The Role of C-X-C Chemokines in *Staphylococcus aureus* Endophthalmitis

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Purpose. To test the hypothesis that the C-X-C chemokines CXCL1, CXCL2, and CXCL10 contribute to inflammation during *Staphylococcus aureus* endophthalmitis.

Methods. *S. aureus* endophthalmitis was induced by intravitreal injection of 5000 colony forming units of *S. aureus* into the eyes of C57BL/6J, CXCL1$^{-/-}$, CXCL2$^{-/-}$, or CXCL10$^{-/-}$ mice. At 12, 24, and 36 hours postinfection, bacterial counts, intraocular inflammation, and retinal function were assessed. Based on these results, the effectiveness of intravitreal administration of anti-CXCL1 in reducing inflammation and improving retinal function was evaluated in *S. aureus*–infected C57BL/6J mice.

Results. We observed significant attenuation of inflammation and improvement in retinal function in CXCL1$^{-/-}$ mice relative to C57BL/6J at 12 hours but not at 24 or 36 hours postinfection with *S. aureus*. Co-administration of anti-CXCL1 antibodies with *S. aureus*, however, did not improve retinal function or reduce inflammation at 12 hours postinfection. In CXCL2$^{-/-}$ and CXCL10$^{-/-}$ mice, retinal function and intraocular inflammation were not significantly different from those of C57BL/6J mice at 12 and 24 hours postinfection. At 12, 24, or 36 hours, an absence of CXCL1, CXCL2, or CXCL10 did not alter intraocular *S. aureus* concentrations.

Conclusions. CXCL1 appears to contribute to the early host innate response to *S. aureus* endophthalmitis, but treatment with anti-CXCL1 did not effectively limit inflammation in this infection. CXCL2 and CXCL10 did not seem to play an integral role in inflammation during the early stages of *S. aureus* endophthalmitis.

Keywords: endophthalmitis, intraocular infection, *staphylococcus aureus*, chemokines, inflammation

Endophthalmitis is an intraocular infection resulting from bacterial entry and invasion of the eye.[1–4] Infection stems from a variety of sources, most notably postoperative complications, ocular injuries, or endogenous sources.[1–7] The majority of endophthalmitis cases, however, occur following surgical procedures, such as cataract surgery, intravitreal injection, or penetrating ocular trauma.[8,9] Symptoms of endophthalmitis range from minor inflammation to fulminant infection accompanied by vision loss, eye pain, and redness.[1–7] Diagnosis of endophthalmitis usually is accomplished via cultures of the vitreous and/or the aqueous humor or via blood cultures if the infection stemmed from an endogenous source.[3] The current treatment strategies for endophthalmitis include intravitreal injections of vancomycin, ceftazidime, amikacin, and/or fourth-generation fluoroquinolones; corticosteroids for anti-inflammation; and/or surgical debridement via vitrectomy if necessary.[3,8] Due to the rapid tissue damage and potentially blinding effects of this intraocular infection, endophthalmitis is a medical emergency that necessitates expeditious diagnosis and treatment.

Staphylococcus aureus ranks among the most frequent and virulent bacterial etiological agents of endophthalmitis, resulting in poor visual outcomes.[10–14] The development of resistance to multiple antibiotics (including cephalosporins, methicillin, imipenem, and aminoglycosides) has resulted in a greater likelihood of treatment failure.[10–15] In addition to its multidrug resistance (MDR), *S. aureus* produces a wide array of secreted toxins that increase the severity of intraocular infections by contributing to retinal function loss and eliciting host inflammatory responses.[16] Individual *S. aureus* pore-forming toxins and their regulators have been ascribed roles in endophthalmitis.[9,17] The combination of the production of secreted toxins and the emergence of MDR isolates in both the clinical and community settings complicate treatment with traditional antibacterial agents. This necessitates

the identification of alternative bacterial targets, as well as an enhanced understanding of potentially targetable host inflammatory processes incited by *S. aureus*.

In animal models of endophthalmitis, *S. aureus* upregulates innate immune signaling pathways and molecules involved in inflammatory cell recruitment.[18,19] TNFα, IL-1β, and C-X-C motif chemokine ligand 1 (CXCL1) were identified in the vitreous at 24 hours following intraocular infection in rats.[18] Expanding on this, key regulators of inflammatory pathways, including STAT1, STAT3, IL-6, NFKB2, JUN, SPP1, IL-1β, CSF1, CXCL2, IGF1, CEBPB, and PTPN1, were upregulated at early time points following infection in mice.[19] In the latter study, pretreatment with Pam3Cys, a Toll-like receptor 2 (TLR2) inhibitor, resulted in downregulation of IL-6, JUN, and CXCL2, as well as Jak/Stat signaling mediators, TLR and IL-10 signaling molecules, and TNFR2 signaling genes.[19] Further, pretreatment of mouse eyes with the glycolysis inhibitor 2-deoxy-glucose prior to infection with *S. aureus* significantly reduced the expression of IL-1β, IL-6, CXCL1, and CXCL2 and inhibited ERK1/2 phosphorylation at 24 hours postinfection.[20] This latter inhibition was postulated to be the mechanism for the anti-inflammatory effect of 2-deoxy-glucose observed in this study. Identification of key inflammatory regulators and downstream immune mediators that are produced during the earlier stages of *S. aureus* endophthalmitis and the utilization of specific inhibitors suggested possible targets for controlling the inflammatory response to *S. aureus* intraocular infection.

In order to more precisely define the individual role of immunomodulators in endophthalmitis, we previously evaluated the inflammatory responses and retinal function in C-X-C chemokine–deficient mice following *Bacillus cereus* infection. Significantly reduced inflammatory responses were observed in CXCL1$^{-/-}$ mice, accompanied by increased preservation of retinal function following infection.[2] Furthermore, treatment of *B. cereus*–infected eyes with anti-CXCL1 neutralizing antibodies resulted in improved retinal function and less overall inflammation.[2] Additionally, the absence of CXCL2 or CXCL10 also resulted in improved retinal function and mitigated inflammation.[21] Collectively, these results suggest that C-X-C chemokines facilitate inflammation and influence outcomes of *B. cereus* endophthalmitis; however, the identities of specific chemokines that are integral to the host immune response to *S. aureus* have yet to be elucidated.

In the current study, we evaluated the contribution of the C-X-C chemokines CXCL1, CXCL2, and CXCL10 in experimental murine *S. aureus* endophthalmitis. Based on our findings that CXCL1-, CXCL2-, and CXCL10-deficient mice exhibited reduced inflammation and improved retinal function during *B. cereus* endophthalmitis, we hypothesized that the absence or inhibition of CXCL1, CXCL2, or CXCL10 would result in reduced inflammation and infection severity.[2,21] Here, we demonstrated that the absence of CXCL1, CXCL2, or CXCL10 did not alter *S. aureus* growth in the eye at any time point tested. Whereas an absence of CXCL1 resulted in improved retinal function and reduced inflammation at 12 hours following intraocular *S. aureus* infection, the absence of CXCL2 or CXCL10 did not exert a demonstrable improvement in retinal function or intraocular inflammation at 12 or 24 hours postinfection. Although these results suggest that targeting CXCL1 might similarly serve to attenuate infection severity, surprisingly, co-administration of anti-CXCL1 antibodies with *S. aureus* did not significantly alter infection outcomes.

Materials and Methods

Mice

All procedures described in this study adhered to the recommendations in the Guide for the Care and Use of Laboratory Animals, the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and the policies set forth by the University of Oklahoma Health Sciences Center Institutional Animal Care and Use Committee. C57BL/6J (cat. no. 000664), CXCL2$^{-/-}$ (C57BL/6NJ-Cxcl2em1(IMPC)J/J; cat. no. 029557), and CXCL10$^{-/-}$ (B6.129S4-Cxcl10tm1adl/J; cat no. 006087) mice were acquired from The Jackson Laboratory (Bar Harbor, ME, USA). CXCL1$^{-/-}$ mice were a kind gift from Sergio Lira, MD (Icahn School of Medicine, Mount Sinai, NY, USA). CXCL1$^{-/-}$, CXCL2$^{-/-}$, and CXCL10$^{-/-}$ mice were bred on the C57BL/6J background. Weaned or vendor-supplied mice were housed under biosafety level 2 microisolation conditions on a 12-hour on/12-hour off light cycle for at least 2 weeks prior to infections to equilibrate their microbiota and to allow for physiological and nutritional stabilization.

Murine *S. aureus* Endophthalmitis Model

S. aureus strain 8325-4 was grown in Brain Heart Infusion (BHI) medium at 37°C for 18 hours and diluted to 10^7 colony forming units (CFU)/mL prior to intravitreal injection. Mice were anesthetized with a combination of ketamine (Ketamine HCl, 85 mg/kg body weight; Covetrus, Portland, ME, USA) and xylazine (AnaSed, 14 mg/kg body weight; Akorn, Decatur, IL, USA). Intravitreal injections were performed with sterile borosilicate glass micropipettes (Kimble Glass Company, Vineland, NJ, USA) beveled to an approximate bore size of 10 to 20 µm (BV-10 KT Brown Type micropipette beveller; Sutter Instrument Company, Novato, CA, USA). Eyes were visualized with a stereomicroscope, and the micropipettes were inserted just posterior to the superior limbus. The right eyes of anesthetized mice were injected with 5000 CFU in 0.5 µL, and the left eyes were used as contralateral, uninfected controls.[19,22]

Scotopic Electroretinography

Infected mice were dark adapted for 6 hours prior to electroretinography (ERG). At 12, 24, or 36 hours postinfection, mice were anesthetized as described above, and topical phenylephrine (10% phenylephrine HCl; Paragon BioTeck, Inc., Portland, OR, USA) to dilate the eyes and topical anesthetic (0.5% proparacaine HCl; Alcon Laboratories, Inc., Fort Worth, TX, USA) were applied to eyes prior to obtaining the ERG recordings. Gold-wire electrodes were placed on the cornea of each eye, and reference electrodes were attached to the head and tail of the mouse. Five white-light flashes (1200 cd·s/m^2) were administered consecutively to the mouse 60 seconds apart (10-ms duration) in order to provoke a retinal response. Scotopic A-wave (corresponding to photoreceptor cell activity) and B-wave (corresponding to Müller, bipolar, and amacrine cell activity) amplitudes were recorded for each eye (Espion E2; Diagnosys LLC, Lowell, MA, USA). Immediately following the ERG, mice were euthanized by CO_2 asphyxiation prior to harvesting the eyes for myeloperoxidase (MPO) and bacterial CFU quantification or histological analysis. The percentage of retinal function retained in the infected eye was calculated in comparison with uninfected left eye controls as

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100 – {[1 – (experimental A-wave or B-wave amplitude/control A-wave or B-wave amplitude)] × 100}. Values represent the mean ± standard error of the mean (SEM) for at least four eyes per group. At least two independent experiments were performed.

Myeloperoxidase Assays

At 12, 24, or 36 hours postinfection, inflammatory cell influx into eyes was assessed indirectly by measurement of MPO concentrations in whole eye homogenates. Eyes were enucleated, placed into separate tubes containing 400 µL of sterile PBS supplemented with proteinase inhibitor cocktail (Roche Diagnostics, Indianapolis, IN, USA) and 1.0-mm sterile glass beads (BioSpec Products, Bartlesville, OK, USA), and homogenized for 60 seconds at 5000 rpm in a Mini-Beadbeater (BioSpec Products). MPO concentrations were measured using a sandwich ELISA (Hycult Biotech, Plymouth Meeting, PA, USA), as previously described.[2,21] Negative controls consisted of uninfected eye homogenates. Values represent the mean ± SEM for at least six eyes per group. Two independent experiments were performed.

Bacterial Quantitation

At 12, 24, and 36 hours postinfection, enucleated eyes were homogenized as described for the MPO assays. Eye homogenates were serially diluted 10-fold and plated in triplicate on BHI agar plates. After overnight incubation, the CFUs per eye were determined as previously described.[23,24] Values represent the mean ± SEM for at least five eyes per group. At least two independent experiments were performed.

Thin-Section Histology

At 12, 24, and 36 hours postinfection, harvested eyes were incubated in high-alcohol Prefer fixative for 30 minutes at room temperature.[21] Eyes were then transferred to 70% ethanol, embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Images are representative of at least three eyes from two independent experiments.

Anti-CXCL1 Treatment

Groups of C57BL/6J mice were anesthetized as described above. For the untreated control group, the right eyes were intravitreally injected with 5000 CFU of *S. aureus* in 1 µL of PBS. For the groups treated with anti-CXCL1, the right eyes were intravitreally injected with either 125 ng or 250 ng of anti-CXCL1 monoclonal antibody (anti-KC/CXCL1 IgG2A MAB 453, clone 48415; R&D Systems, Minneapolis, MN, USA) and 5000 CFU of *S. aureus* in 1 µL of PBS. For the isotype control group, the right eyes were intravitreally injected with 250 ng of the isotype control antibody (nonspecific control IgG2A MAB 006, clone 54447l; R&D Systems) and 5000 CFU of *S. aureus* in 1 µL of PBS. All eyes were injected once. Retinal function, intraocular inflammation, and bacterial growth were assessed at 12 hours postinfection as described above. Values represent the mean ± SEM for at least six eyes per group. Two independent experiments were performed.

Statistics

Data are the arithmetic means ± SEM of all samples in the same experimental group in replicate experiments. Comparative differences between groups were taken to be statistically significant when $P < 0.05$. The Mann–Whitney U test was used to compare experimental groups for ERG, MPO, and bacterial counts per eye. All statistical analyses were performed using Prism 8.4.3 for Windows (GraphPad, San Diego, CA, USA).

Results

Absence of CXCL1 Improved Retinal Function and Reduced Inflammation But Did Not Affect Bacterial Growth During the Early Stages of *S. aureus* Intraocular Infection

Previous studies have shown that an absence of CXCL1, via genetic deficiency or anti-CXCL1 antibody treatment, partially attenuated inflammatory responses during *B. cereus* endophthalmitis.[1,2] To determine whether CXCL1 contributes to the pathogenesis of *S. aureus* endophthalmitis, we sought to evaluate the influence that an absence of CXCL1 might exert on retinal function, inflammation, and bacterial growth in a *S. aureus* endophthalmitis model (Fig. 1). At 12 hours postinfection, the mean A-wave retention of CXCL1$^{-/-}$ eyes was significantly higher than that for C57BL/6J eye (87.27% vs. 58.92%; $P = 0.0159$) (Fig. 1A). The mean B-wave retention was also significantly higher in CXCL1$^{-/-}$ eyes relative to C57BL/6J eyes (75.54% vs. 41.74%; $P = 0.0159$) (Fig. 1B). However, at both 24 hours and 36 hours postinfection, no significant differences between the CXCL1$^{-/-}$ and C57BL/6J groups were observed in A-wave retention ($P = 0.6454$ at 24 hr; $P > 0.9999$ at 36 hr) (Fig. 1A) or in B-wave retention ($P = 0.6454$ at 24 hr; $P > 0.9999$ at 36 hr) (Fig. 1B). These results showed that CXCL1$^{-/-}$ mice retained significantly greater retinal function at 12 hours following infection, whereas retinal function in CXCL1$^{-/-}$ mice was statistically similar to their C57BL/6J counterparts at 24 and 36 hours postinfection.

To assess the contribution of CXCL1$^{-/-}$ to the host inflammatory response, inflammatory cell influx was evaluated as a function of MPO concentrations in the eye at 12, 24, and 36 hours postinfection (Fig. 1C). At 12 hours, mean concentrations of MPO were 67.4 ng/eye in C57BL/6J eyes, whereas a significantly lower mean concentration of 47.97 ng/eye was observed in CXCL1$^{-/-}$ eyes ($P = 0.0001$) (Fig. 1C). At 24 and 36 hours, however, there were no significant differences in measured mean MPO levels between C57BL/6J and CXCL1$^{-/-}$ mice ($P = 0.1978$ at 24 hr; $P = 0.1230$ at 36 hr) (Fig. 1C). Bacterial quantitation of *S. aureus*–infected C57BL/6J and CXCL1$^{-/-}$ mouse eyes revealed that the absence of CXCL1 did not alter bacterial concentrations (Fig. 1D). At 12, 24, and 36 hours, there was no significant difference in bacterial load between C57BL/6J and CXCL1$^{-/-}$ mice at each time point ($P = 0.8413$ at 12 hr; $P = 0.1375$ at 24 hr; $P = 0.2857$ at 36 hr) (Fig. 1D). Bacterial concentrations also remained at a steady state and did not increase throughout the course of the infection.

Histological analysis of eye thin sections from *S. aureus*–infected C57BL/6J and CXCL1$^{-/-}$ eyes showed similar fibrinous deposition in both anterior and posterior chambers, non-edematous corneas, and intact retinas at 12 hours

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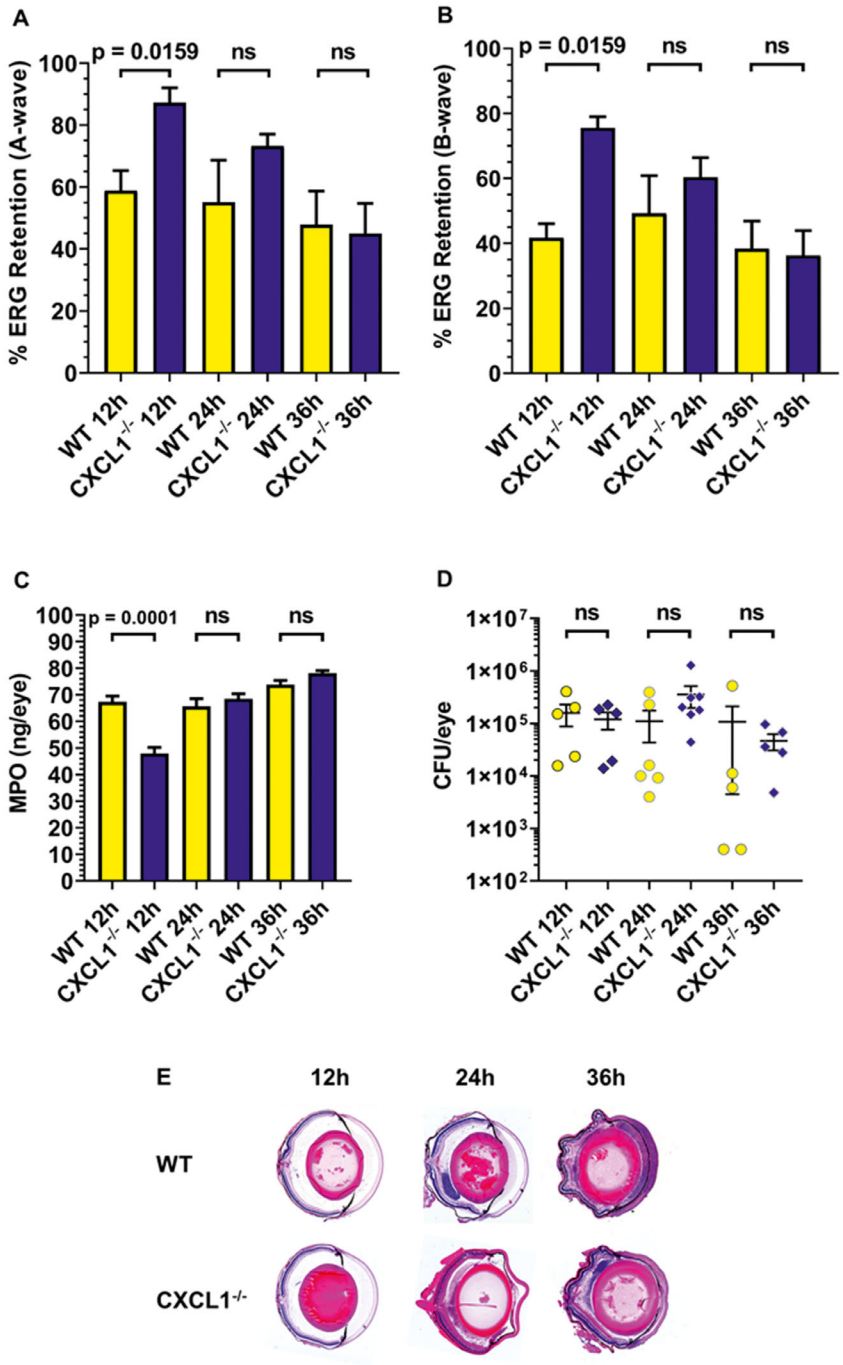

Figure 1. Absence of CXCL1 improved retinal function and reduced inflammation but did not affect bacterial growth at 12 hours after infection with *S. aureus*. Right eyes of C57BL/6J and CXCL1$^{-/-}$ mice were infected with 5000 CFU of *S. aureus* 8325-4. (**A**, **B**) Retinal function was assessed by electroretinography at 12, 24, and 36 hours postinfection. Values represent means ± SEM of $n \geq 4$ eyes per group in two independent experiments ($P \geq 0.05$); ns, not significant. (**C**) Inflammatory cell influx at 12, 24, and 36 hours postinfection was measured as a function of MPO concentrations determined by sandwich ELISA of harvested and homogenized whole eyes. Values represent means ± SEM of $n \geq 10$ eyes per group in two independent experiments ($P \geq 0.05$). (**D**) Eyes were harvested from mice at 12, 24, and 36 hours postinfection, and *S. aureus* CFU/eye was determined. Values represent means ± SEM of $n \geq 5$ eyes per group in two independent experiments ($P \geq 0.05$). (**E**) At 12, 24, and 36 hours postinfection, eyes were harvested and processed for hematoxylin and eosin staining. At 12 hours postinfection, fibrin deposition in the anterior and posterior chambers was observed, and corneas and retinas appeared normal in both C57BL/6J and CXCL1$^{-/-}$ mice. At 24 hours, cellular infiltration and fibrin deposition were apparent in the anterior and posterior chambers. Retinal and corneal edema was more pronounced in CXCL1$^{-/-}$ eyes relative to C57BL/6J eyes; however, the retinal architecture remained intact in eyes from both groups. At 36 hours, cellular infiltration and fibrin deposition, severe retinal and corneal edema, and disruption of the retinal architecture were observed in both C57BL/6J and CXCL1$^{-/-}$ eyes.

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postinfection (Fig. 1E). At 24 hours, both groups showed significant cellular infiltration and fibrin deposition in both the anterior and posterior chambers. CXCL1$^{-/-}$ mouse eyes showed somewhat greater retinal and corneal edema than C57BL/6J eyes; however, the retinal architecture remained intact in eyes from both groups (Fig. 1E). At 36 hours postinfection, cellular infiltration and fibrin deposition were markedly increased in C57BL/6J and CXCL1$^{-/-}$ eyes relative to the eyes at 24 hours. Extensive retinal and corneal edema was present, and partial dissolution of the retinal architecture was observed in both C57BL/6J and CXCL1$^{-/-}$ eyes (Fig. 1E). Together, these results demonstrated that CXCL1$^{-/-}$ eyes infected with *S. aureus* exhibited a greater retainment of retinal function, lower levels of intraocular inflammation, and similar bacterial concentrations relative to C57BL/6J eyes at 12 hours following infection. However, at 24 and 36 hours, these infection parameters were not significantly different between C57BL/6J and CXCL1$^{-/-}$ eyes. These results suggest perhaps an earlier role for CXCL1 in mediating the inflammatory response to *S. aureus* infection and that the absence of CXCL1 was influential in minimizing inflammation and subsequent retinal function loss following *S. aureus* eye infection only during the early stages of intraocular infection.

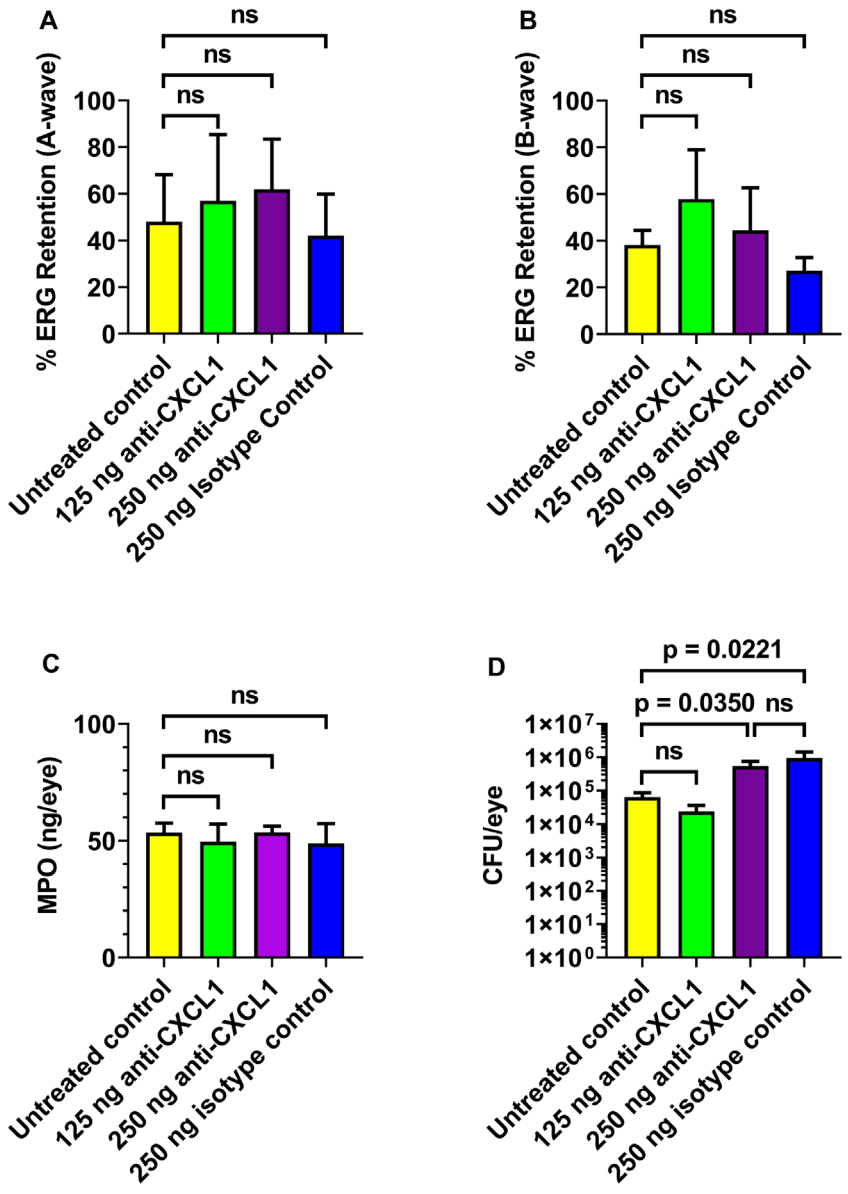

Figure 2. Co-administration of anti-CXCL1 antibodies with *S. aureus* did not influence retinal function, ocular inflammation, or bacterial growth at 12 hours postinfection. Right eyes of C57BL/6J mice were intravitreally injected with 5000 CFU of *S. aureus* 8325-4 (untreated control), 125 ng or 250 ng of anti-CXCL1 monoclonal antibody and 5000 CFU of *S. aureus* 8325-4, or 250 ng of the isotype control antibody and 5000 CFU of *S. aureus* 8325-4. (**A**, **B**) Retinal function was assessed by electroretinography at 12 hours postinfection. (**C**) MPO concentrations to assess inflammatory cell influx were measured by sandwich ELISA of harvested and homogenized whole eyes at 12 hours postinfection. (**D**) Eyes were harvested from mice at 12 hours postinfection, and *S. aureus* CFU/eye was determined. All values represent the mean ± SEM of $n \geq 6$ eyes per group. Two independent experiments were performed ($P \geq 0.05$); ns, not significant.

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Treatment With Anti-CXCL1 Did Not Reduce Intraocular Inflammation or Result in Improved Retinal Function

Because the absence of CXCL1 due to genetic knockout attenuated inflammatory responses and resulted in greater retinal function at a time point during the early stages of infection and because treatment of *Bacillus* endophthalmitis with anti-CXCL1 improved infection outcomes, we sought to determine whether treatment of *S. aureus*–infected C57BL/6J mouse eyes with anti-CXCL1 antibodies would produce a similar effect on inflammatory outcomes and retinal function at 12 hours postinfection (Fig. 2).[21] We first examined the effect of anti-CXCL1 antibody treatment on retinal function via ERG as compared to untreated control eyes (Figs. 2A, 2B). The mean A-wave retention was 48.04%, 57.03%, 61.92%, and 42.12% in the control, 125-ng anti-CXCL, 250-ng anti-CXCL1, and 250-ng isotype control antibody groups, respectively. However, there were no significant differences between the untreated group and any of the treatment groups ($P \geq 0.7143$) (Fig. 2A). The mean B-wave retention was 38.2%, 57.87%, 44.48%, and 27.24% in the control, 125-ng anti-CXCL, 250-ng anti-CXCL1, and 250-ng isotype control antibody groups, respectively. Similarly, no significant differences were detected among any of the treatment groups compared to untreated control eyes ($P \geq 0.4000$) (Fig. 2B). To assess whether treatment with anti-CXCL1 influenced immune cell influx, MPO levels for each treatment group as compared to untreated control eyes were determined. Concentrations of 53.47, 49.62, 53.58, and 48.92 ng/eye were observed in the control, 125-ng anti-CXCL, 250-ng anti-CXCL1, and 250-ng isotype control antibody groups, respectively. Comparison of the control group with each of the other groups revealed no significant differences

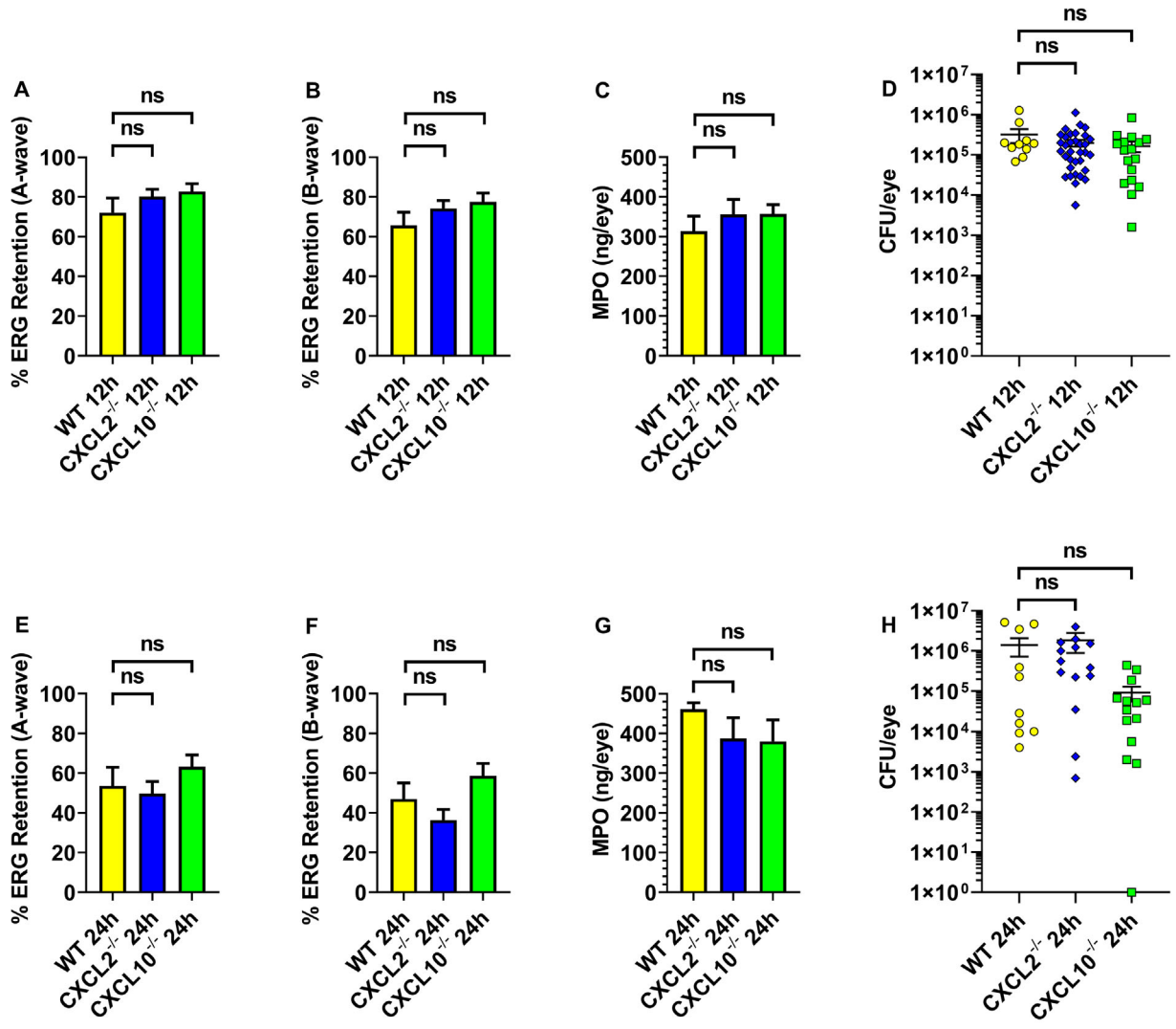

Figure 3. The absence of CXCL2 or CXCL10 did alter retinal function or bacterial growth at 12 or 24 hours following *S. aureus* intraocular infection. Right eyes of C57BL/6J, CXCL2$^{-/-}$, and CXCL10$^{-/-}$ mice were infected with 5000 CFU of *S. aureus* 8325-4. Retinal function was assessed by electroretinography at 12 hours (**A**, **B**) and at 24 hours (**E**, **F**) postinfection. Values represent means ± SEM of $n \geq 10$ eyes per group in two independent experiments ($P \geq 0.05$); ns, not significant. MPO concentrations to assess inflammatory cell influx were measured by sandwich ELISA of harvested and homogenized whole eyes at 12 hours (**C**) and at 24 hours (**G**) postinfection. Values represent means ± SEM of $n = 6$ eyes per group in two independent experiments ($P \geq 0.05$). Eyes were harvested from mice at 12 hours (**D**) and at 24 hours (**H**) postinfection, and *S. aureus* CFU/eye was determined. Values represent means ± SEM of $n \geq 10$ eyes per group in two independent experiments ($P \geq 0.05$).

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($P > 0.9999$) (Fig. 2C). To determine the effect of anti-CXCL1 treatment on intraocular bacterial growth, bacterial concentrations were measured in the control and antibody-treated groups. No significant differences in bacterial load were observed between the control group and the 125-ng anti-CXCL1–treated group ($P = 0.1014$) (Fig. 2D). Interestingly, a significantly higher concentration of *S. aureus* was observed in eyes treated with 250 ng anti-CXCL1 antibody relative to the control group ($P = 0.0350$) (Fig. 2D). However, a similarly higher concentration of *S. aureus* was also observed in mouse eyes treated with 250 ng of the isotype control ($P = 0.0221$ vs. control group) (Fig. 2D), suggesting that the observed effect was not specific to the anti-CXCL1 antibody. In summary, the results showed that administration of anti-CXCL1, at the initiation of infection and at the concentrations used in these experiments, did not significantly improve retinal function, attenuate intraocular inflammation, or alter bacterial growth at 12 hours postinfection. This finding suggests that the concentration of anti-CXCL1 might have been insufficient to neutralize in vivo CXCL1 levels or that other chemokines were involved in regulating inflammatory responses to *S. aureus* endophthalmitis in a redundant manner.

Absence of CXCL2 or CXCL10 Did Not Impact Retinal Function, Intraocular Inflammation, or Bacterial Growth Following *S. aureus* Intraocular Infection

The absence of CXCL2 or CXCL10 in mice with *Bacillus* endophthalmitis resulted in an improvement in retinal function and diminished inflammation.[2,21] We therefore sought to determine whether a genetic absence of CXCL2 or CXCL10 would have similar effects on retinal function, inflammation, and bacterial growth in *S. aureus* endophthalmitis. Retinal function, MPO levels, and bacterial concentrations in CXCL2$^{-/-}$ and CXCL10$^{-/-}$ mouse eyes were compared to those of C57BL/6J eyes at 12 and 24 hours postinfection (Fig. 3). At 12 hours postinfection, there were no statistically significant differences in A-wave retention, B-wave retention, MPO concentrations, or bacterial concentrations between C57BL/6J and CXCL2$^{-/-}$ or CXCL10$^{-/-}$eyes (Figs. 3A, 3B, 3C, 3D, respectively). Mean A-wave retention was 72.01%, 80.23%, and 82.81% for C57BL/6J, CXCL2$^{-/-}$, and CXCL10$^{-/-}$ eyes, respectively ($P \geq 0.2188$) (Fig. 3A). Mean B-wave retention was 65.68%, 74.18%, and 77.44% for C57BL/6J, CXCL2$^{-/-}$, and CXCL10$^{-/-}$ eyes, respectively ($P \geq 0.1261$) (Fig. 3B). The mean MPO concentrations for C57BL/6J, CXCL2$^{-/-}$, and CXCL10$^{-/-}$ eyes were 313.5, 355.5, and 356.9 ng/eye, respectively ($P \geq 0.5887$) (Fig. 3C). Bacterial growth was 3.17×10^5 CFU/eye in C57BL/6J eyes, as compared to 1.98×10^5 CFU/eye in CXCL2$^{-/-}$ or 1.65×10^5 CFU/eye in CXCL10$^{-/-}$ eyes ($P \geq 0.1909$) (Fig. 3D). Similarly, at 24 hours postinfection, no statistically significant differences between C57BL/6J and CXCL2$^{-/-}$ or CXCL10$^{-/-}$ eyes were observed in A-wave retention, B-wave retention, MPO concentrations, or bacterial load (Figs. 3E, 3F, 3G, 3H, respectively). Mean A-wave retention was 53.59% for C57BL/6J, 49.7% for CXCL2$^{-/-}$, and 63.17% for CXCL10$^{-/-}$ eyes ($P \geq 0.5028$) (Fig. 3E). Mean B-wave retention was 46.94% for C57BL/6J, 36.29% for CXCL2$^{-/-}$, and 58.62% for CXCL10$^{-/-}$ eyes ($P \geq 0.3470$) (Fig. 3F). The mean MPO concentrations for C57BL/6J, CXCL2$^{-/-}$, and CXCL10$^{-/-}$ eyes were 461.3, 387.6, and 380.3 ng/eye, respectively ($P \geq 0.5556$) (Fig. 3G). Bacterial concentrations were 1.40×10^6 CFU/eye in C57BL/6J eyes, 1.86×10^6 CFU/eye in CXCL2$^{-/-}$ eyes, and 9.23×10^4 CFU/eye in CXCL10$^{-/-}$ eyes ($P \geq 0.3119$) (Fig. 3H). The above results indicate that CXCL2 or CXCL10 likely do not singularly exert a notable influence on disease severity at early and intermediate time points in *S. aureus* endophthalmitis.

Discussion

The continuing emergence of MDR ocular isolates poses a threat to the successful treatment of *S. aureus* endophthalmitis. The development of novel therapeutic approaches that target *S. aureus* and dampen the host immune response is therefore of paramount importance. Achieving this goal entails understanding the interplay between *S. aureus* virulence factors and the host immune mechanisms governing pathogen detection, recognition, and response. *S. aureus*–secreted toxins and cell-wall components have been shown to be important in the onset of host inflammation, progression of the immune response, and ultimately the visual outcome of intraocular inflammation. Among the cell-wall components of *S. aureus*, peptidoglycan and lipoteichoic acid have been shown to induce IL-1β, TNF-α, IL-6, KC (murine equivalent of CXCL1/IL-8), and MIP-2 after injection into mouse eyes, and peptidoglycan stimulated polymorphonuclear leukocyte infiltration into the retina to a greater extent than any other *S. aureus* virulence factor tested in vivo.[25] Surface protein A, a *S. aureus* virulence factor that binds IgG and interferes with phagocytosis, also stimulated IL-1β, TNF-α, and KC production in mouse eyes.[25] Wall teichoic acids have been established to be proinflammatory in mouse eyes, as a *S. aureus* mutant defective in wall teichoic acid biosynthesis elicited less inflammatory cell influx in vivo than the isogenic parental strain.[26] Both the secreted toxic shock syndrome toxin-1 (TSST-1) and α-toxin elicited the production of IL-1β, TNF-α, and KC, with the greatest impact on IL-1β levels in mouse eyes.[25] Taken together, these studies indicate that *S. aureus* cell-wall components and virulence factors induce overlapping innate immune responses. This presents a challenge to designing therapies that interfere with individual virulence factors and points toward targeting the host immunomodulating factors that are induced by *S. aureus* components. Although the presence of these host immune factors in *S. aureus* endophthalmitis has been established, the contributions of individual immunomodulators to the inflammatory response and disease severity have not been determined. We previously demonstrated in a mouse model of *B. cereus* endophthalmitis that, among the 12 most highly upregulated chemokines/cytokines early in infection, six belonged to the C-X-C motif family of chemokines, including CXCL1, CXCL2, and CXCL10.[27] Subsequent studies with CXCL1-, CXCL2-, and CXCL10-deficient mice suggested important individual roles for these chemokines in inflammation and disease severity.[2,21] In the current study, the effects of the absence of CXCL1, CXCL2, or CXCL10 in bacterial endophthalmitis due to *S. aureus* were determined to explicitly assess the role that these chemokines play in influencing intraocular inflammation and visual outcomes.

CXCL1 is a member of the C-X-C motif chemokines and an important mediator of innate immune responses to infection via neutrophil activation and chemotaxis.[28–30] Our previous studies have demonstrated that CXCL1 is a key

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determinant of host inflammatory responses and disease severity in a mouse model of *B. cereus* endophthalmitis.[21] CXCL1 expression was highly upregulated early following intraocular *B. cereus* infection, with levels 34-fold higher in infected mouse eyes than in uninfected mouse eyes at 4 hours postinfection.[27] Significant decreases in neutrophil recruitment and inflammation, retinal damage, and functional loss were observed in CXCL1$^{-/-}$ mice at 4, 8, and 12 hours postinfection.[2] Congruent with these results, the genetic absence of CXCL1 resulted in improved retinal function, decreased neutrophil recruitment, and attenuated intraocular inflammation at 12 hours following infection with *S. aureus*. These results suggest that targeting CXCL1 early during infection might serve to attenuate infection severity.

The association of IL-8 with chronic inflammatory diseases and cancer has led to the investigation of anti-IL-8 monoclonal antibodies as a potential therapeutic agent. These studies have demonstrated improved clinical outcomes and decreased inflammation in human patients.[29,31] Parkunan et al.[2] demonstrated the efficacy of anti-CXCL1 neutralizing antibodies in reducing intraocular inflammation and improving retinal function after *B. cereus* infection. Co-injection of anti-CXCL1 antibodies with *B. cereus* significantly reduced inflammatory cell infiltration into the eye. Decreased retinal architecture damage and improvements in retinal function were also observed, likely associated with a decrease in proinflammatory cytokines, tissue-damaging enzymes, and oxygen free radicals. These results supported the prospect of targeting CXCL1 as a potential adjunctive immunomodulatory therapy. Surprisingly, in the current study, co-administration of *S. aureus* and anti-CXCL1 antibodies did not result in improved retinal function or inflammatory outcomes at 12 hours postinfection. The ineffectiveness of co-administration of anti-CXCL1 antibodies with *S. aureus* could be attributed to several factors. Antibody binding by *S. aureus* cell-wall–anchored protein A potentially could have prevented antibody diffusion to the retinal tissues, and antibody concentrations might have been insufficient to escape bacterial replication and additional protein A production. *S. aureus* is non-motile, and it is possible that the lack of coordination between diffusion of anti-CXCL1 to the retinal vasculature and bacteria-mediated stimulation of the host immune response at 12 hours postinfection could have abrogated the effectiveness of antibody treatment. Although Kochan et al.[32] observed possible bacteria in the retina 36 hours following *S. aureus* intraocular infection, the presence of *S. aureus* in the retina at earlier time points has not been shown. In contrast, the rapid migration of motile *B. cereus* to the retina and activation of TLRs and downstream mediators prior to antibody diffusion to the retina might have contributed to the success of anti-CXCL1 co-administration in our *B. cereus* model. It is also likely that other functionally redundant chemokines obviated the effects of CXCL1 neutralization, which is supported by the observed lack of significant differences in inflammation and retinal function between C57BL/6J and CXCL1$^{-/-}$ mouse eyes at 24 and 36 hours. Future studies to examine these possibilities will be critical to determine whether targeting CXCL1 is a feasible early intervention therapy for *S. aureus* or other types of endophthalmitis.

In order to address the hypothesis that other C-X-C chemokines also contribute to *S. aureus* endophthalmitis, we compared retina functional outcomes and intraocular inflammation in CXCL2$^{-/-}$ or CXCL10$^{-/-}$ mouse eyes with C57BL/6J eyes. CXCL2 and CXCL10 initiate the inflammatory response via binding and subsequent activation of CXCR2 and CXCR3, respectively, coordinating various cellular functions.[33–35] Additionally, CXCL2 and CXCL10 have been shown to facilitate wound healing, angiogenesis, phagocytosis, and the recruitment of leukocytes.[36–39] CXCL2 was previously shown to be upregulated at early time points after intraocular infection with *S. aureus*.[19] Additionally, the absence of CXCL2 or CXCL10 reduced inflammation and resulted in improved retinal function in *B. cereus* models of endophthalmitis in mice.[21] In the present study, no differences in retinal function, MPO concentrations, or bacterial growth were found between *S. aureus*–infected C57BL/6J and CXCL2$^{-/-}$ or CXCL10$^{-/-}$ mouse eyes. Given the lack of an apparent influence on retinal function, intraocular inflammation, and bacterial growth at 12 and 24 hours, we did not perform experiments at 36 hours postinfection. We also did not pursue studies examining the effect of intraocular anti-CXCL2 or anti-CXCL10 antibody administration in conjunction with *S. aureus* infection. The lack of differences seen in CXCL2$^{-/-}$ or CXCL10$^{-/-}$ mice might be attributable to redundancy in chemokine signaling and function. Binding of CXCL1 to CXCR2, for example, might have accommodated for the lack of CXCL2 or CXCL10.[40] Novosad and colleagues[41] demonstrated the importance of TLR2 in *B. cereus* endophthalmitis in that inflammatory cell infiltration and retinal function loss were delayed in the eyes of TLR2$^{-/-}$ eyes relative to wild-type mouse eyes. However, retinal architecture disruption and MPO levels at later time points in TLR2$^{-/-}$ eyes were comparable to those of wild-type eyes at earlier time points. This delay suggested redundant mechanisms for recognition of *B. cereus* and subsequent inflammatory responses. Indeed, TLR4 was activated by the *B. cereus* surface layer protein A[42] and played a significant role in mediating inflammatory processes following *B. cereus* intraocular infection.[27,43] TLR2 and TLR4 are capable of signaling through distinct and overlapping pathways, with TLR2 initiating downstream signaling through MyD88 adaptor-dependent pathways and TLR4 activating both MyD88-dependent and MyD88-independent (TRIF-dependent) pathways.[44] Talreja et al.[45] showed that TLR2$^{-/-}$ and MyD88$^{-/-}$ mouse eyes exhibited decreased levels of proinflammatory chemokines and cytokines relative to wild-type mouse eyes 24 hours after infection with *S. aureus*. However, redundant signaling pathways became apparent as levels of chemokines and cytokines in TLR2$^{-/-}$ and MyD88$^{-/-}$ mouse eyes were observed to be higher or similar to the levels observed in wild-type eyes at 48 and 96 hours postinfection.[45] These redundant mechanisms might have confounded any anti-inflammatory benefit of the absence of CXCL2 or CXCL10.

In conclusion, the present study revealed that the absence of CXCL1 improved retinal function and decreased overall intraocular inflammation at an early infection time point. This suggests that CXCL1 is influential in the early inflammatory response to *S. aureus* endophthalmitis and might serve as a potential therapeutic target. Differences in the apparent influence of CXCL2 or CXCL10 on retinal function outcomes or intraocular inflammation in a mouse model of *S. aureus* endophthalmitis were not observed in this study when compared to similar studies with a mouse model of *B. cereus* endophthalmitis.[21] The lack of an apparent contribution of CXCL2 or CXCL10 to *S. aureus* endoph-

thalmitis in this model might be due to redundancies in chemokine expression that preserve the host immune response. Furthermore, *S. aureus* is a nonmotile bacterium, as compared to motile *B. cereus*. This difference, along with other morphological differences, might delay immune response activation in *S. aureus* endophthalmitis at these early time points. Ultimately, this may lead to an inflammatory scenario where CXCL1 is the primary necessary chemokine, with others (notably, CXCL2 and CXCL10) likely serving a more supporting, redundant role in inflammation. When controlling for time postinfection, the differences in observed results between endophthalmitis etiologies suggest the involvement of different and overlapping inflammatory pathways and markers. These differences also highlight the importance of developing treatment options that account for differences in the inflammatory response, allowing for efficacious treatment that increases the likelihood of vision preservation, regardless of etiology. Identification of host factors that contribute to infection outcomes and expanding treatment repertoires continues to be of paramount importance in managing these potentially blinding intraocular infections.

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