Innate Immune Interference Attenuates Inflammation In *Bacillus* Endophthalmitis

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Purpose. To explore the consequences of innate interference on intraocular inflammatory responses during *Bacillus* endophthalmitis.

Methods. *Bacillus* endophthalmitis was induced in mice. Innate immune pathway activation was interfered by injecting S layer protein-deficient ($\Delta slpA$) *B. thuringiensis* or by treating wild-type (WT)–infected mice with a TLR2/4 inhibitor (WT+OxPAPC). At 10 hours postinfection, eyes were harvested and RNA was purified. A NanoString murine inflammation panel was used to compare gene expression in WT-infected, WT+OxPAPC, $\Delta slpA$-infected, and uninfected eyes.

Results. In WT-infected eyes, 56% of genes were significantly upregulated compared to uninfected controls. Compared to WT-infected eyes, the expression of 27% and 50% of genes were significantly reduced in WT+OxPAPC and $\Delta slpA$-infected eyes, respectively. Expression of 61 genes that were upregulated in WT-infected eyes was decreased in WT+OxPAPC and $\Delta slpA$-infected eyes. Innate interference resulted in blunted expression of complement factors (C3, Cfb, and C6) and several innate pathway genes (TLRs 2, 4, 6, and 8, MyD88, Nod2, Nlrp3, NF-κB, STAT3, RelA, RelB, and Ptgs2). Innate interference also reduced the expression of several inflammatory cytokines (CSF2, CSF3, IL-6, IL-1β, IL-1α, TNFα, IL-23α, TGFβ1, and IL-12β) and chemokines (CCL2, CCL3, and CXCLs 1, 2, 3, 5, 9, and 10). All of the aforementioned genes were significantly upregulated in WT-infected eyes.

Conclusions. These results suggest that interfering with innate activation significantly reduced the intraocular inflammatory response in *Bacillus* endophthalmitis. This positive clinical outcome could be a strategy for anti-inflammatory therapy of an infection typically refractory to corticosteroid treatment.

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.[1–4] 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 inflammogenic 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 monomolecular 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.[34] SLP appears to be a significant virulence factor in the pathogenesis of *Bacillus* endophthalmitis.

During infection, ocular immune privilege[35] 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 are 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 elicit 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.[41,42] 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.[43–45] There-fore 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.[44] 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.[45]

In endophthalmitis, the use of anti-inflammatory therapeutics with antibiotics has not proven completely effective at improving disease outcomes.[5–8] 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.[9,46] 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. Innate pathway interference was achieved by infection with SLP-deficient *Bacillus* ($\Delta slpA$) 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).
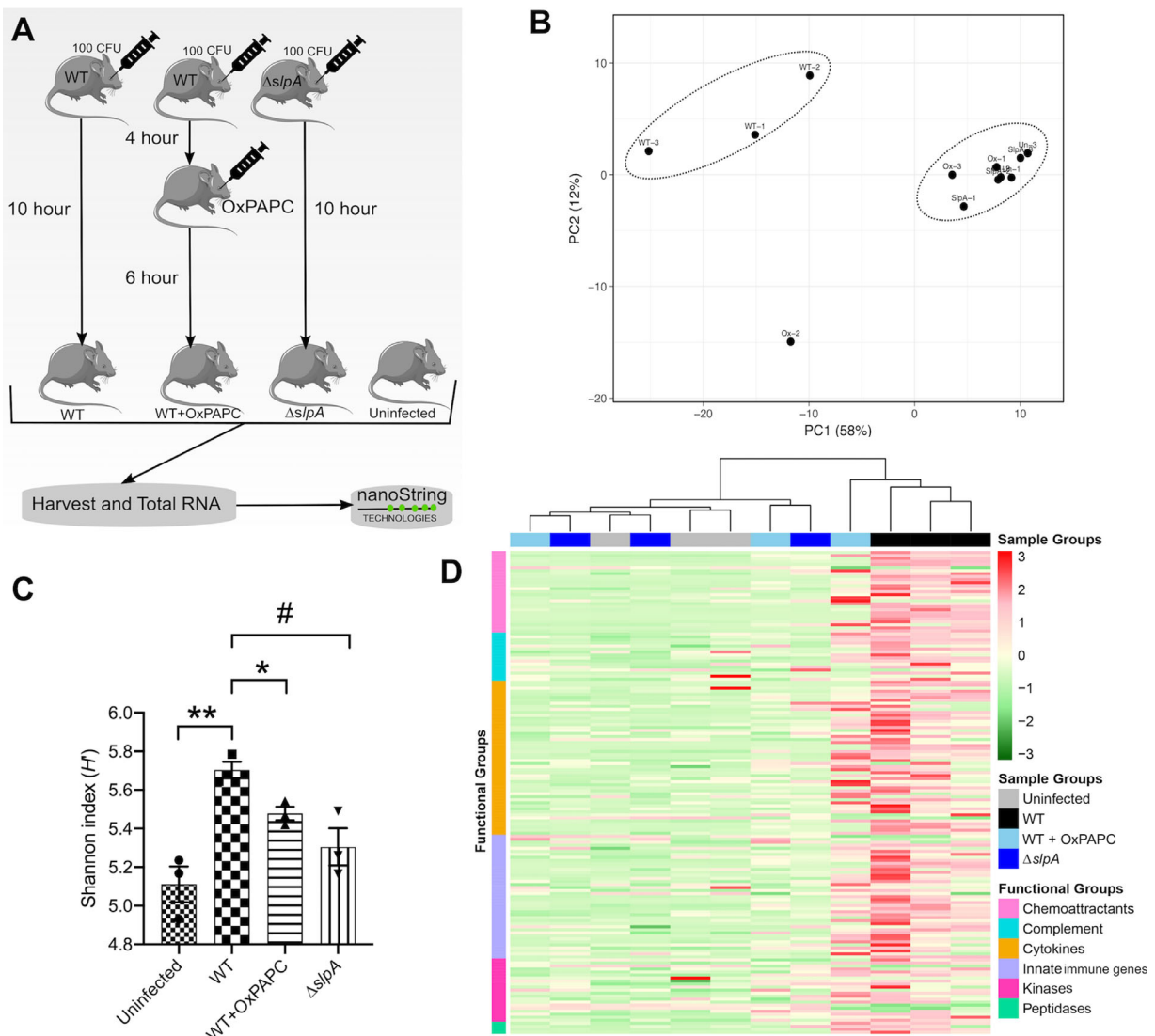
FIGURE 1. Interfering with TLR2 and TLR4 activation affected inflammatory gene expression in the eye. (**A**) Experimental outline of the NanoString experiment. (**B**) PCA analysis of mouse inflammatory gene. All WT-infected eyes cluster together. Except for one WT+OxPAPC eye, all uninfected, $\Delta slpA$-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_ ($\Delta slpA$-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 ($\Delta slpA$) were used to initiate experimental endophthalmitis, as previously described.[33,34] 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.[50] 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 $\Delta slpA$ _B. thuringiensis_/0.5 µl BHI, as previously described.[33,34] 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 CO_2 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-cap 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.[50]

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 eyes. From each sample, 100 ng of RNA was hybridized with NanoString's XT PGX Mmv2 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 Δ*slpA*-infected eyes compared to uninfected eyes.

Statistics

NanoString analysis was performed in NanoString's nSolver Analysis Software v 4.0. After data normalization, Graph-Pad 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 Δ*slpA*-infected eyes. We also compared WT-infected eyes with WT+OxPAPC and Δ*slpA*-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 Δ*slpA*-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 Δ*slpA*-infected eyes. In Figure 1D, we used the R package "pHeatmap" for visualization of gene expression in our groups.[51] Most of the inflammatory genes in three uninfected, three Δ*slpA*-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 all Δ*slpA*-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 Δ*slpA*-infected eyes was only 44 and 34, respectively (Fig. 2Gi) 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-
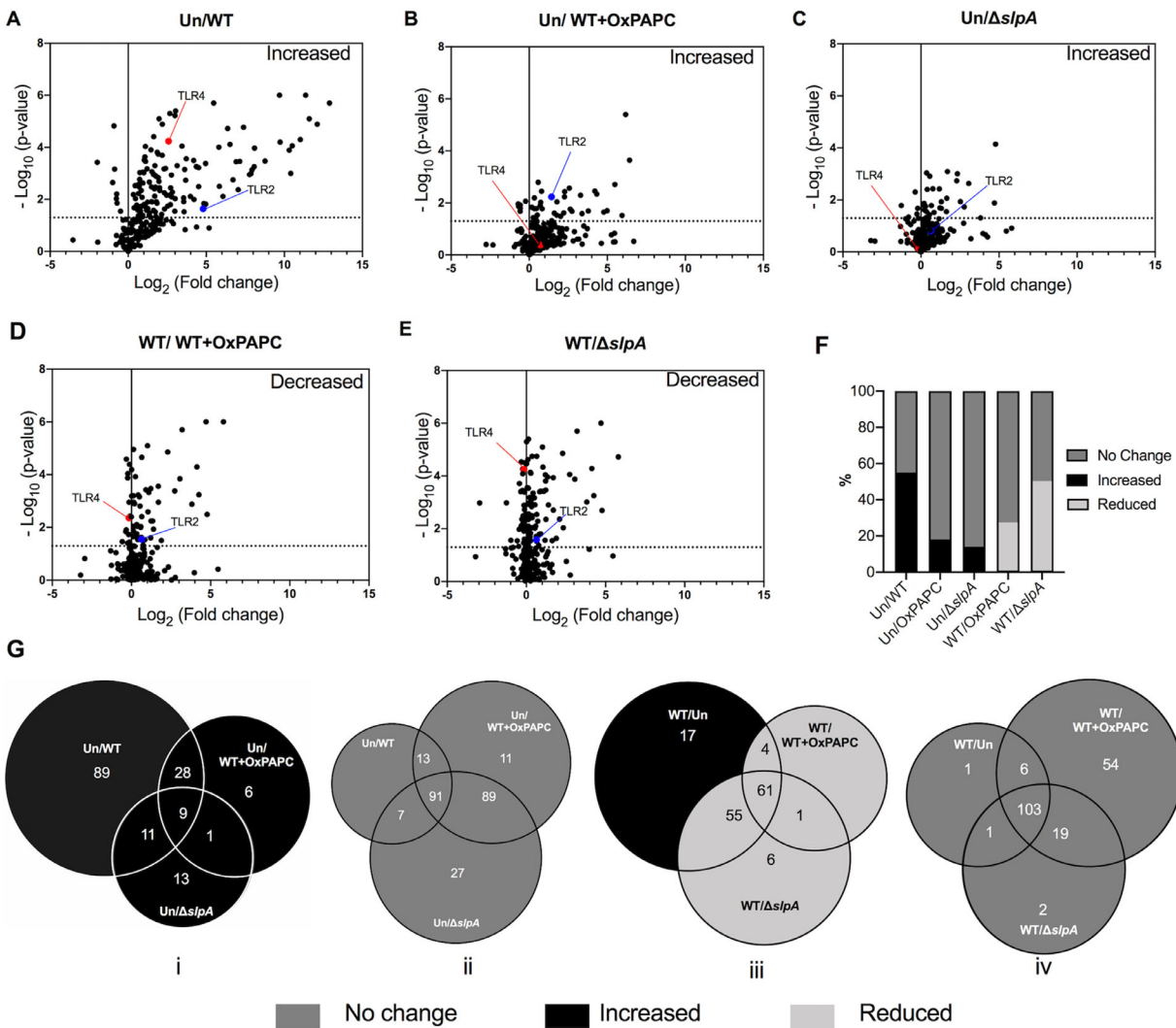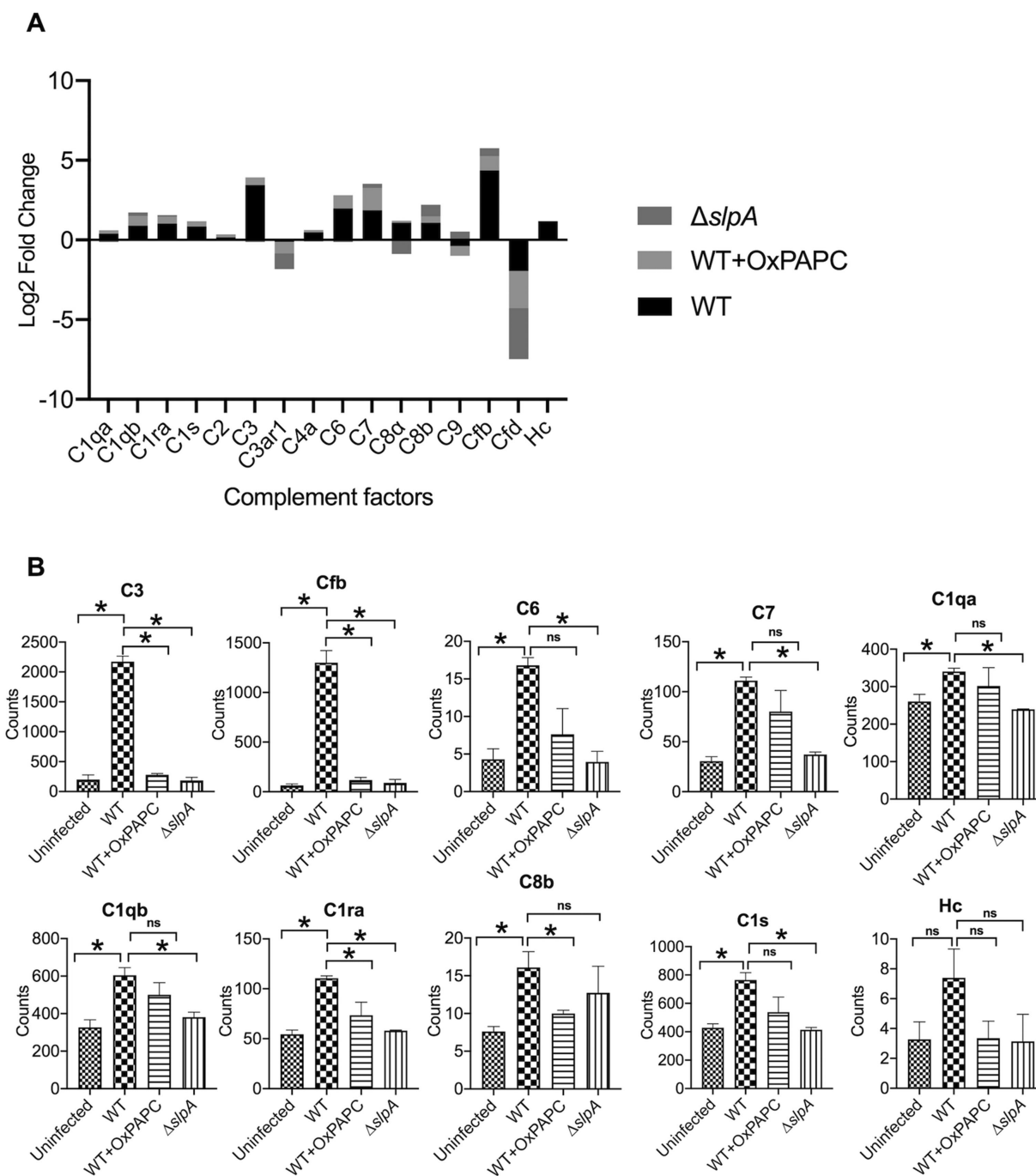
Figure 2. Interfering with TLR2 and TLR4 activation reduced ocular inflammatory responses. Volcano plot analysis of NanoString data derived from WT-infected, WT+OxPAPC, and ∆slpA-infected eyes compared with (**A–C**) uninfected eyes and (**D, E**) WT-infected eyes. For **A to C** and **D to E** the x-axis 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 ∆slpA-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 ∆slpA-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 ∆slpA-infected eyes, respectively, compared to WT-infected eyes (Figs. 2D, 2E, and 2Giii). Compared to TLR2 and TLR4 expression in WT-infected eyes, the expression of TLR2 (blue) and TLR4 (red) in WT+OxPAPC and ∆slpA-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 ∆slpA-infected eyes. One gene, protein kinase C alpha (PRCKα), was significantly downregulated in WT-infected eyes but upregulated in WT+OxPAPC and ∆slpA-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 ∆slpA-infected eyes compared to uninfected eyes, respectively. Compared to WT-infected eyes, expression of 27% and 50% genes was decreased in WT+OxPAPC and ∆slpA-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-

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 Δ*slpA*-infected eyes. (**A**) Fold changes of complement factor expression in WT-infected, WT+OxPAPC, and Δ*slpA*-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 Δ*slpA*-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*[52] and *Pseudomonas aeruginosa*.[53] 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.[54] These findings suggest diversity in the contribution of the comple-

ment cascade in different endophthalmitis models. Figure 3A depicts the log fold change of complement pathway components in WT-infected, WT+OxPAPC, and Δ*slpA*-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 Δ*slpA*-infected eyes (Supplemen-

tary Table S2). Figure 3B demonstrates the transcript levels of differentially expressed complement factors. We detected increased expression of C3, Cfb, C6, C7, C1q subcomponent-alpha polypeptide (a), C1q subcomponent-beta polypeptide (b), C1r subcomponent A (a), C8 beta polypeptide (b), and C1 s subcomponent (s) transcripts in WT-infected eyes compared to uninfected eyes. Compared to WT-infected eyes, expression of C3, Cfb, and C1ra transcripts were significantly reduced in WT+OxPAPC and $\Delta slpA$-infected eyes. Expression of C6, C7, C1qb, and C1s transcripts was also significantly reduced in $\Delta slpA$-infected eyes. C8b expression was only reduced in WT+OxPAPC eyes. Expression of hemolytic complement Hc, was increased 1.3-fold in WT-infected eyes compared to uninfected eyes and decreased 1.2-fold and 1.4-fold in WT+OxPAPC and $\Delta slpA$ infected eyes, respectively, compared to WT-infected eyes. Collectively, these findings suggested that *Bacillus* infection-driven complement gene expression in the eye could be prevented by inhibiting TLR2 and TLR4 activation.

Interfering with TLR2 and TLR4 Activation Impacted Innate Immune Gene Expression in the Eye

Intraocular innate receptors and their adaptors contribute to the pathogenesis of *Bacillus* endophthalmitis and other vision-threatening infections. During *Bacillus* endophthalmitis, severe intraocular inflammation is triggered by TLR2 and TLR4 via MyD88 and TRIF.[36,37,50] Here, we focused on 41 genes involved in innate immune pathways, which include receptors, adaptors, signaling molecules, and hormones. (Supplementary Table S3). Among the 24 differentially expressed innate immune pathway genes, 11 were highly upregulated in WT-infected eyes compared to uninfected eyes and were reduced in WT+OxPAPC and $\Delta slpA$-infected eyes compared to WT-infected eyes (Fig. 4A). Figure 4B depicts a heatmap of the expression of TLRs and other innate receptors. As expected, we observed increased expression of TLR2 and TLR4 transcripts in WT-infected eyes. In addition, we observed elevated expression of TLR6, TLR8, nucleotide-binding oligomerization domain-containing protein (NOD) 2, and LRR- and pyrin domain-containing protein (Nlrp3) in WT-infected eyes. Expression of these innate genes was significantly decreased in WT+OxPAPC or $\Delta slpA$-infected eyes compared to WT-infected eyes. The expression of other innate immune genes, such as MyD88, NF-κB, v-rel reticuloendotheliosis viral oncogene homolog (Rel) A, and v-rel oncogene related (Rel) B, prostaglandin-endoperoxide synthase (Ptsg) 2, signal transducer and activator of transcription (STAT) 2 and 3, and heat shock protein (Hspb) 1 was significantly increased in WT-infected eyes compared to uninfected eyes (Fig. 4C). Expression of these genes was decreased in WT+OxPAPC and $\Delta slpA$-infected eyes compared to WT-infected eyes. Among the 41 innate immune genes we analyzed, expression of Ptgs2, Nlrp3, TLR2, TLR6, and NOD2 were 218-, 140-, 26.8-, 19.8-, and 17.5-fold greater than in uninfected eyes and were the top five upregulated innate immune genes in WT-infected eyes. We observed a negative fold change of these genes in WT+OxPAPC and $\Delta slpA$-infected eyes compared to WT-infected eyes (Supplementary Table S3). Here, we identified several innate immune genes whose expression was not changed at 4 hours postinfection in *Bacillus* endophthalmitis. Furthermore, we observed dampened expression

of those innate immune genes when TLR2 and TLR4 activation did not occur. These results suggested that the innate immune gene expression in *Bacillus* endophthalmitis occurring during robust inflammation could be prevented by inhibiting TLR2 and TLR4 activation.
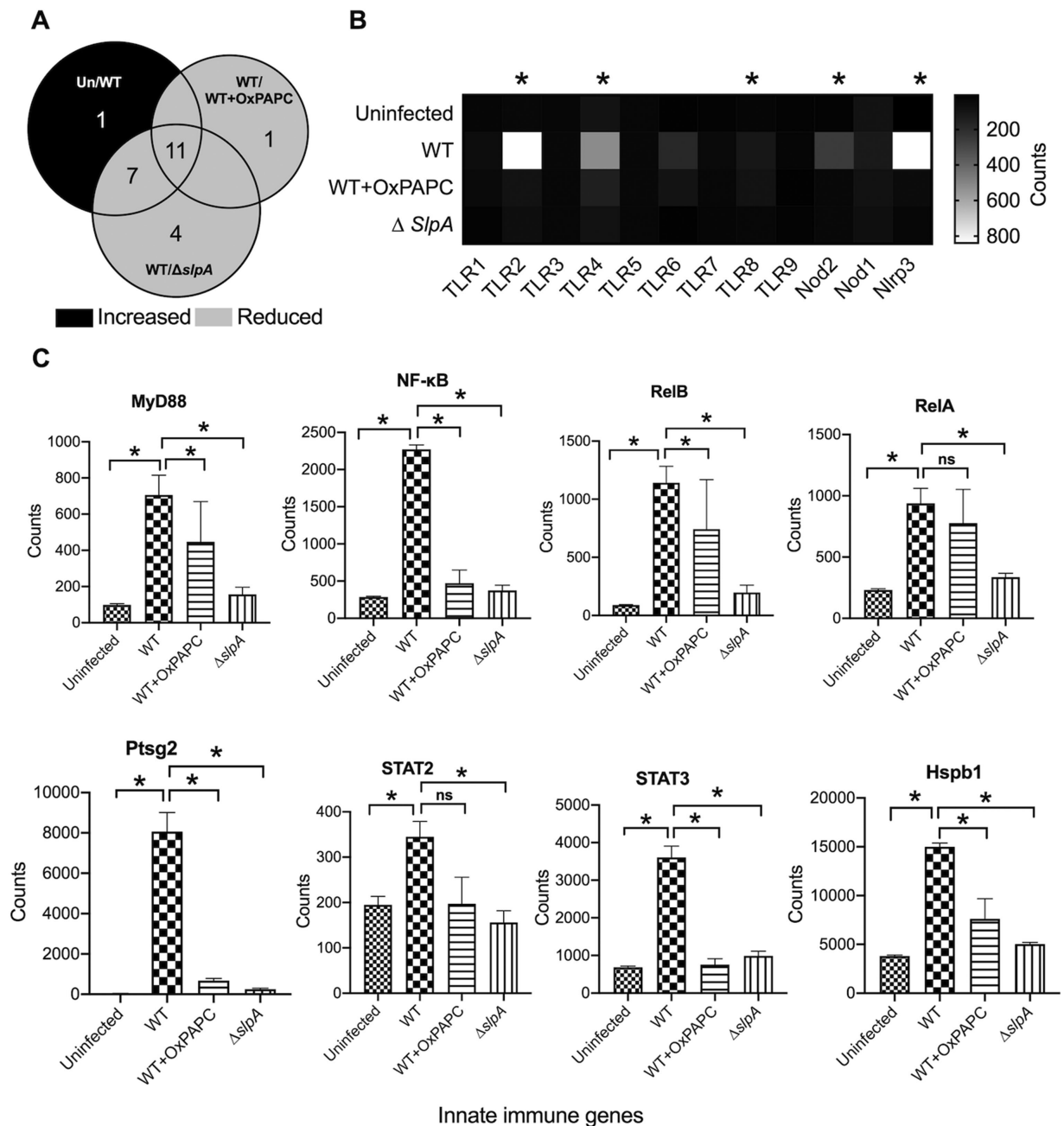
Interfering with TLR2 and TLR4 Activation Reduced Cytokine Gene Expression in the Eye

During endophthalmitis, cytokines and chemokines drive ocular inflammation. In TNF$\alpha^{-/-}$ and CXCL1$^{-/-}$ mice, neutrophil recruitment was suppressed. However, this resulted in an elevated bacterial load and disease severity in TNF $\alpha^{-/-}$ mice,[44] 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 $\Delta slpA$-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 $\Delta slpA$-infected eyes compared to WT-infected eyes. Expression of TGFβ1 and IL-12β was reduced only in $\Delta slpA$-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 $\Delta slpA$-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 Chemoattractant Gene Expression in the Eye

A hallmark of *Bacillus* endophthalmitis is robust migration of inflammatory cells into the posterior chamber. Chemokines act as chemoattractants to guide the migra-
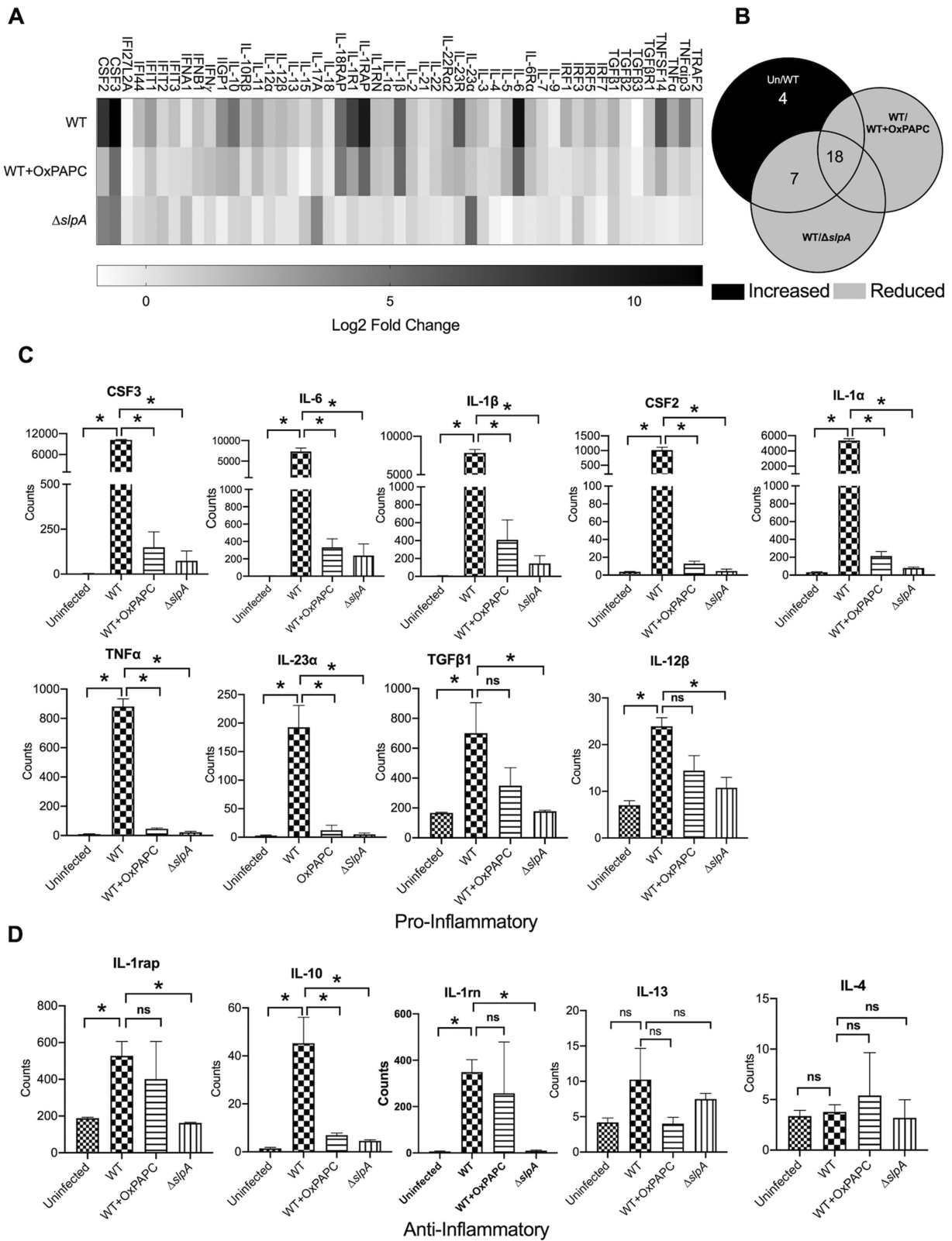
FIGURE 4. Interfering with TLR2 and TLR4 activation impacted innate immune gene expression in the eye. Expression of innate immune genes in mouse eyes. (**A**) Venn diagram showing the number of innate immune genes differentially upregulated between the groups. (**B**) Heatmap showing the expression 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.

tion of these cells. 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 Δ*slpA*-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 Δ*slpA*-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, ΔslpA-infected eyes were analyzed. (**A**) Log fold change of 51 mouse inflammatory cytokines from WT-infected, WT+OxPAPC, and ΔslpA-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 ΔslpA-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 ΔslpA-infected eyes. Values represent the mean ± SEM of transcript counts at 10 hours after infection (N = 3). *P < 0.05 was considered significant.
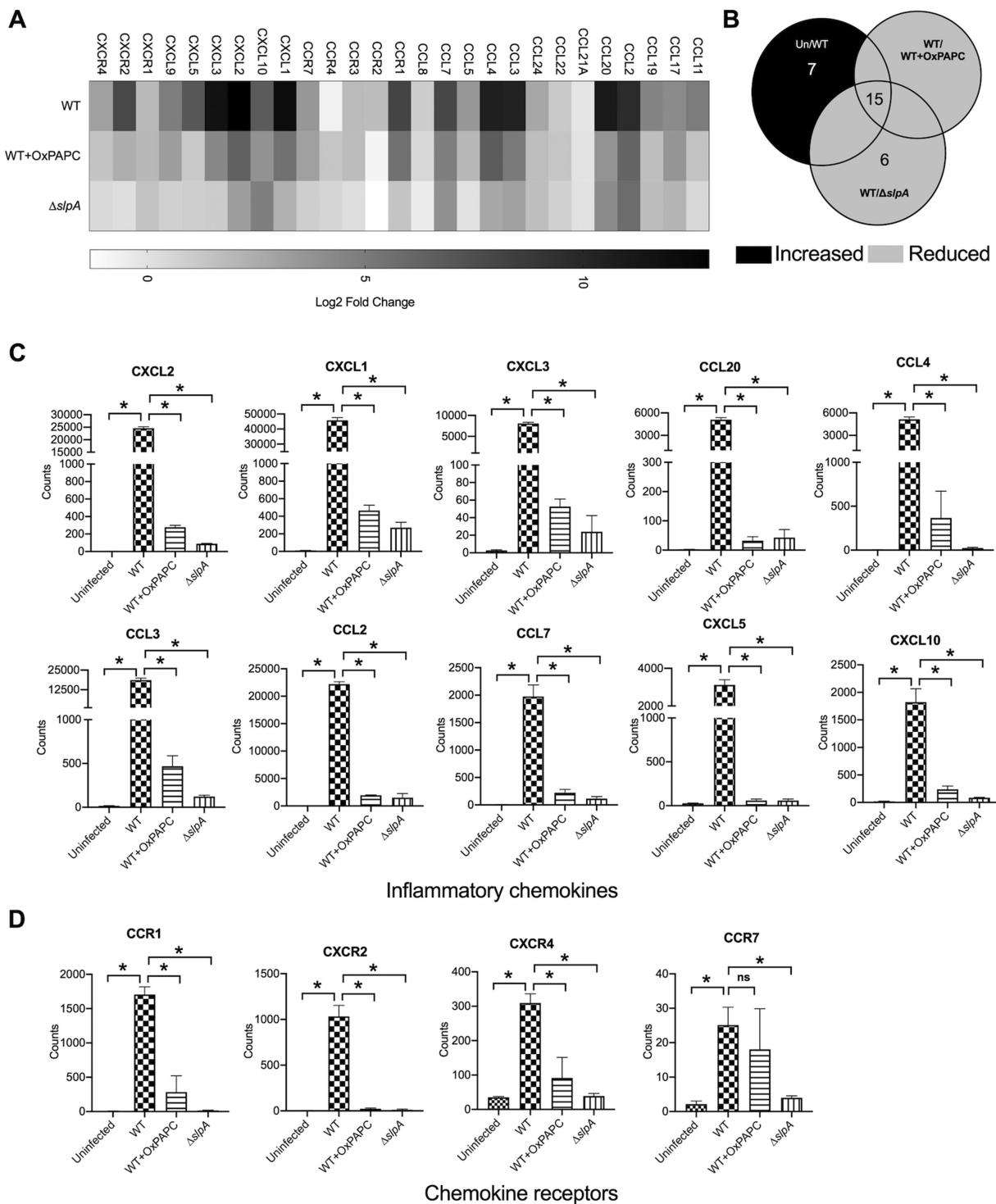
FIGURE 6. Interfering with TLR2 and TLR4 activation reduced chemoattractant gene expression in the eye. NanoString analysis of CXC and CC chemokine groups analyzed in WT-infected, WT+OxPAPC, and Δ*slpA*-infected mouse eyes. (**A**) Log fold changes of inflammatory chemokines from WT-infected, WT+OxPAPC, and Δ*slpA*-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 Δ*slpA*-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 Δ*slpA*-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 Δ*slpA*-infected eyes compared to WT-infected eyes. CCR7 expression was significantly increased in WT-infected eyes compared to uninfected eyes, and significantly reduced in Δ*slpA*-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*,[50] 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.[35]

We reported that *Bacillus* reach their maximum growth faster than most other Gram-positive endophthalmitis pathogen in the eye.[9] *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.[1–8] Corticosteroids use in endophthalmitis is routine, but their usefulness has not been without controversy.[55–61] 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,[11] 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.[26,62,63] We recently reported that an absence of *Bacillus* SLP reduced disease severity and damage to the eye in murine experimental endophthalmitis.[33] 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.[34] 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.[50] The reduced inflammation and improved clinical outcomes in the absence of TLR2[36] or TLR4[37] or after TLR2 and TLR4 inhibition[34] 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,[58] 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.[64] Unlike other alternatives, NanoString does not require polymerase activity, and therefore the chance of introducing bias is lower.[65–67] 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, Δ*slpA*-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 Δ*slpA*-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 Δ*slpA*-infected eyes compared to WT-infected eyes (Supplementary Table S1). Only one gene, protein kinase C alpha (PRKCα), was decreased in WT-infected eyes compared to uninfected eyes but significantly increased in Δ*slpA*-infected and WT+OxPAPC eyes. In the retina, PRKCα modulates rod photoceptor and bipolar cell function. PRKCα and few other isoforms of PRKC are reported to be present in ganglion, amacrine, and RPE cells. PRKCα contributes to cellular proliferation, differentiation, motility, apoptosis, and inflammation.[68–71] It has been

reported that TLR1/2-driven activation of AP-1, MAPK, and NF-kB and secretion of IL-6, IL-10, and TNF-α by dendritic cells requires the activation of PRKCα.[70] Overexpression of PRKCα resulted in loss of tight junctions, which reduced the transepithelial permeability in an epithelial cell line LLC-PK1.[72] PRKCα 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.[73] 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.[74] The absence of PRKC isoform δ in mice resulted in reduced inflammatory mediator production and neutrophil infiltration in an asbestos-associated disease model.[75] We observed decreased expression of PRKCα in WT-infected eyes compared to uninfected eyes, which suggested a potential anti-inflammatory property of PRKCα. 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.[76] 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*.[50] 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.[52,53] However, for *S. aureus* endophthalmitis, complement was not protective.[54] 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.[36,37] TLR2 is also important for inflammatory responses in *S. aureus* endophthalmitis.[77] TLR6 forms a heterodimer with TLR2 and recognizes diacylated lipopeptides such as lipoteichoic acid on the cell wall of Gram-positive bacteria. Synergistic interactions of TLR2/6 and TLR9 provide resistance against lung infection.[78,79] TLR8, which is an innate endosomal receptor, usually senses viral infection.[80] 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.[81] 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.[50] 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. Inflammasomes are important because they aid in maintaining homeostasis with commensals and protecting tissues against pathogens.[82,83] Although we reported that the absence of Nlrp3 in Nlrp3$^{-/-}$ global knockout mice did not affect the clinical outcomes in *Bacillus* endophthalmitis at eight hours postinfection,[61] a recent report suggested that *B. cereus* nonhemolytic 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.[84] Here, we observed significant increased expression of TLR2, TLR4, and Nlrp3 in WT-infected eyes compared to uninfected eyes. Expression of these innate receptors were reduced after TLR2 and TLR4 inhibition or infection with the $\Delta slpA$ 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.[38] The importance of MyD88 has also been reported for *S. aureus* endophthalmitis.[85] Arachidonic acid–derived lipid mediators control cell metabolism, proliferation, migration, and apoptosis.[86] Prostaglandin-endoperoxide synthase (Ptgs) 2 converts arachidonic acid to prostaglandin endoperoxide H2 (PGE2), which immunomodulates neutrophil activation.[87] Ptgs2 was upregulated in a TLR4-dependent manner in the retinas of mice infected with *Bacillus*.[50] 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.[33] 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-kB activation and promotes the inflammatory response. Retinal transcriptome analysis of *S. aureus* endophthalmitis showed elevated expression of STAT3 in a TLR2-dependent manner.[88] SOCS3, which is a negative regulator of cytokine signaling, inhibits STAT3 phosphorylation and prevents over-activation of proinflammatory genes.[89] 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.[33] 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.[90] 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.[91,92] 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.[43] 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,[50] 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.[93] IL-6 expression increases during the course of bacterial endophthalmitis[43,77] in a TLR2- and TLR4-dependent manner.[36,37] 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.[88] Surprisingly, the absence of IL-6 in IL-6$^{-/-}$ mice did not affect the overall outcome of *Bacillus* endophthalmitis.[45] 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] Our results suggest that the expression of anti-inflammatory cytokines may not affect the clinical outcome in our model because the expression of anti-inflammatory cytokines in WT-infected eyes did not affect the expression of IL-6, TNF-α, or IL-1β.

Chemokines are inflammatory mediators that serve as chemoattractants and are divided into two significant subfamilies (CXC and CC chemokines) based on the spacing of the first pair of N-terminal cysteines.[96–98] The CXC chemokines recruit neutrophils, while CC chemokines attract monocytes.[96–98] Activation of TLR2 on retinal microglia,[99] and Muller glia[100,101] by *S. aureus* resulted in the synthesis of CXC chemoattractants. Among the 248 mouse inflammatory genes analyzed, CXCL2, CXCL1, and CXCL3 were the most highly expressed chemokines in WT-infected eyes (Supplementary Table S5). CXCL2, CXCL1, and CXCL3 are highly homologous and are powerful neutrophil chemoattractants.[96–98] We also observed increased expression of the CC chemokines CCL2/MCP-1, CCL3/macrophage inflammatory protein (MIP)-1α, CCL4/MIP-1β, CCL7/monocyte-chemotactic protein 3 (MCP3), and CCL20/macrophage inflammatory protein-3 (MIP3a) in WT-infected eyes. CCL2 does not function as a chemoattractant for neutrophils but does recruit basophils, dendritic cells, monocytes, and memory T-cells to tissues where inflammation is induced by injury or infection.[102] CCL2 expression in Muller cells regulate the infiltration of monocytes and microglia following retinal injury.[102] CCL2 has been associated with acute inflammatory conditions[98] and recruits and activates neutrophils by binding to CCR1, CCR4, and CCR5.[102,103] CCL20 strongly attracts lymphocytes and weakly attracts neutrophils.[104] Expression of CCL2 and CCL20 can be induced by micro-

bial factors such as lipopolysaccharides and inflammatory cytokines such as IL-6 and TNFα.[104–108] 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, CCL7, CCL4, 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.[103] 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.[45] 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.[47,49,110–115] 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.[47] Oxidized phospholipids also significantly reduced LPS-induced cytokine production, barrier disruption, and tissue inflammation in rats.[111] 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

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

Gene Symbol	Gene Title	Accession	Fold Change		
			Un/WT	WT/WT+OxPAPC	WT/$\triangle slpA$
CXCL2	Chemokine (C-X-C motif) ligand 2	NM_009140.2	7605	−87	−277
CXCL1	Chemokine (C-X-C motif) ligand 1	NM_008176.1	4403	−98	−169
CXCL3	Chemokine (C-X-C motif) ligand 3	NM_203320.2	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
IL-6	Interleukin 6	NM_031168.1	1361	−21	−30
CCL3	Chemokine (C-C motif) ligand 3	NM_011337.1	1269	−39	−151
IL1β	Interleukin 1 beta	NM_008361.3	848	−18	−53
CCL2	Chemokine (C-C motif) ligand 2	NM_011333.3	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-X-C motif) receptor 2	NM_009909.3	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	FBJ osteosarcoma oncogene	NM_010234.2	82	−13	−13
IL23α	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 $\triangle slpA$-infected eyes relative to uninfected and WT-infected eyes are shown. A negative fold change represents reduced expression relative to WT-infected eyes.

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 chemoattractants 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,[33] chemical inhibition of TLR2 and TLR4 after infection,[34] mice deficient in TLR2,[36] TLR4,[37] MyD88,[38,85] and TRIF,[38] mice pre-treated with TLR2 agonists,[77,99] mice deficient in CXCL1,[45] and anti-CXCL1 administration[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.

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