Factors Impacting Corneal Epithelial Barrier Function against *Pseudomonas aeruginosa* Traversal

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Purpose. Mechanisms determining epithelial resistance versus susceptibility to microbial traversal in vivo remain poorly understood. Here, a novel murine model was used to explore factors influencing the corneal epithelial barrier to *Pseudomonas aeruginosa* penetration.

Methods. Murine corneas were blotted with tissue paper before inoculation with green fluorescent protein–expressing *P. aeruginosa.* The impact of blotting on epithelial integrity was evaluated by susceptibility to fluorescein staining and histology. Using fluorescence imaging, blotted corneas were compared to nonblotted corneas for susceptibility to bacterial binding and epithelial penetration after 5 hours or were monitored for disease development. In some experiments, inoculation was performed ex vivo to exclude tear fluid or corneas were pretreated with EGTA to disrupt Ca^{2+}-dependent factors. The role of surfactant protein D (SP-D), which inhibits *P. aeruginosa* cell invasion in vitro, was examined using knockout mice.

Results. Blotting enabled fluorescein penetration through the epithelium into the underlying stroma without obvious disruption to corneal morphology. Although blotting enabled bacterial binding to the otherwise adhesion-resistant epithelial surface, adherent bacteria did not penetrate the surface or initiate pathology. In contrast, bacteria penetrated blotted corneas after EGTA treatment and in SP-D knockouts. Visible disease occurred and progressed only in aged, blotted, and EGTA-treated, SP-D knockout mice.

Conclusions. Neither fluorescein staining nor bacterial adhesion necessarily predict or enable corneal susceptibility to bacterial penetration or disease. Corneal epithelial defenses limiting traversal by adherent bacteria include EGTA-sensitive factors and SP-D. Understanding mechanisms modulating epithelial traversal by microbes could improve our understanding of susceptibility to infection and may indicate new strategies for preventing disease. (*Invest Ophthalmol Vis Sci.* 2011;52:1368–1377) DOI:10.1167/iovs.10-6125

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After corneal injury or during contact lens wear, *Pseudomonas aeruginosa* can cause sight-threatening corneal disease.[1–3] However, the healthy human cornea is similar to our other exposed surfaces in its remarkable resistance to infection by this, and other, opportunistic bacterial pathogens. Indeed, we have shown that inoculation of large numbers of *P. aeruginosa* onto healthy animal corneas in vivo results in rapid bacterial clearance without inflammation or infection.[4] Because of this resistance, researchers who study corneal infection commonly use "invasive" methods to enable disease, including scarification before inoculation or intrastromal injection of the inoculum.[5–8]

It is assumed that the corneal epithelium provides the major barrier to microbes in vivo and that the scarification and intrastromal injection methods work because they enable bacteria to bypass it. Indeed, in vitro studies have clearly shown the potential for corneal epithelial cells to act as a barrier to microbes—for example, through their tight junctions.[9,10] However, corneal epithelial cells grown in vitro still permit traversal by *P. aeruginosa*, even if grown as multilayers,[11,12] illustrating the importance of other in vivo factors in defense against infection. Related to this, we have shown that tear fluid[13] at the surface of the corneal epithelium plays roles in defense through mechanisms independent of bacteriostatic activity.[14] We have also shown that the basal lamina, the basement membrane below the epithelium, prevents bacteria from entering the corneal stroma.[12] The role of the corneal epithelium itself in defense against bacterial penetration, and infection, of the cornea in vivo has not been well studied. Paradoxically, this is probably *because* existing infection models deliberately bypass it to enable disease to be studied.

Results of in vitro studies suggest that epithelial tight junctions, present at the corneal surface and within the epithelium[15] and critical for cell polarity, are of significance in protecting against *P. aeruginosa*.[16,17] In vitro studies also suggest that factors secreted by corneal epithelial cells (including mucins, defensins, surfactant proteins, and extracellular matrix proteins) can modulate bacterial adhesion, invasion, and traversal of these and other cells found in vivo.[12,18–23] Although these in vitro-derived insights will likely be important in vivo, their actual in vivo relevance cannot be explored using existing animal models that bypass the epithelium.

The aim of this study was to develop an infection model that does not require bypassing the epithelium to determine the minimal parameters that enable susceptibility to infection, with a goal of better understanding the defenses that protect us against infection during health.

Thus, we developed a method for studying epithelial barrier function against bacteria in vivo using tissue paper blotting of the epithelial surface to enable bacterial adhesion. We then used this method to demonstrate roles for both ethylene glycol tetraacetic acid (EGTA)-sensitive factors and SP-D in epithelial defense against *P. aeruginosa* traversal. That information will

form the foundation for further studies focused on the mechanisms of disease and developing methods to prevent it.

Materials and Methods

Bacteria

P. aeruginosa invasive strain PAO1 expressing green fluorescence protein (GFP) on a plasmid pSMC2 (PAO1-GFP)[4,24] was used for all experiments. Bacteria were grown on tryptic soy agar plates supplemented with carbenicillin 300 μg/mL at 37°C for approximately 16 hours. Inocula were prepared by suspending bacteria in supplemented hormonal epithelial media (SHEM) without antibiotics[25] to a concentration of approximately 10^{11} cfu/mL (a thick bacterial suspension, approximately 10^9 cfu in a 5-μL drop). Bacterial concentrations were confirmed by viable counts.

Animals

Wild-type C57BL/6 (Charles River Laboratories Inc., Wilmington, MA) or Black-Swiss (Taconic Farms, Inc., Hudson, NY) mice and age-matched *SP-D*$^{-/-}$ gene knockout mice of the same background were used. Animals were subject to anesthesia by intraperitoneal injection (50 μL/25 g body weight) with 21 mg/mL ketamine, 2.4 mg/mL xylazine, and 0.3 mg/mL acepromazine before procedures. All experiments involved at least three to five animals per group and were repeated at least twice. Procedures were performed in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research and were approved by the Animal Care and Use Committee of the University of California at Berkeley.

Tissue Paper–Blotting Method In Vivo

One cornea each of 6- to 10-week-old (age matched within experiments) anesthetized animals was rinsed with PBS to wash away tear fluid, then blotted three times with 1-ply tissue paper (Kimwipe; Kimberly-Clark, Irving, TX). Impact on barrier function to small molecules was compared to nonblotted controls using a 5 μL drop of 0.35% wt/vol ophthalmic fluorescein sodium solution. Extent of fluorescein retention (staining) was examined immediately (within 15 minutes) using a stereomicroscope (Stemi 2000-C; Carl Zeiss, Thornwood, NY) with an attached three-chip cooled camera (Optronics, Goleta, CA). Other eyes were examined using confocal microscopy (details described below) to determine the depth of fluorescein penetration.

In Vivo and Ex Vivo Bacterial Traversal Studies

To study in vivo susceptibility of corneas to traversal, blotted eyes (and nonblotted controls) were immediately inoculated with 5 μL concentrated bacterial suspension (5 μL contains approximately 10^9 cfu). Animals were euthanatized at 5 hours after inoculation by lethal injection, and eyes were then carefully enucleated and rinsed with PBS (approximately 10 mL) before processing for immunofluorescence imaging (as described in detail) to visualize bacterial traversal.

In other experiments, bacterial inoculation was carried out ex vivo to exclude tear fluid (and other in vivo factors extraneous to the eyeball). Mice were euthanatized (as described) immediately after PBS rinsing and tissue paper-blotting of the cornea (under anesthesia). Enucleated whole eyeballs were then incubated in 200 μL of highly concentrated bacterial suspension (approximately 10^{11} cfu/mL) a various times from 5 to 8 hours at 35°C before rinsing to remove nonadherent bacteria and processing for imaging. In some experiments, rinsed and blotted eyes were treated with EGTA solution (100 mM in PBS) for 1 hour before bacterial challenge to disrupt calcium-dependent factors (e.g., cell-to-cell junctions). EGTA was removed by thorough washing with PBS before incubation with bacteria.

In Vivo Infection Studies

The right eye of each mouse was tissue paper-blotted and inoculated as described, but instead of euthanatizing animals at 5 hours after inoculation, they were monitored daily for 2 to 3 weeks for evidence of visible pathology. In other experiments, aged (35- to 36-week-old) mice were used or eyes were treated with EGTA (100 mM) for 3 hours before inoculation. A 2-mm punched-out section of a human soft contact lens was used to retain the EGTA at the ocular surface for the 3-hour period. Disease severity was graded using a 16-point scoring system, as previously described.[26]

Histology

Blotted and uninoculated eyeballs were fixed in 4% (wt/vol) paraformaldehyde, rinsed with PBS, then dehydrated using a graded ethanol series (35%, 50%, 70%, 80%, 95%, and pure ethanol) for 10 minutes each. Eyes were then infiltrated sequentially in 2:1, 1:1, 1:2 ethanol/resin (Ted Pella, Redding, CA) for 1 hour each and were transferred to pure resin to polymerize at 60°C for 2 days. Cross-sections (1-μm thick) were prepared using an ultramicrotome (Ultracut R; Leica, Bannockburn, IL) to capture the front of the cornea where the blotting was applied. Sections were stained with toluidine blue (0.1% wt/vol) and evaluated by light microscopy (Olympus, Melville, NY).

Immunohistochemistry

Enucleated eyeballs were fixed in paraformaldehyde (4% wt/vol) followed by overnight immersion in sucrose solution (30% wt/vol in PBS). Eyes were embedded in optimal cutting temperature (OCT) medium, frozen at −20°C, and sectioned at 8 μm in thickness. Cryosections were blocked for 1 hour at room temperature (RT) with PBS containing BSA (3% wt/vol), goat serum (3% wt/vol), and Triton X-100 (0.1% vol/vol). Sections were then incubated with primary antibody: rabbit polyclonal anti-laminin (1:200 in blocking buffer; SB Biotechnology Inc., Santa Cruz, CA) for 1 hour at RT (see Figs. 3, 4A–D, 6A–D), or rabbit polyclonal anti-ZO1(1:200 in blocking buffer) (Zymed; Invitrogen, Carlsbad, CA) for 1 hour at RT (see Figs. 4E, 4F), then washed three times in PBS-Tween (0.1% vol/vol) for 5 minutes before labeling with secondary antibody: goat anti-rabbit IgG conjugated to rhodamine red (1:5000 in blocking buffer) (Molecular Probes, Eugene, OR) for 1 hour at RT. After three more PBS-Tween washes, samples were mounted and counterstained with medium containing DAPI (Vectashield; Vector Laboratories, Burlingame, CA) to label cell nuclei (blue) and viewed using an epifluorescence microscope (IX70; Olympus, Center Valley, PA), and captured images were processed with deconvolution software (Volocity; Improvision Inc., Lexington, MA).

Laser Scanning Confocal Microscopy and Quantification of Bacterial Penetration Ex Vivo

Enucleated eyes (subject to various treatments) were rinsed once with PBS, then submerged in Ham's F12 cell culture medium and imaged (using a 63×/0.95 NA water-dipping lens) with an upright confocal system (LSM 510; Carl Zeiss GmbH, Jena, Germany). The 488-nm laser line was used to examine the depth of fluorescein penetration and for quantification of GFP–*P. aeruginosa* penetration of the cornea ex vivo (z-stack analysis, customized software; available on request).

Statistical Analysis

Mann-Whitney *U* or Student's *t*-tests were used to determine the significance of differences between groups, depending on the distribution of data. $P < 0.05$ was considered significant.

Results

Tissue Paper Blotting Enables Fluorescein Staining, but Not *P. aeruginosa* Keratitis

Tissue paper-blotted corneas of wild-type (C57BL/6) mice stained extensively with fluorescein (Fig. 1A). Confocal microscopy of stained, blotted eyeballs (Fig. 1B) confirmed fluorescein penetration through the full thickness of the epithelium

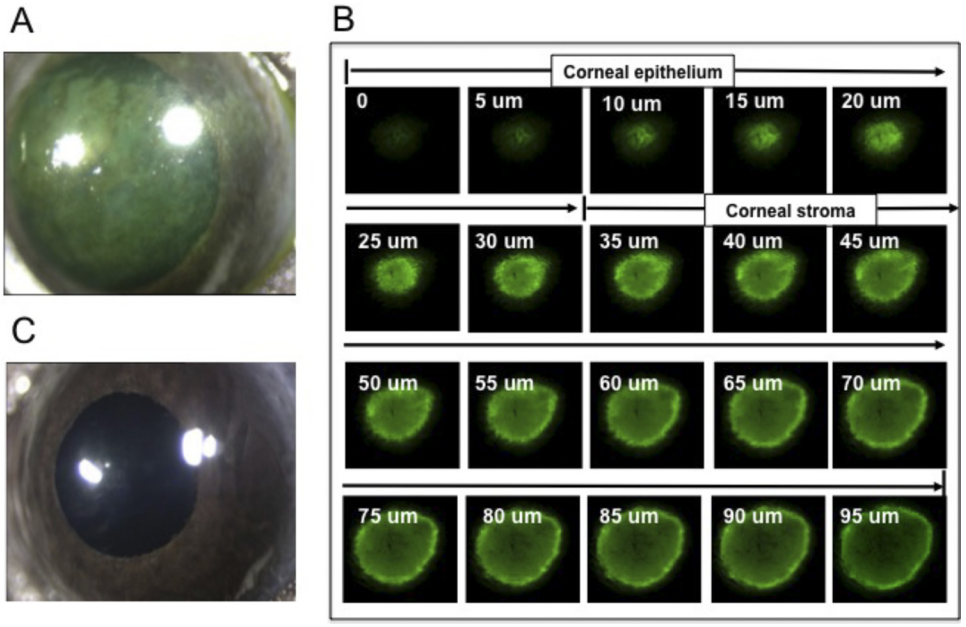

FIGURE 1. Impact of tissue paper blotting on corneal susceptibility to fluorescein staining and infection. Fluorescein was added to wild-type (C57BL/6) corneas immediately after the blotting procedure. Blotted corneas showed extensive fluorescein staining (**A**). Confocal microscopy *z*-stack of blotted and fluorescein-stained mouse cornea showed fluorescein penetration of the corneal epithelium and stroma to a depth of approximately 100 μM (**B**). Other blotted corneas were immediately inoculated with GFP-expressing *P. aeruginosa* strain PAO1. Blotting did not enable susceptibility to infection at 24 hours after infection (**C**).

and into the corneal stroma (100 μm below the corneal surface). As expected, fluorescein did not stain the corneal epithelium of control (unblotted) wild-type mice in vivo (data not shown).

Fluorescein staining in humans is often used as a means to assess corneal epithelial integrity, but its specific relationship to epithelial barrier function against microbes and its susceptibility to infection has not been established. To explore this, blotted (fluorescein positive) and non-blotted (fluorescein negative) corneas of wild-type mice were compared for their susceptibility to infection. Eyes were inoculated with *P. aeruginosa* and were then monitored for at least 4 weeks for evidence of pathology. None of the eyes became infected (scores of 0 in each mouse in each group) despite the use of large inocula ($\sim 10^9$ CFU bacteria in 5 μL; Fig. 1C). This strain of *P. aeruginosa* (PAO1) is virulent in the corneal scarification/healing model, even if used at significantly lower inocula.[27] Histologic evaluation of a blotted murine cornea (Fig. 2A) showed that the corneal epithelium remained multilayered after blotting and appeared to have normal architecture (Fig. 2B).

Tissue Paper Blotting Allows *P. aeruginosa* to Attach to, but Not Traverse, the Corneal Epithelium In Vivo and Ex Vivo

As expected, bacterial adherence was not detected on the surfaces of unblotted healthy corneas of wild-type mice inoculated in vivo for 5 hours with concentrated bacterial suspension (Fig. 3A). In contrast, corneas that were blotted with tissue paper before inoculation became regionally susceptible to bacterial attachment (Fig. 3B). Quantification of bacterial adherence to blotted corneas (28 random sections were used for each mouse) revealed 3.53 $\pm$ 0.38 bacteria per section versus none for corneas that were not blotted.

Although bacteria could adhere to blotted corneas, they were unable to penetrate beyond the superficial surface. This was confirmed using a large number of sections over multiple random regions of each cornea.

We previously reported that tear fluid can inhibit bacterial traversal of cultured corneal epithelial cells in vitro.[13] Thus, we tested the hypothesis that tear fluid was inhibiting the penetration of adherent bacteria in vivo. To exclude tear fluid from the inoculation step, eyes were removed immediately after rinsing and blotting, and the entire eyeball was placed into 200 μL concentrated bacterial suspension (approximately 10^{11} cfu/mL) for 5 hours. This ex vivo inoculation method yielded the same results as in vivo inoculation—that is, bacteria adhered only to blotted corneal epithelia (not to

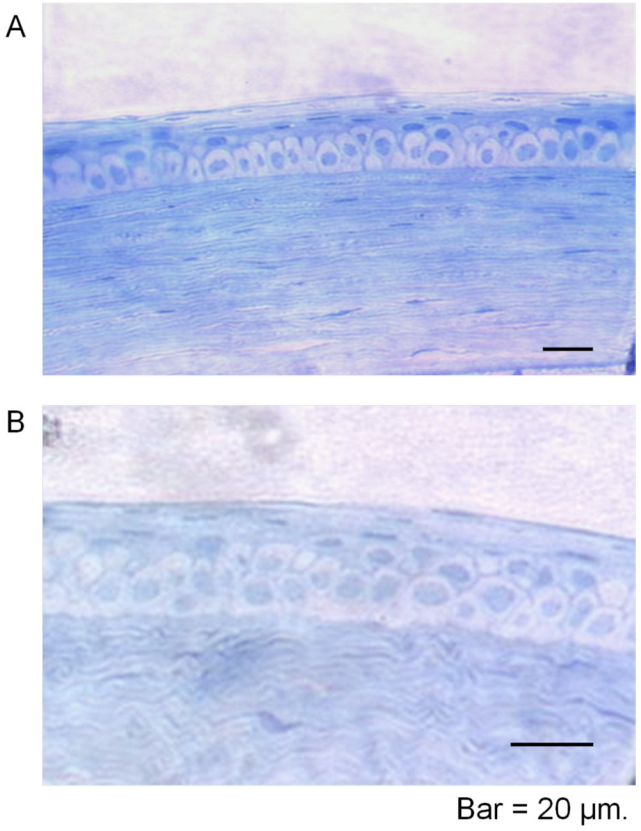

FIGURE 2. (**A**) Transverse section through rinsed and tissue paper–blotted mouse cornea (C57BL/6). Toluidine-blue staining illustrated that the corneal epithelium remained multilayered and morphologically indistinguishable from a normal healthy cornea (**B**) after these treatments.

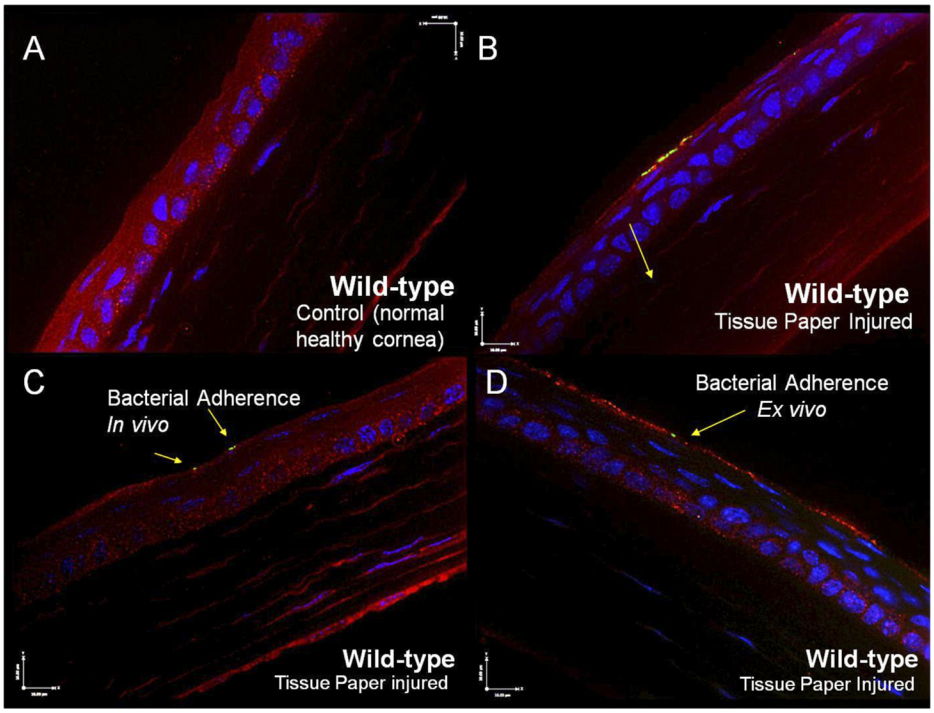

FIGURE 3. (**A**, **B**) Bacterial distribution within wild-type (C57BL/6) cornea inoculated with GFP-expressing *P. aeruginosa* strain PAO1for 5 hours in vivo, as assessed using fluorescence imaging. Healthy corneas were clear of inoculated bacteria (**A**). Blotted corneas showed bacterial adherence to the superficial surface (*arrow*), but bacteria were not detected below the surface within deeper layers of the corneal epithelium (**B**). (**C**, **D**) Impact of ex vivo inoculation on susceptibility of the blotted cornea to *P. aeruginosa* penetration. The impact of excluding tear fluid was examined by comparing in vivo inoculation to inoculation of excised eyeballs in vitro. Corneas of wild-type (C57BL/6) mice were tissue paper blotted, then inoculated with GFP-expressing *P. aeruginosa* strain PAO1 (*green*) either in vivo (**C**) or after excision ex vivo (**D**) for 5 hours. In both situations, bacteria adhered to the corneal epithelium (*arrows*) but did not traverse. Scale bar, 10 μm. *Green*: bacteria (GFP); *blue*: cell nuclei (DAPI); *red*: laminin (rhodamine).

unblotted corneas) but did not penetrate beyond the epithelial surface (Figs. 3C, 3D). Remarkably, bacteria still showed minimal penetration of blotted wild-type corneas even when the incubation time was extended to 8 hours (see later experiments, Fig. 6E).

EGTA Treatment after Blotting Disrupts ZO-1 Staining and Enables Epithelial Susceptibility to Bacterial Traversal

The fact that blotting results in fluorescein penetration into the stroma under the corneal epithelium shows that barriers to this molecule are disrupted by the blotting process even though barriers to bacteria remain. An obvious difference between fluorescein and bacteria is their relative size, raising the possibility of size-selective barriers within the corneal epithelium. Indeed, a previous study demonstrated the presence of proteins normally associated with calcium-dependent tight junctions within suprabasal regions of the corneal epithelium,[15] and the authors proposed that the region might function as a barrier to leukocyte trafficking.

Thus, blotted eyeballs were subsequently treated for 1 hour with EGTA solution (100 mM in PBS), with EGTA a Ca^{2+} chelator capable of disrupting junctional barriers and other calcium-dependent processes. In contrast to PBS-pretreated (1-hour) blotted controls, which showed only bacterial adherence to the epithelial surface (Fig. 4A), EGTA-pretreated, blotted corneas permitted deep bacterial penetration through the epithelium (Figs. 4B–D). This result showed that there are calcium-dependent factors that can function as a barrier to bacterial traversal (but not to fluorescein) that remain after blotting.

To begin to explore the mechanism for EGTA-mediated loss of barrier function to bacteria, we next studied the impact of EGTA on the distribution of ZO-1, a tight-junction–associated protein, using immunohistochemistry. Results confirmed that ZO-1 was localized between cells in deeper layers of the corneal epithelium of blotted, PBS-treated, control corneas (Fig. 4E) and that ZO-1 localization became diffused by EGTA treatment after blotting (Fig. 4F). These findings suggested that there could have ZO-1–containing junctions between cells that functioned to limit bacterial penetration.

Subsequent quantification of *P. aeruginosa* penetration of similarly treated corneas ex vivo using confocal microscopy clearly showed enhanced bacterial penetration of corneas treated with EGTA (100 mM in PBS, 1 hour) compared with PBS controls (Fig. 5).

SP-D Participates in Defending the Corneal Epithelium against Bacterial Penetration In Vivo

We previously reported that SP-D is expressed and secreted by corneal epithelial cells in vivo and in vitro,[20] is upregulated in response to *P. aeruginosa* antigens in vitro,[28] inhibits bacterial invasion of cells in vitro,[20] and is involved in the clearance of *P. aeruginosa* from the healthy ocular surface.[4] SP-D activities are dependent on calcium.[29] Thus, we explored the role of SP-D in epithelial barrier function to bacteria.

As expected, control wild-type mice remained resistant to *P. aeruginosa* traversal after blotting (Fig. 6A). In contrast, the epithelium of age- and background-matched (6- to 8-week-old C57BL/6) SP-$D^{-/-}$ mice became permissive to bacterial penetration (Figs. 6B–D). While bacteria were found below the superficial surface, bacteria did not traverse as deeply in the time frame of the experiments as they did after EGTA treatment (which enabled some bacteria to penetrate to the level of the basal lamina), suggesting that calcium-dependent factors other than SP-D likely contributed to barrier function in wild-type mice.

In other experiments, *P. aeruginosa* traversal of wild-type versus SP-$D^{-/-}$ tissue paper–blotted corneas was quantified ex vivo using confocal microscopy (Fig. 6E). Although in a different background (Black Swiss) from in vivo experiments and over a longer (8-hour) period, a similar pattern of results was obtained, that is, *P. aeruginosa* showed greater traversal of SP-$D^{-/-}$ than did wild-type blotted corneas.

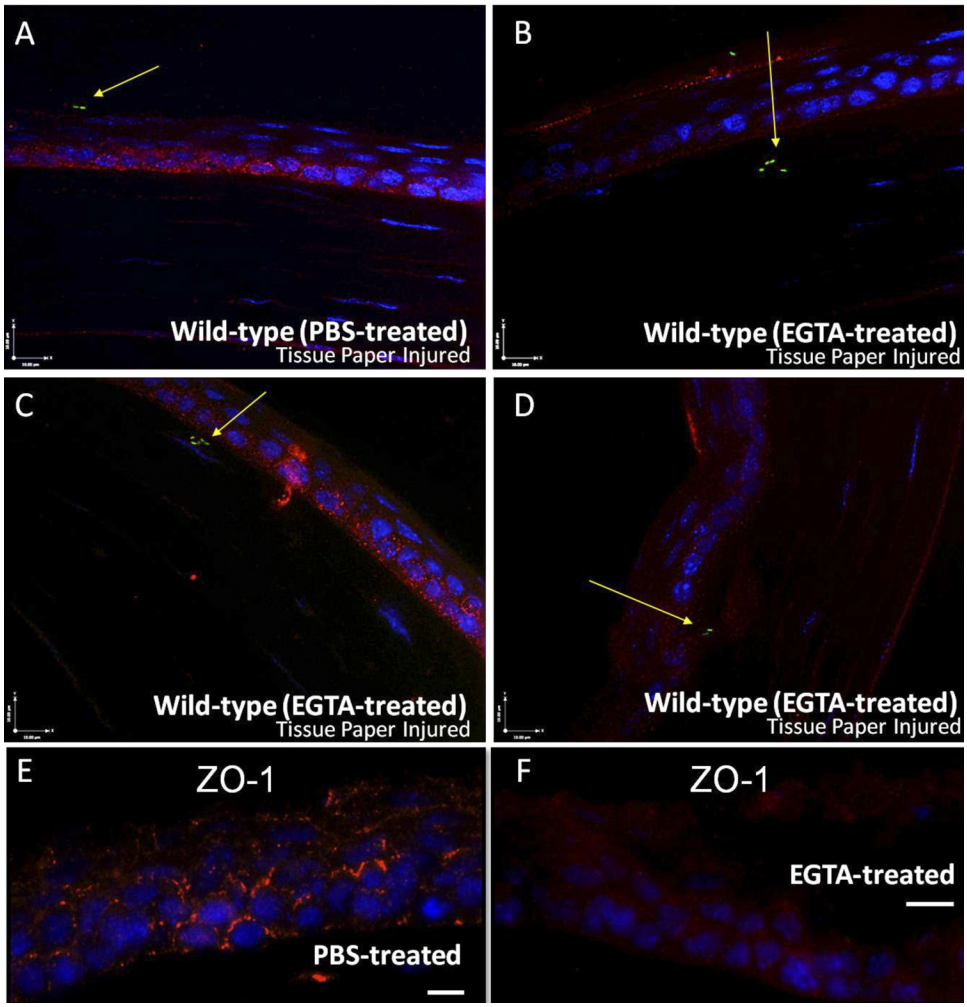

Figure 4. (**A–D**) Impact of EGTA pretreatment on the susceptibility to *P. aeruginosa* traversal. Blotted murine (C57BL/6) eyeballs were pretreated ex vivo for 1 hour with PBS (**A**) or with 100 mM EGTA in PBS (**B–D**) before incubation with GFP-expressing bacteria. After 5 hours, significant numbers of bacteria (*arrows*) traversed the corneal epithelia of EGTA-pretreated, but not PBS control, eyes. Scale bar, 10 μm. *Green*: bacteria (GFP); *blue*: cell nuclei (DAPI); *red*: laminin (rhodamine). (**E**, **F**) Immunofluorescence (ZO-1 labeling) of cryosectioned mouse corneas. Enucleated, blotted, and PBS rinsed murine (Black Swiss) eyeballs were pretreated ex vivo for 1 hour with PBS or with100 mM EGTA in PBS. Blotted, PBS-treated corneas showed ZO-1 labeling throughout the epithelium (**E**), which was diffused in the blotted, EGTA-pretreated corneas (**F**). Scale bar, 10 μm. *Blue*: nuclei (DAPI); *red*: ZO-1 (rhodamine).

Susceptibility to Keratitis in Aged Mice Using Methods That Enable Bacteria to Traverse the Corneal Epithelium

Although both EGTA treatment and SP-D deficiency disrupted the epithelial barrier to bacteria in blotted corneas, neither method enabled susceptibility to actual disease in vivo in young (6- to 8-week-old) wild-type animals (data now shown). Thus, we explored the impact of combining these treatments with the goal of developing an infection model that minimizes invasive manipulation while determining parameters that define resistance versus susceptibility to disease initiation.

Corneas of wild-type and SP-D$^{-/-}$ C57BL/6 mice were tissue paper blotted then treated with EGTA for 3 hours before rinsing and inoculation with *P. aeruginosa* (see Methods). This combination of treatments in 6- to 8-week-old mice did not result in infection in any of the animals tested (data not shown). Thus, the same set of experiments was carried out using aged mice (approximately 35–36 weeks old), which are known to have reduced inflammatory and immune responses to *P. aeruginosa* keratitis.[30]

With aged mice, some eyes in both the wild-type and the SP-D–deficient groups showed evidence of keratitis at 1 day after inoculation (Figs. 7, 8). Although similar numbers were infected in both groups, there were differences between them both in disease severity and in disease progression over time. Wild-type mice tended to remain stable or recovered. In contrast, SP-D knockout mice continued to worsen to severe disease (Fig. 8). Occasionally, eyes did not develop infection in either group. Repeat experiments showed almost identical patterns of disease progression and scores, with significant differences between SP-D disease scores (more severe) and wild-type on each day (Fig. 8).

Thus, superficial blotting, EGTA treatment, and older age combined were sufficient to enable susceptibility to disease in a percentage of animals without a need for scarification. SP-D deficiency was not required for disease initiation, but it contributed to making disease more severe and progressive. These data, which show a difference in disease severity at later time points for SP-D–deficient mice, are consistent with results of a separate study that also explored the role of SP-D but that used a different infection model (scarification) and a different mouse (Black Swiss) background.[31]

Discussion

In this study, we investigated factors influencing barrier function of the corneal epithelium to *P. aeruginosa* in vivo and ex vivo by introducing subtle forms of injury/compromise and studying their impact. Results showed that superficial blotting of otherwise healthy mouse corneas using tissue paper was sufficient to enable fluorescein to penetrate the stroma and *P. aeruginosa* to adhere but was not sufficient to allow *P. aeruginosa* to penetrate the epithelium and subsequently cause keratitis. Bacterial traversal did occur when blotted corneas were then EGTA treated before the addition of bacteria or if SP-D knockout animals were used, but neither situation resulted in actual pathology in young (6- to 8-week-old) mice. Visible

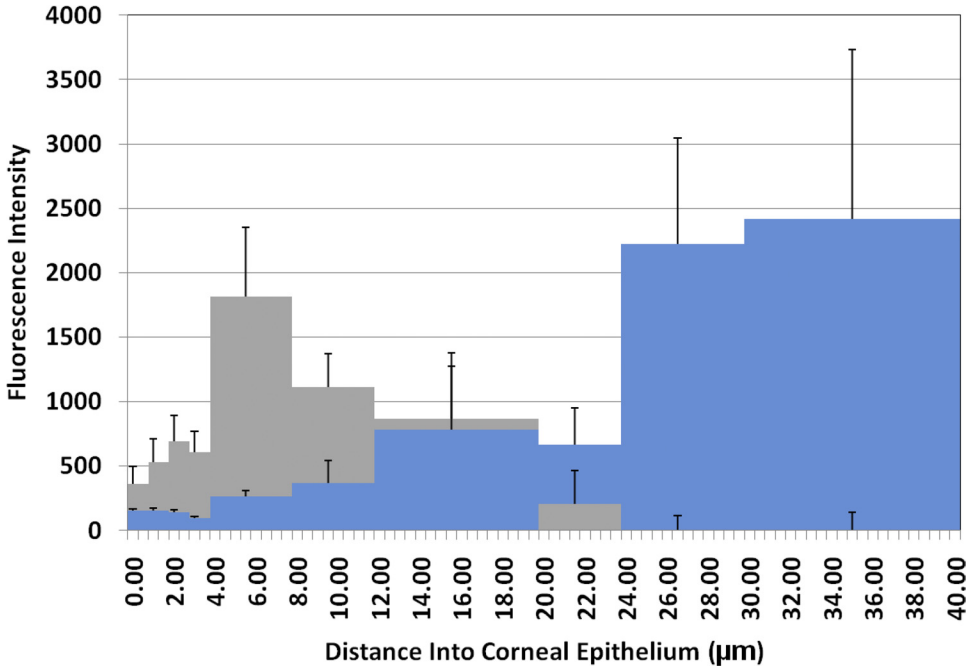

FIGURE 5. Quantification of GFP-labeled *P. aeruginosa* penetration of the blotted murine cornea (Black Swiss) ex vivo with and without EGTA pretreatment. After euthanatization, eyes were enucleated, tissue paper blotted, and PBS rinsed before pretreatment ex vivo for 1 hour with PBS or EGTA (100 mM in PBS). After removal of the EGTA solution and a PBS wash, eyes were incubated with GFP-expressing *P. aeruginosa* (200 μL of a concentrated bacterial suspension approximately 10^{11} cfu/mL). After 6 hours of exposure to bacteria, confocal microscopy showed greater bacterial traversal of the corneal epithelium in EGTA-pretreated eyes (*blue*) compared with PBS-treated controls (*gray*).

pathology did occur in aged mice when corneas were blotted and EGTA treated before inoculation, but sustained disease occurred only in aged, blotted, EGTA-treated mice that were also SP-D deficient. These results highlight that defenses against infection in the cornea are extremely robust and multifactorial and that there is significant redundancy built into the system. This is consistent with the requirement for overt injury (i.e., scarification)[7,32–34] or stromal injection to bypass the epithelial barrier to establish corneal infection in previously described animal models.

Fluorescein staining is commonly used as a clinical tool to indicate compromise to the corneal epithelium[35–37] and is often assumed to correlate with a higher risk of corneal infection. A key finding of this study was that extensive fluorescein staining could occur without susceptibility to infection. Similarly, our earlier studies showed that mouse corneas in the process of healing after scratch injury recovered resistance to keratitis within 12 hours despite continued fluorescein staining.[27] Using a rat model, we recently reported that fluorescein staining induced by tissue paper blotting does not reduce the timing, rate, or severity of contact lens–induced infection.[38] We have also collected unpublished data (2010) that show that dry eye-induced fluorescein staining does not correlate with susceptibility to infection. Thus, it appears (at least in rodent models) that fluorescein staining is an unreliable predictor of susceptibility to infection in multiple circumstances that include superficial injury, healing after deep penetrating injury, dry eye, and contact lens wear.

Although blotting did not enable bacteria to infection, it did render the normally resistant corneal epithelium susceptible to bacterial adherence. Factors at the ocular surface that function to limit bacterial adhesion include secreted and membrane-associated mucins.[18,22] It is likely, therefore, that the mechanism by which blotting enables adhesion is by removal or otherwise compromise of the mucin barrier or other cell surface–associated antiadhesion factors.[4,20–22,39] However, blotting could also remove surface epithelial cells (as does impression cytology) and thereby expose incompletely differentiated cells with cell surfaces that have not yet acquired mature surface properties[40] and are consequently more susceptible to bacterial adhesion. The fact that bacterial adhesion to blotted corneas occurred in patches rather evenly across the corneal surface suggests this possibility.

We previously reported that *P. aeruginosa* can traverse corneal epithelial cells grown in vitro[13] but that human tear fluid can protect against this and other virulence strategies of *P. aeruginosa*.[14,13,20,21] Results of the present study showed that mouse corneal epithelium could resist *P. aeruginosa* traversal even when blotted to enable bacterial adhesion and even if tear fluid (and other in vivo factors) were excluded by inoculating the eyeball in vitro. Thus, corneal epithelial cells exposed to the in vivo environment likely express additional defenses that are protective against *P. aeruginosa* traversal compared with cells grown in culture in vitro. Putative additional defenses could be deposited from tear fluid or they could be made by cells in response to in vivo factors. Indeed, we have found that exposure of human corneal epithelial cells to human tear fluid for 6 hours induces profound changes to gene expression (unpublished data, 2010). Further, we recently reported[12] that corneal epithelial cells grown on basement membrane proteins (similar composition to the basal lamina under the corneal epithelium in vivo) showed increased resistance to *P. aeruginosa* and that though much of this effect was due to direct "filtering" by the basement membrane, we also observed retention of bacteria at the apical cell surface.

Defenses against bacterial traversal within the corneal epithelium could include either physical or biochemical barriers. The data showed that SP-D$^{-/-}$ mice were more susceptible to traversal, showing SP-D contributes either directly or indirectly to defense. We previously showed SP-D is upregulated by *P. aeruginosa* antigens (flagellin and LPS)[28] and that it can protect against *P. aeruginosa* invasion of epithelial cells.[20] In addition, it can have direct antimicrobial activity against *P. aeruginosa*.[41] Additional work will be needed to determine which, if any, of these activities contributes to the mechanisms by which SP-D modulates *P. aeruginosa* traversal in the corneal epithelium.

The calcium chelator EGTA compromised defense against traversal. Given that SP-D activities are calcium dependent,[29] the mechanism for EGTA effects could include loss of SP-D activity. However, the effects of EGTA on traversal were more extreme than the effects of SP-D depletion; thus other calcium-

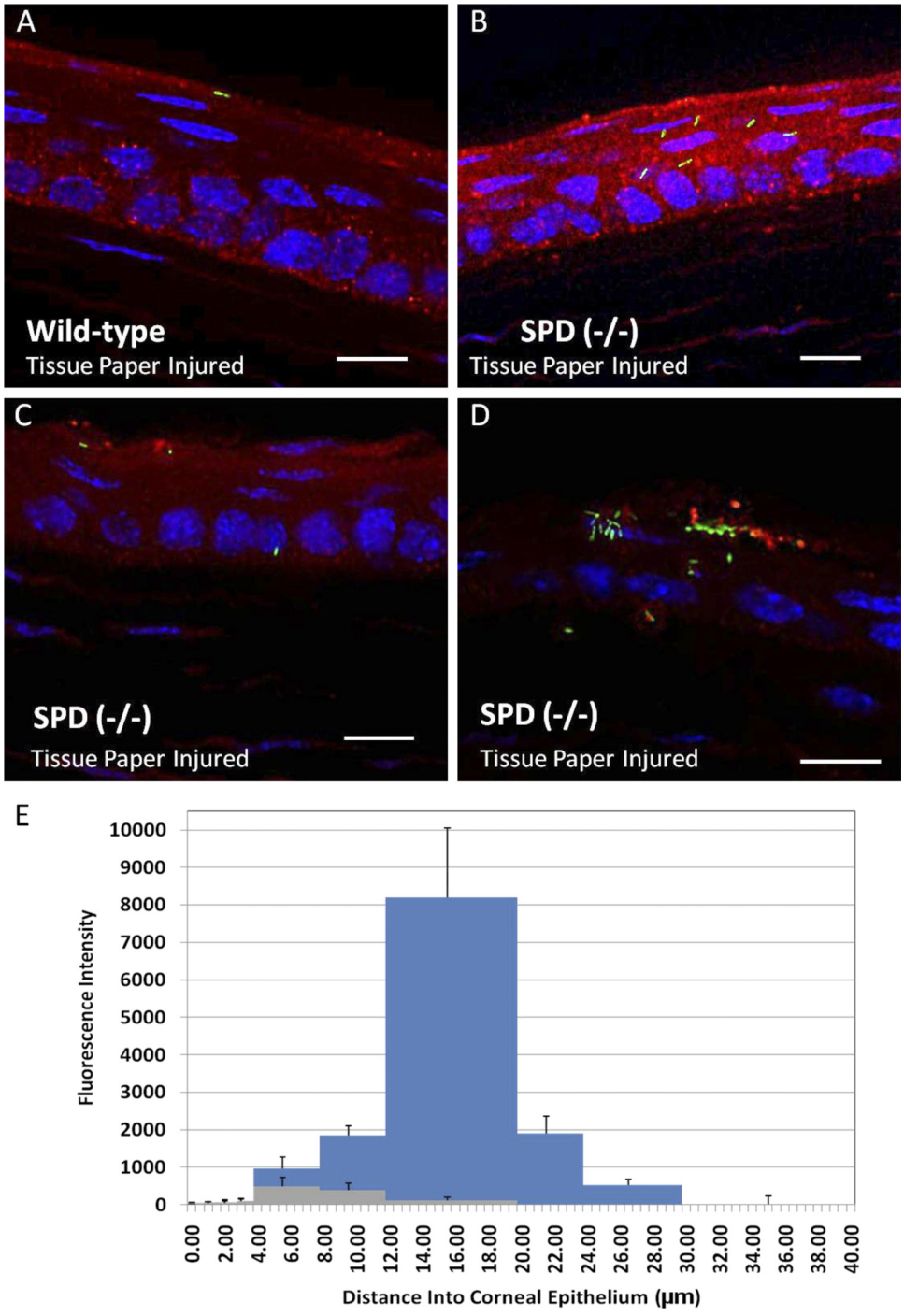

Figure 6. (**A–D**) Impact of SP-D on epithelial susceptibility to *P. aeruginosa* traversal. Corneas of wild-type C57BL/6 (**A**) and SP-D$^{-/-}$ (**B–D**) mice were tissue paper blotted before inoculation in vivo with GFP-expressing *P. aeruginosa* strain PAO1. Fluorescence microscopy showed that after 5 hours, bacteria had partially traversed the corneal epithelium of SP-D$^{-/-}$, but not wild-type, mice. Scale bar, 10 μm. (*green*) Bacteria (GFP). *Blue*: cell nuclei (DAPI); *red*: laminin (rhodamine). (**E**) Quantification of GFP-labeled *P. aeruginosa* penetration of a blotted cornea ex vivo comparing wild-type (Black Swiss) and SP-D$^{-/-}$ mice. After euthanatization, eyes were enucleated, tissue paper blotted, and PBS rinsed before incubation with GFP-expressing *P. aeruginosa* (200 μL of a concentrated bacterial suspension approximately 10^{11} cfu/mL). After 8 hours of exposure to bacteria, confocal microscopy showed greater bacterial traversal of the corneal epithelium in SP-D$^{-/-}$ eyes (*blue*) compared with wild-type (*gray*).

dependent factors may also be involved in defense against traversal. Many cellular functions, including some regulating responses to pathogens, can be calcium dependent. These include Ca^{2+}-dependent tight junction complexes found within the epithelium of the cornea[15] and other tissues,[42] thought to provide physical barriers to the passage of cells and molecules. Disruption of junctions by EGTA could have enabled paracellular transport of *P. aeruginosa* or it might have increased cellular susceptibility to *P. aeruginosa* internalization by exposing basolateral cell surfaces.[16,43] Indeed, EGTA used at the same concentrations and conditions that allowed *P. aeruginosa* traversal also diffused ZO-1, a tight junction associated protein that was localized between cells throughout the epithelium in blotted corneas that were not EGTA treated. Although detection of ZO-1 between cells in deeper layers of the epithelium does not prove the existence of functional junctions in that region capable of excluding the passage of bacteria, EGTA diffusion of the ZO-1 correlating with reduced resistance to traversal suggests the possibility is worthy of further investigation. Indeed, the existence of suprabasal junctions functioning as barriers to the passage of molecules and cells has been previously proposed by others.[15]

Although our data show that *P. aeruginosa* has the capacity to traverse the corneal epithelium under certain conditions of compromise, the relative contributions of internalization and paracellular transport remain to be elucidated. Cellular internalization by *P. aeruginosa* can occur in vivo and in vitro,[16,44] and mutants defective in cellular exit after internalization have

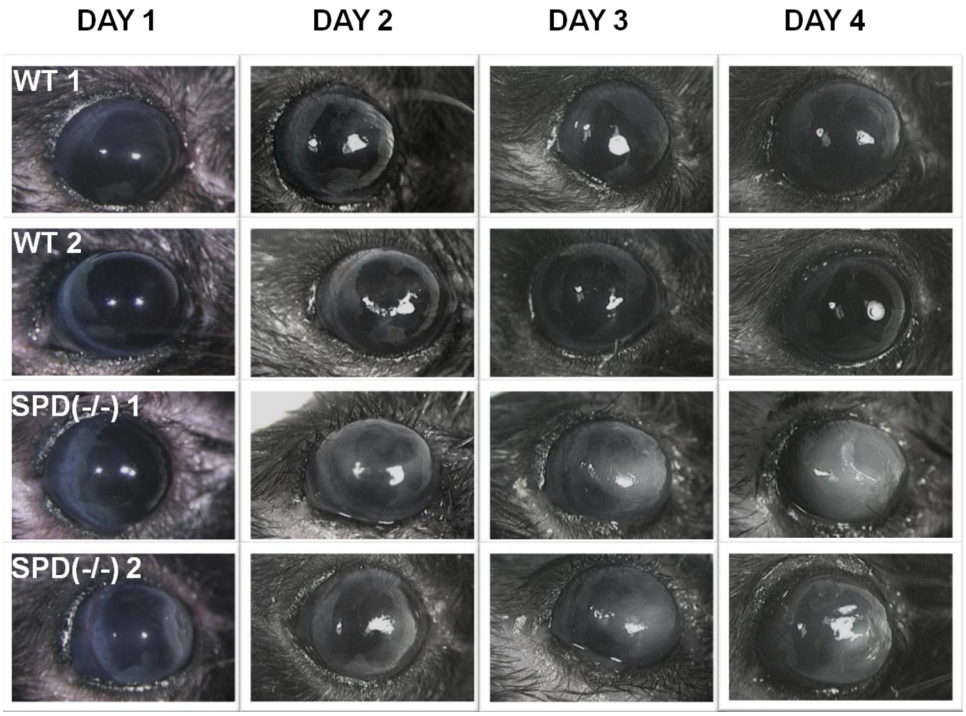

FIGURE 7. Examples of corneal disease found in aged (35–36 weeks) wild-type (C57BL/6) mice and SP-D$^{-/-}$ mice 1 to 4 days after inoculation with *P. aeruginosa* strain PAO1 (approximately 10^9 cfu in 5 μL). Corneas were blotted and then treated with EGTA (100 mM in PBS) for 3 hours before inoculation. Two-millimeter cutouts from a soft contact lens were used to retain EGTA at the ocular surface for the 3-hour period. Disease in SP-D$^{-/-}$ mice became progressively more severe with time; in contrast, wild-type eyes with visible pathology at 24 hours showed little further progression or recovered.

reduced capacity for epithelial traversal in vitro.[11] Interestingly, it has recently been shown that *P. aeruginosa* can alter epithelial polarity[17] to convert apical surfaces to a basolateral phenotype, and it can also disrupt epithelial tight junction complexes to allow the traversal of airway epithelial monolayers.[45] Although these virulence strategies might have augmented effects of EGTA and SP-D deficiency observed in the present study to enable bacteria to penetrate the corneal epithelium, they alone were not sufficient to initiate the traversal of either normal or blotted corneal epithelium in vivo or ex vivo.

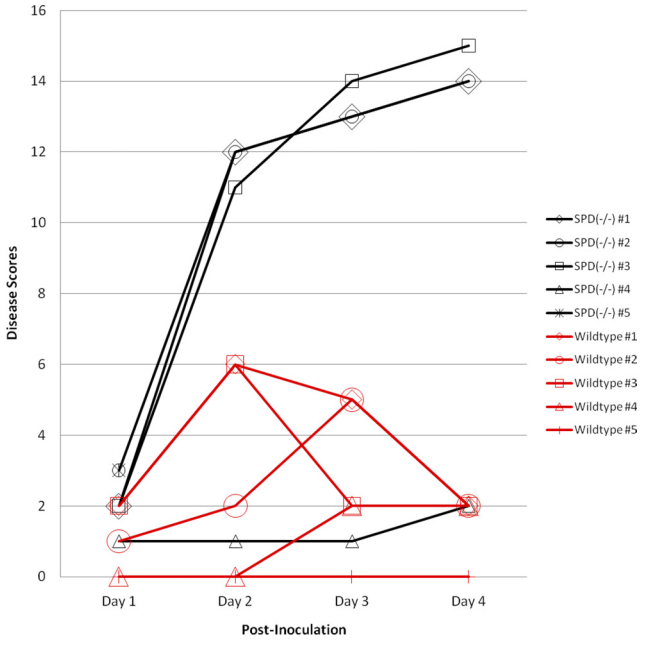

FIGURE 8. Progression of corneal disease (individual disease scores) in aged mice (C57BL/6) compared with aged-matched SP-D$^{-/-}$ mice after inoculation with *P. aeruginosa* (approximately 10^9 cfu in 5 μL). Before inoculation, eyes were pretreated with EGTA (Fig. 7). Significant differences in disease scores were observed between wild-type and SP-D$^{-/-}$ groups on each day (*t*-tests: day 1, $P = 0.016$; day 2, $P = 0.027$; day 3, $P = 0.045$; day 4, $P = 0.03$). One mouse (SP-D$^{-/-}$ mouse 5) died after day 1.

With the exception of the aged mouse, keratitis did not develop in vivo under any of the conditions tested despite the enabling of epithelial traversal by blotting and EGTA treatment. Lack of disease in young animals might relate to the fact that the basal lamina under the epithelium functions as another barrier to bacteria.[12] When intact, this basement membrane limits the number of traversing bacteria that access the stroma, likely explaining the need for overt scarification or stromal injection to enable disease caused by *P. aeruginosa* in young mice. Age-related changes in epithelia can influence barrier function,[46] including the loss of a Reg (regulatory) protein from the epithelium and the basement membrane.[47]Also of possible relevance, aged mice have been shown to have greater susceptibility and reduced immunity to *P. aeruginosa* than younger animals, correlating with decreased proinflammatory cytokine and ICAM-1 expression.[30]

SP-D deficiency did not increase the likelihood of disease initiation, but it increased disease severity at later time points (Figs. 7, 8). The lack of a role at early time points is not altogether surprising considering that corneas were EGTA treated and that activities of SP-D are calcium dependent.[29] Increased severity after disease initiation in SP-D$^{-/-}$ mice at later time points (when EGTA no longer had an influence) could relate to the known binding, aggregative, opsonic, and immunomodulatory activities of SP-D.[29,48] A previous study that used the scarification model to induce *P. aeruginosa* keratitis also found increased disease severity at later time points in SP-D$^{-/-}$ animals.[31]

It was interesting that wild-type mice tended to recover after disease initiation considering that C57BL/6 mice progress to perforation when infection is induced using the scarification model. Whether this discrepancy relates to disparate immunologic responses when infection is induced using different

methods or to differences in the ages of the mice is to be determined.

In conclusion, we have developed and used novel in vivo methods for studying epithelial traversal by bacteria and the defenses that protect against it. The results highlight the multifactorial and redundant nature of corneal epithelial defenses against bacterial traversal and subsequent infection. Indeed, they show that neither fluorescein staining nor microbe adhesion necessarily predict epithelial susceptibility to microbe traversal and that, in turn, epithelial traversal does not necessarily predict susceptibility to actual disease. Defenses against traversal were found to depend on SP-D and were susceptible to EGTA. Studies expanding on the mechanisms by which these manipulations impact epithelial interactions with bacteria should improve our understanding of how epithelia normally protect against infection when healthy and of the mechanisms involved in susceptibility during compromise. They could also lead to the development of new interventions to prevent the initiation of infection at epithelial surfaces. The most common cause of *P. aeruginosa* infection in the cornea is contact lens wear, which follows use of contact lenses that sit at the corneal epithelium/tear interface, sometimes in association with various chemicals. The relevance of the data contained within this report to the pathogenesis of contact lens–related infections is to be determined.

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