

## $\alpha$ B-Crystallin Protects Retinal Tissue during *Staphylococcus aureus*-Induced Endophthalmitis<sup>∇</sup>

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**Bacterial infections of the eye highlight a dilemma that is central to all immune-privileged sites. On the one hand, immune privilege limits inflammation to prevent bystander destruction of normal tissue and loss of vision. On the other hand, bacterial infections require a robust inflammatory response for rapid clearance of the pathogen. We demonstrate that the retina handles this dilemma, in part, by activation of a protective heat shock protein. During *Staphylococcus aureus*-induced endophthalmitis, the small heat shock protein  $\alpha$ B-crystallin is upregulated in the retina and prevents apoptosis during immune clearance of the bacteria. In the absence of  $\alpha$ B-crystallin, mice display increased retinal apoptosis and retinal damage. We found that *S. aureus* produces a protease capable of cleaving  $\alpha$ B-crystallin to a form that coincides with increased retinal apoptosis and tissue destruction. We conclude that  $\alpha$ B-crystallin is important in protecting sensitive retinal tissue during destructive inflammation that occurs during bacterial endophthalmitis.**

Endophthalmitis is a vision-threatening bacterial infection that occurs when bacteria are introduced into the posterior of the eye following cataract surgery or a deep puncture wound. *Staphylococcus aureus* is the most common cause of destructive endophthalmitis following cataract surgery and accounts for 10% of all endophthalmitis cases (7, 12, 13, 16). Upon entering the posterior segment of the eye, *S. aureus* proliferates rapidly and produces exotoxins that directly damage retinal tissue (5, 7). In addition, the host mounts a massive neutrophil-mediated immune response in which degranulation of neutrophils releases cytotoxic mediators that further contribute to retinal-tissue damage (7, 8, 14). Typical treatment of endophthalmitis involves intravitreal injection of antibiotics, combined with vitrectomy in severe cases (7, 12). However, while intravitreal antibiotic treatment is effective in clearing the infection, in many cases it does not mitigate the tissue damage induced by the bacterial exotoxins and the by-products of the host immune response (7, 12).

The eye is an immune-privileged site where inflammation is inhibited to preserve vision by preventing damage to the delicate ocular tissues (34, 41). However, immune privilege is not absolute and can be breached, leaving the eye vulnerable to immune-mediated inflammation. Ocular infections, in particular, can overwhelm ocular immune privilege and trigger vigorous inflammation within the eye, raising a very important question (33): how does the eye protect the ocular tissues from

nonspecific damage when immune privilege is overcome and inflammation is induced within the eye, as in the case of bacterial endophthalmitis? We propose that in addition to the multiple mechanisms that inhibit inflammation, an additional layer of immune privilege consists of special protective mechanisms that preserve the highly vulnerable ocular tissue when inflammation does occur. The present study examines one of these protective mechanisms and how it is compromised by pathogenic bacteria.

Using a murine model of *S. aureus* endophthalmitis, we identified a small heat shock protein,  $\alpha$ B-crystallin, that plays a critical role in protecting the retina during endophthalmitis.  $\alpha$ B-crystallin is a member of the small heat shock family of proteins and is expressed in long-lived tissues, such as muscle, brain, and lens (6, 9, 30, 38, 39, 42). Like other small heat shock proteins,  $\alpha$ B-crystallin was first identified as a molecular chaperone that prevents aggregation of cellular proteins (21, 36, 37). More recently,  $\alpha$ B-crystallin was shown to prevent apoptosis by inhibiting the activation of caspase 3, a protease essential for apoptosis (1, 21, 22, 23, 26, 37). Furthermore, recent studies have demonstrated that  $\alpha$ B-crystallin is expressed in the retina (in the ganglion cells, inner and outer nuclear layers, inner segments, and retinal pigment epithelium), where it is upregulated and prevents apoptosis in response to oxidative stress (43, 44). We hypothesized that one role for  $\alpha$ B-crystallin is to protect the delicate retinal tissue from apoptosis induced by bacterial exotoxins, as well as bystander cell death induced by the host inflammatory response during *S. aureus*-induced endophthalmitis.

In the present study, we demonstrate that apoptosis is a critical component of the pathogenesis of *S. aureus*-induced endophthalmitis, leading to the loss of retinal function. In the early response to a pathogen,  $\alpha$ B-crystallin is upregulated in

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the retina, which limits apoptosis. However, *S. aureus* produces a protease that cleaves and inactivates  $\alpha$ B-crystallin, deranging the normal response of the host and increasing retinal-cell vulnerability to apoptosis. This study illuminates a novel protective mechanism used by the host to prevent retinal damage during endophthalmitis and identifies a new role for a protease of *S. aureus* in the pathogenesis of infection.

## MATERIALS AND METHODS

**Animals.** Female C57BL/6J mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Male and female 129S6/SvEvTac control mice were purchased from Taconic Farms (Germantown, NY). The  $\alpha$ B-crystallin knockout (KO) mice (129S6/SvEvTac) were obtained from the National Eye Institute (6). The genotypes of the  $\alpha$ B-crystallin KO mice were confirmed by PCR according to a previously published protocol (6). All animals were treated according to the guidelines of the Association for Research in Vision and Ophthalmology Resolution on the Use of Animals in Research. All procedures involving mice were approved by the Schepens IACUC.

**Induction of endophthalmitis.** Mice received an intravitreal injection of 0.5  $\mu$ l of sterile physiological saline (Hospira, Lake Forest, IL), in which 500 or 5,000 CFU *S. aureus* (strain RN6390) were suspended, into the left eye according to a previously described protocol (13). Briefly, the bacteria were grown for 4.5 h at 37°C with shaking to log phase and diluted to the appropriate concentrations. An intravitreal injection of *S. aureus*, just posterior to the limbus-parallel conjunctival vessels, was performed using a borosilicate microcapillary pipette pulled to a tip size of 50  $\mu$ m. The right eye of each mouse was untreated and served as an internal control for electroretinography (ERG) studies. Before injection, *S. aureus* was grown in standard Bacto brain heart infusion (BHI) broth (BD Bioscience, Sparks, MD), harvested in log phase, and diluted in physiological saline to 500 CFU/0.5  $\mu$ l or 5,000 CFU/0.5  $\mu$ l.

**Clinical scoring.** At 24, 48, 72, and 96 h following injection, clinical examinations were performed by slit lamp biomicroscopy. The mice were assigned a clinical score of 0 to 3, with a clinical score of 0 corresponding to a clear anterior chamber, clear vitreous, and clear view of the retina; 1 corresponded to mild aqueous flare, mild vitreal haze, and the view of the retina slightly obscured; 2 corresponded to moderate aqueous flare, dense vitreal haze, poor pupil dilation, and the view of the retina moderately obscured; 3 represented intense aqueous flare, opaque vitreous, and the view of the retina completely obscured.

**Histological analysis.** Mice were euthanized at 24, 48, 72, and 96 h following injection, and following euthanasia, the eyes were enucleated using Stevens curved sharp-tip scissors. The eyes were fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (HE). Apoptotic cells were identified using a terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) in situ cell death detection kit (TMR red; Roche Applied Science). The sections were mounted using DAPI (4',6'-diamidino-2-phenylindole) pannuclear stain (Vectashield). Following staining, the ratio of TUNEL-positive to total DAPI-positive cells was calculated in six visual fields at  $\times 100$  magnification. These calculations were repeated in three sections per experimental eye, with at least four animals per group per time point. An anti-CD45 antibody was used to distinguish bone marrow-derived inflammatory cells from the resident cells of the retina. Some slides were double stained with TUNEL and anti-CD45 staining. CD45 staining was performed using a three-step staining procedure with rat anti-mouse CD45 antibody (BD Biosciences, San Jose, CA), biotin-conjugated anti-rat immunoglobulin G2b (BD Bioscience, Sparks, MD) as the secondary antibody, and streptavidin-horseradish peroxidase (BD Bioscience, Sparks, MD), together with the DAB detection system (Vector Laboratories, Burlingame, CA).

**Intraocular bacterial quantification.** Mice were euthanized at 24, 48, 72, and 96 h following injection, and the eyes were enucleated as described above. The eyes were placed in 1.0 ml sterile phosphate-buffered saline on ice and homogenized by bead beating (FastPrep FP120; Thermo Fisher Scientific, Waltham, MA) with 1.0-mm glass beads for 90 seconds at maximum speed. The homogenates were serially diluted and plated on BHI agar plates.

**ERG.** ERG was performed on the mice at 24, 48, 72, and 96 h postinoculation using a slightly modified protocol (13). Briefly, the mice were dark adapted for at least 4 h. The mice were then anesthetized, and the pupils were dilated using 1% tropicamide ophthalmic solution (Bausch & Lomb, Tampa, FL). Following anesthesia, the body temperature was maintained at 37°C using a microwave heat pad. Gold wire electrodes (0.25 mm; Alfa Aesar, Ward Hill, MA) were placed on the cornea after application of a hypromellose ophthalmic prism solution

(Akorn, Inc.) and connected to a visual electrodiagnostic system (UTAS-E 3000; LKC Technologies, Gaithersburg, MD). Needle electrodes placed in the anterior scalp and the tail served as reference and ground leads, respectively. The b-wave amplitude (measured from the trough of the a-wave to the peak of the b-wave) in response to a bright flash in a Ganzfeld illumination sphere was recorded for the injected left eye and the contralateral (internal-control) right eye simultaneously. A total of 30 readings at 0.6 cd-s/m<sup>2</sup> flash intensity with a 1-second interval between flashes were taken and averaged. The retinal function was defined as the ratio of the b-wave amplitude (from the trough of the a-wave to the peak of the b-wave) of the experimental treated eye divided by the value for the contralateral untreated eye.

**Posterior-segment protein analysis.** Mice were euthanized at 24, 48, 72, and 96 h following injection, and following euthanasia, the eyes were enucleated and snap-frozen in liquid nitrogen; eyes that received no injections were enucleated as 0-h controls. The eyes were dissected frozen in a cold room on dry ice, and the posterior segment (vitreous, retina, retinal pigment epithelium, sclera, choroids, and optic nerve) of each eye was collected. Posterior segments were homogenized in 8 M urea/2% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} buffer with 1 $\times$  Complete minicocktail protease inhibitor (Roche Applied Science, Indianapolis, IN). The protein concentration was quantified using a Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA). Posterior-segment proteins were analyzed by Western blotting. The proteins were separated on 12% Tris-glycine gels (Invitrogen, Carlsbad, CA) and transferred onto Invitrolon polyvinylidene difluoride membranes (Invitrogen, Carlsbad, CA). The membranes were blocked with 5% milk and probed for  $\alpha$ B-crystallin, caspase 3, and actin using monoclonal mouse anti- $\alpha$ B-crystallin (Assay Designs, Ann Arbor, MI), polyclonal rabbit anti-cleaved caspase 3 (Cell Signaling Technology, Danvers, MA), and polyclonal goat anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA) with the appropriate secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA): goat anti-mouse, goat anti-rabbit, and bovine anti-goat, respectively.

**In vitro  $\alpha$ B-crystallin assay.** Recombinant  $\alpha$ B-crystallin (50 ng) (Assay Designs, Ann Arbor, MI) was incubated overnight with 24  $\mu$ l of either (i) sterile BHI broth (Becton, Dickinson and Company, Sparks, MD), (ii) nonsterile *S. aureus* supernatant, or (iii) nonsterile *S. aureus* supernatant with 1 $\times$  Complete minicocktail protease inhibitor (Roche Applied Science, Indianapolis, IN). Samples were analyzed by Western blotting and probed using monoclonal mouse anti- $\alpha$ B-crystallin with goat anti-mouse secondary antibody, as described above.

**Statistical analysis.** Where normally distributed, the data were analyzed with an unpaired *t* test with a *P* value of  $\leq 0.05$  as the basis for rejection of the null hypothesis; nonparametric data were analyzed using a Mann-Whitney *U* test. Where appropriate, the Q test was used to eliminate one outlier per experimental group. Statistical analysis and graphing were performed using Prism and Microsoft Excel.

## RESULTS

**Induction of bacterial endophthalmitis.** We used a murine model of *S. aureus*-induced endophthalmitis (13) to determine whether  $\alpha$ B-crystallin plays a significant role in protecting ocular tissue from apoptosis in response to infection. Since the *S. aureus* endophthalmitis model was developed recently, the first series of experiments served as an independent confirmation that the laboratory model was reproducible.

Similar to what was previously reported, eyes infected with 500 CFU of *S. aureus* developed a mild infection in which the intraocular bacterial load peaked at 48 h (maximum,  $1.6 \times 10^4$  CFU/eye) but the infection was cleared by 96 h in 85% of the mice, resulting in destruction of the eyes in only 15% of the mice (Fig. 1). By contrast, eyes infected with 5,000 CFU of *S. aureus* developed a progressive infection that increased in severity over 96 h (maximum,  $1.4 \times 10^7$  CFU), resulting in complete destruction of the eyes in 70% of the mice. As expected, no ocular damage was observed in the control mice that received an intravitreal injection of sterile saline.

In the eyes of mice that cleared an infection of 500 CFU of *S. aureus*, slit lamp examinations revealed minimal corneal opacity and poor pupil dilation that peaked at 48 h and sub-

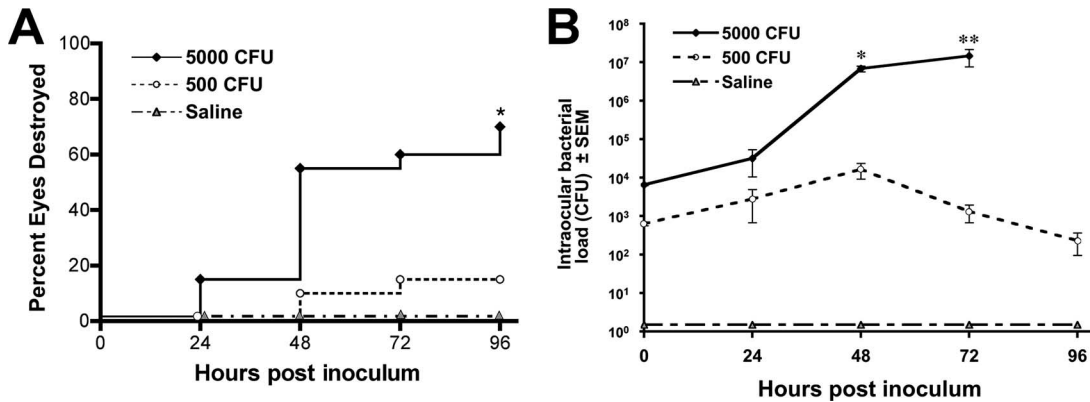


FIG. 1. Clinical examination and bacterial quantification. C57BL/6J mice received intravitreal injections of either sterile saline or 500 CFU or 5,000 CFU *S. aureus*. (A) Eyes were examined by slit lamp and given a score of 0, 1, 2, or 3 (0, cleared, and 3, destroyed). At 96 h, 70% of eyes receiving 5,000 CFU and 15% of eyes receiving 500 CFU were destroyed (score, 3). Sterile saline, *n* = 9; 500 and 5,000 CFU, *n* = 30; \* *P* < 0.001. (B) The bacterial load in eyes injected with 500 CFU peaked at 48 h ( $1.6 \times 10^4$  CFU/eye); eyes injected with 5,000 CFU did not clear the infection, and the bacterial load increased to  $1.4 \times 10^7$  at 72 h. No bacteria grew in eyes injected with sterile saline at any time point. Saline, *n* = 3; 500 CFU, *n* ≥ 9; 5,000 CFU, *n* ≥ 9; \*, *P* < 0.0001; \*\*, *P* = 0.01.

sequently resolved (Fig. 2). Histological analysis revealed mild inflammation with a minimal vitreous infiltrate at 24 and 48 h that resolved and coincided with slight retinal folding in the inner and outer nuclear layers. By contrast, mice that received 5,000 CFU of *S. aureus* displayed severe inflammation, characterized by significant corneal opacity, corneal edema, and poor pupil dilation within 48 h. Histological analysis revealed a robust inflammatory infiltrate, retinal hemorrhaging, severe retinal folding, and visible bacterial growth in the vitreous at 24 to 48 h with complete destruction of the retina by 72 to 96 h (Fig. 2). Control mice receiving an injection of sterile saline did not exhibit any signs of inflammation at any time postinjection (Fig. 2).

Retinal function, as determined by the b-wave amplitude

measured by ERG, corresponded with the clinical and histological findings. Control mice that received sterile-saline intravitreal injections displayed a transient loss of retinal function at 24 h postinjection, returning to 91% of normal by 96 h (Fig. 3B). Mice that received 500 CFU of *S. aureus* displayed a loss in retinal function to 48% of normal at 24 h that returned to 83% of normal by 96 h as the bacterial infection was cleared. By contrast, mice that received 5,000 CFU of *S. aureus* displayed a complete loss of retinal function at 24 h that never recovered. Representative ERG recordings at 96 h revealed a total loss of the b-wave amplitude in eyes that received 5,000 CFU of *S. aureus*; by contrast, mouse eyes that received 500 CFU of *S. aureus* displayed 83% of the normal (contralateral-eye) b-wave amplitude at 96 h (Fig. 3A).

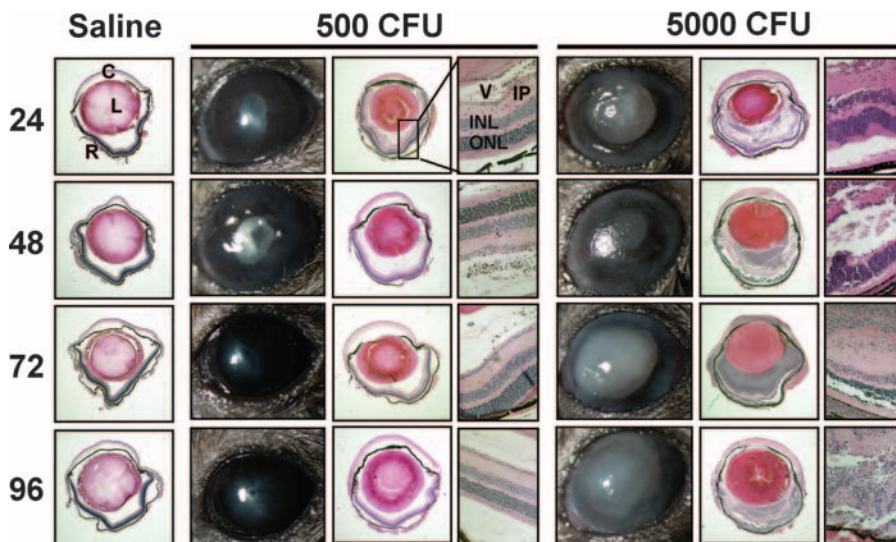


FIG. 2. Representative clinical pictures and histology. C57BL/6J mice received intravitreal injections of either sterile saline or 500 CFU or 5,000 CFU *S. aureus*. At 24, 48, 72, and 96 h, the eyes were photographed and then removed, sectioned, and stained for HE. Mild inflammation was observed in eyes that received 500 CFU, and severe retinal damage was observed in eyes that received 5,000 CFU. Retinal detachment in saline- and 500-CFU-injected eyes is an artifact of tissue sectioning. HE pictures were taken of whole eyes at  $\times 2$  magnification, and the retina at  $\times 20$  magnification. C, cornea; L, lens; R, retina; V, vitreous; IP, inner plexiform; INL, inner nuclear layer; ONL, outer nuclear layer.

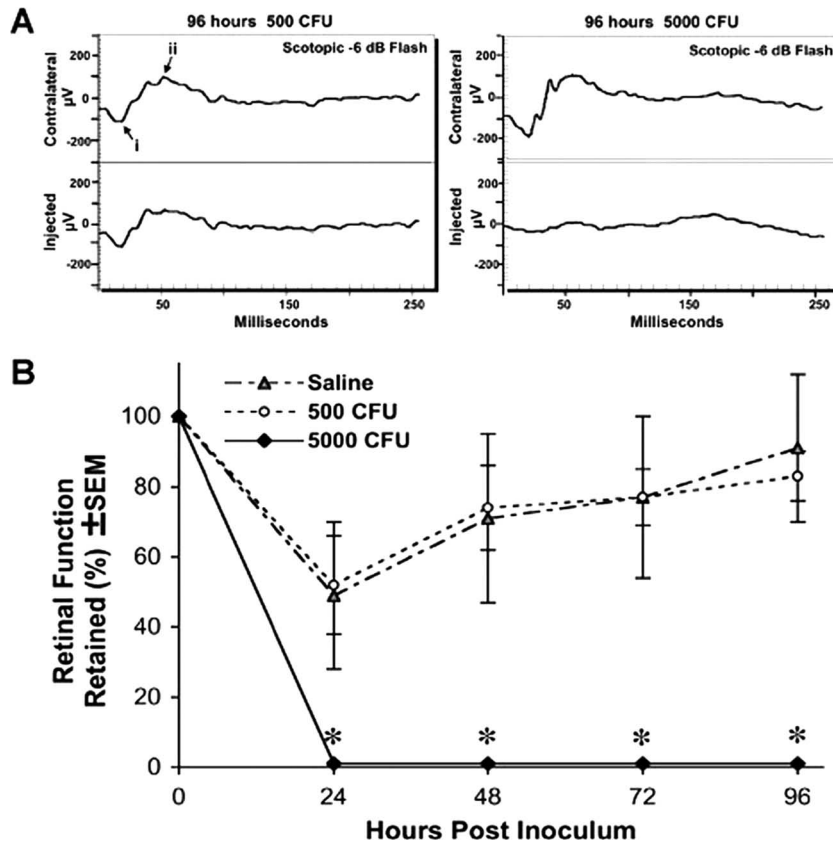


FIG. 3. ERG assessment of retinal function. C57BL/6J mice received intravitreal injections of sterile saline or 500 CFU or 5,000 CFU *S. aureus*. (A) Representative ERG response to 0.6 cd-s/m<sup>2</sup> flash intensity at 96 h following 500 CFU or 5,000 CFU *S. aureus* injection (right and left, respectively). The b-wave amplitude (from the trough of the a-wave [i] to the peak of the b-wave [ii]) was determined, and the ratio of injected eye to contralateral internal control eye was calculated. (B) The 5,000-CFU group lost all retinal function, while the 500-CFU and saline groups displayed only a transient loss of retinal function. The data are expressed as percent retinal function in the experimental left eye compared to the control right eye. Saline,  $n = 3$ ; 500 CFU,  $n = 10$ ; 5,000 CFU,  $n = 10$ . \*,  $P < 0.001$  for 5,000 CFU versus saline and 5,000 CFU versus 500 CFU.

**Expression of  $\alpha$ B-crystallin during endophthalmitis.** To determine whether  $\alpha$ B-crystallin was upregulated during endophthalmitis, the posterior segment of each eye was isolated following an intravitreal injection of either saline or 500 or 5,000 CFU of *S. aureus*. Eyes that did not receive an intravitreal injection (0 h) yielded a 22-kDa  $\alpha$ B-crystallin band (Fig. 4A). Interestingly, an intravitreal injection of sterile saline alone induced a significant increase in  $\alpha$ B-crystallin expression that peaked at 48 h (more than 250% of the unperturbed normal eye) and remained elevated at 96 h. This up-regulation of  $\alpha$ B-crystallin at 48 h coincided with the recovery of retinal function (Fig. 3B and 4B). Mice that received 500 CFU of *S. aureus* displayed a similar upregulation of  $\alpha$ B-crystallin at 48 h postinfection, but a smaller, 18-kDa  $\alpha$ B-crystallin band began to appear at 72 h (Fig. 4A and B). However, the 18-kDa band was always expressed at much lower levels than full-length (22-kDa)  $\alpha$ B-crystallin. This smaller, 18-kDa band may represent a cleavage product of  $\alpha$ B-crystallin. By contrast, in mice that received 5,000 CFU of *S. aureus*, the 22-kDa  $\alpha$ B-crystallin band decreased below baseline expression at 72 and 96 h postinfection, and the smaller, 18-kDa band increased steadily from 24 to 96 h, so that the level of the 18-kDa  $\alpha$ B-crystallin was significantly higher ( $P < 0.05$ ) than the level of full-length (22-kDa)  $\alpha$ B-crystallin. The increase in the 18-kDa band co-

incided with the increase in the bacterial load and retinal damage in these eyes. These data imply that the upregulation of full-length  $\alpha$ B-crystallin is associated with retinal protection, while cleavage of  $\alpha$ B-crystallin is associated with retinal destruction and loss of retinal function.

**Expression of active caspase 3.** Recent studies demonstrated that  $\alpha$ B-crystallin prevents apoptosis by binding to caspase 3 and directly blocking its activation (21, 22, 23).  $\alpha$ B-crystallin can also indirectly block the activation of caspase 3 by (i) binding to the proapoptotic factors Bax and Bcl-X(S), (ii) preventing their translocation into the mitochondria, and (iii) restricting the release of cytochrome *c* (27). Both the direct and indirect antiapoptotic activities may be terminated if  $\alpha$ B-crystallin is cleaved. The next series of experiments determined whether the loss of full-length  $\alpha$ B-crystallin (the 22-kDa band) and the appearance of the smaller (18-kDa band)  $\alpha$ B-crystallin protein coincided with the activation of caspase 3. Western blot analysis was performed on the posterior-segment protein lysates using a caspase 3-specific antibody that recognizes only the active form of caspase 3. As predicted, there was no detectable active caspase 3 at any time point in the eyes of mice receiving either sterile saline or 500 CFU of *S. aureus* (Fig. 4D). However, the percentage of eyes expressing active caspase 3 in mice receiving 5,000 CFU of *S. aureus* increased

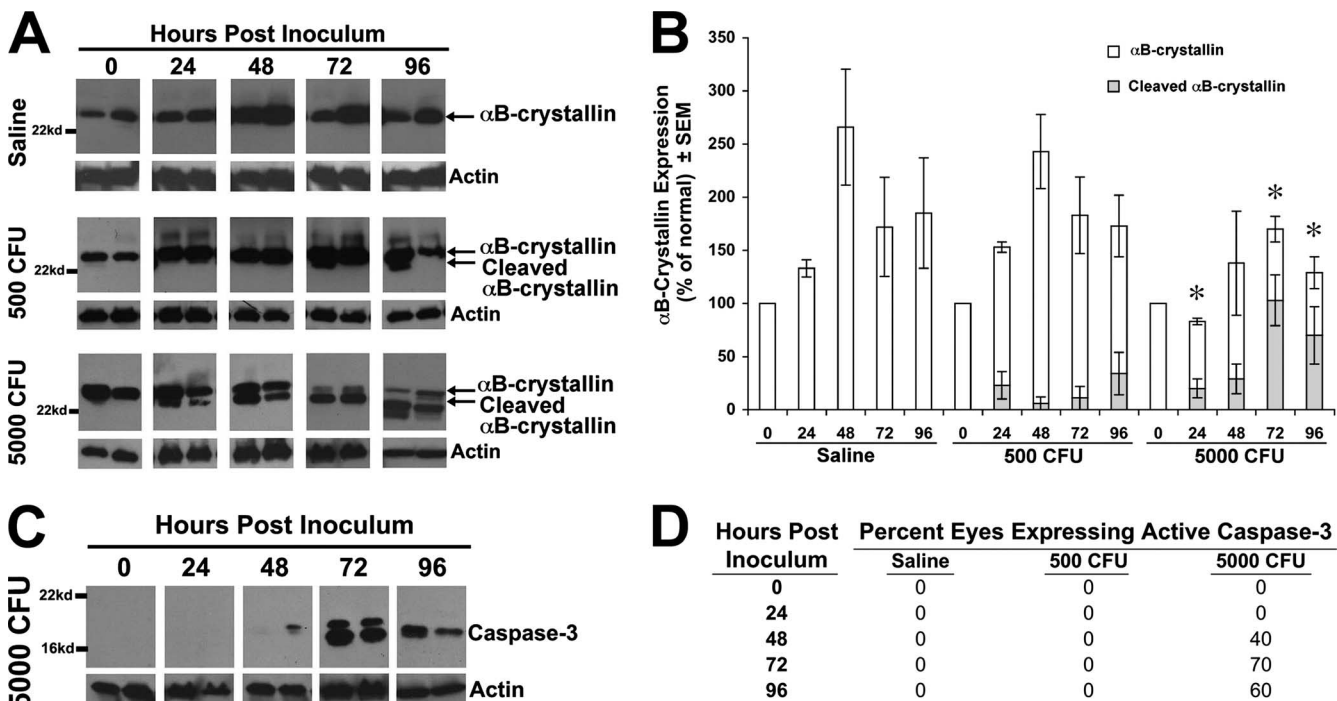


FIG. 4. Expression of αB-crystallin and active caspase 3. C57BL/6J mice received intravitreal injections of sterile saline or 500 CFU or 5,000 CFU *S. aureus*. (A) Representative Western blot results probing for αB-crystallin (22 kDa) and actin (43 kDa) loading control. Two samples per group per time point are shown. (B) Densitometry of Western blot results, probing for αB-crystallin. The data are shown as the mean [(αB-crystallin/actin) injected sample]/[(αB-crystallin/actin) normal control]. Saline, *n* = 3; 500 CFU, *n* = 4; 5,000 CFU, *n* ≥ 5. Standard error of the mean: \*, *P* < 0.05 for 5,000 versus saline or 500 CFU full αB-crystallin. (C) Representative Western blot results using an active caspase 3-specific antibody in eyes that received 5,000 CFU *S. aureus*. (D) Percentages of eyes by Western blot analysis expressing active caspase 3 at 24, 48, 72, and 96 h in C57BL/6J mice following sterile-saline or 500- or 5,000-CFU *S. aureus* injection. Saline, *n* = 3; 500 CFU, *n* = 4; 5,000 CFU, *n* ≥ 5.

steadily following the intravitreal injections (Fig. 4C), so that by 96 h, 60% of the mice expressed active caspase 3 (Fig. 4D). The appearance of active caspase 3 beginning in eyes that received 5,000 CFU at 48 h coincided with the increased level of the smaller, 18-kDa αB-crystallin band and the decrease in the full-length 22-kDa band, suggesting that the antiapoptotic activity of αB-crystallin may be lost when it is cleaved.

**In vitro cleavage of αB-crystallin.** *S. aureus* also produces a variety of exotoxin proteases that contribute to virulence and are capable of breaking down both host and pathogen matrix proteins (5, 10, 24, 40). The best-studied *S. aureus* extracellular proteases include SspA, SspB, and aureolysin, which have been linked to virulence and are involved in activating other exotoxins (40). In order to determine whether *S. aureus* produces an exotoxin that cleaves αB-crystallin, recombinant αB-crystallin was incubated overnight with an *S. aureus* supernatant and analyzed by Western blotting. The data in Fig. 5 reveal that *S. aureus* produces a protease that cleaves recombinant αB-crystallin in vitro, and the cleavage band (~18 kDa) was the same size as the band observed in the posterior segments of mice receiving 5,000 CFU of *S. aureus*. The addition of a protease inhibitor cocktail to the *S. aureus* supernatant blocked the cleavage and formation of the 18-kDa band (Fig. 5). These data indicate that an extracellular protease produced by *S. aureus* cleaves αB-crystallin in vitro and imply that a similar cleavage may occur in vivo during bacterial endophthalmitis.

To determine the protease class responsible for cleaving

αB-crystallin, recombinant αB-crystallin was incubated overnight with an *S. aureus* supernatant in the presence or absence of specific inhibitors of serine, cysteine, or metalloproteases (Pefabloc SC, E-64, and EDTA, respectively). Unexpectedly, all three protease inhibitors blocked the cleavage of αB-crystallin (data not shown), indicating that a complex network of proteases are involved in cleaving αB-crystallin.

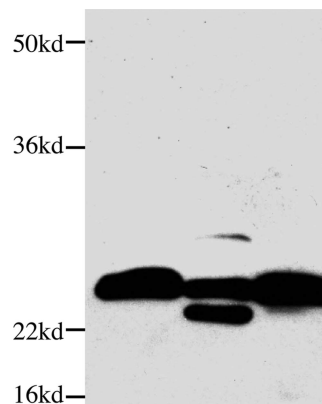


FIG. 5. Cleavage of recombinant αB-crystallin by *S. aureus* supernatant. Western blot analysis of recombinant αB-crystallin treated with (i) sterile BHI broth, (ii) *S. aureus* culture supernatant, or (iii) *S. aureus* culture supernatant plus protease inhibitor cocktail.

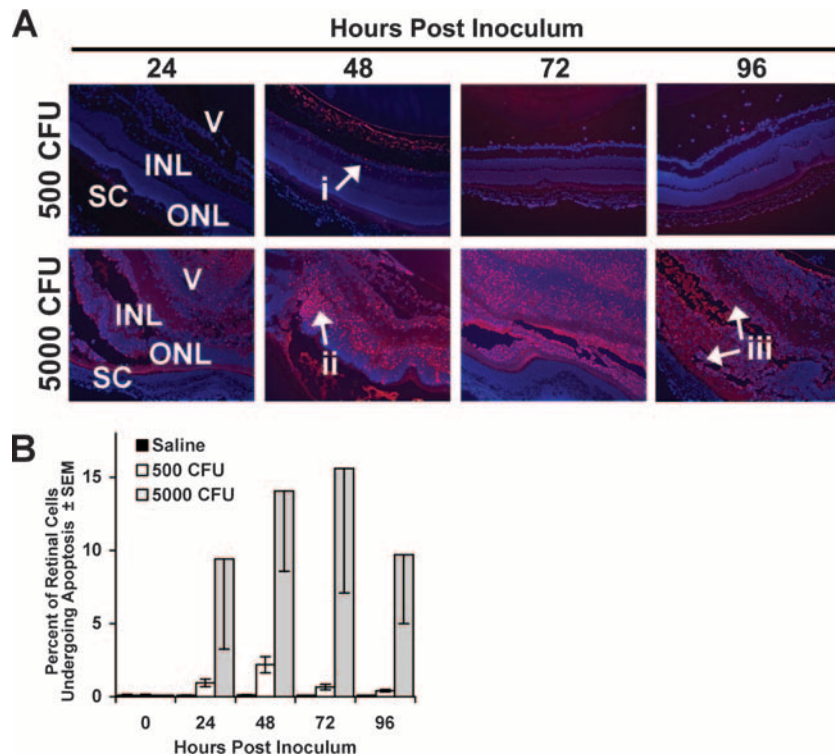


FIG. 6. Retinal apoptosis during endophthalmitis. C57BL/6J mice received intravitreal injections of either sterile saline or 500 CFU or 5,000 CFU *S. aureus*. (A) Representative TUNEL staining ( $\times 10$  magnification) in C57BL/6J mouse retinas (red fluorescence signifies TUNEL staining). No apoptosis was observed in mice receiving saline injections. Very little apoptosis (i) was observed at any of the time points in mice receiving 500 CFU. High levels of apoptosis were observed in the inner nuclear layer at 24 to 48 h (ii) and throughout the retina at 72 to 96 h (iii) in mice receiving 5,000 CFU *S. aureus*. V, vitreous; INL, inner nuclear layer; ONL, outer nuclear layer; SC, sclera/choroid. (B) Percentages of TUNEL-positive cells in the retina. Saline,  $n = 2$ ; 500 CFU,  $n = 4$ ; 5,000 CFU,  $n \geq 5$ .

**Retinal apoptosis during endophthalmitis.** Our data indicate that cleavage of  $\alpha$ B-crystallin coincides with increased activation of caspase 3. Therefore, we predicted that the loss of the full-length  $\alpha$ B-crystallin and subsequent increase in the cleaved  $\alpha$ B-crystallin would correspond with increased apoptosis in the retina. Retinal apoptosis following intravitreal infection was determined by TUNEL staining of retinal sections. TUNEL staining identified very few apoptotic cells in retinas of eyes that received either sterile saline or 500 CFU of *S. aureus*. The proportion of cells in the retina undergoing apoptosis never increased above 2 to 3% of total cells (Fig. 6A and B). However, the fraction of apoptotic cells in the retina increased steadily in mice that received 5,000 CFU, with 9% apoptotic retinal cells at 24 h, peaking at 16% at 72 h.

Double staining with an anti-CD45 antibody revealed that the cells undergoing apoptosis in the saline and 500-CFU groups were primarily CD45-positive (CD45<sup>+</sup>) infiltrating inflammatory cells (Fig. 7). By contrast, the cells undergoing apoptosis in the retinas of the mice that received 5,000 CFU were primarily CD45 negative. We conclude that during destructive bacterial endophthalmitis, cleavage of  $\alpha$ B-crystallin coincides with increased retinal-cell apoptosis.

**Endophthalmitis in  $\alpha$ B-crystallin KO mice.** Our data demonstrate that increased expression of  $\alpha$ B-crystallin coincides with reduced retinal apoptosis and reduced retinal damage, while cleavage of  $\alpha$ B-crystallin coincides with increased retinal apoptosis and increased retinal damage. Based upon these

data, we hypothesize that upregulation of full-length  $\alpha$ B-crystallin is required for prevention of apoptosis in the retina and preservation of retinal function, even during infections that are successfully cleared. To test this hypothesis, we compared the development of bacterial endophthalmitis in  $\alpha$ B-crystallin KO mice with that in wild-type mice that received intravitreal injections of 500 CFU of *S. aureus*.

$\alpha$ B-crystallin KO mice and wild-type mice displayed no significant difference in their ability to clear an infection of 500 CFU of *S. aureus*. Seventy-seven percent of the eyes from  $\alpha$ B-crystallin KO mice and 83% of the eyes from wild-type mice cleared the infection by 96 h (Fig. 8A). Clearance of the bacteria was confirmed by determining the bacterial load at 96 h (data not shown). Interestingly, although there was no difference in the ability to clear the bacteria in  $\alpha$ B-crystallin KO mice, retinal function was significantly reduced ( $P < 0.05$ ) at 48 h in  $\alpha$ B-crystallin KO eyes (49% of normal) compared with wild-type eyes (69% of normal), and they were slower to recover retinal function (Fig. 8B). To determine whether the difference in retinal function at 48 h was due to increased retinal apoptosis in  $\alpha$ B-crystallin KO mice, histological sections of eyes from wild-type and  $\alpha$ B-crystallin KO mice were double stained for TUNEL and CD45 at 48 h postinfection. Increased levels of apoptosis of CD45-negative cells were observed in the retinas of  $\alpha$ B-crystallin KO mice compared with the wild-type control mice (Fig. 8C). Apoptosis in the  $\alpha$ B-crystallin KO eyes was concentrated in pockets and coincided

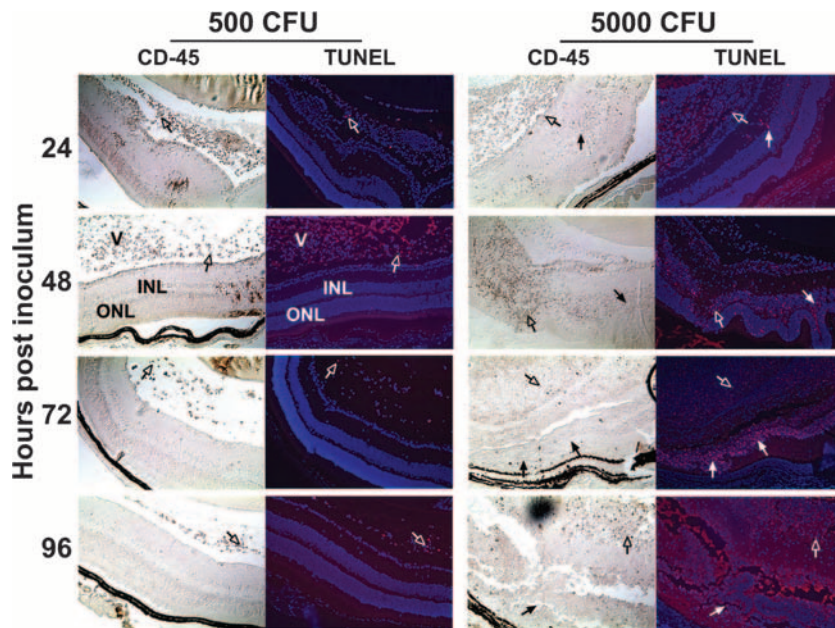


FIG. 7. TUNEL and CD45 double staining. C57BL/6J mice received intravitreal injections of either sterile saline or 500 CFU or 5,000 CFU *S. aureus*. The open arrows indicate apoptosis of infiltrating CD45-positive immune cells; the closed arrows indicate apoptosis of CD45-negative retinal cells. Magnification,  $\times 10$ . V, vitreous; INL, inner nuclear layer; ONL, outer nuclear layer; SC, sclera/choroid.

with increased retinal damage, as demonstrated by retinal folds and hemorrhaging. However, at 48 h postinjection of saline alone, no apoptosis was observed in the retinas of wild-type or  $\alpha$ B-crystallin KO mice. The results from the saline controls indicated that an intravitreal injection of saline alone did not induce apoptosis in either the wild-type or the  $\alpha$ B-crystallin KO mice. Therefore, the increased apoptosis observed in the retinas of  $\alpha$ B-crystallin KO mice was specifically induced by the bacterial infection and not by the intravitreal injection alone. Together, these data indicate that  $\alpha$ B-crystallin prevents retinal apoptosis during a bacterial infection and that in the absence of  $\alpha$ B-crystallin, there is increased apoptosis and increased retinal damage, which occur even when the bacterial infection is successfully cleared.

## DISCUSSION

Our studies identified upregulation of  $\alpha$ B-crystallin as a defense mechanism of the immune-privileged eye that protects delicate retinal tissue from bacterial and host-mediated bystander damage, as occurs during *S. aureus* endophthalmitis. Furthermore, our studies revealed that *S. aureus* produces a protease capable of cleaving  $\alpha$ B-crystallin into a form identified as being ineffective in preventing retinal apoptosis and destructive endophthalmitis.

A unique property of the eye is the ability to protect against pathogens while simultaneously protecting the delicate visual axis from the sight-destroying effects of immunogenic inflammation (for a review, see reference 41). This property is called immune privilege and was first described by Medawar in 1948. Medawar demonstrated that foreign tissue allografts survive for a prolonged period of time in immune-privileged sites such as the eye, whereas placement of identical allografts at conventional (nonprivileged) body sites led to immune rejection

(28). Work over the last several decades has revealed that ocular immune privilege restricts both adaptive and innate immune effectors in a way that balances the challenge of pathogenic infection against the challenge of inflammation-induced vision loss (41). The present study shows that  $\alpha$ B-crystallin contributes to the protection of the retina from the inflammatory effects of infection and that this protection is undermined by *S. aureus*, a leading cause of severe endophthalmitis.

A member of the small heat shock protein family,  $\alpha$ B-crystallin was first identified as a lens protein involved in maintaining lens clarity (18). However, in 1989, Bhat and Nagineni and Dubin et al. demonstrated extralenticular expression of crystallin (4, 11). Over the last decade, several investigators demonstrated that  $\alpha$ B-crystallin is an important molecular chaperone that is induced by a variety of stress stimuli and confers cellular protection by suppressing the nonspecific aggregation of denatured proteins (17, 25). More recently, studies have demonstrated that  $\alpha$ B-crystallin also inhibits apoptosis by blocking the proteolytic activation of caspase 3 (21, 22). Furthermore, while  $\alpha$ B-crystallin is expressed constitutively within the neural retina and retinal pigment epithelium, oxidative stress dramatically increases the level of expression (43, 44). In vitro studies have demonstrated that overexpression of  $\alpha$ B-crystallin in retinal pigment epithelium cells coincides with reduced caspase 3 activity and apoptosis in response to oxidative stress (44). These studies imply that upregulation of  $\alpha$ B-crystallin in the retina amplifies resistance to cell death, allowing the cells to adapt to changes in their environment and to survive otherwise-lethal conditions.

In this model of *S. aureus*-induced endophthalmitis, the antiapoptotic effect of  $\alpha$ B-crystallin was most evident during a mild infection of the eye that was successfully cleared by the

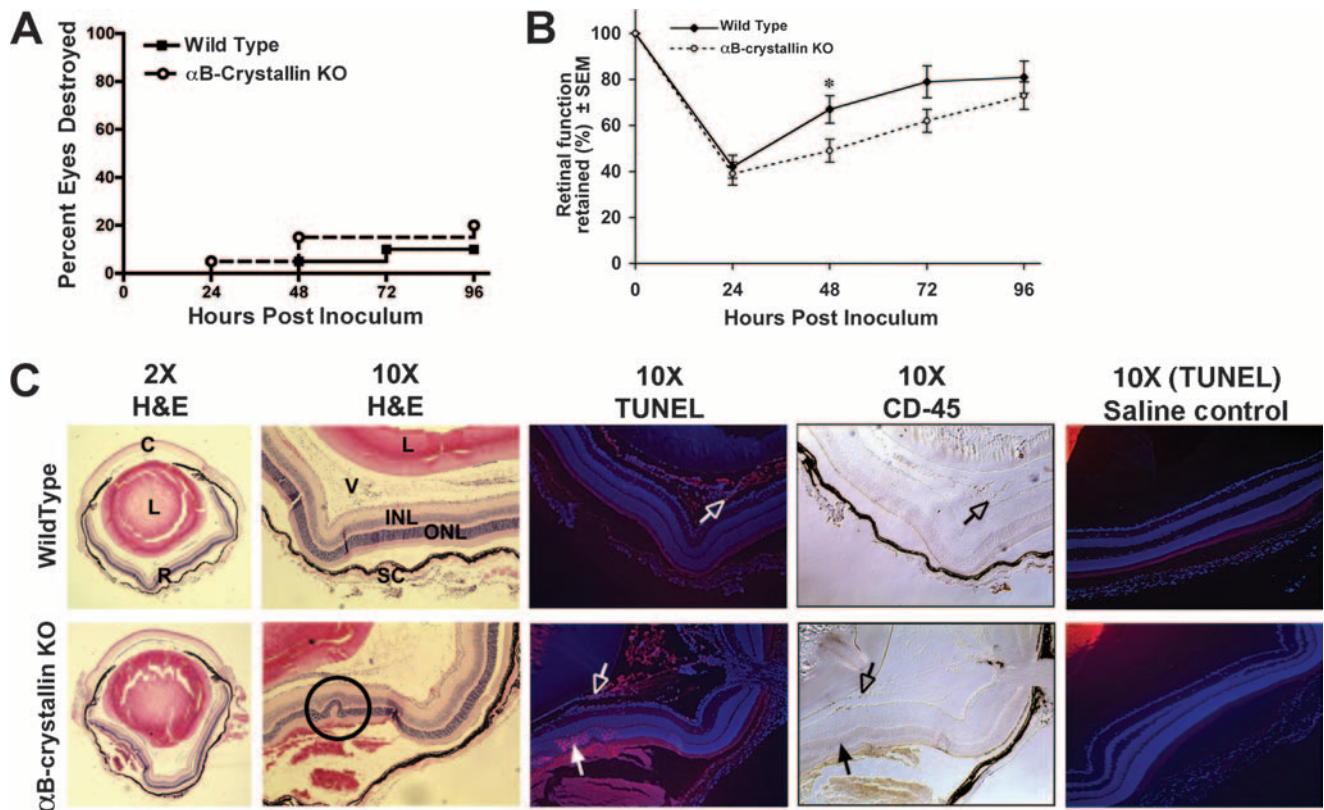


FIG. 8. Clinical examination, bacterial clearance, and retinal apoptosis.  $\alpha$ B-crystallin KO and wild-type mice received 500-CFU *S. aureus* intravitreal injections. (A) Eyes were examined by slit lamp and given a score of 0, 1, 2, or 3 (0, cleared, and 3, destroyed). At 96 h, there was no significant difference in percentages of destroyed (score, 3) eyes (23% of  $\alpha$ B-crystallin KO; 17% of wild-type). (B) Retinal function, as determined by ERG, was significantly depressed at 48 h (49% of normal) in  $\alpha$ B-crystallin KO mice compared to wild-type controls (69% of normal) ( $n = 10$ ; \*,  $P = 0.05$ ). (C) HE and TUNEL staining in eyes enucleated at 48 h revealed increased retinal folding and bleeding (circled in  $\times 10$  HE image) and retinal apoptosis in  $\alpha$ B-crystallin KO mice compared to the wild type (closed arrows, CD45 negative). Low levels of CD45-positive inflammatory-cell apoptosis were observed in both wild-type and KO eyes (open arrows, CD45 positive). No apoptosis was observed in wild-type or  $\alpha$ B-crystallin KO mice that received saline only. C, cornea; L, lens; R, retina; V, vitreous; INL, inner nuclear layer; ONL, outer nuclear layer; SC, sclera/choroid.

immune system. An intravitreal injection of 500 CFU of *S. aureus* induced a low level of inflammation, as well as upregulation of  $\alpha$ B-crystallin in the retina. The inflammatory response successfully cleared the infection within 72 h with little apoptosis. Studies using the  $\alpha$ B-crystallin KO mice further confirmed the protective effects of  $\alpha$ B-crystallin. While  $\alpha$ B-crystallin KO mice did not show impaired ability to clear infection, KO mice showed increased retinal damage and apoptosis compared to wild-type mice.

In addition to demonstrating the role of  $\alpha$ B-crystallin in protecting the retina during infection, we also identified a novel mechanism by which *S. aureus* may impede this protection. The heat shock protein  $\alpha$ B-crystallin is constitutively expressed in the retina and, as others have demonstrated, is upregulated in response to many forms of stress (43, 44). However, unique to our studies is the finding that *S. aureus* produces a protease that cleaves and inactivates  $\alpha$ B-crystallin, thus eliminating its protective effect. When the host did not readily clear an *S. aureus* infection, we observed that retinal  $\alpha$ B-crystallin was cleaved, and this cleavage coincided with increased retinal apoptosis, significant tissue damage, and loss of retinal function. The cleavage of  $\alpha$ B-crystallin was observed only fol-

lowing infection with *S. aureus*, and no cleavage of  $\alpha$ B-crystallin was observed in wild-type mice treated with saline alone. In addition, in vitro studies determined that *S. aureus* produced one or more proteases that cleave  $\alpha$ B-crystallin. Although we have not elucidated the cleavage site, based on the molecular weight of the cleavage product, we predict that cleavage occurs in either the N-terminal or the C-terminal domain of  $\alpha$ B-crystallin, which are involved in oligomerization and solubility of oligomers, respectively (23, 32). The data from Kamradt et al. indicates that, if the oligomerization of  $\alpha$ B-crystallin is impaired (by N-terminal domain mutation), then this impaired form of  $\alpha$ B-crystallin allows increased activation of caspase 3 and increased apoptosis (23). Therefore, if the cleavage of  $\alpha$ B-crystallin observed in infected retinas interferes with N-terminal oligomerization, then this will coincide with increased caspase 3 activation and increased apoptosis. These data reveal another pathogenic mechanism by which *S. aureus* increases the vulnerability of retinal cells to apoptotic cell death. Conceivably, other endophthalmitis pathogens, such as *Pseudomonas* and *Bacillus*, produce proteases with similar activities.

These data also raise important questions about the pathogenesis of *S. aureus* endophthalmitis. Specifically, how do bacteria



induce apoptosis of cells in the retina? Bantel et al. reported that *S. aureus* induces apoptosis in Jurkat cells in vitro without bacterial internalization (2). This was accomplished via the release of the bacterial toxin alpha-toxin, which forms pores within the plasma membranes of target cells (2). Genestier and colleagues identified another pore-forming toxin, Pantone-Valentine leukocidin (PVL), which induces apoptosis in neutrophils (15). Genestier proposed that PVL forms large pores in the membranes of neutrophils, resulting in necrosis. They hypothesized, however, that sublytic concentrations of PVL form a few small pores that allow the entry of *S. aureus*-derived toxins and proteases. This induced apoptosis via the intrinsic death pathway, in which cytochrome *c* was released within the mitochondria, triggering the pathway that leads to the activation of downstream caspases, such as caspase 3. Support for the prospect that *S. aureus* is capable of introducing protein factors that manipulate the biology of target cells stems from the observation that the *S. aureus* C3-like ADP-ribosyltransferase is capable of accessing its intracellular substrate in an otherwise-intact cell (31). However,  $\alpha$ B-crystallin inhibits the activation of caspase 3, increasing the resistance of retinal cells to apoptosis. We propose that cleaved  $\alpha$ B-crystallin is nonfunctional and unable to prevent activation of caspase 3. Therefore, entry of the protease into retinal cells through mechanisms such as membrane pores could lead to cleavage of  $\alpha$ B-crystallin, making the retinal cells more susceptible to the apoptosis that is triggered by *S. aureus* toxins, such as alpha-toxin and PVL.

Alternatively, *S. aureus* may induce apoptosis following internalization into retinal cells. While *S. aureus* is not generally considered an intracellular pathogen, many host cells, including corneal epithelial cells, internalize *S. aureus* (19). Furthermore, internalization of *S. aureus* can induce apoptosis in many cell types, including epithelial cells, endothelial cells, and keratinocytes (3, 20, 29, 35). If retinal cells internalize *S. aureus*, bacterial toxins and the proteases could be released through pores in a phagocytic vacuole, potentially making the cell more vulnerable to apoptosis. Studies are ongoing in our laboratory to (i) identify the *S. aureus*-derived protease that cleaves  $\alpha$ B-crystallin and (ii) determine whether retinal cells internalize *S. aureus* during endophthalmitis and/or whether *S. aureus* secretes bacterial toxins that enter retinal cells through membrane pores.

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