Fas Ligand but Not Complement Is Critical for Control of Experimental Staphylococcus aureus Endophthalmitis

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PURPOSE. To determine the role of complement and Fas Ligand (FasL) in the host defense against Staphylococcus aureus endophthalmitis.

METHODS. C3−/−, FasL defective gld, and C57/BL6 (wild-type [WT]) mice were infected intravitreally with 500 and 5000 CFU S. aureus, and the course of infection was followed by determining the intraocular bacteria counts, retinal function by ERG, and morphologic damage and inflammation by histopathology and flow cytometry.

RESULTS. In WT eyes injected with 500 CFU, S. aureus grew to 1 × 10^4 CFU/mL by 24 hours, but was cleared by 96 hours. In the WT eyes injected with 5000 CFU, S. aureus grew to 2 × 10^6 CFU/mL by 72 hours, resulting in corneal perforation. C3−/− eyes injected with 500 CFU reached transiently higher levels than their WT counterparts (P < 0.001), but eventually followed a similar course. Bacterial counts in gld eyes infected with 500 CFU were similar to those in WT eyes infected with 5000 CFU. In WT and C3−/− eyes injected with 500 CFU, retinal function decreased only transiently and recovered to 66% in 72 hours. In WT eyes injected with 5000 CFU and gld eyes infected with 500 CFU, retinal function was completely lost by 24 hours. By 24 hours, WT and C3−/− eyes injected with 500 CFU were infiltrated with a similar number of granulocytes, but recruitment was significantly impaired in gld eyes (P < 0.005). Cell counts in WT and C3−/− eyes decreased thereafter without overt retinal disease. In eyes injected with 5000 CFU and gld eyes infected with 500 CFU, inflammatory cells completely filled the intraocular space by 48 hours. Retinal and uveal tissue was destroyed by that time.

CONCLUSIONS. The tipping point for a good versus a bad outcome in this murine model of endophthalmitis lies between 500 and 5000 CFU S. aureus. This point is identical in animals deficient in complement activation, suggesting that complement does not play a significant role in the ocular defense against intraocular bacteria. In contrast, FasL was found to be critical for clearance, since animals deficient in FasL signaling were unable to control infection with 500 CFU. (Invest Ophthalmol Vis Sci. 2005;46:2479–2486) DOI:10.1167/iovs.04-1139

Intraocular bacterial infection (endophthalmitis) is a vision-threatening complication of penetrating eye injury and intraocular surgery. Staphylococcus aureus is the most common cause of severe endophthalmitis after cataract surgery. Several studies have shown that agents associated with postoperative and posttraumatic endophthalmitis, including S. aureus,5,6 Bacillus cereus,5,6 and Enterococcus faecalis,6,7 once introduced into the eye, are infective in the eye in extremely small doses (typically on the order of 100 colony forming units; [CFU]).2,3,5–7 This observation demonstrates that the eye has limited capacity to respond to microorganisms, which may be related to its characteristic immune privilege. Although much has been learned in recent years about the contribution of bacterial virulence factors to the pathogenesis of endophthalmitis,2,4,6–8 comparatively little is known about the host response in the disease. Because the eye has been shown to be an immunomodulated environment with restricted ability to mount an adaptive immune response,9 it was of interest to determine how factors involved in immune privilege affect the innate response to infection.

Immune privilege evolved as a concept to describe the limited immune response to antigens introduced into specific body sites, such as the eye and the brain, compared with other sites, such as the subcutaneous space.9 It is generally believed that immune privilege in the brain and eye evolved to limit bystander damage resulting from systemic inflammation, because much of the neural function depends on the activities of nonregenerating postmitotic cells of the central nervous system.9–11 Many soluble factors, including components of the complement system11 and at least one membrane-bound factor, FasL,12,13 have been shown to contribute to immune privilege. Although most research on immune privilege has centered on the adaptive response to antigen, it has been suggested that immune privilege extends to innate immunity.14 However, immune privilege has not been examined in the context of the innate response to bacterial infection.

One of the first lines of innate immune defense to bacterial infection is the complement system. It is known that several aqueous humor factors possess complement-inhibitory activity.15–17 The level of complement components in the eye are several orders of magnitude lower than in the serum,16 even in inflamed eyes with a compromised blood–retinal/blood–aqueous barrier as a consequence of intraocular inflammation.19 However, the concentration of complement in the eye is sufficient to elicit severe experimental uveitis in response to zymosan injected into the anterior chamber of the rat.16 Experiments using guinea pigs, rendered deficient in complement by treatment with cobra venom factor, suggested a role for complement in defense against S. aureus intraocular infection.20 In toto, these observations suggest that although immune privilege restricts the innate defense, it does not limit complement activity, allowing for defense against pathogens in the eye. Moreover, it simultaneously suppresses the adaptive
immune system preventing the potentially harmful antigen-specific delayed-type hypersensitivity (DTH) responses.11 FasL has also been found to be critical for immune privilege, in that it suppresses the adaptive immune response to corneal grafts.12 Moreover, FasL has been shown to induce apoptosis in infiltrating inflammatory cells.21 However, FasL has not been examined to determine its contribution to innate immunity in bacterial infection. Therefore, it was also of interest to determine its role in creating an intraocular environment that is permissive for bacterial infection.

**Materials and Methods**

**Animal Husbandry**

Female C57BL/6J mice were obtained from The Jackson Laboratory (Bar Harbor, ME). B6 129S4-C3mitCrr(C57/–/–), B6Smn.C3-Tnfsf9gld(gld), and B6.MRL-Tnfsf9gldtm1bpr (lpr) breeder trios were also obtained from The Jackson Laboratory and bred at the Dean A. McGee Eye Institute (DMEI) animal facility (Oklahoma City, OK). Genotypes were verified by either C3 ELISA from serum, aqueous, and vitreous (for C5/–/– as described later) or tail PCR (gld), according to Jackson Laboratory protocols; http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objectype=prot_list). All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. All experimental protocols were approved by the Animal Care and Use Committee of The University of Oklahoma Health Sciences Center (OUHSC) and the Dean A. McGee Eye Institute. When appropriate, mice were anesthetized by intraperitoneal injection of ketamine (KetaVed; Vedco, St. Joseph, MO) and xylazine (TranqulVed, Vedco). Animals were euthanized at the appropriate time points by CO2 asphyxiation after anesthesia.

**Murine Model of Endophthalmitis**

The vitreous from eyes of 6- to 8-week-old mice was inoculated by insertion of a borosilicate microcapillary, pulled to a tip size of 50 μm, immediately behind the limbus-parallel conjunctival vessels, which correspond to the narrow murine pars plana. Right eyes of mice were immediately behind the limbus-parallel conjunctival vessels, which correspond to the narrow murine pars plana. Right eyes of mice were inoculated with 0.5 μL of a bacterial suspension containing either 500 or 5000 CFU of *S. aureus* RN 6390 diluted in physiological saline. Left eyes received injections of filter-sterilized supernatants from the respective suspensions when appropriate. *S. aureus* RN 6390 was chosen because it is a well-characterized strain of the agr 1 group, which has been used in staphylococcal pathogenesis research in models of endocarditis,24 subcutaneous abscess,25 arthritis,26 osteomyelitis,27 and endophthalmitis.2,3 Before infection, *S. aureus* RN 6390 was grown overnight in standard brain-heart infusion (Difco Laboratories, Detroit, MI). Experiments were performed with a minimum of five animals per experimental group and repeated at least twice.

**Quantification of Bacterial Growth**

Eyes were enucleated after euthanasia, by severing first the conjunctiva and extraocular muscles parallel to the limbus and then the optic nerve, using fine Vannas iris scissors and Colibri forceps. Residual adnexal tissue was trimmed before eyes were placed in cold phosphate-buffered saline (PBS). Enucleated eyes were disrupted and homogenized, to release bacteria for enumeration by bead-beating with 1.0-mm glass beads (Mini-BeadBeader; Biospec Products, Bartlesville, OK) in 400 μL PBS for 1 minute at maximum speed. The homogenates were serially diluted, plated onto blood agar plates, and incubated overnight at 37°C.

**Flow Cytometric Analysis**

After euthanasia and enucleation of eyes, the anterior segment of the eye, consisting of cornea, iris, and lens, was dissected from the postero-
RESULTS

Characterization of the Murine Endophthalmitis Model

To determine the role of factors involved in immune privilege in innate defense against bacterial infection, a mouse model of endophthalmitis was developed. In this model, two infective doses were identified that resulted in an infection that resolved over 3 to 4 days or progressed and destroyed the organ. C57BL/6J mouse eyes infected intravitreally with 500 CFU S. aureus were observed to reach maximum intraocular bacterial concentrations of $1.3 \times 10^7$ CFU/mL by 24 hours, but eventually controlled the infection and reduced bacterial numbers to $5.7 \times 10^3$ CFU/mL within 72 hours. In contrast, C57BL/6J mice infected intravitreally with an inoculum of 5000 CFU were not able to control the infection. Intraocular bacterial counts reached a plateau at levels above $1 \times 10^9$ CFU/mL by 48 hours and did not decrease thereafter (Fig. 1).

The difference in intraocular bacterial growth was mirrored by the clinical course of infection. In the eyes of C57BL/6J mice infected with 500 CFU, only mild signs of inflammation were present throughout the 3-day course of infection, and these eyes appeared normal at 72 hours (Fig. 2A). Retinal function during infection, as determined by electroretinography, decreased transiently to 32% of preinfection levels but returned to 66% by 72 hours when bacterial counts had decreased below the initial inoculum level (Fig. 3). In contrast, eyes of C57BL/6J mice infected with 5000 CFU inoculum, rapidly lost b-wave amplitude to 5% of the preinfection level by 24 hours, and eventually lost all measurable retinal function (Fig. 3). These eyes displayed more severe inflammation, and corneal perforation was noted in some eyes (Fig. 2B).

Consistent with these findings, eyes of C57BL/6J mice infected with 500 CFU displayed undisturbed retinal architecture (Fig. 4A) with few residual inflammatory cells and undisturbed retinal architecture, whereas C57BL/6J mouse eyes infected with 5000 CFU exhibited severe signs of endophthalmitis, with massive infiltration of all ocular spaces and structures, and dense, visible aggregates of bacteria, as well as destruction of the retina (Fig. 4B).

Evaluation of the Role of Complement in Innate Defense against Intraocular Infection

Previous experiments have shown that guinea pigs20 artificially depleted of complement by systemic injection of cobra venom factor (CVF) have increased susceptibility to intraocular infection. We have observed similar results with CVF-treated mice (data not shown). To determine whether this effect results from loss of complement or possibly from the pleiotropic effects of CVF-mediated complement activation and consump-
Figure 3. C57BL/6j (B6) mice infected with 500 CFU *S. aureus* retained retinal responsiveness to light, whereas B6 mice infected with 5000 CFU lost all retinal function. ERG assessment of retinal response expressed as a percentage of the response of the healthy contralateral eye to light stimulation is shown. After a transient loss of 67% at 24 hours, retinal function in B6 mice infected with 500 CFU stabilized at between 41% of baseline at 48 hours and 66% at 72 hours. B6 mice infected with 5000 CFU *S. aureus* had 5% residual function at 24 hours, and no detectable function at 48 and 72 hours.

Figure 4. *S. aureus* ocular infection of C57/BL6J (B6) or C3−/− mice led to little anatomic destruction, whereas eyes of B6 mice infected with 5000 CFU and eyes of Fast-defective gld mice infected with 500 CFU were destroyed by the infection. Histologic appearance of eyes 72 hours after infection is shown. B6 (A) and C3−/− (C) mouse eyes infected with 500 CFU had few infiltrating inflammatory cells and no microscopic retinal damage, whereas B6 (B) and gld (D) eyes were massively infiltrated and displayed severe damage to ocular structures. Hematoxylin and cosin stain, original magnification, ×20.

Figure 5. Ocular infections in C3−/− mice inoculated with 500 CFU *S. aureus* transiently reach higher bacterial counts than did similar infections in C57BL/6j (B6) mice, but the infection ultimately was controlled. *S. aureus* grew to similar levels in C3−/− and WT C57BL/6j mice at the 12-hour time point. Although intraocular bacterial concentrations in B6 mice transiently stabilized at 24 hours at a level of 1 × 10⁷/mL and decreased rapidly to 6 × 10⁶/mL at 48 hours and 6 × 10^⁵/mL at 72 hours, intraocular bacterial concentrations in similarly infected C3−/− mice increased to a maximum of 1 × 10⁸/mL at 24 hours, but then declined to 2 × 10⁶/mL at 48 hours and 3 × 10⁵/mL at 72 hours. These differences between C3−/− and B6 mice were significant at 24 (P < 0.001) and 48 (P < 0.02) hours, but no longer at 72 hours (P = 0.111).

As shown in Figure 5, *S. aureus* intraocular growth was similar in C3−/− and WT C57BL/6j mice at the 12-hour time point. The number of *S. aureus* CFU in C3−/− mice infected with 500 CFU exceeded those in similarly infected WT C57BL/6j at 24 (P < 0.001) and 48 (P < 0.002) hours, but these differences were transient, as the levels of infection were indistinguishable at 72 hours (P = 0.11). At 24 hours after infection, C3−/− mice infected with 500 CFU yielded organisms in a number comparable to that in eyes of WT C57BL/6j mice that had been infected with one order of magnitude more organisms, 5000 CFU, at 24 hours (P = 0.13). However, the number of organisms in the eyes of WT mice infected with the higher inoculum remained fairly stable at subsequent time points. In summary, the bacterial density in *S. aureus*-infected eyes of C3−/− mice transiently exceeded that in infected WT C57BL/6j counterparts at 24 hours after which clearing occurred, producing similar counts at 72 hours.

Both C57BL/6j and C3−/− eyes yielded a similar clinical presentation. Mild signs of inflammation were observed to be present in C3−/− eyes infected with 500 CFU at 24 and 48 hours, but they appeared normal at 72 hours (Fig. 2C). When compared with C57BL/6j mice infected with a similar number of bacteria (Fig. 2A), no difference in the course of infection was observed at any point in time.

Likewise, retinal function in C3−/− mice infected with 500 CFU *S. aureus* also followed a course that was indistinguishable from infected C57BL/6j animals (Fig. 6). After a transient loss of b-wave amplitude (57% and 67%) at 24 hours, retinal function of C3−/− and WT mice infected with 500 CFU *S. aureus* eventually stabilized at levels of 66% to 67% of baseline complement activation pathway. Absence of complement from serum and aqueous and vitreous humor of C3−/− animals was verified by C3 ELISA. Levels of C3 in the eyes of naïve WT mice were found to be a fraction of that in the serum. In the vitreous, levels of 0.12% of the respective serum concentration were determined, and only 0.03% in the aqueous (data not shown). However, no C3 was identified in serum, aqueous or vitreous of C3−/− animals (data not shown).
at 72 hours. In contrast, C57BL/6j mice infected with 5000 CFU S. aureus exhibited loss of retinal function over the first 24 hours and had no detectable function at 48 and 72 hours. Eyes of C3 −/− mice infected with 500 CFU S. aureus appeared normal at 72 hours with few residual inflammatory cells in the anterior chamber and vitreous, and no other signs of ocular inflammation (Fig. 4C). Histopathologically, this result is comparable to the one observed in the infected C57BL/6j counterparts (Fig. 4A).

**Evaluation of the Role of Fasl Expression in the Eye in Creating an Immune-Privileged Site Susceptible to Infection**

To determine the extent to which Fas-Fasl signaling contributes to susceptibility to bacterial growth, B6Smn.C3-Tnfsf6glgd (gld) mice with a point mutation that changes an amino acid in the extracellular domain of the protein, thus rendering it defective in signaling,29 and B6.MRL-Tnfrsf6lpr (lpr) mice, with a transposable element insertion in intron 2 of the Fasl gene that prevents full-length transcription and expression of Fas,30 were infected with 500 CFU S. aureus. Unlike C57 −/− or WT mice, in which the infection resolved, gld mice were observed to be susceptible to the lower amount of inoculum. By 72 hours, bacterial counts in the eyes of gld mice infected with 500 CFU S. aureus were approximately 7 × 10^8 CFU/mL, with 2.1 × 10^9 CFU/mL in the eyes of lpr mice compared with 1 × 10^3 CFU/mL in the eyes of C57BL/6j mice infected with 500 CFU (P = 0.0012 and P = 0.0072), respectively; (Fig. 7). The high level of S. aureus growth in gld and lpr mice infected with 500 CFU was comparable to that detected in C57BL/6j mice infected with the higher amount of inoculum of 5000 CFU.

Reflecting the effect of higher bacterial counts, gld mice infected with 500 CFU S. aureus exhibited loss of retinal function that was similar to C57BL/6j WT mice infected with 5000 CFU (Fig. 8). Fasl-defective mice retained only 7% of b-wave amplitude at 24 hours and exhibited no detectable retinal function thereafter. This course was indistinguishable from the WT C57BL/6j mice infected with 5000 CFU inoculum at all time points. Eyes of gld mice infected with 500 CFU S. aureus also showed histologic signs of severe intraocular inflammation and tissue destruction (Fig. 4D). This appearance was similar to C57BL/6j mice that had been infected with 5000 CFU inoculum.

To determine whether the susceptibility of gld mouse eyes to S. aureus infection reflected a limitation in the influx of phagocytic cells at an early time, inflammatory cells infiltrating the eyes of gld mice were examined at 24 hours by flow cytometry. Half as many granulocytes (defined as fluorescent stain-negative, CD45-positive, and Gr1-positive) infiltrated the eyes of gld mice in response to infection with 500 CFU S. aureus at 24 hours as was observed in similarly infected C57BL/6j mice (P < 0.005; (Fig. 9).
DISCUSSION

To develop new or optimized antimicrobial and anti-inflammatory therapies for treating endophthalmitis, it is of interest to determine which elements of immune privilege promote or restrict bacterial clearance from the eye. Therefore, to take advantage of many of the sophisticated tools and reagents available, a murine endophthalmitis model was developed. Using this model, we found that an infecting inoculum of 500 CFU of *S. aureus* reproducibly resulted in an endophthalmitis in C57BL/6J mice that evolved over a 3 day course, but ultimately resolved with significant residual retinal function remaining. 

It is known that several regulatory factors in the eye account for low complement activity. The present study demonstrates that the central complement system component C3 is present at highly reduced levels in the anterior and posterior compartments of the murine eye. This observation is consistent with a tight regulation of the presence of central elements of the immune system in privileged compartments. Despite the low level of complement in the eye, findings in studies in CVF complement-depleted guinea pigs indicated that complement in the eye is critical for limiting growth of *S. aureus*. Similar observations have been made using CFV-treated C57BL/6J mice in the present study. However, in C3–/– mice, complement was found to be comparatively inconsequential to the outcome of endophthalmitis. Among the reasons for obtaining disparate results is the fact that CVF factor activation of complement has profound effects on physiology. As noted by others, it is associated with serious side effects and a high mortality rate. CVF injection into rats has profound pulmonary effects, including neutrophil aggregation and activation, intrapulmonary capillary sequestration of neutrophils, and vascular injury, which have been exploited in models of acute lung injury. This massive mobilization of neutrophils leads to transient neutropenia and subsequent granulocytopsis. The immune status of animals complemented with CVF therefore does not appear to be otherwise comparable to untreated animals, which is an important caveat. The finding that complement component C3 was comparatively inconsequential in the outcome of endophthalmitis was unexpected, particularly in view of the previous reports and prevailing views of the importance of complement in limiting the proliferation of bacteria at sites of infection, including the eye. The limited contribution of complement in this context, however, is consistent with its highly restricted level in the eye as well as the presence of factors shown to limit complement activity.

Because FasL has been shown to be central to immune privilege by suppressing the adaptive immune response, it was of interest to determine whether the permissive intraocular environment was retained in the absence of Fasl. Unexpectedly, it was found that Fasl expression on ocular tissues was actually essential for efficient clearance of *S. aureus* from the intraocular space. An inoculum of 500 CFU *S. aureus* could be readily cleared by the WT C57BL/6J mice, but not by FasL-defective gld mice. Instead, the course of disease in the gld mice was similar to the course in WT C57BL/6J mice infected with 5000 CFU *S. aureus*, which plateau at stationary-phase levels. The dependence of *S. aureus* clearance on Fasl was evident in bacterial counts, retinal function, and histopathology.

It was observed that a significantly greater number of phagocytes were recruited to the intraocular infection site in mice expressing Fasl, when compared with either Fasl-deficient gld mice or Fasl-defective la/la mice deficient in Fas. This difference may be attributable to a chemotactic effect of soluble Fasl. Fasl can be converted to its soluble form by host proteases, and perhaps also bacterial proteases produced during the infection, but this remains speculative. However, another study failed to observe a chemotactic effect of soluble Fasl, but instead demonstrated that it suppressed the response to subcutaneously injected tumor cells. In that study, membrane-attached Fasl was observed to be immunostimulatory, finding that was later reproduced in a study that examined the immune response to tumor cells injected into the anterior chambers of mouse eyes. These findings suggest an alternate mechanism from chemotaxis, by which Fasl is critical for the resolution of ocular infection. They support a model in which Fasl activates cells, either resident cells or possibly the first wave of neutrophils, to release chemoattractive factors, such as MIP-2. These chemoattractants may subsequently recruit granulocytes, as was observed at early time points in the Fasl-expressing animals. An analogous role of resident cells has also been shown in other inflammatory disease models. In endophthalmitis, it then becomes a question of why resident dendritic cells and macrophages do not secrete chemoattractive factors in the uninfected eye, where Fasl is also present. It may be that dendritic cells and macrophages are kept in check by the immunosuppressive environment in the uninflamed eye and only become activated when the blood–retinal barrier is compromised, allowing for equilibration of soluble factors in the serum; resident dendritic cells and macrophages may encounter Fasl only when mobilized during an immune response and migrating toward bacteria in the vitreous; or Fasl expression may be upregulated during the inflammatory reaction to the invading microorganism.

In summary, the data in the present study show that, contrary to expectations, complement is relatively unimportant in limiting bacterial growth in the eye and, consequently, in
slowing the course of disease. Further, although Fasl has been shown to limit the immune response in the eye, it promotes clearance of S. aureus from this site of infection. These findings highlight the importance of testing various factors, shown to contribute to limiting the adaptive response in immune privilege, directly before ascribing roles in the innate response to bacterial infection.

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**References**


