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ExoS and ExoT ADP Ribosyltransferase Activities Mediate Pseudomonas aeruginosa Keratitis by Promoting Neutrophil Apoptosis and Bacterial Survival

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Abbreviations used in this article: ADPR, ADP ribosyltransferase; GAP, GTPase-activating protein.

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Pseudomonas aeruginosa is a leading cause of blinding corneal ulcers worldwide. To determine the role of type III secretion in the pathogenesis of P. aeruginosa keratitis, corneas of C57BL/6 mice were infected with P. aeruginosa strain PAO1 or PAK, which expresses ExoS, ExoT, and ExoY, but not ExoU. PAO1- and PAK-infected corneas developed severe disease with pronounced opacification and rapid bacterial growth. In contrast, corneas infected with ΔpscD or ΔpscI mutants that cannot assemble a type III secretion system, or with mutants lacking the translocator proteins, do not develop clinical disease, and bacteria are rapidly killed by infiltrating neutrophils. Furthermore, survival of PAO1 and PAK strains in the cornea and development of corneal disease was impaired in ΔexoS, ΔexoT, and ΔexoST mutants of both strains, but not in a ΔexoY mutant. ΔexoST mutants were also rapidly killed in neutrophils in vitro and were impaired in their ability to promote neutrophil apoptosis in vivo compared with PAO1. Point mutations in the ADP ribosyltransferase (ADPR) regions of ExoS or ExoT also impaired proapoptotic activity in infected neutrophils, and exoS(T/ADPR-) mutants replicated the ΔexoST phenotype in vitro and in vivo, whereas mutations in rho–GTPase-activating protein showed the same phenotype as PAO1. Together, these findings demonstrate that the pathogenesis of P. aeruginosa keratitis in ExoS- and ExoT-producing strains is almost entirely due to their ADPR activities, which subvert the host response by targeting the antibacterial activity of infiltrating neutrophils.


Corneal infection with Pseudomonas aeruginosa is a major cause of visual impairment and blindness worldwide, occurring either as a result of trauma or in association with contact lens wear (1–6). Results of our studies and of others (7–10) show that P. aeruginosa keratitis is dependent on TLR4 expression. We also showed that the host response in the cornea is initiated following TLR4 and TLR5 activation of resident stromal macrophages, which signal through TIRAP/MyD88 and TRIF to produce IL-1α, IL-1β, and the neutrophil chemokines CXCL1 and CXCL2 in the corneal stroma (9). IL-1α and IL-1β exacerbate this response by activating IL-1R1/MyD88 responses, leading to neutrophil infiltration to the corneal stroma, bacterial killing, and tissue damage, which is manifest as corneal opacification (9). More recently, we demonstrated that production of IFN-γ during P. aeruginosa keratitis stimulates expression of the TLR4 coreceptor, MD-2 on corneal epithelial cells, conferring LPS responsiveness and thereby contributing to the inflammatory response in the cornea (11). Overall, these studies demonstrate a role for TLRs and innate immunity in the pathogenesis of P. aeruginosa keratitis, which is similar, although not identical to the role for neutrophils and TLRs in P. aeruginosa-induced lung disease (12–15).

Like many Gram-negative pathogens, P. aeruginosa uses a type III secretion system to deliver effector proteins into targeted host cells. P. aeruginosa type III effector proteins are thought to prevent wound healing and phagocytosis, as well as promote systemic spread of the organism (16). The type III secretion system of Gram-negative bacteria is a complex, needle-like organelle designed to inject bacterial toxins and other proteins directly into host cells (reviewed in 16, 17). It is comprised of a basal body, which spans the bacterial cell envelope, and is connected to a needle that protrudes from the surface of the bacterium. Injection of effector proteins into targeted host cells is contact dependent and requires the action of the two translocator proteins, PopB and PopD. These translocator proteins form a pore in the host-cell membrane through which the effector proteins are injected (16, 17).

Four effector proteins (exoenzymes) have been described in P. aeruginosa, as follows: ExoS, ExoT, ExoU, and ExoY. ExoU has potent phospholipase activity and causes rapid lysis of host cells (18), and ExoY is an adenylate cyclase (16). ExoS and ExoT are closely related and have GTPase-activating protein (GAP) and ADP ribosyltransferase (ADPR) activities (19). The Rho-GAP domains of these two proteins target a similar subset of GTPases, including Rho, Rac1, and CDC42 (20, 21). Together Rho-GAP and ADPR activities alter host cell cytoskeletal function, resulting in impaired cell migration and adhesion, in addition to blocking phagocytosis, disrupting epithelial cell barriers, and preventing wound healing (16, 19, 22).

Almost every strain of P. aeruginosa contains exoT, and ~86% of strains harbor the gene for ExoY; however, for reasons that are not yet clear, most P. aeruginosa isolates express either ExoS or ExoU, but not both effectors (23). ExoS- and ExoU-expressing...
isolates are recovered from infected corneas at similar frequencies (24). In corneal infections, type III secretion appears to be important for ExoU-producing, cytotoxic variants of P. aeruginosa (25), whereas ExoS-producing strains did not depend on type III secretion to cause disease (26–28). This observation was curious in that lack of ExoT, which is produced by both ExoU- and ExoS-producing strains of P. aeruginosa, only had a virulence phenotype in the ExoU-producing, but not the ExoS-producing strain (28). These data also contrast with observations made in the lung model, in which type III secretion is an important virulence factor for both ExoS- and ExoU-producing strains of P. aeruginosa (29, 30). Neutrophils are the predominant infiltrating cell type in lung infections, and it has been proposed that killing of infiltrating neutrophils creates an immunocompromised milieu in the lung in which P. aeruginosa can thrive (31). We and others reported that infection with either ExoU- or ExoS-expressing strains causes keratitis in murine models, and that bacterial clearance and the severity of infection are dependent on the innate immune response, including neutrophil infiltration to the corneal stroma and bacterial survival (7–9, 11, 26). The obvious importance of neutrophils in clearing P. aeruginosa infections, both in the lung and in the eye, and the identification of neutrophils as the primary mediator of type III secretion in the lung model of infection prompted us to revisit the role of type III secretion in eye infections, particularly for ExoS-producing strains of P. aeruginosa.

In the current study, we use strains PAO1 and PAK, which express ExoS, ExoT, and ExoY, but not ExoU (32), and demonstrate that type III secretion is required for corneal disease elicited by these two strains of P. aeruginosa. Moreover, we identified the ADPR activities of ExoS and ExoT as the essential mediators promoting neutrophil apoptosis and bacterial survival in neutrophils in vitro and in vivo.

**Materials and Methods**

**Generation of type III secretion mutants**

All strains and plasmids used in this study are listed in Table I. Chromosomal mutants were all derived from the same parental PAO1 strain or PAK strain, as indicated in Table I, and were generated by allelic exchange. PAO1 strains vary widely in their expression of virulence genes. This difference has, in part, been linked to nfκB mutants that overexpress mxeEF-OprN (33). The strain used in this study is chloramphenicol sensitive, and therefore most likely does not overexpress the mxeEF-OprN efflux pump. Notably, many PAO1 strains express the type III secretion system poorly (34); however, the strain used in this study expresses the type III secretion genes well (35). All primers used for plasmid construction are listed in Table II. Flanks specifying the appropriate mutation were amplified using chromosomal DNA as template (unless specified otherwise), joined by splicing by overlap extension PCR, and cloned into the appropriate plasmid (either the allelic exchange vector pEXG2 or the shuttle vector pSVS35) using the indicated restriction enzymes. Primers specifying the exoT R149K and E383D/E385D mutations were derived from a previous study by the Barbieri laboratory (36). We reported that the ΔexoST, ΔexoTY, ΔexoSY, and ΔSTOX mutant strains are secretion competent and that they export the expected effector proteins (also, all four strains still export PopN) (35). The single deletion mutants are precursors to these double- and triple-null mutants.

**Western blotting**

ExoS and ExoT expression was measured by Western blot analysis as described (35). Briefly, 1 ml log-phase bacterial culture was centrifuged, and culture supernatants were precipitated with 10% TCA. Cell pellets and supernatant proteins were separated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with rabbit antisera (1:5000). Primary Abs were detected using HRP-conjugated secondary Abs and a chemiluminescent detection reagent (SuperSignal West Pico, Pierce).

**Mouse strains**

C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME), TLR4+/− and MyD88−/− mice were obtained with permission from S. Akira (Research Institute for Microbial Diseases, Osaka University), and TLR5−/− mice were obtained from R. Flavell (Howard Hughes Medical Institute, Yale University, New Haven, CT). TLR4−/−/MyD88−/− mice were generated at Case Western Reserve University animal facility, and Mafia mice were generated by S. Burnett (University of Utah, Provo, UT), and are now available from The Jackson Laboratory. All animals were housed under specific pathogen-free conditions in microisolator cages and maintained according to institutional guidelines and the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

**In vivo model of corneal infection**

Mice were anesthetized by i.p. injection of 0.4 ml 2,2,2-tribromoethanol (1.2%). Central corneas were scarified with three parallel 1-mm-long abrasions using a 26-gauge needle. A 2.5-μl aliquot containing ~1 × 10⁷ PAO1 was applied to the scarified cornea, as described in our previous study (9). Sterile PBS was applied to the abraded cornea as a trauma control. A sterile trephine (Miltex, Tuttlingen, Germany) was used to generate a 2-mm-diameter punch of silicone hydrogel contact lens (Night and Day; CIBA Vision, Duluth, GA), which was placed over the central cornea to maintain placement of the bacterial suspension. The contact lenses were removed after 2 h, and mice were allowed to recover from the anesthesia.

**Quantification of corneal opacification**

Image analysis was used to generate an objective measure of corneal opacification based on our studies on fungal keratitis (37). Mouse corneas were illuminated using a gooseneck fiber optic light source, and constant light levels were maintained during image acquisition. Twenty-four–bit color images were captured with a SPOT RTCKe camera (Diagnostic Instruments, Sterling Heights, MI) connected to a Leica MZF III stereo microscope, and all images were captured using the same exposure time. Image analysis was performed using Metamorph Imaging software (Molecular Devices, Downingtown, PA) and described in detail in Supplemental Fig. 1. Briefly, a circular region of constant area was centered on the images of corneas of infected mice, and Metamorph software was used to generate the percentage of corneal opacity and the total corneal opacity. A threshold value was set from naive C57BL/6 eyes or eyes with no apparent disease, and corneal opacity was based on values above the threshold, with the more opaque regions displaying a greater integrated pixel intensity value. Areas of glare were demarcated and then set to zero, thus effectively eliminating the iris from the subsequent analysis process.

**Mafia mouse model for depletion of macrophages and dendritic cells**

Mafia mice express enhanced GFP and a membrane-bound suicide protein comprising the human low-affinity nerve growth factor receptor, the FK506-binding protein, and a cytoplasmic domain of Fas (38, 39). AP20187 is a covalently linked dimerizer (Ariad Pharmaceuticals, Cambridge, MA) that cross-links the FK506-binding protein region of the suicide protein and induces caspase 8-dependent apoptosis, as described (38, 40). Intrapitoneal injection of AP20187 selectively depletes macrophages and dendritic cell populations. These mice are on a C57BL/6 background and have a normal phenotype in the absence of the dimerizer.

**Bacterial quantification**

Whole eyes were homogenized under sterile conditions using the Mixer Mill MM300 (Retsch, Newtown, PA) at 33 Hz for 4 min. Serial log dilutions were performed and bacteria were plated onto brain heart infusion agar (BD Biosciences, Sparks, MD). Plates were incubated at 37°C for 18 h, and the number of CFU was determined by direct counting.

**Histology and immunohistochemistry**

Eyes were enucleated and placed in 10% formalin/PBS for 24 h and embedded in paraffin, and 5-μm sections were stained by H&E. Neutrophils in the corneal stroma were detected after incubation with rat anti-mouse neutrophil Ab (LeuM1 R14; Abcam, Cambridge, MA; 1:100 in 1% FCS-PBS). Sections were washed and incubated with FITC-conjugated rabbit anti-rat Ab (Vector Laboratories, Burlingame, CA) diluted 1:200 in 1% FCS-PBS for 45 min. Neutrophils in the corneal stroma were enumerated by fluorescence microscopy, as described (9).
In vitro neutrophil survival assay

To obtain marine neutrophils, mice were injected with 1 ml 9% casein, 16 and 3 h prior to peritoneal lavage, and cells were layered onto a sterile 90% Percoll gradient (GE Healthcare, Piscataway, NJ). The neutrophil population was recovered from the second layer on the gradient, as determined by cytology, and routinely yielded 95−100% pure neutrophils, as described in our previous studies (41). Viability was 95%, as determined by trypan blue exclusion. Neutrophils (5 × 10^6 cells/ml) were incubated in DMEM at 37 °C with 5% FBS, 0.1% sodium azide. Cells were then washed twice with PBS; cells were immediately lysed using 0.1% Triton X-100; and CFU was determined at this time. Remaining cultures were incubated an additional 90 min (total incubation time = 2.5 h), washed, and lysed, and CFU were quantified.

Analysis of total and percentage of annexin V-positive corneal neutrophils by flow cytometry

Corneas were excised 24 h postinfection, and residual iris material was removed. Individual corneas were incubated with NIMP-R14 + and anti-mouse annexin V-allophycocyanin–conjugated Ab (eBioscience). Neutrophils were gated, and annexin V expression was analyzed. Histograms showing the percentage of annexin-positive or -negative NIMP-R14+ neutrophils were generated using C-Flow software (Accuri).

Statistical analysis

Student t test or ANOVA with Tukey’s multiple comparison test was performed using GraphPad Prism (San Diego, CA). Statistical significance was defined as a p value <0.05.

Results

Type III secretion system is essential for development of P. aeruginosa keratitis

As a first step in dissecting the role of the type III secretion in the development of P. aeruginosa keratitis, mice were infected with the PAO1 parent strain of P. aeruginosa, the ΔpscD mutant, which does not assemble a type III secretion apparatus, and the ΔpscD mutant that was complemented with a plasmid expressing PscD (ΔpscDpscD) as described in Tables I and II.

We found that infection with PAO1 caused increasing corneal opacification over time, with severe disease apparent at 72 h (Fig. 1A–C). In contrast, infection with the ΔpscD mutant induced much less corneal disease. Complementation with a plasmid expressing PscD (ΔpscDpscD) completely restored the capacity of the ΔpscD mutant to cause clinical disease. To determine the role of type III secretion on bacterial survival in the cornea, eyes were homogenized and CFU were quantified. Fig. 1D shows that, at 72 h, CFU in PAO1-infected corneas were significantly elevated compared with the inoculum. In contrast, CFU in mice infected with the ΔpscD strain were lower than the inoculum, indicating that these bacteria were being killed. Infection with the complemented ΔpscDpscD strain restored the wild-type phenotype, thereby demonstrating an essential role for type III secretion in bacterial survival in the cornea. Repeat experiments showed that the role of

Table I. Strains and plasmids

<table>
<thead>
<tr>
<th>Strain No.</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
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<td>PAO1F (wild-type PAO1)</td>
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<tr>
<td>RP1871</td>
<td>PAO1F ΔpscD</td>
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</tr>
<tr>
<td>RP1883</td>
<td>PAO1F ΔexoS</td>
<td>This study</td>
</tr>
<tr>
<td>RP1945</td>
<td>PAO1F ΔexoT</td>
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</tr>
<tr>
<td>RP1923</td>
<td>PAO1F ΔexoY</td>
<td>This study</td>
</tr>
<tr>
<td>RP1947</td>
<td>PAO1F ΔexoST</td>
<td>This study</td>
</tr>
<tr>
<td>RP1949</td>
<td>PAO1F ΔexoS ΔexoT ΔexoY (Δ3TOX)</td>
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<tr>
<td>RP2750</td>
<td>PAO1F ΔpopBD</td>
<td>This study</td>
</tr>
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<td>RP5479</td>
<td>PAO1F exoS(GAP-)</td>
<td>This study</td>
</tr>
<tr>
<td>RP5481</td>
<td>PAO1F exoS(ADPR-)</td>
<td>This study</td>
</tr>
<tr>
<td>RP5572</td>
<td>PAO1F exoS(GAP-)(ADPR-)</td>
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<td>PAO1F exoT(GAP-)</td>
<td>This study</td>
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<td>PAO1F exoT(ADPR-)</td>
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<td>PAO1F exoS(GAP-)(ADPR-)</td>
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<td>PAO1F exoT(GAP-)(ADPR-)</td>
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<td>PAK ΔexoT</td>
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<td>RP1603</td>
<td>PAK ΔexoS ΔexoT</td>
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<td>RP1311</td>
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<td>pPSV35</td>
<td>Expression plasmid with lacP, lacUV5 promoter, gent resistance, colE1, and Pseudomonas origins of replication, oriT</td>
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<tr>
<td>pPA1838</td>
<td>pscD cloned under control of lacUV5 promoter</td>
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<tr>
<td>pEXG2-ΔpopBD</td>
<td>pEX2G2 with popBD deletion allele (popB codon 3 to popD codon 268)</td>
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<td>pEX-ΔexoT</td>
<td>pEXGW plasmid with exoT deletion allele</td>
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<tr>
<td>pEX-ΔexoY</td>
<td>pEXGW plasmid with exoY deletion allele</td>
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<tr>
<td>pEXG2-exoS(GAP-)</td>
<td>pEX2G2 with exoS(R149K) mutant</td>
<td>35</td>
</tr>
<tr>
<td>pEXG2-exoS(ADPR-)</td>
<td>pEX2G2 with exoS(E379D,E381D) mutant</td>
<td>35</td>
</tr>
<tr>
<td>pEXG2-exoS(GAP-)(ADPR-)</td>
<td>pEX2G2 with exoS(E379D,E381D) mutant</td>
<td>35</td>
</tr>
<tr>
<td>pEXG2-exoT(GAP-)</td>
<td>pEX2G2 with exoT(R149K) mutant</td>
<td>This study</td>
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<tr>
<td>pEXG2-exoT(ADPR-)</td>
<td>pEX2G2 with exoT(E383D,E385D) mutant</td>
<td>This study</td>
</tr>
<tr>
<td>pEXG2-exoT(GAP-)(ADPR-)</td>
<td>pEX2G2 with exoT(R149K,E383D,E385D) mutant</td>
<td>This study</td>
</tr>
</tbody>
</table>
type III secretion on bacterial survival was highly reproducible (Table III).

Histological sections showed a prominent cellular infiltrate in PAO1-infected corneas after 24 h (Fig. 1E), whereas infection with the ΔpscD strain induced less of an infiltrate. The ΔpscD/pscD strain in which the type III secretion apparatus was restored showed the wild-type phenotype, and immunostaining of these sections using the neutrophil-specific NIMP-R14 14 Ab revealed a prominent neutrophil infiltrate in the cornea (Fig. 1F). Given that neutrophils were recruited to the avascular cornea, we assayed production of the neutrophil chemokines CXCL1/1/KC and CXCL2/MIP-2 at 3 h postinfection, which is prior to neutrophil infiltration, or 24 h following infection when neutrophils are present. Our previous studies showed that CXCL1 was produced by corneal fibroblasts and macrophages at early time points, whereas CXCL2 was produced by neutrophils and macrophages after 24 h (42). Corneas were dissected and homogenized, and chemokine production was measured by ELISA. Fig. 1 shows that CXCL1 was elevated at 3 h postinfection corneas infected with either PAO1 or ΔpscD, and CXCL2/MIP-2 3 h postinfection was significantly lower in the ΔpscD strain compared with PAO1-infected corneas, which is consistent with the lower neutrophil infiltrate and CFU in the absence of type III secretion.

**ΔpscD mutants replicate in MyD88−/− corneas**

To determine whether the decreased ΔpscD mutant numbers in the cornea are due to an inability to replicate in this tissue or to resist killing by infiltrating phagocytic cells, we infected MyD88−/− corneas. As MyD88−/− mice have impaired neutrophil recruitment to the corneal stroma in response to an ExoU-expressing strain (9), we used these mice for infection with the ΔpscD mutant strain, and examined corneal opacification, histopathology, and bacterial survival, as described above. Fig. 2A–C shows significantly less corneal opacity (area and intensity) in PAO1-infected MyD88−/− compared with C57BL/6 mice. In contrast, although the percentage of area of opacity was significantly less in MyD88−/− than C57BL/6 corneas mice (Fig. 2D), further at high magnification, numerous bacteria can be detected in the corneal stroma of MyD88−/−/ mice (inset). Quantification of bacteria in MyD88−/− and C57BL/6 corneas (Fig. 2E) shows significantly higher CFU in MyD88−/−/ corneas infected with either PAO1 or ΔpscD mutants.

These findings demonstrate that type III secretion is not required for *P. aeruginosa* survival and growth in the corneal stroma in the absence of infiltrating cells, and is therefore consistent with a role for type III secretion in inhibiting bacterial killing by infiltrating cells.

**PopBD, ExoS, and ExoT, but not ExoY, are required for development of P. aeruginosa keratitis**

The translocation pore can elicit killing of macrophages in vitro even in the absence of effector proteins (30), and can similarly mediate killing of neutrophils in a pore formation-dependent manner.
To identify the relative contribution of the individual effector proteins to pathogenesis, as well as the translocation pore itself, we infected corneas of wild-type C57BL/6 mice with mutant strains in which these T3SS components had been inactivated.

To assess the contribution of the translocon, corneas of C57BL/6 mice were abraded and infected with PAO1 or mutant bacteria lacking pscD or the pore-forming translocator proteins (ΔpopBD). Clinical disease and CFU were examined, as described above. We found that, as with the ΔpscD strain, corneas infected with ΔpopBD mutants had less opacification and lower CFUs than PAO1 (Fig. 3A), indicating that the translocation apparatus is essential for development of PAO1-induced keratitis.

To determine the relative contribution of the effector proteins in corneal disease, we infected wild-type mice with a strain lacking the genes for all three effector proteins, exoS, exoT, and exoY (Δ3TOX), or with a ΔexoY mutant (Fig. 3B). Removal of all three effector proteins resulted in a virulence phenotype that was indistinguishable from that of the ΔpscD or ΔpopBD mutant bacteria, suggesting that survival of PAO1 in the cornea is mediated by delivery of effector proteins, rather than translocon-mediated killing of neutrophils. In contrast, we found that deletion of ΔexoY mutants was not significantly different from PAO1 in survival or in inducing corneal opacification, indicating no apparent role for ExoY in P. aeruginosa keratitis.

We further dissected the role of individual effector proteins by examining the virulence phenotype of strains lacking exoS and/or exoT. Fig. 3C shows significantly lower CFU when mice were infected with the ΔexoS, ΔexoT, or ΔexoST strains compared with PAO1. Corneal opacification was also lower in ΔexoS-, ΔexoT-, and ΔexoST-infected mice, as shown in representative images below. Furthermore, deletion of exoS or exoT individually resulted in an intermediate defect in colonization relative to wild-type bacteria and a strain lacking both exoS and exoT, suggesting that these two effectors contribute in a nonredundant manner to disease. However, whereas infection with the strain lacking both exoS and exoT consistently resulted in fewer CFU recovered from the double-deletion mutant when compared with the individual ΔexoS and ΔexoT mutant strains, this difference was not statistically significant.

Taken together, these findings indicate that ExoS and ExoT are the primary effectors responsible for the survival of PAO1 in infected corneas, and that ExoS and ExoT have nonredundant activities that promote bacterial survival and growth in the cornea. The observation that the ΔexoST double mutant has the same

**Table III. Role of type III secretion in bacterial survival in corneas of C57BL/6 mice**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>PA Strain</th>
<th>Inoculum</th>
<th>Day</th>
<th>CFU</th>
<th>No. Mice</th>
<th>p Value</th>
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<td>1</td>
<td>PAO1</td>
<td>1 × 10⁶</td>
<td>2</td>
<td>1.8 × 10⁷</td>
<td>4</td>
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<tr>
<td>2</td>
<td>PAO1</td>
<td>1 × 10⁵</td>
<td>2</td>
<td>2.8 × 10⁸</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>PAO1</td>
<td>1 × 10⁶</td>
<td>2</td>
<td>2.0 × 10⁷</td>
<td>4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>4</td>
<td>PAO1</td>
<td>1 × 10⁶</td>
<td>3</td>
<td>4.3 × 10⁷</td>
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<td>&lt;0.01</td>
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<td>PAO1</td>
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<td>2</td>
<td>2.0 × 10⁸</td>
<td>6</td>
<td>&lt;0.01</td>
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</table>
phenotype as the strain lacking all three effectors is further evidence that there is no role for ExoY in corneal disease.

Given the discrepancy between our data and the published lack of type III secretion-related phenotype in strain PAK (28), we also examined the role of type III secretion in PAK-mediated corneal infection. C57BL/6 corneas were abraded and infected with parent strain PAK, the type III secretion mutant ΔpscD (equivalent to strain ΔpscD in the PAO1 background), and ΔexoS, ΔexoT, or ΔexoST mutants (Fig. 3D). Consistent with our findings in strain PAO1, corneal opacification and CFU were significantly lower in all four mutants compared with PAK, and as with PAO1, the ΔexoST double mutant had lower CFU and opacification scores than single mutants.

Together, these findings demonstrate that strains PAO1 and PAK rely on an intact type III secretion system, and ExoS and ExoT in particular in P. aeruginosa keratitis.

**ΔexoST mutants survive and replicate in mice with impaired neutrophil and macrophage infiltration**

As ΔexoS and ΔexoT mutants are rapidly killed in C57BL/6 corneas, we next examined whether mutants lacking ExoS and ExoT can replicate in the cornea in the absence of infiltrating neutrophils and macrophages, which would indicate a role for these exotoxins in preventing bacterial killing by these cells. To this end, we used three transgenic mouse strains that have impaired neutrophil and macrophage recruitment to the cornea in *P. aeruginosa* keratitis (9, 39). MyD88Δ−/− and TLR4Δ−/− mice were infected with ΔexoST, and CFU was quantified after 48 h. We found that in contrast to C57BL/6 mice, CFU of ΔexoST mutants increased in MyD88Δ−/− and TLR4Δ−/− corneas (Fig. 4).

As a third approach to examine the ability of ΔexoST to grow in the absence of infiltrating cells, we used transgenic Mafia mice that are on a C57BL/6 background and express fas under control of the c-fms promoter. In these mice, macrophages and dendritic cells expressing fas can also be selectively depleted by injection of the AP20187 dimerizer that induces fas-mediated apoptosis (38). Our previous studies using these mice showed that macrophages are the predominant resident cells in the corneal stroma and have an essential role in CXC chemokine production and recruitment of neutrophils (9, 39). Mafia mice were either treated with the dimerizer or left untreated prior to corneal infection with ΔexoST bacteria. After 24 h, eyes were homogenized and CFU were assayed, as before. Fig. 4 shows that, as with C57BL/6 mice, ΔexoST were decreased in Mafia mice not given the dimerizer; however, we found a 3-log increase in CFU in infected AP20187-treated Mafia mice, consistent with an essential role for macrophages.

Taken together, these findings indicate that ExoS and ExoT prevent bacterial killing by macrophages and neutrophils that are recruited to the cornea following infection.

**ADPR, but not rho-GAP activities of ExoS and ExoT, is essential for *P. aeruginosa* intracellular survival in neutrophils**

ADPR and rho-GAP point mutations in ExoS and ExoT were generated as described in Materials and Methods. Total cell lysates and culture supernatants were processed for Western blot analysis, and polyclonal rabbit serum was used to detect ExoS, ExoT, and the cell-associated protein RAN polymerase A (RpoA, fractionation control). Fig. 5A shows that whereas ExoS and ExoT proteins were absent in the ΔexoS and ΔexoT null mutants, strains with point mutations had intact ExoS and ExoT proteins. ExoS and ExoT were also sequestered into the culture supernatant (Fig. 5B).

To determine whether ExoS and ExoT mediate resistance to neutrophil killing, peritoneal neutrophils and macrophages were incubated with PAO1 or ΔpscD, ΔexoS, ΔexoT, or ΔexoST mutant strains for 30 min to allow for bacterial attachment and phagocytosis. Cells were then incubated for 30 min in the presence of gentamicin to kill extracellular bacteria; the cultures were washed and lysed; and bacterial growth/survival was assessed by CFU analysis.

The number of intracellular bacteria after 30 min in gentamicin was similar for all strains (1 × 10⁵ CFU/ml; data not shown). PAO1 survived the additional hour after the initial time point, with 1 × 10⁵ CFU recovered. Fig. 5C shows that CFU from neutrophils infected with ΔpscD, ΔexoS, ΔexoT, or ΔexoST strains were significantly lower than PAO1, indicating bacterial killing. In contrast, there was no difference in bacterial survival in macrophages between PAO1 and any of the null mutants (Fig. 5D), indicating that there is no apparent role for type III secretion in *P. aeruginosa* survival in macrophages.

ExoS and ExoT each have N-terminal rho-GAP and C-terminal ADPR activities. To determine whether rho-GAP or ADPR activities contribute to intracellular survival in neutrophils, *P. aeru-
uginosa mutants were generated in which either the rho-GAP activity (G-) or the ADPR activity (A-) was inactivated through point mutations introduced into the chromosomal copy of the corresponding effector gene. Survival of intracellular PAO1 or mutant strains in peritoneal neutrophils was examined. At time = 0 after the initial gentamicin treatment, there was no difference in CFU among the strains (data not shown); however, after 90 min, CFU from neutrophils infected with either the exoS(A-) mutant or the exoS(G/A-) double-mutant strains were significantly lower than PAO1 and similar to the ΔexoS strain, whereas CFU from neutrophils infected with the exoS(G-) mutant were not significantly different from PAO1 (Fig. 5E), indicating that the ADPR activity of ExoS is essential for P. aeruginosa survival in neutrophils.

Similarly, exoT(A-) and exoT(G/A-) mutants had significantly lower CFU compared with PAO1 and the exoT(G-) mutant, demonstrating that the ADPR activity of ExoT is also essential for survival in neutrophils, whereas the rho-GAP mutant was not significantly different from the PAO1 parent strain (Fig. 5F). To determine whether there is a synergistic effect between ExoS and

![FIGURE 3](image-url)

**FIGURE 3.** The role of popBD, ExoS, ExoT, and ExoY postinfection with bacterial strains PAO1 or PAK. A, Representative corneas, quantitative corneal opacity, and CFU in C57BL/6 mice 48 h postinfection with $1 \times 10^5$ PAO1, ΔpscD, or mutants in the translocon apparatus (ΔpopBD); B, Corneas infected with PAO1, ΔexoS,T,Y (Δ3TOX), or ΔexoT mutants; C, Corneal infection with PAO1, Δ3TOX, ΔexoS, ΔexoT, or ΔexoST mutants; D, Corneas infected with strain PAK, type III secretion mutant ΔpscJ, or ΔexoS, ΔexoT, or ΔexoST PAK mutants. Data points represent individual corneas, which were analyzed by ANOVA, and p values for pairwise comparisons from the post hoc tests are shown. Overall ANOVA p value was <0.0001 for each set of studies. These experiments were repeated twice with similar results. Original magnification ×20.

![FIGURE 4](image-url)

**FIGURE 4.** Survival of ΔexoST mutants in mice with impaired neutrophil and macrophage recruitment to the cornea. Corneas of C57BL/6, MyD88−/−, TLR4/5−/−, and macrophage-depleted Mafia mice previously shown to have impaired cellular recruitment (8) were infected with $1 \times 10^5$ PAO1-derived ΔexoST mutants, and CFU were examined after 48 h. Data points represent CFU of individual corneas from one of two repeat experiments, analyzed by ANOVA, and p values for pairwise comparisons from the post hoc test are shown.
ExoS ADPR activities, neutrophils were infected with the exoS(A-), exoT(A-), or exoS(A-)/exoT(A-) double mutants. We found that CFU recovered postinfection with the double mutant was not significantly different from the single mutants (Fig. 5G), indicating that both ExoS and ExoT ADPR activities are required equally for bacterial survival in neutrophils. Furthermore, whereas the CFU recovered from neutrophils infected with the ΔexoST double-deletion mutant were consistently lower than those recovered from neutrophils infected with the double ADPR- mutant, the difference was not statistically significant, suggesting that whereas the rho-GAP activities may have a minor role in survival in neutrophils, the phenotype relies primarily on the ADPR activities of ExoS and ExoT.

Finally, to determine whether the ExoS and ExoT Rho-GAP activities are redundant, neutrophils were infected with exoS(G-), exoT(G-), or exoS(G-)/exoT(G-) double mutants, and CFU was assessed. However, as shown in Fig. 5H, there was no significant difference between the double mutants and PAO1, indicating that there is no role for Rho-GAP activity in bacterial survival in neutrophils. Together, these findings demonstrate that survival in neutrophils is dependent on the ADPR activity of ExoS and ExoT.

ADPR, but not rho-GAP activities of ExoS and ExoT, is essential for neutrophil recruitment to the corneal stroma and neutrophil apoptosis

Given that ExoS and ExoT ADPR activity appears to mediate bacterial survival in neutrophils, and that ExoS and ExoT cause apoptosis in epithelial cells (HeLa cell line) (44, 45), we examined whether ExoS and ExoT induce neutrophil apoptosis during corneal infection.

In the first set of experiments, C57BL/6 corneas were infected with PAO1, ΔexoS, ΔexoT, or ΔexoST strains, and, after 24 h, corneas were digested with collagenase, and the total number of neutrophils per cornea and the percentage of apoptotic neutrophils were assessed by flow cytometry using NIMPR14. As shown in Fig. 6A (upper panel), the total number of neutrophils from corneas infected with ΔexoS, ΔexoT, or ΔexoST was significantly lower than corneas infected with PAO1, which is most likely due to decreased bacterial survival in neutrophils, as shown in Fig. 5.

To identify apoptotic neutrophils in the cornea, infiltrating cells were incubated with NIMPR14 together with an Ab to annexin V. NIMPR14+ cells were gated, and the percentage of annexin V-positive neutrophils was determined by flow cytometry. As shown in Fig. 6A, central panel), which is consistent with a role for ExoS and ExoT in neutrophil apoptosis. Representative flow cytometry scans are shown in the lower panels.

To determine the relative contribution of rho-GAP (G) and ADPR (A) activities of ExoS and ExoT on neutrophil infiltration to the corneal stroma and apoptosis, C57BL/6 corneas were infected with ExoS rho-GAP or ADPR mutants, ExoT rho-GAP or ADPR mutants, or ExoST ADPR mutants. As shown in Fig. 6B, there was
no significant difference in either neutrophil recruitment to the corneal stroma (upper panel), or in the percentage of annexin-positive neutrophils between PAO1 and exoS(G-) mutants (center and lower panels). In marked contrast, the number of infiltrating neutrophils and the number of annexin-positive neutrophils in exoS(A-)-infected corneas were significantly less than in PAO1-infected corneas. Furthermore, there was no significant difference in apoptosis among exoS(A-), exoS(G/A-), and ΔexoS mutants, indicating that the ADPR activity accounts for the proapoptotic effect of ExoS on neutrophils with no apparent role for rho-GAP activity.

Similarly, the exoT(G-) mutant strain was not significantly different from PAO1 in either neutrophil infiltration or the percentage of annexin-positive neutrophils in the cornea, whereas the exoT(A-) mutant induced significantly less neutrophil recruitment to the cornea and less apoptosis of these neutrophils (Fig. 6C). Also, the exoT(G/A-) double mutant had the same phenotype as the exoT(A-) and the ΔexoT strain, demonstrating that, as with ExoS, the ADPR activity accounts for the proapoptotic activity of ExoT. To ascertain whether there is synergistic activity between the ADPR activities of ExoS and ExoT, corneas were infected with the exoS(A-) mutant, the exoT(A-) mutant, the exoS(A-)/exoT(A-) double mutant, or the ΔexoST mutant, and the percentage of annexin-positive cells was examined, as before. As shown in Fig. 6D, the percentage of annexin-positive cells in the exoS(A-)/exoT(A-) double mutant and the ΔexoST mutant was significantly lower than with either of the single ADPR-mutant strains, which is consistent with a synergistic role for ADPR activity of ExoS and ExoT in neutrophil apoptosis.

Together, these data reveal an essential role for ADPR, but not rho-GAP activity of ExoS and ExoT in promoting neutrophil apoptosis in corneal infections. The increased neutrophil numbers in the presence of ADPR activity are most likely due to increased survival of bacteria, which induces further neutrophil recruitment.

ADPR activity of ExoS and ExoT mediates corneal opacification and bacterial survival in the cornea

Given the predominant role for ADPR on neutrophil apoptosis and bacterial survival, we next examined the role of ADPR in P. aeruginosa keratitis. Corneas of C57BL/6 mice were abraded and infected with PAO1 or exoS(A-), exoT(A-), exoS(A-)/exoT(A-), or ΔexoST mutants, and corneal opacification and bacterial recovery were examined after 48 h. As shown in Fig. 7, each of the ADPR mutants caused less corneal opacification than PAO1. Furthermore, there was significantly less CFU recovered from ADPR mutants than from PAO1-infected corneas. Whereas there appeared to be a reduction in recovered CFU when comparing the ΔexoST and exoST(A-) mutants, this difference was not statistically significant.

These results demonstrate that ExoS and ExoT ADPR activities are essential for bacterial survival in the cornea and development of corneal disease. As with the neutrophil survival assays, the role of the RhoGAP activities of ExoS and ExoT appears to only play a minor role in survival in the cornea because the difference in...
The role of ExoS and ExoT ADPR in corneal disease and bacterial survival. Corneas of C57BL/6 mice were abraded and infected with $1 \times 10^7$ PAO1, exoS(A-), exoT(A-), exoST(A-), or ΔexoST. A, Corneal opacification in representative mice examined 48 h postinfection. Original magnification ×20. B, Percentage of corneal opacity; C, Average corneal opacity; D, CFU recovered from infected eyes; each data point represents an individual animal showing a significant difference between PAO1 and each of the mutants. Data were analyzed by ANOVA, and $p$ values for pairwise comparisons from the post hoc test are shown. Overall $p$ value was <0.001, although there was no significant difference between exoST (A-) and ΔexoST mutants. This experiment was repeated twice with similar results. 

FIGURE 7. The role of ExoS and ExoT ADPR in corneal disease and bacterial survival. Corneas of C57BL/6 mice were abraded and infected with $1 \times 10^7$ PAO1, exoS(A-), exoT(A-), exoST(A-), or ΔexoST. A, Corneal opacification in representative mice examined 48 h postinfection. Original magnification ×20. B, Percentage of corneal opacity; C, Average corneal opacity; D, CFU recovered from infected eyes; each data point represents an individual animal showing a significant difference between PAO1 and each of the mutants. Data were analyzed by ANOVA, and $p$ values for pairwise comparisons from the post hoc test are shown. Overall $p$ value was <0.001, although there was no significant difference between exoST (A-) and ΔexoST mutants. This experiment was repeated twice with similar results. 

Discussion

The corneal surface is well equipped to prevent bacterial adhesion and infection given that tears contain β-defensins and other antibacterial agents, and mucins and surfactants on the external layer of the corneal epithelium inhibit attachment to the corneal epithelial cells (46–48). In addition, corneal epithelial cells form tight junctions, which are an effective barrier to bacterial invasion. Infection of the underlying corneal stroma, which is associated with disease, therefore either requires a breach of the epithelial barrier by trauma (as used in most mouse models) or is associated with contact lens wear and P. aeruginosa biofilm formation (49, 50).

Overall, the results generated in the current study increase our understanding of the pathogenesis of this disease by demonstrating that the type III secretion system in ExoS/T-expressing strains subverts the host response in the corneal stroma. Specifically, the two type III secretion-delivered effector proteins ExoS and ExoT are essential for establishing a productive infection in a mouse model of keratitis. Using mutant mice defective in macrophage and neutrophil functions, we also showed that the type III secretion system, and ExoS and ExoT in particular, are only required for pathogenesis in the presence of a cellular infiltrate, as ΔexoS and ΔexoT mutants were able to replicate in macrophage-depleted Mafa mice (which also have impaired neutrophil recruitment), and in MyD88<sup>−/−</sup> and TLR4<sup>−/−</sup> corneas. Moreover, we found that ExoS- and ExoT-expressing bacteria cause neutrophil apoptosis, and that ExoS and ExoT are essential for survival in neutrophils in vitro. Together, these observations support the conclusion that the principal role of type III secretion is to subvert the host response by targeting the antibacterial activity of infiltrating neutrophils.

The role of ExoS and ExoT in P. aeruginosa keratitis is almost entirely due to the ADPR activities, which appear to have nonredundant roles in bacterial survival in neutrophils, and in induction of neutrophil apoptosis. Both ExoS and ExoT ADPR activities were required for survival in isolated peritoneal neutrophils, which would explain the nonredundant requirement for ExoS and ExoT in our initial infection experiments. In marked contrast to ADPR, inactivation of the Rho-GAP activities of ExoS and ExoT did not significantly affect the ability of P. aeruginosa to survive in neutrophils. However, although not statistically significant, we consistently saw a reduction of recovered CFU in vivo when comparing the ΔexoST double-null mutant with either ΔexoS and ΔexoT (Fig. 3), or the strain in which both ADPR activities had been inactivated (Fig. 7), suggesting that the Rho-GAP activities of these two enzymes may have a minor function in promoting survival in the cornea.

In corneal and lung epithelial cells, Fleiszig and coworkers (51, 52) showed that P. aeruginosa is sequestered in membrane blebs, formation of which depends on the ADPR activity of ExoS. Whereas we focused on the activity of ExoS in promoting survival in neutrophils, this sequestration in nonphagocytic cells could also serve to evade killing by neutrophils and is consistent with the importance of the ADPR activity of ExoS for establishing a productive infection. Our studies also show that corneal epithelial cells can be activated by P. aeruginosa through TLR4/MD-2 to produce CXC1 and CXCL2, which work together with CXCL5 produced by corneal fibroblasts to recruit neutrophils to the corneal stroma (11, 42).

Both ExoS and ExoT have been implicated in induction of apoptosis in vitro (45, 53, 54), although there is some controversy with regard to the role of ExoT in eliciting apoptosis, because it actually prevented cell death in infected Chinese hamster ovary cells (55). Furthermore, differences in cell type are most likely involved also as ExoT-mediated apoptosis was detected in HeLa cells. In the current study, we demonstrate that ExoS and ExoT promote apoptosis in infiltrating neutrophils in vivo. Consistent with prior reports showing ExoST-dependent apoptosis of HeLa cells (45, 54), induction of apoptosis relied on the ADPR activity of both effectors. It is tempting to speculate that apoptosis of infiltrating phagocytes contributes to persistence of P. aeruginosa in the eye. However, induction of apoptosis is most likely not the only factor influencing survival in neutrophils, as these effectors may also interfere with an activity that is directly involved in clearing phagocyted bacteria, such as fusion with neutrophil granules or generation of an oxidative burst. Even if induction of apoptosis does not influence the immediate survival of P. aeruginosa in neutrophils, it may still contribute to persistence in the tissue at the population level, by killing the primary cell type responsible for the clearance of these bacteria. The ADPR activities of ExoS and ExoT may therefore promote keratitis by inhibiting the antibacterial effects of neutrophils in the corneal stroma, by promoting evasion of neutrophils through sequestration within external corneal epithelial cells (52, 56), and by promoting neutrophil apoptosis.

Although Fleiszig and coworkers (52) showed a clear role for ExoS in P. aeruginosa survival in epithelial cells, they reported that the PAK ΔexoS mutant could still cause corneal disease in a mouse keratitis model (28). It is likely that the primary reason for the difference between those findings and results of the current study, which clearly demonstrate that delivery of ExoS and ExoT
is essential in the invasive PAO1 and PAK strains of *P. aeruginosa* (in the absence of ExoU), is due to the higher inoculum used in that study, which most likely masked the effect of ExoS (S. Fleizsig, personal communication).

One emerging area of consensus is that the relative importance of the virulence factors expressed by a given *Pseudomonas* isolate depends on the site of infection. For example, ExoS and ExoT have nonredundant functions in our model of keratitis, whereas deletion of *exoT* alone has no phenotype in lung or burn-wound infections (57–59). It is possible that ExoS activity is either redundant with that of ExoT, or has a distinct virulence phenotype in infections (57–59). It is possible that ExoS activity is either redundant with that of ExoT, or has a distinct virulence phenotype depending on the strain used (30, 58–60). In contrast to the keratitis model using the PA103 strain of *P. aeruginosa* (28), ExoU is the dominant effector in an acute lung model of infection, as deletion of *exoU* results in a significant reduction in virulence, whereas deletion of *exoT* results in a phenotype only in the context of the *exoU* mutant strain (60). Effectors therefore have niche-specific roles, even though cleavage of *P. aeruginosa* relies on neutrophils in both the lungs and the cornea (9, 29, 31, 61). The molecular basis for these differences awaits further studies directly comparing infection in these tissues.

In conclusion, given that the pathogenesis of *P. aeruginosa* keratitis is a consequence of the host response and bacterial virulence factors, our experiments demonstrate a clear role for the type III secretion and ExoS and ExoT in subverting the host response to promote bacterial survival and development of corneal disease. Although the molecular basis for these observations has yet to be determined, we conclude that the ADPR activities of ExoS and ExoT mediate survival of *P. aeruginosa* in neutrophils and also promote apoptotic cell death, both of which likely contribute to corneal disease.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**

Figure S1. Quantification of corneal opacification - Metamorph Imaging software (Molecular Devices, Downington PA) was used to quantify the Percent Area of Opacity and the Integrated Corneal Opacity using the equipment described in the Methods. Firstly, a circular region of the same constant area was centered on the image of each cornea to standardize the area (upper panels). Secondly photos of corneas images were converted into pseudocolor images (in Metamorph) (lower panels) thereby allowing a color visualization of corneal opacity using a scale (shown on left) ranging from purple (no opacity) to red (maximum opacity) that corresponds to increasing pixel intensity level. Areas of glare (red arrows) were also demarcated and then set to zero, thereby eliminating glare from the subsequent analysis. To set the threshold for no opacity (transparency), we either used images of naïve corneas that were captured at the same time, or corneas within the data set that have distinct regions with no apparent disease (indicated by purple and blue pseudocolors of the ΔpscD mutant). The mean value obtained from at least four naïve or non-diseased corneas was considered the threshold value. All values above this threshold were included in analyses of Percent Corneal Opacity (= area over the threshold value / area of circle – glare x100). Total Corneal Opacity (= integrated pixel intensity above the threshold) was calculated using Metamorph software. This quantitative approach does not account for other indicators of disease such as vascularization, perforation or hypopion, but does generate an objective measure of corneal opacity.