1 **INNATE IMMUNE INTERFERENCE ATTENUATES INFLAMMATION**

2 **IN *BACILLUS* ENDOPHTHALMITIS**

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ABSTRACT

PURPOSE. *Bacillus* endophthalmitis is a sight-threatening bacterial infection that sometimes requires enucleation. Inflammation in this disease is driven by activation of innate Toll-like receptor (TLR) pathways. Here, we explored the consequences of innate immune interference on intraocular inflammatory responses during *Bacillus* endophthalmitis.

METHODS. Endophthalmitis was induced in mice by injecting 100 CFU *Bacillus thuringiensis* in to the mid-vitreous. We interfered with activation of the TLR2 and TLR4 pathways by 1) injecting a group of mice with S layer protein-deficient ($\Delta slpA$) *B. thuringiensis* or 2) injecting a group of wild type (WT)-infected mice with a TLR2/4 inhibitor, oxidized phospholipid (OxPAPC). At 10 hours postinfection, infected eyes were removed and total RNA was purified. mRNA expression was then analyzed by NanoString using a murine inflammation panel. We compared findings with expression data from eyes infected with eyes injected with WT *B. thuringiensis*, eyes injected with OxPAPC alone, and uninfected eyes.

RESULTS: Interference of TLR2 and TLR4 pathways resulted in differential expression of mouse inflammatory genes compared to expression in WT-infected eyes. In WT-infected eyes, 56% of genes were significantly upregulated compared to that of uninfected controls. However, 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. The expression of 61 genes which were significantly upregulated in WT-infected eyes was decreased in WT+OxPAPC or $\Delta slpA$-infected eyes. Interference with activation of the TLR2 and TLR4 pathways resulted in blunted expression of complement factors (C3, Cfb, and C6) and several innate genes such as TLR2, TLR4, TLR6, TLR8, MyD88, Nod2, Nlrp3, NF-κB, STAT3, RelA, RelB, and Ptgs2. Interference with activation of the TLR2 and TLR4 pathways also reduced the expression of

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53   several inflammatory cytokines such as CSF3, IL-6, IL-1β, CSF2, IL-1α, TNFα, IL-23α, TGFβ1,

54   and IL-12β and chemokines CCL2, CCl3, CXCL1, CXCL2, CXCL3, CXCL5, CXCL9, and

55   CXCL10. All of the aforementioned genes were significantly upregulated in WT-infected eyes.

56

57   **CONCLUSIONS.** These results suggest that interfering with the activation of innate

58   immune pathways during *Bacillus* endophthalmitis significantly reduced the intraocular

59   inflammatory response. This positive clinical outcome could be a strategy for anti-inflammatory

60   therapy of an infection typically refractory to corticosteroid treatment.

61

62   **Key Words:** endophthalmitis, inflammatory response, TLR, *Bacillus*, inflammation, retina,

63   blindness

64

65   **INTRODUCTION:**

66   Bacterial endophthalmitis is a dangerous ocular infection that is considered a medical

67   emergency. This intraocular infection is often caused by Gram-positive bacteria [1-4]. The infecting

68   organism can be introduced into the immune-privileged posterior chamber of the eye by an injury

69   (post-traumatic), after a surgical procedure (post-operative), or by metastasis of bacteria into the

70   eye from a distant infection site (endogenous) [5-8]. Regardless of the route of infection, the signs

71   and symptoms are quite similar, ranging from red eyes and swollen eyelids to severe intraocular

72   pain and vision loss [1-4]. The severity of the disease can range from mild inflammation that resolves

73   with treatment, to a fulminant, rapidly progressing infection that is refractory to currently available

74   treatment options. *Bacillus* causes the most severe form of bacterial endophthalmitis and is often

75   associated with worse patient outcomes than infections caused by other pathogenic species [6,8,9-

76   11]. The pathogenicity of *Bacillus* endophthalmitis is associated with the inflammogenicity of the

77   bacterial cell wall and the production of secreted toxins and enzymes [12-18]. The very severe nature

78   of *Bacillus* endophthalmitis dictates the need for prompt and rapid therapy to stop the disease

79   progression. However, *Bacillus* endophthalmitis may result in vision loss within 12-24 hours of

80   infection, despite therapeutic intervention that may otherwise effectively attenuate infection

81   caused by other ocular pathogens such as *Staphylococcus aureus* or *Streptococcus pneumoniae*

82   [9].

83

84   *Bacillus* is a Gram-positive, motile, spore-forming rod, and is distributed throughout nature,

85   especially in soil [19-21]. Metabolically-inactive *B. cereus* triggered an inflammatory response in a

86   rabbit experimental endophthalmitis model, suggesting a significant role of cell wall components

87   in inciting inflammation [11]. In addition to the thick outer layer containing peptidoglycan, lipoteichoic

88   acid, and lipoproteins, the unique architecture of the *Bacillus* envelope also includes flagella and

89   a monomolecular crystalline array of proteinaceous subunits termed surface layers or S-layer [22-

90   25]. If present, S-layer protein (SLP) is one of the most abundant proteins in the bacterial cell

91   envelope and provides the organism with a selective advantage in diverse habitats [26]. SLP

92   contributes to colonization by promoting bacterial adherence and biofilm formation [27,28], as well

93   as protecting the bacteria against complement killing and phagocytosis [29-32]. We recently

94   demonstrated that the absence of SlpA resulted in significant retention of retinal function, reduced

95   inflammatory cell influx, and muted disease severity in a mouse experimental endophthalmitis

96   model of *Bacillus* endophthalmitis [33]. We also reported *Bacillus* SlpA as a stimulator of nuclear

97   factor kappa-light-chain-enhancer of activated B cells (NF-κB) in human retinal Muller cells *in vitro*

98   [34]. SLP also protects *Bacillus* from phagocytosis by neutrophils and retinal cells and also impacts

99   bacterial adherence to retinal cells [34]. SLP appears to be an important virulence factor in the

100   pathogenesis of *Bacillus* endophthalmitis.

101

102   During infection, ocular immune privilege [35] is compromised by an overwhelming, acute

103   inflammation resulting from interactions between innate receptors and microbial ligands. We

104   reported that Toll-like receptors (TLR) 2 and 4, but not TLR5, were essential for rapid intraocular

105   inflammation during *Bacillus* infection [36-38]. Being a Gram-positive pathogen, *Bacillus* does not

106   produce lipopolysaccharide (LPS), so the specific ligand on *Bacillus* that interacts with TLR4

107   remained unknown. We reported that *Bacillus* SLP activated both TLR2 and TLR4 [34]. When

108   activation of these TLRs was blocked by injection of oxidized phospholipid (OxPAPC) during

109   experimental endophthalmitis, disease severity and overall inflammation were reduced and retinal

110   function was retained compared to that in untreated control mice [34]. OxPAPC is produced by the

111   oxidation of 1-palmitoyl-2-arachidonyl-sn- glycero-3-phosphorylcholine (PAPC) and has been

112   shown to inhibit the signaling induced by bacterial lipopeptide and lipopolysaccharide (LPS),

113   known agonists for TLR2 and TLR4. OxPAPC competes with CD14, lipid binding protein (LBP),

114   and MD2, and thus blocks signaling in TLR2 and TLR4 pathways [39,40].

115

116   TLR-ligand interactions during intraocular infection generates inflammatory mediators that

117   recruit neutrophils into the eye [41,42]. When TLRs recognize surface molecules on pathogenic

118   bacteria, signals are transmitted via the adaptor molecules to the signaling molecules which are

119   located in the cytoplasm. Signals from cytoplasm reach the nucleus and activate the transcription

120   of inflammatory mediators. We reported that both myeloid differentiation primary response gene-

121   88 (MyD88) and Toll/interleukin-1 receptor (TIR) domain-containing adaptor-inducing interferon-

122   β (TRIF) regulate this inflammation signaling cascade during murine experimental

123   endophthalmitis [38]. Neutrophils are the primary innate responders and may cause bystander

124   damage to the retina during an intraocular infection [43-45]. Therefore, inflammatory mediators that

125   recruit neutrophils are crucial for disease outcome. During experimental *Bacillus* endophthalmitis,

126   the absence of tumor necrosis factor (TNF) α resulted in reduced neutrophil infiltration into the

127   eye, which resulted in an elevated bacterial load and damage to ocular tissues [44]. The absence

128   of TNFα was compensated for by elevated expression of other mediators such as chemokine (C-

129   X-C motif) ligand (CXCL) 1, CXCL2/MIP1α, and IL-6, which might have contributed to delayed

130   recruitment of neutrophils and overall pathogenesis [44]. We reported that the absence of CXCL1

131   or anti-CXCL1 treatment led to a blunted inflammatory response but improved clinical outcome,

132   suggesting a potential benefit for targeting neutrophil chemoattractants to curb inflammation-

133   mediated damage [45].

134

135   In endophthalmitis, the use of anti-inflammatory therapeutics with antibiotics has not

136   proven completely effective at improving disease outcomes [5-8]. The primary function of the innate

137   inflammatory response is to detect invading pathogens and clear them as quickly as possible.

138   However, the highly inflammogenic *Bacillus*-induced ocular inflammatory response is often so

139   robust that it is difficult to control and ultimately results in vision loss [9,46]. At present, a universal

140   therapeutic regimen to prevent vision loss in *Bacillus* endophthalmitis is not available. Since the

141   depletion of individual proinflammatory mediators could be compensated for by the synthesis of

142   other mediators, finding anti-inflammatory agents with the potential to block multiple inflammatory

143   pathways might hold the potential for future anti-inflammatory therapeutics.

144

145   An acute and potentially damaging inflammatory response in the eye is one of the major

146   problems of bacterial endophthalmitis. We reported that an absence of TLR2 or TLR4 in knockout

147   mice or inhibition of TLR2 and TLR4 activation by OxPAPC improved the clinical outcome of

148   experimental *Bacillus* endophthalmitis [33,36,37]. OxPAPC-mediated interference of innate activation

149   in response to pathogens and pathogen associated molecular patterns (PAMPs) are well

150   recognized [39,40,47,48]. In this study, we investigated how interfering with innate immune pathways

151   during the early stage of *Bacillus* infection would impact inflammation during the later stage of

152   infection. Since inhibition of TLR signaling serves as a promising treatment option for

153   inflammatory diseases [49], we hypothesized that innate interference could be protective for a

154   rapidly blinding disease like *Bacillus* endophthalmitis. Innate pathway interference was achieved

155   by infection with SLP-deficient *Bacillus* (∆*slpA*) or treatment of experimental *Bacillus*

156   endophthalmitis with OxPAPC. NanoString analysis of a mouse inflammation panel revealed that

157   innate interference considerably reduced inflammatory gene expression in the whole infected eye.

158   Our findings suggest that the attenuation of innate activation may be a viable anti-inflammatory

159   option for the treatment of *Bacillus* and perhaps other types of endophthalmitis.

160

161   **MATERIALS AND METHODS**

162

163   **Ethical statement**

164   The experiments in this study involved the use of mice. All animal experiments were

165   performed in strict accordance with the recommendations in the Guide for the Care and Use of

166   Laboratory Animals and the Association for Research in Vision and Ophthalmology Statement for

167   the Use of Animals in Ophthalmic and Vision Research. The protocols were approved by the

168   Institutional Animal Care and Use Committee of the University of Oklahoma Health Sciences

169   Center (protocol numbers 15-103, 18-043, and 18-087).

170

171   **Bacterial strains**

172   *B. thuringiensis* subsp. Galleriae NRRL 4045 (WT) or its isogenic S-layer protein-deficient

173   mutant (∆*slpA*) was used to initiate experimental endophthalmitis in mice, as previously described

174   [33,34]. WT *B. thuringiensis* and ∆*slpA B. thuringiensis* were grown to early stationary phase in brain

175   heart infusion (BHI; VWR, Radnor PA) broth for 18 hours and diluted to 100 CFU/0.5 µl for

176   injection into the mid-vitreous.

177

178   **Mice and Intraocular Infection**

179   C57BL/6J mice purchased from Jackson Laboratories (Bar Harbor ME, Stock No. 000664)

180   were used for all experiments. Upon arrival, they were housed on a 12 hour on/12 hour off light

181   cycle in biohazard level 2 conditions and acclimated for at least two weeks to equilibrate their

182   microbiota. Mice were 8-10 weeks of age at the time of the experiments. A combination of

183   ketamine (85 mg/kg body weight; Ketasthesia, Henry Schein Animal Health, Dublin, OH) and

184   xylazine (14 mg/kg body weight; AnaSed, Akorn Inc., Decatur, IL) were used to sedate the mice.

185   Four groups of C57BL/6J mice with three mice in each group were used in these experiments

186   (Figure 1A). The first two groups were infected with 100 CFU WT *B. thuringiensis*/0.5 μl BHI, and

187   the third group was infected with 100 CFU Δ*slpA B. thuringiensis*/0.5 μl BHI,as previously

188   described [33,34]. At 4 hours postinfection, the second group of WT *B. thuringiensis*-infected mice

189   was intravitreally treated with the synthetic TLR2/4 inhibitor OxPAPC (Invivogen; 30 ng/μl)

190   (WT+OxPAPC) [34]. Uninfected mice were used as control .

191

192   **Harvesting mouse eyes and RNA preparation**

193   At 10 hours postinfection, all mice were euthanized by CO_2 inhalation. Experimental and

194   control eyes were harvested and transferred to individual 1.5ml screw-cap tubes containing sterile

195   1mm glass beads (BioSpec Products, Inc., Bartlesville OK) and 400μl lysis buffer (RLT) from an

196   RNeasy kit (Qiagen, Germantown, MD). Eyes were homogenized as previously described using

197   a Mini-BeadBeater (Biospec Products Inc., Bartlesville, OK) for 120 seconds (2 pulses of 60

198   seconds each). Eye homogenates were centrifuged and transferred into another screw-cap tube

199   containing 0.1 mm glass beads (Biospec Products Inc., Bartlesville, OK) and homogenized for 60

200   seconds in the Mini-BeadBeater. Tissue lysates were recovered by centrifugation and processed

201   for total RNA purification using the RNeasy kit according to the manufacturer's instructions.

202   Genomic DNA was removed using the TURBO DNA-free kit (ThermoFisher Scientific, Inc.,

203   Waltham, Massachusetts). Eluted RNA was cleaned and concentrated using RNA Clean &

204 Concentrator (Zymo Research, CA, USA). The concentration and purity were confirmed on a

205 Nanodrop spectrophotometer [50].

206

NanoString Analysis

208 In this technique, a biotin-labeled capture probe and a fluorescent-labeled reporter probe

209 hybridize to specific target transcripts. The nCounter NanoString analysis system counts the

210 immobilized RNAs using their barcodes. The NanoString assay was performed using total RNA

211 samples from whole mouse eyes. From each sample, 100ng of RNA was hybridized with

212 NanoString's XT PGX Mmv2 Inflammation code set containing 248 mouse inflammatory genes

213 and six housekeeping genes using NanoString's nCounter XT CodeSet Gene Expression Assay

214 Protocol. After hybridization for 16 hours at 65°C, the samples were loaded onto an nCounter

215 Cartridge and run on the NanoString Sprint platform. Data was normalized using NanoString's

216 nSolver Analysis Software v 4.0. Normalized data of 248 inflammatory genes was then used to

217 analyze the expression profile in WT-infected, WT-infected and OxPAPC-treated (WT+OxPAPC),

218 and Δ*slpA*-infected eyes compared to uninfected eyes.

219

Statistics

221 NanoString analysis was performed in NanoString's nSolver Analysis Software v 4.0. After

222 data normalization, GraphPad Prism 7 (Graph-Pad Software, Inc., La Jolla, CA) was used for the

223 comparative analysis. Multiple t-tests were performed to compare the means of individual genes

224 from two different groups. Gene expression in uninfected eyes was compared with that of WT-

225 infected, WT+OxPAPC, and Δ*slpA*-infected eyes. We also compared WT-infected eyes with

226 WT+OxPAPC and Δ*slpA*-infected eyes. P values less than 0.05 were considered to be significant.

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229

230   **RESULTS**

231

232   **Interfering with TLR2 and TLR4 activation affected inflammatory gene expression in the**

233   **eye.**

234   *Bacillus* induces a robust intraocular inflammatory response that irreversibly damages

235   nonregenerative retinal tissues and results in a devastating clinical outcome. *Bacillus* SLP likely

236   triggers the host inflammatory pathway by activating both TLR2 and TLR4 [33,34]. To explore the

237   interference of innate activation on intraocular inflammatory responses during the later stages of

238   *Bacillus* endophthalmitis, we performed NanoString analysis on total RNA isolated from our

239   experimental groups. Figure 1A represents the schematics of our experimental approach. In

240   Figure 1B, we performed Principal Component Analysis (PCA) to understand the variability of

241   gene expression in our experimental groups. The normalized mouse inflammatory genes were

242   plotted in ClustVis, a web tool for visualizing the clustering of multivariate data. All replicates of

243   WT-infected eyes clustered together. Except for one eye in the WT+OxPAPC group, replicates of

244   three uninfected, three Δ*slpA*-infected, and two WT+OxPAPC eyes were clustered together,

245   indicating transcriptional similarities among these groups. In Figure 1C, we calculated the

246   Shannon diversity index to understand the diversities of expressed genes between groups.

247   Compared to uninfected eyes, gene expression was more diverse in WT-infected eyes. However,

248   compared to WT-infected eyes, gene expression was less diverse in WT+OxPAPC or Δ*slpA*-

249   infected eyes. In Figure 1D, we employed the R package "pHeatmap" for visualization of gene

250   expression in our groups [51]. Most of the inflammatory genes in three uninfected, three Δ*slpA*-

251   infected, and two WT+OxPAPC eyes had low levels of expression. In contrast, most of the genes

252   in three WT-infected eyes and one WT+OxPAPC eye had high levels of expression. The branch

253   lengths on the top of the heat map indicate the correlation of gene expression. The longer

254   branches indicate a lower correlation. All uninfected, two WT+OxPAPC, and all Δ*slpA*-infected

255   eyes were highly correlated with each other, whereas all WT-infected and one WT+OxPAPC eye

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256   were highly correlated with one another. On the Y-axis, we grouped and color-coded these

257   inflammatory genes based on their function: chemoattractants, complement, innate immune

258   genes, cytokines, kinases, and peptidases. Together, the distinct gene expression clusters and

259   expression patterns demonstrate that mouse ocular inflammatory gene expression was muted if

260   SLP was absent in the infecting strain or if infected eyes were treated with the TLR inhibitor

261   OxPAPC.

262

263   **Interfering with TLR2 and TLR4 activation reduced ocular inflammatory responses.**

264   We further probed our NanoString data to quantify genes that were altered in our

265   experimental groups. Figure 2A-C and 2G i-ii demonstrate that among the 248 mouse

266   inflammatory genes, 137 genes were significantly upregulated in WT-infected eyes compared to

267   uninfected eyes. However, the number of significantly upregulated genes in WT+OxPAPC eyes

268   and ∆*slpA*-infected eyes was only 44 and 34, respectively (Figure 2G i) compared to that of

269   uninfected eyes. Compared to uninfected eyes, expression of 9 genes, which were common to

270   all non-control groups, was significantly increased (Figure 2G i). Similarly, expression of 91 genes

271   which were common to all non-control groups remained unchanged compared to that of

272   uninfected eyes (Figure 2G ii). We found 66 and 123 genes significantly decreased in

273   WT+OxPAPC and ∆*slpA*-infected eyes, respectively, compared to WT-infected eyes (Figure 2D,

274   E, and G iii). Compared to TLR2 and TLR4 expression in WT-infected eyes, the expression of

275   TLR2 (blue) and TLR4 (red) in WT+OxPAPC and ∆*slpA*-infected eyes was decreased, suggesting

276   that OxPAPC indeed interfered with the innate activation of those two pathways. In Figure 2G iii,

277   the expression of 61 significantly upregulated genes in WT-infected eyes was blunted in

278   WT+OxPAPC and ∆*slpA*-infected eyes. One gene, protein kinase C alpha (PRCKα), was

279   significantly downregulated in WT-infected eyes but upregulated in WT+OxPAPC and ∆*slpA*-

280   infected eyes (Supplementary Table 1). There were no differences in expression of 103 genes in

281   the 4 groups (Figure 2G iv). Compared to uninfected eyes, 55% of total genes were differentially

282 expressed in WT-infected eyes. In contrast, only 18% and 14% of genes were differentially

283 expressed in WT+OxPAPC and Δ*slpA*-infected eyes compared to uninfected eyes, respectively.

284 Compared to WT-infected eyes, expression of 27% and 50% genes were decreased in

285 WT+OxPAPC and Δ*slpA*-infected eyes, respectively (Figure 2F). Taken together, these findings

286 further suggested that inflammation was not as robust when SlpA was absent in *Bacillus* or when

287 TLR2 and TLR4 was inhibited by OxPAPC.

288

289 **Interfering with TLR2 and TLR4 activation blunted complement gene expression in the eye.**

290 Activation of the complement cascade is one of the first lines of defense in bacterial

291 infection. Studies about the impact of complement factors in the pathogenesis of intraocular

292 diseases is limited. Cobra venom factor-decomplemented Guinea pigs had impaired host defense

293 during intraocular infection with *S. epidermidis* [52] and *Pseudomonas aeruginosa* [53]. Host defenses

294 were restored when complement levels returned to normal in those guinea pigs. In contrast, the

295 absence of complement component C3 did not alter inflammation in a mouse model of

296 experimental *S. aureus* endophthalmitis [54]. These findings suggest diversity in the contribution of

297 the complement cascade in different endophthalmitis models. Figure 3A depicts the log fold

298 change of complement pathway components in WT-infected, WT+OxPAPC, and Δ*slpA*-infected

299 eyes compared to uninfected eyes. Expression of complement factors b (Cfb), C3, C6, and C7 in

300 WT-infected eyes was 19.6-, 9.9-, 2.9-, and 2.6-fold higher, respectively, compared to uninfected

301 eyes. In contrast, compared to WT-infected eyes, the expression of these genes was decreased

302 in both WT+OxPAPC and Δ*slpA*-infected eyes (Supplementary Table 2). Figure 3B demonstrates

303 the transcript levels of differentially expressed complement factors. We detected increased

304 expression of C3, Cfb, C6, C7, C1q subcomponent-alpha polypeptide (a), C1q subcomponent-

305 beta polypeptide (b), C1r subcomponent A (a), C8 beta polypeptide (b), and C1 s subcomponent

306 (s) transcripts in WT-infected eyes compared to uninfected eyes. Compared to WT-infected eyes,

307 expression of C3, Cfb, and C1ra transcripts were significantly reduced in WT+OxPAPC and

308 Δ*slpA*-infected eyes. Expression of C6, C7, C1qb, and C1s transcripts was also significantly

309 reduced in Δ*slpA*-infected eyes. C8b expression was only reduced in WT+OxPAPC eyes.

310 Expression of hemolytic complement Hc, was increased 1.3-fold in WT-infected eyes compared

311 to uninfected eyes and decreased 1.2-fold and 1.4-fold in WT+OxPAPC and Δ*slpA* infected eyes

312 respectively, compared to WT-infected eyes. Collectively, these findings suggested that *Bacillus*

313 infection-driven complement gene expression in the eye could be prevented by inhibiting TLR2

314 and TLR4 activation.

315

316 **Interfering with TLR2 and TLR4 activation impacted innate immune gene expression in the**

317 **eye.**

318   Intraocular innate receptors and their adaptors contribute to the pathogenesis of *Bacillus*

319 endophthalmitis and other vision-threatening infections. During *Bacillus* endophthalmitis, severe

320 intraocular inflammation is triggered by TLR2 and TLR4 via MyD88 and TRIF [36,37,50]. Here, we

321 focused on 41 genes involved in innate immune pathways, which include receptors, adaptors,

322 signaling molecules, and hormones. (Supplementary Table 3). Among the 24 differentially

323 expressed innate immune pathway genes, 11 were highly upregulated in WT-infected eyes

324 compared to uninfected eyes, and were reduced in WT+OxPAPC and Δ*slpA*-infected eyes

325 compared to WT-infected eyes (Figure 4A). Figure 4B depicts a heatmap of the expression of

326 TLRs and other innate receptors. As expected, we observed increased expression of TLR2 and

327 TLR4 transcripts in WT-infected eyes. In addition, we observed elevated expression of TLR6,

328 TLR8, nucleotide-binding oligomerization domain-containing protein (NOD) 2, and LRR- and pyrin

329 domain-containing protein (Nlrp3) in WT-infected eyes. Expression of these innate genes was

330 significantly decreased in WT+OxPAPC or Δ*slpA*-infected eyes compared to WT-infected eyes.

331 The expression of other innate immune genes, such as MyD88, NF-κB, v-rel reticuloendotheliosis

332 viral oncogene homolog (Rel) A, and v-rel oncogene related (Rel) B, prostaglandin-endoperoxide

333 synthase (Ptsg) 2, signal transducer and activator of transcription (STAT) 2 and 3, and heat shock

334   protein (Hspb) 1 was significantly increased in WT-infected eyes compared to uninfected eyes

335   (Figure 4C). Expression of these genes was decreased in WT+OxPAPC and Δ*slpA*-infected eyes

336   compared to WT-infected eyes. Among the 41 innate immune genes we analyzed, expression of

337   Ptgs2, Nlrp3, TLR2, TLR6, and NOD2 were 218-, 140-, 26.8-, 19.8-, and 17.5-fold greater than in

338   uninfected eyes and were the top five upregulated innate immune genes in WT-infected eyes. We

339   observed a negative fold change of these genes in WT+OxPAPC and Δ*slpA*-infected eyes

340   compared to WT-infected eyes (Supplementary Table 3). Here, we identified several innate

341   immune genes whose expression was not changed at 4 hours postinfection in *Bacillus*

342   endophthalmitis. Furthermore, we observed dampened expression of those innate immune genes

343   when TLR2 and TLR4 activation did not occur. These results suggested that the innate immune

344   gene expression in *Bacillus* endophthalmitis occurring during robust inflammation could be

345   prevented by inhibiting TLR2 and TLR4 activation.

346

347   **Interfering with TLR2 and TLR4 activation reduced cytokine gene expression in the eye.**

348   During endophthalmitis, cytokines and chemokines drive ocular inflammation. In TNF$\alpha^{-/-}$

349   and CXCL1$^{-/-}$ mice, neutrophil recruitment was suppressed. However, this resulted in an increase

350   in bacterial load and disease severity in TNF$\alpha^{-/-}$ mice[44], but no change in bacterial load and a

351   reduction in disease severity in CXCL1$^{-/-}$ mice[45]. These contrasting outcomes suggest a potential

352   role of additional inflammatory cytokines during infection. Here, we focused on the expression of

353   51 mouse inflammatory cytokines during *Bacillus* endophthalmitis. Figure 5A depicts the log-fold

354   changes of these genes in our experimental groups. Eighteen inflammatory cytokine genes were

355   significantly increased in WT-infected eyes compared to uninfected eyes. These genes were

356   decreased in WT+OxPAPC or Δ*slpA*-infected eyes compared to WT-infected eyes (Figure 5B,

357   Supplementary Table 4). Figure 5C depicts the expression of IL-6, TNFα, and IL-1β and several

358   other pro-inflammatory cytokines in our infection and treatment groups. In addition to IL-6, TNFα,

359   and IL-1β, the expression of colony-stimulating factor (CSF)3, CSF2, IL-1α, IL-23α, transforming

360   growth factor (TGF)β1, and IL-12β were significantly increased WT-infected eyes compared to

361   uninfected eyes. Expression of CSF3, IL-6, IL-1β, CSF2, IL-1α, TNFα, and IL-23α was

362   significantly decreased in both WT+OxPAPC and Δ*slpA*-infected eyes compared to WT-infected

363   eyes. Expression of TGFβ1 and IL-12β was reduced only in Δ*slpA*-infected eyes compared to

364   WT-infected eyes.

365

366   Pro-inflammatory cytokines act in concert with specific cytokine inhibitors to regulate the

367   human immune response. The expression of anti-inflammatory cytokines IL-1 receptor accessory

368   protein (rap), IL-10, and IL-1 receptor antagonist (rn) was significantly increased in WT-infected

369   eyes compared to uninfected eyes, but significantly reduced in Δ*slpA*-infected eyes compared to

370   WT-infected eyes. The expression of IL-10 was reduced only in WT+OxPAPC eyes compared to

371   WT-infected eyes (Figure 5D). The expression of anti-inflammatory cytokines IL-13 and IL-4 were

372   not changed in all groups. Relative to uninfected eyes, expression of CSF3, IL-6, IL-1β, CSF2, IL-

373   1α, and TNFα was 50-fold or higher (Supplementary Table 4). These findings not only identified

374   several inflammatory cytokines which were not expressed at 4 hours postinfection in *Bacillus*

375   endophthalmitis, but also identified blunted expression of others when TLR2 and TLR4 activation

376   was blocked. Collectively, these findings suggested that the innate inhibition could be a viable

377   strategy to avert inflammatory cytokine production during *Bacillus* endophthalmitis.

378

379   **Interfering with TLR2 and TLR4 activation reduced chemoattractant gene expression in the**

380   **eye.**

381   A hallmark of *Bacillus* endophthalmitis is robust influx of inflammatory cells into the

382   posterior chamber of the eye. Chemokines act as chemoattractants to guide the migration of these

383   cells. Figure 6A depicts the log fold changes of 27 inflammatory chemokines and receptors that

384   belong to CXC and CC groups. Fifteen chemokines were significantly increased in WT-infected

385   eyes compared to uninfected eyes. Compared to WT-infected eyes, expression of these 15

386   chemokines was significantly reduced in WT+OxPAPC or Δ*slpA*-infected eyes (Figure 6B,

387   Supplementary Table 5). Figure 6C depicts the expression of chemoattractants in our infection

388   and treatment groups. Expression of CXCL1, CXCL2, CXCL10, CCL2, CCL3, CXCL3, CCL20,

389   and CCL4 was elevated in WT-infected eyes compared to uninfected eyes. Expression of these

390   genes was reduced 10-fold or greater in WT+OxPAPC or Δ*slpA*-infected eyes compared to WT-

391   infected eyes. Figure 6D depicts analysis of the expression of chemokine receptors. Expression

392   of chemokine (C-C motif) receptor (CCR)1, chemokine (C-X-C motif) receptor (CXCR) 2, CXCR4,

393   and CCR7 was greater in WT-infected eyes compared to uninfected eyes, but was significantly

394   reduced in WT+OxPAPC or Δ*slpA*-infected eyes compared to WT-infected eyes. CCR7

395   expression was significantly increased in WT-infected eyes compared to uninfected eyes, and

396   significantly reduced in Δ*slpA*-infected eyes compared to WT-infected eyes. There was no change

397   in CCR7 expression between WT-infected and WT+OxPAPC eyes. Expression of CXCL2,

398   CXCL1, CXCL3, CCL20, CCL4, CCL3, and CCL2 was 500-fold or higher relative to that of

399   uninfected eyes (Supplementary Table 5). In addition to confirming our retina-specific

400   transcriptome data at 4 hours postinfection with *Bacillus* [50], these findings identified the

401   expression of additional chemoattractants in the whole eye during the later stages of infection.

402   Altogether, these results demonstrated that the chemokine response is blunted when SlpA is

403   absent in *Bacillus* and when TLR2 and TLR4 are inhibited by OxPAPC. Collectively, these results

404   suggested the inhibition of TLR2 and TLR4 pathways might be a viable strategy to block the

405   chemoattractant synthesis during *Bacillus* endophthalmitis, arresting the influx of inflammatory

406   cells into the eye.

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411   **DISCUSSION**

412

413   The inflammatory response is generally defined as a protective reaction to stimulation of

414   the host defense system to invading pathogens or endogenous signals. When this protective

415   response goes unchecked, dysregulated inflammatory responses occur and can worsen the

416   disease severity. Cells involved in immune responses produce plethora of mediators including

417   cytokines, chemokines, antibodies, and complement to aid in the war against invading pathogens.

418   Although inflammatory responses protect tissue against these insults, the inflammation itself could

419   cause damage. Immune privileged tissues resist immunogenic inflammation through multiple

420   mechanisms. A well-controlled system to regulate the inflammatory response in the eye is

421   necessary [35].

422

423   We reported that *Bacillus* reach their maximum growth faster than most other Gram-

424   positive endophthalmitis pathogen in the eye [9]*. Bacillus* endophthalmitis is well recognized for

425   creating an aggressive acute inflammatory response that results in retinal damage with rapid loss

426   of vision within 12-24 hours despite antibiotic and anti-inflammatory treatment. Avirulent *S.*

427   *epidermidis* is generally cleared by an active but not overly robust inflammatory response. Current

428   therapies for this severe blinding infection include intravitreal antibiotics that can sterilize the eye

429   if administered at an early phase of infection [1-8]. The use of corticosteroids for anti-inflammatory

430   therapy in endophthalmitis is common, but their efficacy in this disease is controversial [55-61].

431   Therefore, identifying critical elements of inflammatory pathways that can be targeted to inhibit

432   the intraocular inflammation are viable prospects for future therapeutics in *Bacillus*

433   endophthalmitis.

434

435   One of the earliest steps of any inflammatory response is the recognition of the invading

436   organisms by the host innate defense system. The retina is comprised of different types of cells

437   necessary for the visual cycle, maintaining retinal integrity, and homeostasis. These cells also act

438   as a defensive barrier to protect against invading pathogens by expressing innate receptors. We

439   reported that during infection, *Bacillus* migrates in close proximity to the inner limiting membrane

440   (ILM) of the retina [11], facilitating potential interactions between the retina and invading microbes.

441   As such, the outer most layer of the bacterium may be the first to interact with innate receptors

442   on cells lining the ILM.

443

444   S-layer protein (SLP), the outer most layer of the *Bacillus* envelope, serves as a protective

445   barrier for the pathogen. Besides maintaining cell wall integrity, SLP provides additional benefits

446   for the microbes to survive in diverse host environments, but can also activate the immune system

447   [26,62,63]. We recently reported that the absence of *Bacillus* SLP reduced disease severity and

448   damage to the eye in murine experimental endophthalmitis [33]. Activation of innate immunity and

449   its effects on host tissue play a crucial role in the severity and poor visual outcomes of *Bacillus*

450   endophthalmitis. In a follow-up study, we reported *Bacillus* SLP as an activator of the TLR2 and

451   TLR4 pathways [34]. In a recent transcriptome study of retinas of eyes infected with *Bacillus*, we

452   reported elevated expression of 72 genes at 4 hours postinfection and decreased expression of

453   these 72 genes in the absence of TLR4 [50]. The reduced inflammation and improved clinical

454   outcomes in the absence of TLR2 [36] or TLR4 [37] or following TLR2 and TLR4 inhibition [34] suggests

455   innate immune pathway inhibition as a prospective option for the anti-inflammatory arm of

456   treatment of *Bacillus* intraocular infection.

457

458   The nature of ocular tissue damage arising from an excessive inflammatory response in

459   bacterial endophthalmitis is not completely understood. Based on our recent studies

460   demonstrating positive clinical outcomes when TLR2 and TLR4 pathways are blocked, we further

461   probed this treatment strategy by using NanoString to compare gene expression in whole eyes in

462   our experimental groups. NanoString is better for targeted transcriptomics since there is no need

463   for preparing gene libraries, enzymes, and processing as in other next generation sequencing

464   (NGS) techniques [64]. Unlike other alternatives, NanoString does not require polymerase activity,

465   and therefore the chance of introducing bias is lower [65-67]. NanoString can identify RNAs in a

466   heterogeneous sample, even one that contains cells from different species. In this study, we

467   focused on 248 mouse genes potentially associated with the inflammatory response in

468   experimental murine *Bacillus* endophthalmitis. Distinct clusters of infection groups in our principal

469   component analysis suggested a similar pattern of gene expression within each group. Except for

470   one eye in the WT+OxPAPC group, we observed specific clusters in our groups. One explanation

471   for this outlier could be a microinjection error during intraocular OxPAPC delivery. Overall,

472   however, the clustering of uninfected, Δ*slpA*-infected, and WT+OxPAPC eyes suggested

473   transcriptional similarities among these groups.

474

475   Most of the genes analyzed were differentially expressed in WT-infected eyes compared

476   to uninfected eyes. Expression of most of these genes were reduced in WT+OxPAPC, and Δ*slpA*-

477   infected eyes compared to WT-infected eyes. Expression of 61 genes which were significantly

478   increased in WT-infected eyes compared to uninfected eyes were decreased in WT+OxPAPC or

479   Δ*slpA*-infected eyes compared to WT-infected eyes (Supplementary Table 1). Only one gene,

480   protein kinase C alpha (PRKCα), was decreased in WT-infected eyes compared to uninfected

481   eyes but significantly increased in Δ*slpA*-infected and WT+OxPAPC eyes. PRKCα is an essential

482   modulator of rod photoceptor and bipolar cell function. PRKCα and few other isoforms of PRKC

483   are reported to be present in ganglion, amacrine, and RPE cells. PRKCα has a role in a variety

484   of cellular functions, including proliferation, apoptosis, differentiation, motility, and inflammation

485   [68-71]. It has been reported that TLR1/2-driven activation of MAPK, NF-kB, and AP-1, as well as

486   secretion of TNF-α, IL-6, and IL-10 by dendritic cells requires the activation of PRKCα [70].

487   Overexpression of PRKCα resulted in loss of tight junctions, which reduced the transepithelial

488   permeability in an epithelial cell line LLC-PK1 [72]. Nitric oxide is an effector molecule of innate

489   immunity and inflammatory modulator in different inflammatory conditions, and PRKCα has been

490   linked to the regulation of nitic oxide production in vascular smooth muscle, murine macrophages,

491   and murine microglia [73]. Activation of protein kinase C (PKC) induced the production of various

492   inflammatory cytokines from human bronchial epithelia cells, suggesting that PKC activation might

493   be crucial for the initial defense mechanism in the airway against pathogens [74]. The absence of

494   PRKC isoform δ in mice resulted in reduced inflammatory mediator production and neutrophil

495   infiltration in an asbestos-associated disease model [75]. We observed decreased expression of

496   PRKCα in WT-infected eyes compared to uninfected eyes, which suggested a potential anti-

497   inflammatory property of PRKCα. However, this was not reflected in the pathogenesis *in vivo*,

498   since we found increased PMN influx in WT-infected eyes. PKC is expressed differentially in

499   various mammalian tissues, and is highly enriched in brain and lymphoid organs [76]. Together, this

500   suggests that the role of PRKC in inflammation may be tissue and organ-specific.

501

502   Improving treatment options is a main focus of our research in endophthalmitis. This is

503   especially critical for *Bacillus* endophthalmitis in which the majority of infected eyes lose significant

504   vision. The contribution of the host immune response in mediating bystander damage and visual

505   function loss is still not well understood. Complement is an integral component of the innate host

506   defense against infection and a prime mediator of tissue inflammation. We recently reported that

507   pentraxin 3 (PTX3), which activates the classical complement pathway via C1q to facilitate

508   pathogen recognition and clearance, was expressed as early as 4 hours in retinas of mouse eyes

509   infected with *Bacillus* [50]. In this study, TLR2 and TLR4 interference resulted in decreased

510   expression of PTX3 and other complement pathway-related genes. In endophthalmitis caused by

511   *S. epidermidis* and *Pseudomonas aeruginosa*, complement exhibited a beneficial effect in ocular

512   defense [52,53]. However, for *S. aureus* endophthalmitis, complement was not protective [54]. Since

513   the interplay between complement and bacteria is relevant in inflammation, studying the role of

514 complement pathway-associated genes in the pathogenesis of a rapidly blinding infection like

515 *Bacillus* endophthalmitis may be valuable.

516

517   Innate recognition activation of associated pathways are complex events requiring

518 coordinate regulation of multiple cellular signaling components. We previously reported the role

519 of TLR2 and TLR4 in the pathogenesis of *Bacillus* endophthalmitis [36,37]. TLR2 is also important

520 for inflammatory responses in *S. aureus* endophthalmitis [77]. TLR6 forms a heterodimer with TLR2

521 and recognizes diacylated lipopeptides such as lipoteichoic acid on the cell wall of Gram-positive

522 bacteria. Synergistic interactions of TLR2/6 and TLR9 provide resistance against lung infection

523 [78,79]. TLR8, which is an innate endosomal receptor, usually senses viral infection [80]. However,

524 TLR8 may also detect RNA from *S. aureus*, which occurs when the bacterium is internalized and

525 degraded inside a phagocytic cells. Activation of TLR2 could serve as an inhibitor of TLR8

526 activation inside the cell [81]. This counter-inhibition might serve as a safety mechanism to avoid

527 exaggerated immune activation. Since TLR2 and TLR8 sense different PAMPs, this cross-

528 regulation represents a fine-tuning to the excessive immune response towards different classes

529 of pathogens. Neither TLR6 nor TLR8 expression was detected at 4 hours postinfection in retinas

530 of *B. cereus*-infected mice[50]. TLR6 and TLR8 were expressed, but not to a great degree at 10

531 hours in *B. cereus*-infected mouse eyes in this study, so this expression may emanate from

532 tissues or cells other than in the retina. Inflammasomes are critical as they protect the tissue

533 against pathogens and maintain homeostasis with commensal microbes [82,83]. While we reported

534 that the absence of Nlrp3 in Nlrp3^{-/-} global knockout mice did not affect the clinical outcomes in

535 *Bacillus* endophthalmitis at 8 hours postinfection [61], a recent report suggested that non-hemolytic

536 enterotoxin (NHE) from *B. cereus* activated the NLRP3 inflammasome and pyroptosis in primary

537 BMDMs, and that NHE and hemolysin BL functioned synergistically to induce inflammation in

538 mice [84]. Here, we observed significant increased expression of TLR2, TLR4, and Nlrp3 in WT-

539 infected eyes compared to uninfected eyes. Expression of these innate receptors were reduced

540 after TLR2 and TLR4 inhibition or infection with the Δ*slpA* mutant. These results suggested that

541 interference of innate pathways affected the expression of other innate receptors in the eye,

542 perhaps further limiting inflammation.

543

544 An absence of MyD88 or TRIF in mice resulted in improved clinical outcomes of *Bacillus*

545 endophthalmitis [38]. The importance of MyD88 has also been reported for *S. aureus*

546 endophthalmitis [85]. Lipid mediators derived from arachidonic acid control cell proliferation,

547 apoptosis, metabolism, and migration [86]. Prostaglandin-endoperoxide synthase (Ptgs) 2 converts

548 arachidonic acid to prostaglandin endoperoxide H2 (PGE2), which has an immunomodulatory

549 effect and serves to prevent the activation of neutrophils [87]. Ptgs2 was upregulated in a TLR4-

550 dependent manner in the retinas of mice infected with *Bacillus* [50]. Activation of TLRs and their

551 adaptors transfer the activation signal via a series of factors that ultimately activate inflammatory

552 transcription factor NF-κB in the nucleus. We recently reported that *Bacillus* SLP activated NF-κB

553 in human retinal Muller cells [33]. Here, we observed increased expression of MyD88, Ptgs2, RelA,

554 RelB, STAT2, STAT3, and Hspb1 in WT-infected eyes compared to that of uninfected eyes. We

555 also observed blunted expression of these innate immune genes when TLR2 and TLR4 pathways

556 were interfered with. Unfortunately, TRIF expression was not included in the NanoString panel,

557 so this was not tested. Phosphorylation of STAT3 and Hspb1 influences NF-kB activation and

558 promotes the inflammatory response. Retinal transcriptome analysis of *S. aureus* endophthalmitis

559 showed elevated expression of STAT3 in a TLR2-dependent manner [88]. SOCS3, which is a

560 negative regulator of cytokine signaling, inhibits STAT3 phosphorylation and prevents typically

561 excessive activation of proinflammatory genes [89]. The enhanced expression of STAT3 in our

562 study may help to explain why increased TLR4-dependent expression of SOCS3 failed to

563 negatively regulate inflammatory gene expression.

564

565   The *Bacillus* cell wall is highly inflammogenic. *Bacillus* SLP triggers the expression of

566   inflammatory mediators CXCL1, TNFα, CCL2, and IL-6 in human retinal Muller cells [33].

567   Inflammatory cytokines, which are polypeptides, can act as intercellular messengers and mediate

568   the process of inflammation and repair. In the eye, in response to an infection or injury,

569   inflammatory cytokines can be produced by corneal epithelium, RPE, microglia, macrophages,

570   endothelial cells, and other immune cells [90]. Here, we analyzed the expression of 51 inflammatory

571   cytokines and found 50-fold or greater expression of IL-1α, IL-1β, IL-6, CSF2, CSF3, and TNFα

572   in WT-infected eyes (Supplementary Table 4). CSF is a glycoprotein that stimulates the

573   production of neutrophil granulocytes and their release into the blood and the survival,

574   proliferation, differentiation, and function of neutrophil precursors and mature neutrophils [91,92].

575   Quite a bit is known about CSF's role in treating neutropenia and hematopoietic mobilization, but

576   information about its role in ocular infection is limited. Neutrophils enter the eye as early as 4

577   hours postinfection and are the primary immune cells that migrate into the vitreous after infection

578   [43]. Because of its role in neutrophil migration, the increased expression of CSF in the eye may

579   contribute to the rapid migration of neutrophils into the eye in experimental *Bacillus*

580   endophthalmitis. Because we did not observe an increase in the expression of CSF at 4 hours

581   postinfection in retinas of infected mouse eyes [50], the source of CSF may not be the retinal cells

582   at this early stage of infection.

583

584   IL-6 is both a pro-inflammatory and anti-inflammatory cytokine, depending upon the

585   environment [93]. IL-6 expression increases during the course of bacterial endophthalmitis [43,77] in a

586   TLR2- and TLR4-dependent manner [36,37]. Expression of IL-6 and IL-1β was also increased with

587   time in retinas of *S. aureus*-infected mice; however, compared to wild type, expression of these

588   mediators was reduced when TLR2 was absent [88]. Surprisingly, the absence of IL-6 in IL-6^{-/-} mice

589   did not affect the overall outcome of *Bacillus* endophthalmitis [45]. Here, interference with TLR 2

590   and TLR4 also resulted in reduced expression of the anti-inflammatory cytokine IL-10 at 10 hours

591   postinfection. Intravenous administration of IL-10, a potent inhibitor of cytokine production,

592   reduced neutrophil chemotaxis in LPS-induced uveitis [94,95]. Our results suggest that the

593   expression of anti-inflammatory cytokines may not affect the clinical outcome in our model

594   because the expression of anti-inflammatory cytokines in WT-infected eyes did not affect the

595   expression of IL-6, TNF-α, or IL-1β.

596

597   Chemokines are inflammatory mediators that serve as chemoattractants and are divided

598   into two significant subfamilies (CXC and CC chemokines) based on the spacing of the first pair

599   of N-terminal cysteine residues [96-98]. The CXC chemokines usually recruit neutrophils, whereas

600   CC chemokines tend to attract monocytes and involve in the pathogenesis of immune mediated

601   inflammation [96-98]. Activation of TLR2 on retinal microglia [99], and Muller glia [100,101] by *S. aureus*

602   resulted in the synthesis of CXC chemoattractants. Among the 248 mouse inflammatory genes

603   analyzed, CXCL2, CXCL1, and CXCL3 were the most highly expressed chemokines in WT-

604   infected eyes (Supplementary Table 5). CXCL2, CXCL1, and CXCL3 are highly homologous and

605   are powerful neutrophil chemoattractants [96-98]. We also observed increased expression of the CC

606   chemokines CCL2/MCP-1, CCL3/macrophage inflammatory protein (MIP)-1α, CCL4/MIP-1β,

607   CCL7/monocyte-chemotactic protein 3 (MCP3), and CCL20/Macrophage Inflammatory Protein-3

608   (MIP3A) in WT-infected eyes. CCL2 does not function as a chemoattractant for neutrophils, but

609   does recruit basophils, monocytes, memory T-cells, and dendritic cells to the sites of inflammation

610   induced by either tissue injury or microbial infection [102]. CCL2 expression in Muller cells regulate

611   the infiltration of monocytes and microglia following retinal injury [102]. CCL2 has been associated

612   with acute inflammatory conditions [98], and recruits and activates neutrophils by binding to CCR1,

613   CCR4, and CCR5 [102,103]. CCL20 strongly attracts lymphocytes and weakly attracts neutrophils [104].

614   Expression of CCL2 and CCL20 can be induced by microbial factors such as lipopolysaccharides

615   and inflammatory cytokines such as IL-6 and TNFα [104-108]. We reported TLR4-dependent

616   expression of CCL2 and CCL3 in the retinas of *Bacillus*-infected mouse eyes at 4 hours

617   postinfection [50]. Here, we demonstrated blunted expression of CCL2, CCL3, CCL7, CCL4, and

618   CCL20 when TLR2 and TLR4 activation was interfered with. CXCR2, CXCR4, CCR1 are the

619   major CXC and CC chemokine receptors that bind and respond to chemokines to mobilize

620   immune cells. CXCR1 and CXCR2 interact with CXCL1-8 and are expressed on the neutrophil

621   surface [103]. Therefore, the increased expression of CXC and CC receptors and chemoattractants

622   in WT-infected eyes may be related to the presence of neutrophils in the intraocular environment

623   at this stage of infection. We reported that the absence of CXCL1 in CXCL1$^{-/-}$ mice or intraocular

624   injection of anti-CXCL1 improved the clinical outcome of *Bacillus* endophthalmitis, resulting in

625   minimal inflammation and retained retinal function [45]. The absence of TLR2 resulted in reduced

626   expression of CXCL2 in mouse retinas during experimental *S. aureus* endophthalmitis [88].

627   Although CCL2 is not known to recruit neutrophils, treatment with anti-CCL2 or anti-CCL3

628   significantly reduced neutrophil infiltration into the cornea, resulting in decreased corneal damage

629   in a mouse model of *Pseudomonas aeruginosa* keratitis [109]. The expression of more than half of

630   the chemokines that we analyzed was blunted when TLR2 and TLR4 activation was interfered

631   with (Supplementary Table 5). Together, these findings suggested a therapeutic potential for

632   targeting CC and CXC chemokines in controlling ocular inflammation during *Bacillus*

633   endophthalmitis, and possibly other forms of endophthalmitis.

634

635   During ocular infection with an avirulent organism, the innate immune response is usually

636   sufficient to clear the infection. However, ocular infections caused by a more virulent pathogen

637   such as *Bacillus* are not easily cleared, and the robust innate response induced by *Bacillus* cell

638   wall components can lead to significant host-mediated damage. Therapeutics designed to

639   counteract the activities of individual bacterial products might not prevent the damage caused by

640   numerous other toxic microbial factors, nor reduce inflammation-induced injury. The fact that the

641   absence of *Bacillus* S-layer had such a profound effect on intraocular inflammation and infection

642   highlights S-layer as an important virulence factor in this disease. The ability of *Bacillus* S-layer

643   to activate both TLR2 and TLR4 may partially explain why *Bacillus* endophthalmitis results in such

644   an explosive inflammatory response.

645

646   Innate inhibition by interfering the TLR pathways has been promising in several

647   inflammation-associated diseases such as Gram-negative bacterial sepsis, acute lung

648   inflammation injury, atherosclerosis, acute and chronic inflammatory pain [47,49,110-115]. The negative

649   regulation of inflammation by oxidized phospholipid has been effective for the treatment of these

650   diseases. Oxidized phospholipids have been shown to inhibit LPS-induced pyroptosis, IL-1β

651   release, and septic shock, providing a basis for therapeutic innate immune interference in Gram-

652   negative bacterial sepsis [47]. Oxidized phospholipids also markedly attenuated LPS-induced tissue

653   inflammation, barrier disruption, and cytokine production in rats [111]. Since the inflammatory

654   response triggered by the innate immune response to *Bacillus* endophthalmitis may cause severe

655   damage to ocular tissues, targeting these pathways could be a viable anti-inflammatory approach

656   which also preserves retinal function. We identified 25 inflammatory genes (Table 1) which were

657   upregulated 50-fold or higher after infection with WT *Bacillus*, and demonstrated reduced

658   expression of these 25 inflammatory genes after OxPAPC-mediated TLR2 and TLR4 inhibition or

659   infection with a SLP-deficient *Bacillus*. The expression of 12 chemoattractants among these 25

660   genes was likely the driver of excessive neutrophil infiltration during *Bacillus* infection. Together,

661   these results suggest that *Bacillus* SLP potentially triggered the expression of these inflammatory

662   genes, which could be prevented by inhibiting the activation of TLR2 and TLR4 pathways.

663   Although we demonstrated improved clinical outcomes in *Bacillus* endophthalmitis when infected

664   eyes were treated with OxPAPC, we did not test co-administration with antibiotics. The

665   administration of anti-inflammatory drugs alone is not an acceptable standard of care for

666   endophthalmitis.

667

668   Present treatment options, including intravitreal therapeutics and vitrectomy, are often

669   unsuccessful at preventing irreversible damage to the retina that can result in total loss of vision.

670   A complete understanding of the role of the host response in the disease process is required to

671   design rational treatment strategies to improve the visual outcome of this disease. Infection with

672   an SLP-deficient pathogen [33], chemical inhibition of TLR2 and TLR4 after infection [34], mice

673   deficient in TLR2 [36], TLR4 [37], MyD88 [38,85], and TRIF[38], mice pre-treated with TLR2 agonists [77,99],

674   mice deficient in CXCL1[45], and anti-CXCL1 administration [45] each resulted in reduced

675   inflammation and better clinical outcomes. Collectively, these findings suggest targeting innate

676   immune activation and their response elements as potential therapeutic strategies. Here, we

677   identified several innate inflammatory genes which could be investigated further as potential anti-

678   inflammatory targets in endophthalmitis. Further studies are necessary to determine whether

679   expression of these genes contributes directly to the pathogenesis of *Bacillus* endophthalmitis.

680   All things considered, this study demonstrated a viable strategy to minimize the otherwise robust

681   and sight-threatening inflammation in *Bacillus* endophthalmitis and perhaps this infection caused

682   by other pathogens.

683

684   **ACKNOWLEDGMENTS**

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Table 1. 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/ ∆*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 Δs*lpA*-infected eyes relative to uninfected and WT-infected eyes are shown. A negative fold change represents reduced expression relative to WT-infected eyes.

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1007   **Figure Legends**

1008   **Figure 1: Interfering with TLR2 and TLR4 activation affected inflammatory gene expression**

1009   **in the eye** (A) Experimental outline of the NanoString experiment. (B) PCA analysis of mouse

1010   inflammatory gene. All WT-infected eyes cluster together. Except for one WT+OxPAPC eye, all

1011   uninfected, Δ*slpA*-infected, and two WT+OxPAPC eyes clustered together. (C) Variations in the

1012   diversity of gene expression profiles across different groups. Shannon index was used to calculate

1013   the diversity. Statistical significance was calculated using unpaired t-test. *P=0.0152, **P=0.0043,

1014   and #P=0.0198. (D) Heatmap for the visualization of gene expression. The red and green color

1015   gradient represents the upregulation and downregulation of genes. Red indicates increased,

1016   whereas green represents reduced gene expression. Sample groups were color-coded by grey

1017   (uninfected), black (WT-infected), light blue (WT+OxPAPC), and deep blue (Δ*slpA*-infected).

1018   Functional groups were color-coded in the Y axis as follows: chemoattractants (light pink),

1019   complement (light blue), cytokines (yellow), innate elements (purple), kinases (deep pink), and

1020   peptidases (green).

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1022   **Figure 2. Interfering with TLR2 and TLR4 activation reduced ocular inflammatory**

1023   **responses.** Volcano plot analysis of NanoString data derived from WT-infected, WT+OxPAPC,

1024   and Δ*slpA*-infected eyes compared with (A-C) uninfected eyes and (D-E) WT-infected eyes. For

1025   A-C and D-E the x-axis indicates the log fold change relative to uninfected eyes and WT-infected

1026   eyes, respectively. The y-axis shows the negative log10 p-value. Each dot represents an

1027   individual gene. TLR2 and TLR4 are indicated by a blue and red dot, respectively. Significance

1028   was assessed using multiple t-tests, and p values of < 0.05 (dotted line) were considered to be

1029   significant. Any gene above the dotted line was either significantly (A-C) increased or (D-E)

1030   decreased. (F) Percent of differentially expressed mouse inflammatory genes in WT+OxPAPC

1031   and Δ*slpA*-infected eyes relative to uninfected eyes or WT-infected eyes. (G) Venn diagram (i-iv)

1032   showing the number of differentially expressed genes in WT+OxPAPC and Δ*slpA*-infected eyes

1033 compared to uninfected or WT-infected eyes. Shading indicates no change in expression (dark

1034 gray), increased expression (black), and reduced expression (light gray).

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1036 **Figure 3. Interfering with TLR2 and TLR4 activation blunted complement gene expression**

1037 **in the eye.** Analysis of complement factor expression in WT-infected, WT+OxPAPC, and Δ*slpA*-

1038 infected eyes. (A) Fold changes of complement factor expression in WT-infected, WT+OxPAPC,

1039 and Δ*slpA*-infected eyes compared to uninfected eyes. (B) Compared to WT-infected eyes,

1040 decreased expression of C3, Cfb, C6, C7, C1qa, C1qb, C1ra, C8b, and C1s transcripts were

1041 observed in WT+OxPAPC or Δ*slpA*-infected eyes. Values represent the mean±SEM of

1042 complement counts at 10 hours postinfection for three different mouse eyes and p-value of < 0.05

1043 was considered significant.

1044

1045 **Figure 4. Interfering with TLR2 and TLR4 activation impacted innate immune gene**

1046 **expression in the eye.** Expression of innate immune genes in mouse eyes. (A) Venn diagram

1047 showing the number of innate immune genes differentially upregulated between the groups. (B)

1048 Heatmap showing the expression of extracellular and intracellular innate receptors. TLR2, TLR4,

1049 TLR6, TLR8, intracellular receptor Nod2, and Nlrp3 were significantly upregulated in WT

1050 untreated eyes compared to uninfected eyes. (C) Innate immune genes such as MyD88, NF-κB,

1051 RelA, RelB, Ptsg2, Hspb1, STAT2, STAT3, and Hspb1 were significantly upregulated in WT-

1052 infected eyes compared to uninfected eyes. Values represent the mean±SEM of transcript counts

1053 at 10 hours postinfection for 3 different mouse eyes and *P< 0.05 was considered significant.

1054

1055 **Figure 5. Interfering with TLR2 and TLR4 activation reduced cytokine gene expression in**

1056 **the eye.** Expression of inflammatory cytokines from WT-infected, WT+OxPAPC, Δ*slpA*-infected

1057 eyes were analyzed. (A) Log fold change of 51 mouse inflammatory cytokines from WT-infected,

1058   WT+OxPAPC, and Δ*slpA*-infected eyes, compared to uninfected eyes. The darkest shading

1059   corresponds to the greatest fold changes**.** (B) Venn diagram showing the number of cytokine

1060   genes differentially expressed between the groups. (C) Elevated expression of pro-inflammatory

1061   cytokines CSF3, IL-6, IL-1β, CSF2, IL-1α, TNFα, IL-23α, TGFβ1, and IL-12β in WT-infected eyes

1062   compared to uninfected eyes. Compared to WT-infected eyes, expression of these pro-

1063   inflammatory cytokines was significantly reduced in WT+OxPAPC or Δ*slpA*-infected eyes. (D)

1064   Compared to uninfected eyes, expression of anti-inflammatory cytokines IL-1rap, IL-10, and IL-

1065   1rn was elevated in WT-infected eyes, but was reduced significantly in WT+OxPAPC or Δ*slpA*-

1066   infected eyes. Values represent the mean±SEM of transcript counts at 10 hours post infection

1067   (N=3). *P< 0.05 was considered significant.

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1069   **Figure 6. Interfering with TLR2 and TLR4 activation reduced chemoattractant gene**

1070   **expression in the eye.** NanoString analysis of CXC and CC chemokine groups analyzed in WT-

1071   infected, WT+OxPAPC, and Δ*slpA*-infected mouse eyes. (A) Log fold changes of inflammatory

1072   chemokines from WT-infected, WT+OxPAPC, and Δ*slpA*-infected eyes, compared to uninfected

1073   eyes. The darker shading corresponds to the greatest log-fold changes**.** (B) Venn diagram

1074   showing the number of chemoattractants differentially expressed between the groups. (C) At 10

1075   hours postinfection, expression of CXCL2, CXCL1, CXCL3, CCL20, CCL4, CCL3, CCL2, CCL7,

1076   CXCL10, and CXCL5 were upregulated in WT-infected, and significantly reduced in WT+OxPAPC

1077   and Δ*slpA*-infected eyes. (D) Expression of chemokine receptor CCR1, CXCR2, CXCR4, and

1078   CCR7 was also significantly upregulated in WT-infected eyes and reduced in WT+OxPAPC and

1079   Δ*slpA*-infected eyes**.** Values represent the mean±SEM of transcript counts at 10 hours

1080   postinfection for three individual mouse eyes. *P< 0.05 was considered significant.

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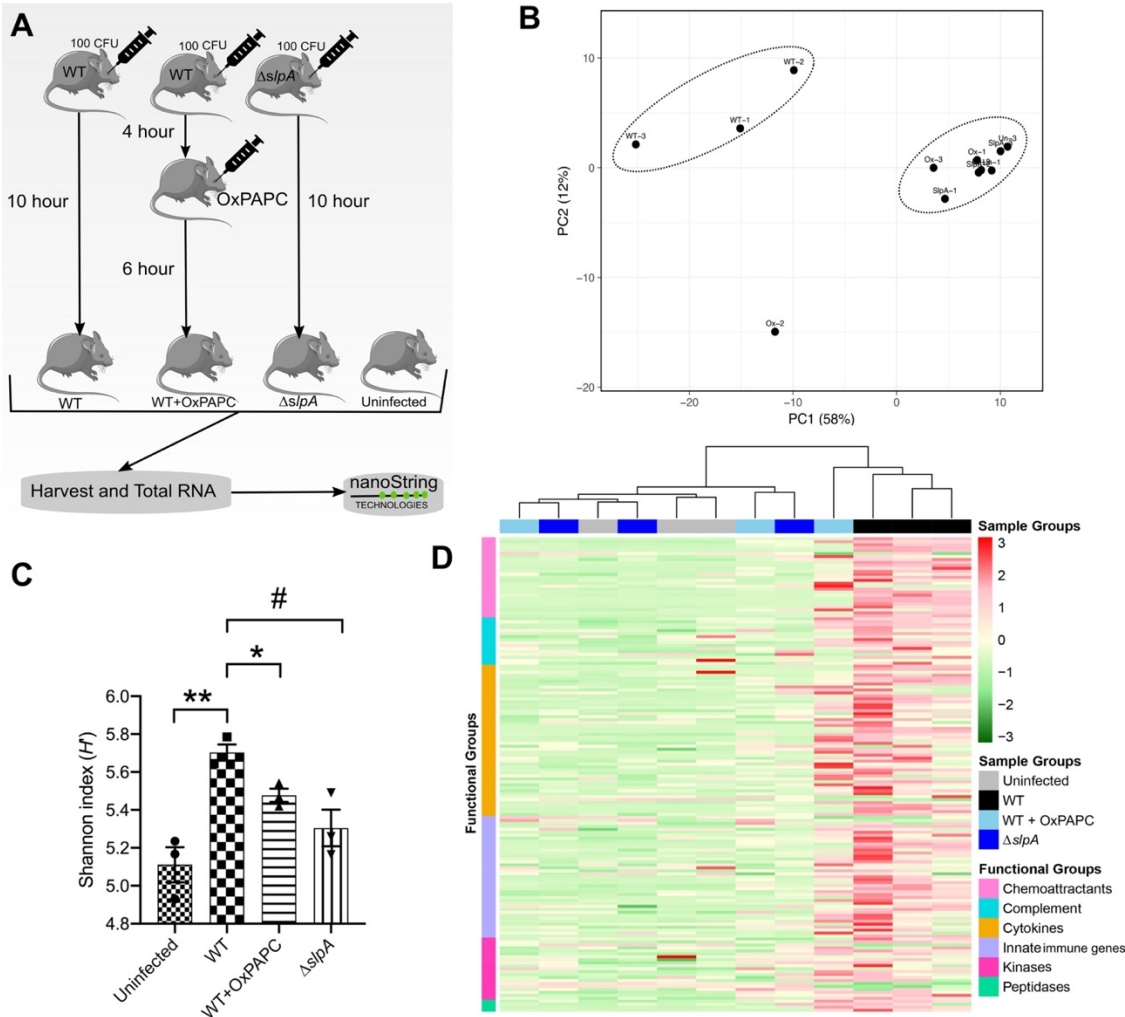1084 **Figure 1: Interfering with TLR2 and TLR4 activation affected inflammatory gene expression**

1085 **in the eye.**

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1095 **Figure 2. Interfering with TLR2 and TLR4 activation reduced ocular inflammatory**

1096 **responses.**

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1106   **Figure 3. Interfering with TLR2 and TLR4 activation blunted complement gene expression**

1107   **in the eye.**

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1113   **Figure 4. Interfering with TLR2 and TLR4 activation impacted innate immune gene**

1114   **expression in the eye.**

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1120 **Figure 5. Interfering with TLR2 and TLR4 activation reduced chemoattractant gene**

1121 **expression in the eye.**

1122

1123

1124 **Figure 6. Interfering with TLR2 and TLR4 activation reduced chemoattractant gene**

1125 **expression in the eye.**

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