3 chemokines in the regulation of polymorphonuclear neutrophils recruitment during corneal infection in mice. *Immunol Cell Biol.* 2007;85:525–531.

97. Kochan T, Singla A, Tosi J, Kumar A. Toll-like receptor 2 ligand pretreatment attenuates retinal microglial inflammatory response but enhances phagocytic activity toward *Staphylococcus aureus.* *Infect Immun.* 2012;80(6):2076–2088.

98. Schutyser E, Struyf S, Menten P, et al. The hierarchy of proinflammatory cytokines in ocular inflammation. *Curr Eye Res.* 2016;43:553–565.

99. Gschwandtner M, Derler R, Midwood KS. More than just attractive: how CCL2 influences myeloid cell behavior beyond chemotaxis. *Front Immunol.* 2019;10:2759–2759.

100. Schutyser E, Struyf S, Van Damme J. The CC chemokine CCL20 and its receptor CCR6. *Cytokine Growth Factor Rev.* 2003;14:409–426.