A Role for Tumor Necrosis Factor-α in Experimental Bacillus cereus Endophthalmitis Pathogenesis

Raniyab T. Ramadan,1 Andrea L. Moyer,2 and Michelle C. Callegan1,2,3,4

PURPOSE. To determine the contribution of tumor necrosis factor-alpha (TNFα) in the pathogenesis of experimental Bacillus cereus endophthalmitis.

METHODS. Experimental B. cereus endophthalmitis was induced in wild-type control (B6.129F1) and age-matched homozygous TNFα knockout mice (TNFα−/−, B6.129S6-Tnftm1Gk1/J). At various times after infection, eyes were analyzed by electroretinography and were harvested for quantitation of bacteria, myeloperoxidase, proinflammatory cytokines and chemokines, and histologic analysis.

RESULTS. B. cereus replicated more rapidly in the eyes of TNFα−/− mice than in the eyes of B6.129F1 mice. Retinal function decreased more rapidly in TNFα−/− mice than in B6.129F1 mice. Retinal layers were not as structurally intact at 6 and 12 hours after infection in TNFα−/− eyes as in B6.129F1 eyes. Histologic analysis suggested less polymorphonuclear leukocyte (PMN) infiltration into the vitreous of TNFα−/− mice than of B6.129F1 mice. B6.129F1 eyes also had greater myeloperoxidase concentrations than did eyes of TNFα−/− mice. In general, concentrations of proinflammatory cytokines and chemokines (IL-1β, KC, IL-6, and MIP-1α) were greater in eyes of TNFα−/− mice than of B6.129F1 mice.

CONCLUSIONS. TNFα is important to intraocular pathogen containment by PMNs during experimental B. cereus endophthalmitis. In the absence of TNFα, fewer PMNs migrated into the eye, facilitating faster bacterial replication and retinal function loss. Although greater concentrations of proinflammatory cytokines were synthesized in the absence of TNFα, the resultant inflammation was diminished, and an equally devastating course of infection occurred. (Invest Ophthalmol Vis Sci. 2008;49:4482-4489) DOI:10.1167/iovs.08-2085

Disclosure: R.T. Ramadan, None; A.L. Moyer, None; M.C. Callegan, None.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Corresponding author: Michelle C. Callegan, Department of Ophthalmology, University of Oklahoma Health Sciences Center, 608 Stanton L. Young Boulevard, DMEI 418, Oklahoma City, OK 73104; michelle-callegan@ouhsc.edu.
endophthalmitis, but, based on experimental studies, it is reasonable to hypothesize that proinflammatory cytokines are key mediators of acute inflammation during this infection.

The inflammatory pathways involved in B. cereus-induced intraocular inflammation remain to be fully elucidated. However, such a rapid response strongly suggests that acute-phase mediators and immune cells are involved. As stated, under experimental conditions, TNFα is upregulated in the eye in parallel with the influx of PMNs, but the contribution of TNFα to the pathogenesis of endophthalmitis has not been determined. We hypothesized that TNFα is an important proinflammatory cytokine that contributes to the recruitment of PMNs into the eye during experimental endophthalmitis. To test this hypothesis, we analyzed infection in wild-type control and homozygous TNFα knockout mice. The results demonstrated that TNFα was important in bacterial growth control through the acute inflammatory response to B. cereus endophthalmitis. In the absence of TNFα, inflammation was muted, resulting in more rapid bacterial replication and retinal function loss. Compensating proinflammatory cytokines and chemokines were synthesized in the eye in the absence of TNFα, resulting in less inflammation but an equally devastating course of infection.

METHODS

Mice and Infections

Breeding pairs of background mice (B6.129F1) and homozygous TNFα−/− mice (B6.129S6-Tnfαm−/−/J) were obtained from Jackson Laboratories (Bar Harbor, ME). C57BL/6J mice were also used for comparisons with B6.129F1 mice for some experiments. Mice were bred and cared for in housing facility conditions according to institutional guidelines and guidelines provided by the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Male and female mice from the breeding colonies were used between 6 to 8 weeks of age, with the appropriate age-matched controls. Polymerase chain reaction (PCR) was performed to confirm the homozygosity of littersmates (data not shown).

Mice were infected intravitreally with wild-type B. cereus, as previously described. Brieﬂy, mice were anesthetized generally with a ketamine/xylazine cocktail (85 mg/kg [Ketaved; Phoenix Scientiﬁc, St. Joseph, MO]/14 mg/kg [Rompun; Bayer Corp., Shawnee Mission, KS] body weight) and topically with 0.5% proparacaine HCl (Ophthetic; Allergan, Hormigueros, Puerto Rico). Bacteria were injected into the vitreous with a sterile glass capillary needle containing 100 CFU B. cereus strain ATCC 14579 in 0.5 μL brain-heart infusion medium. At various times after infection, endophthalmitis was analyzed by biomicroscopy, quantitation of intraocular bacterial growth, proinflammatory cytokines and chemokines, myeloperoxidase (MPO, to estimate PMN infiltration), and electroretinography (ERG).

Electroretinography

Retinal function was assessed by ERG, as previously described. After injection of B. cereus, mice were dark adapted for at least 6 hours. Before ERG, mice were anesthetized with ketamine/xylazine, as described, and pupils were dilated with 10% topical phenylephrine (Akorn, Inc., Buffalo Grove, IL). Gold-wire electrodes were placed on each cornea, and a reference electrode was placed in the mouth. The stimulus used to evoke the response was delivered by a white sphere that mimicked a Ganzfeld. The interval between 2 flashes (10 ms duration) was 60 seconds to prevent light adaptation. A-wave and B-wave amplitudes were measured from the initiation of the light flash to the trough of the A-wave and the trough of the A-wave to the peak of the B-wave, respectively. Five readings were recorded and averaged. Percentages of retinal function retained compared with controls were calculated as described previously. Values represent the mean ± SEM for n = 6 samples per time point.

Bacterial Growth

Globes were homogenized with 1-mm sterile glass beads (BioSpec Products, Inc., Bartlesville, OK) in 400 μL PBS. Bacteria were quantified by track plating serial 10-fold dilutions onto brain-heart infusion agar. Values represent the mean ± SEM for n = 6 eyes per time point.

Cytokines and Chemokines

Eyes were analyzed for the presence of representative proinflammatory cytokines and chemokines shown to be upregulated in various experimental models of ocular infection and inflammation. Globes were removed and homogenized with 1-mm glass beads in a protease inhibitor cocktail (Triton X-100, 0.5 M EDTA, 10 mM sodium orthovanadate [Sigma, St. Louis, MO], and Protease Inhibitor [Calbiochem, La Jolla, CA] in PBS, pH 7.4). Supernatants were then analyzed for IL-1β, MIP-1α, KC, and IL-6 by ELISA (Quantikine Kits; R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions. Concentrations in supernatants of tissue homogenates were compared with those of a standard curve. Values represent the mean ± SEM for n = 6 eyes per time point.

PMNs and Myeloperoxidase

To compare the numbers of circulating PMNs, whole blood was harvested and PMNs were quantified with a hemocytometer. Values represent the mean ± SEM for n = 3 mice per genotype.

To estimate the extent of PMN infiltration into the eye, MPO was quantified. Mouse eyes were removed and homogenized with 1-mm glass beads in a lysis buffer (200 mM NaCl, 5 mM EDTA, 10 mM Tris, 1% glycine [vol/vol], 1 mM phenylmethylsulfonyl fluoride, 1 μg/mL leupeptin, 28 μg/mL aprotinin), and supernatants were analyzed for MPO levels by sandwich ELISA (Mouse MPO ELISA Test Kit; Cell Sciences, Canton, MA). Values represent mean ± SEM for n = 6 eyes per time point.

Histology

Globes were harvested, and eyes were fixed in Perfix and incubated for 24 hours. Globes were embedded in paraffin, sectioned, and stained with hematoxylin and eosin by standard procedures. Histology sections were scored by a masked observer and graded on a scale of 0 to 4+ in terms of severity. Sections presented are representative of n = 3 eyes per time point.

Anti-TNFα and B. cereus Endophthalmitis

A pilot study was undertaken to analyze the potential anti-inflammatory effects of anti-TNFα (infliximab; Remicade; Centocor Inc., Horsham, PA). Anti-TNFα (50 ng/0.5 μL) was injected immediately before B. cereus infection. MPO concentrations (n = 3) were analyzed 10 hours after infection.

Statistical Analysis

Student’s t-test was used for statistical comparisons between mouse strains at each time point. Wilcoxon rank sum test was used for statistical comparison between infection groups. P ≤ 0.05 was considered significant.

RESULTS

Bacterial Growth

B. cereus grew logarithmically in eyes of B6.129F1 wild-type control mice, TNFα−/− mice, and C57BL/6J background control mice (Fig. 1). Rates of growth in eyes of C57BL/6J and B6.129F1 wild-type controls were similar (P > 0.5 at all time points). However, B. cereus grew faster in eyes of the TNFα−/− strain, with greater numbers of viable B. cereus recovered per eye 6, 8, and 12 hours after infection compared with those
concentrations of TNFα and 12 hours after infection (P < 0.05 at all time points). Values represent the mean ± SEM of n ≥ 5 eyes per time point.

recovered from B6.129F1 wild-type control eyes (P < 0.05). In the absence of TNFα, faster intraocular bacterial replication occurred.

Retinal Function

Retinal function analysis findings of endophthalmitis in eyes of TNFα−/− and B6.129F1 wild-type mice are summarized in Figure 2. Amplitudes of A and B waves declined at significantly greater rates in infected eyes of TNFα−/− mice than in eyes of B6.129F1 mice (P < 0.01 at 6 and 8 hours after infection). Taken together, retinal function loss in eyes of the TNFα−/− mice was approximately threefold greater at 6 hours than that of the wild-type B6.129F1 mice. By 12 hours after infection, retinal function was lower than 5% in all infected eyes of either mouse strain. In the absence of TNFα, retinal function declined more rapidly, likely because of increased intraocular bacterial replication.

Whole Blood PMN and Intraocular Inflammation

PMNs were quantified in whole blood of TNFα−/− and B6.129F1 wild-type mice. Manual counts detected similar numbers of PMN from the blood of TNFα−/− mice (6.92 ± 0.05 log10 PMN/mL) and wild-type mice (6.84 ± 0.1 log10 PMN/mL; P = 0.052).

Intraocular inflammation was analyzed by quantifying the MPO of infiltrating PMNs (Fig. 3) and proinflammatory cytokines (Fig. 4) in eyes during endophthalmitis. At 0 hour and 4 hours after infection, MPO concentrations were similar in TNFα−/− and B6.129F1 eyes (P > 0.05). MPO concentrations of TNFα−/− and B6.129F1 eyes were greater at 4 hours after infection than at 0 hour after infection (P < 0.01), with significant increases within each group at each time point thereafter. MPO concentrations were significantly lower in eyes of TNFα−/− mice than in eyes of B6.129F1 mice at 6, 8, and 12 hours after infection (P < 0.05 at all time points). In the absence of TNFα, PMN influx into the infected eyes of TNFα−/− mice was smaller than that into infected wild-type eyes.

In general, proinflammatory cytokine and chemokine concentrations were greater in TNFα−/− eyes than in wild-type B6.129F1 eyes. In TNFα−/− eyes, concentrations of KC were greater than in eyes of B6.129F1 at 4, 8, and 12 hours after infection (P < 0.05 at all time points). Concentrations of MIP-1α and IL-6 were greater in TNFα−/− eyes than in those of B6.129F1 eyes at 8 and 12 hours after infection (P < 0.05). Concentrations of IL-1β were greater in TNFα−/− eyes than in B6.129F1 eyes at 12 hours only (P < 0.05). The upregulation of other proinflammatory cytokines and chemokines in the absence of TNFα resulted in lower numbers of infiltrating PMN in infected eyes of TNFα−/− mice.

Histology

Whole eye and retinal histology of infected TNFα−/− and B6.129F1 wild-type mice demonstrated evolving endophthalmitis similar to that previously reported in this model13 (Fig. 5). Six hours after infection in B6.129F1 wild-type eyes, most infiltrating PMNs observed were located close to the optic nerve head (histology score range, 1–2). At this time, the retinal architecture was slightly disrupted (histology score, 1). Twelve hours after infection, a significant loss of retinal architecture was observed, and significant numbers of PMNs were seen in the posterior segment, near the ciliary body, and in the anterior chamber (histology score range, 2–3). Six hours after infection in TNFα−/− mouse eyes, few PMNs were seen in the posterior segment (histology score range, 0–1), and retinal architecture was disrupted to a greater degree than in wild-type eyes (histology score, 2). Twelve hours after infection, retinas of infected eyes of TNFα−/− mice were disrupted and detached, with greater numbers of B. cereus accumulating near the retina and optic nerve, and PMNs were present in both segments (histology score range, 3–4). In TNFα−/− mice, ret-
inal disruption evolved more rapidly, but PMN influx was smaller than that seen in B6.129F1 wild-type mice.

**Anti-TNFα and *B. cereus* Endophthalmitis**

Ten hours after infection, MPO concentrations were decreased by approximately 40% in eyes injected with 50 ng anti-TNFα alone compared with those of uninjected control eyes.

**DISCUSSION**

Pathogen recognition and a well-regulated inflammatory response to infection are essential in clearing invading organisms with minimal damage to surrounding tissue. A tightly controlled response is even more critical in the eye, where non-regenerative cells and tissues responsible for vision reside. Experimental models of bacterial endophthalmitis have demonstrated that once a pathogen is introduced into the posterior segment, an acute response occurs, including synthesis of proinflammatory cytokines and influx of PMNs into the eye.\(^{12-14}\) In the case of virulent pathogens such as *S. aureus* and *B. cereus*, low numbers of bacteria can be cleared effectively by an adequate inflammatory response.\(^{13,28}\) Once an inoculum threshold is passed, bacterial growth and toxin production overwhelm the inflammatory response. In an exhaustive attempt to clear the infection, PMNs fill the posterior and anterior segments.

Regulation of inflammation is the key to removing the pathogen without harming the eye, but bystander damage from infiltrating cells can occur. For *S. aureus* endophthalmitis, the depletion of neutrophils early in the inflammatory response reduced the severity of host inflammation but severely hampered bacterial clearance, resulting in a more severe infection.\(^{12}\) In the present study, the depression of initial cellular influx in the absence of TNFα resulted in higher bacterial numbers and faster retinal function loss. Explosive inflammation is characteristic of *B. cereus* endophthalmitis. However, because of rapid bacterial growth, migration, and toxin production by *B. cereus* in the eye,\(^1\) attempts at infection control by the host are typically futile.

**FIGURE 3.** Infiltration of PMN into mouse eyes during *B. cereus* endophthalmitis. B6.129F1 wild-type and TNFα−/− mouse eyes were injected with 100 CFU *B. cereus*. PMN infiltration was analyzed by quantifying MPO in whole eyes. MPO concentrations were greater in B6.129F1 eyes than in TNFα−/− eyes at 6, 8, and 12 hours after infection (\(P < 0.05\)), suggesting greater numbers of PMN in wild-type eyes than in TNFα−/− eyes. Values represent the mean ± SEM for \(n \geq 6\) per group.

**FIGURE 4.** Proinflammatory cytokine expression during *B. cereus* endophthalmitis. B6.129F1 wild-type and TNFα−/− mouse eyes were injected with 100 CFU *B. cereus*. Whole eyes were analyzed for proinflammatory cytokines and chemokines by ELISA. In general, levels of MIP1α, IL-6, KC, and IL-1β were greater in TNFα−/− mouse eyes than in B6.129F1 eyes during infection. Values represent the mean ± SEM of \(n = 6\) eyes per time point.
We reported that one of the earliest cytokines detected during experimental *B. cereus* endophthalmitis was TNFα.\textsuperscript{13} TNFα was detected in whole eyes during experimental *B. cereus* endophthalmitis as early as 4 hours after infection, when PMNs were first observed infiltrating into the posterior segment. During many different types of infections, TNFα initiates a cascade of proinflammatory cytokine synthesis and contributes to increased vascular permeability and upregulation of cell adhesions, effectively recruiting macrophages and neutrophils to the infection site. Because TNFα levels increased in parallel with increasing numbers of intraocular PMNs during experimental *B. cereus* endophthalmitis, we sought to determine to what extent TNFα contributed to this initial inflammatory response by comparing infections in wild-type and TNFα\textsuperscript{−/−} mice. Studies of nonocular infections in TNFα\textsuperscript{−/−} or TNFα-receptor knockout mouse strains have demonstrated the value of TNFα in the containment of a wide range of ocular pathogens, including *S. aureus*,\textsuperscript{29,30} *Candida*,\textsuperscript{31} and pneumococcus.\textsuperscript{32,33}

To ensure that experimental endophthalmitis was reproducible in mouse strain B6.129F1, the wild-type strain used in these studies, bacterial growth rates and pathologic conditions were compared with those of C57BL/6J mice. Ocular TNFα concentrations were similar in these two mouse strains during experimental infection (data not shown). Retinal function loss and MPO levels in B6.129F1 eyes were comparable to those of C57BL/6J eyes, as reported previously.\textsuperscript{13} Intraocular *B. cereus* growth and clinical signs of infection were similar at all time points, further validating the use of this genetic background for these studies.

*B. cereus* grew more rapidly and to greater numbers in eyes of TNFα\textsuperscript{−/−} mice than in eyes of B6.129F1 wild-type mice. As expected, retinal function declined more rapidly in TNFα\textsuperscript{−/−} eyes than in B6.129F1 mice. Histologic evidence demonstrated that retinas were damaged and detached to a greater degree in TNFα\textsuperscript{−/−} eyes than in eyes of B6.129F1 mice. These results suggested that in the absence of TNFα, bacterial growth was unimpeded, facilitating greater retinal damage and function loss. Greater numbers of bacteria likely resulted in higher concentrations of toxins produced in the eye, resulting in faster retinal damage and loss of function. The importance of
toxins to the intracellular virulence of \textit{B. cereus} endophthalmitis has been well documented.\textsuperscript{1,7–9}

In infected TNFα/–/– mouse eyes, inflammation was muted compared with eyes of B6.129F1 mice. PMNs migrated in fewer numbers into the eyes of TNFα/–/– mice than of B6.129F1 mice, as demonstrated by MPO assay and histology. Taken together, these data indicated that in the absence of TNFα, fewer PMNs migrating into the posterior segment resulted in higher intraocular bacterial loads and, subsequently, more significant retinal damage. This result suggests that TNFα contributed to the early recruitment of PMNs into the eye and subsequent pathogen control during endophthalmitis. By virtue of its role in affecting blood-retinal barrier integrity,\textsuperscript{34,35} the absence of TNFα might have resulted in less barrier permeability and in the migration of fewer PMNs into the eye. The absence of TNFα has been demonstrated to decrease tight junction-associated permeability\textsuperscript{36–37} and to result in less PMN infiltration\textsuperscript{38} in experimental models of acute lung inflammation and restraint stress (small intestine analyzed). Manual counts detected similar numbers of PMNs from the blood of TNFα/–/– and B6.129F1 mice; hence, differences in intraocular PMN quantities were not the result of differences in whole blood PMNs between the mouse strains. Our PMN numbers in TNFα/–/– mice were similar to those reported by Kuprash et al.,\textsuperscript{58} who detected statistically higher whole blood white blood cells and neutrophils in TNFα/–/– mice compared with C57BL/6 mice. Because the numbers of PMNs were greater 4 hours after infection in the eyes of both mouse strains than in freshly infected eyes at 0 hour, PMN quantities at 4 hours likely represented cells that were recruited to the eye as a result of infection.

In the absence of TNFα, other proinflammatory cytokines and chemokines were synthesized during infection to facilitate the recruitment of PMNs into the eye. In this study, KC was detected 4 hours after infection, and higher concentrations of KC were detected in TNFα/–/– eyes than in B6.129F1 eyes. Because KC and MPO levels paralleled one another and increased beginning at 4 hours after infection in a manner similar to that of TNFα in C57BL/6J mice,\textsuperscript{13} KC may also be an important recruiting cytokine in the eye during the initial stages of experimental \textit{B. cereus} endophthalmitis. KC and its homologs have been detected in experimental and human ocular infections, including keratitis caused by fungi,\textsuperscript{59} adenovirus,\textsuperscript{40} \textit{Pseudomonas},\textsuperscript{24,41} \textit{Staphylococcus},\textsuperscript{12} acute bacterial conjunctivitis,\textsuperscript{43} and uveitis.\textsuperscript{44} Further studies in transgenic mice deficient in KC can confirm the contribution of this cytokine to the pathogenesis of bacterial endophthalmitis.

In terms of proinflammatory cytokine synthesis, IL-6, KC, MIP-1α, and IL-1β were synthesized to higher levels in TNFα/–/– mice than in B6.129F1 mice. IL-1β and IL-6 were below the limits of detection 4 hours after infection but were detected at 8 hours in the eyes of both mouse strains, 4 hours later than the initial influx of PMNs into the posterior segment. MIP-1α levels were just above the limit of detection 4 hours after infection, but levels increased thereafter. IL-1β levels were significantly higher in TNFα/–/– eyes than in B6.129F1 eyes at 12 hours only. Higher concentrations of cytokines/chemokines may be synthesized to compensate for the absence of the most potent recruiting cytokine, TNFα. However, greater cytokine concentrations may not necessarily translate to greater numbers of PMNs in the eye, as was demonstrated in this model, particularly if the absence of TNFα resulted in greater impermeability of the blood-ocular barrier. Studies have demonstrated the production of increased levels of other proinflammatory cytokines/chemokines in the absence of TNFα in experimental models of infection and inflammation.\textsuperscript{59–61} Because IL-6, MIP-1α, and IL-1β were produced at a later stage of infection, when significant numbers of PMNs were already present in the eye, these cytokines could be a product of the infiltrating inflammatory cells themselves and perhaps played only a minor role in initial PMN recruitment. However, PMNs are not the only potential source of cytokines in the eye during intraocular inflammation. Other resident ocular cells, such as retinal or optic nerve head glia and microglia, may also synthesize cytokines/chemokines. These particular cell types have been shown to synthesize proinflammatory cytokines during various states of infection, inflammation, and retinal stress.\textsuperscript{59–61} The specific cells involved in cytokine and chemokine synthesis during intraocular bacterial infection are yet to be identified.

Using single-gene knockouts in mediators of the host response is sufficient for analyzing a deficiency in one mediator, but this approach has its disadvantages. For example, the same types of cells synthesize IL-1β and TNFα. These cytokines act on comparable cell types during inflammation and also signal through the NF-κB pathway. Hypothetically, the absence of one cytokine may induce the synthesis of other compensating cytokines (in a manner similar to that seen in this study), confounding the role of the original cytokine of interest in infection. Functional redundancy has been reported for TNFα and IL-1 in experimental autoimmune uveitis, where the deletion of receptors for both cytokines was more effective in reducing infiltrating cell numbers than the deletion of each receptor alone.\textsuperscript{54} In the context of experimental \textit{B. cereus} endophthalmitis, IL-1β was first detected well after TNFα and KC were initially detected and PMNs were present in the posterior segment, suggesting a minimal role for IL-1β during the initial stages of inflammation.

Because the absence of TNFα was demonstrated in this infection model to dampen the initial inflammatory response during \textit{B. cereus} endophthalmitis, it was of interest to analyze whether therapy targeting TNFα would effectively attenuate inflammation. Our preliminary data demonstrated the anti-inflammatory potential of anti-TNFα when injected immediately before \textit{B. cereus} infection. Infliximab has attenuated intraocular inflammation in experimental models of choroidal neovascularization\textsuperscript{55,56} and endotoxin-induced uveitis\textsuperscript{57} and in patients with uveitis.\textsuperscript{58–62} Infliximab was recently shown to be nontoxic at levels up to 1.7 mg in rabbit eyes.\textsuperscript{63} These findings suggest the potential for the attenuation of inflammation during endophthalmitis by targeting TNFα and perhaps other cytokines, but this sort of therapy would likely be best suited for the initial stages of infection.\textsuperscript{64} Continuing studies will determine the therapeutic potential of cytokine targeting in conjunction with early antibiotic treatment in reducing inflammation during bacterial endophthalmitis.

Acknowledgments

The authors thank Billy D. Novosad (Oklahoma University Health Sciences Center [OUHSC], Department of Microbiology/Immunology), Dustin Woods (OU School of Medicine), and Mark Dittmar (Dean A. McGee Eye Institute Animal Research Facility) for their invaluable technical assistance; Paula Pierce (Excalibur Pathology, Moore, OK) for histology expertise; and Brandt Wiskur (OK Center for Neuroscience), Muayyad Al-Ubaidi (OUHSC), John Ash (OUHSC), James Chodosh (OUHSC), Eric Howard (OUHSC), and Michael Gilmore (Scheepens Eye Research Institute, Boston, MA) for their helpful comments.

References


