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Mutation of the Phospholipase Catalytic Domain of the *Pseudomonas aeruginosa* Cytotoxin ExoU Abolishes Colonization Promoting Activity and Reduces Corneal Disease Severity

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Abstract

We have previously shown that ExoU, a type III secreted cytotoxin of *Pseudomonas aeruginosa*, causes acute cytotoxicity towards corneal epithelial cells *in vitro*, and contributes to corneal disease pathology and ocular colonization *in vivo*. Subsequently, we reported that ExoU represses phagocyte infiltration of infected corneas *in vivo*. ExoU has patatin-like phospholipase activity that is required for cytotoxic activity *in vitro* (mammalian cell injury and death) and for disease in a murine model of pneumonia. We hypothesized that the phospholipase activity was required for ExoU-mediated corneal disease and ocular colonization. Using the murine scarification model, corneal disease pathology was examined after inoculation with ~10^6 cfu of a *P. aeruginosa* effector mutant (PA103Δ*exoU*e*xoT*::Tc) complemented with either *exoU* (pUCP*exoU*), phospholipase-inactive *exoU* (pUCP*exoU*D344A) or a plasmid control (pUCP18). Eyes were photographed and disease severity scored at 24 and 48 h post-infection. Viable bacteria colonizing infected eyes were quantified at 6 and 48 h. Complementation with *exoU* caused significantly more pathology (increased disease severity scores) and enabled bacteria to better colonize (by ~1000-fold) at 48 h as compared to phospholipase-inactive *exoU* which did not differ from plasmid control. Surprisingly, *exoU* did not contribute to early (6 h) colonization. *In-vitro* assays confirmed that the phospholipase domain of *exoU* was required for cytotoxicity towards human corneal epithelial cells. Taken together these data show that the phospholipase activity of the *P. aeruginosa* cytotoxin, ExoU, plays a role in the pathogenesis of corneal infection *via* mechanism(s) occurring after initial colonization of a susceptible cornea.

Keywords

P. aeruginosa; keratitis; type III secretion; ExoU; phospholipase; virulence

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1. Introduction

Pseudomonas aeruginosa keratitis is an acute sight-threatening infection that can occur as a complication of soft contact lens wear or corneal injury (Cheng et al., 1999; Alexandrakis et al., 2000; Lam et al., 2002; Watt and Swarbrick 2005; Verhelst et al., 2006; Bharathi et al., 2007; Pachigolla et al., 2007). Corneal isolates of *P. aeruginosa* can be divided into invasive or cytotoxic strain types based upon how they interact with epithelial cells (Fleiszig et al., 1996; Fleiszig et al., 1997; Cowell et al., 2003). We have shown previously that cytotoxic isolates are genetically different from invasive strains in that they encode the type III secreted toxin ExoU (Fleiszig et al., 1997). Possession of the *exoU* gene was identified as one of several virulence traits encoded by epidemic clones of *P. aeruginosa* isolated from patients with bacterial keratitis (Lomholt et al., 2001).

ExoU is delivered into mammalian cells upon contact with bacteria, and is required for acute (within 3 h) injury and death of several mammalian cell types *in vitro*, including epithelial cells and macrophages (Fleiszig et al., 1996; Fleiszig et al., 1997; Garrity-Ryan et al., 2000; Finck-Barbancon and Frank 2001). Cytotoxic isolates can also kill epithelial cells on the surface of intact corneas *ex vivo* (Fleiszig et al., 1998). Using a murine scarification model of keratitis, we previously demonstrated that ExoU also makes a significant contribution to ocular colonization and corneal disease, and that it represses phagocyte infiltration of the central region of infected corneas *in vivo* (Lee et al., 2003; Zolfaghar et al., 2006). Others have shown that complementation of *exoU* into an invasive strain of *P. aeruginosa* enhances its virulence in a murine model of acute pneumonia (Allewelt et al., 2000).

It has been shown that ExoU exhibits a patatin-like phospholipase activity (Sato et al., 2003; Sato and Frank 2004; Sato et al., 2005) in combination with host factors, e.g. superoxide dismutase (Sato et al., 2006). Multiple regions of ExoU have been found to be required for cytotoxic activity (Rabin and Hauser 2005). The N-terminal domain of the protein encodes the phospholipase activity. Mutations of the catalytic serine (S142) or aspartate (D344) residues abolishes both the phospholipase activity and cytotoxicity towards mammalian and yeast cells (Phillips et al., 2003; Sato et al., 2003; Rabin and Hauser 2005). Mutation of the N-terminal region of ExoU also reduces *P. aeruginosa* virulence of in a murine model of acute pneumonia (Pankhaniya et al., 2004).

Here, we hypothesized that ExoU-mediated colonization and disease-promoting activity in the cornea would require its phospholipase activity. This was tested by comparing corneal disease pathology and ocular colonization by a double effector mutant of the cytotoxic *P. aeruginosa* strain PA103 (PA103Δ*exoUexoT::Tc*) complemented with a functional *exoU* gene on the plasmid pUCP18 (pUCP*exoU*) with disease caused by the same strain when complemented with *exoU* containing a mutation in the catalytic aspartate region of the phospholipase domain (pUCP*exoU*D344A), or a control (pUCP18). Testing in an effector null background was done to remove any potential complicating factors due to ExoT, another type-III secreted effector encoded by strain PA103, that we have previously shown to act redundantly with ExoU in contributing to *P. aeruginosa*-induced pathology in the cornea (Lee et al., 2003).

2. Materials and Methods

2.1. Bacteria

The experiments described in this study were done using an effector null mutant of *P. aeruginosa* strain PA103 (PA103Δ*exoUexoT*::Tc) (PA103ΔUT). This mutant lacks both ExoU and ExoT, the two effectors of the type III secretion system known to be produced by this strain (Vallis et al., 1999). The PA103ΔUT mutant was complemented with plasmid

pUCP18 containing either the *exoU* gene (pUCP*exoU*) which fully restores cytotoxic activity towards eukaryotic cells, or the *exoU* gene in which the aspartate catalytic site of the N-terminal phospholipase domain is mutated (pUCP*exoU*D344A), and thus it lacks phospholipase and *in vitro* cytotoxic activity, or an empty vector control (pUCP18) (Sato et al., 2003). Plasmid complemented mutants were grown on trypticase soy agar (TSA) supplemented with carbenicillin 300 μg/ml overnight (~18 h) at 37 °C. For use in experiments, bacteria were resuspended in tissue culture medium (equal parts DMEM/Hams F12) to an optical density (at 650 nm) of ~0.1 to 0.2 corresponding to a viable count of ~2–3 × 10^8 cfu/mL. Control experiments in this, and previous studies (Vallis et al., 1999; Lee et al., 2003) have confirmed that these plasmid-complemented strains grow equally well *in vitro* with or without exposure to mammalian cells.

2.2 Corneal epithelial cell culture and in vitro cytotoxicity assay

Telomerase-immortalized human corneal epithelial cells (HCEC) were cultured in 24-well tissue culture plates as previously described (Robertson et al., 2005). Cytotoxic activity of *P. aeruginosa* towards HCEC was examined by Trypan Blue staining (Fleiszig et al., 1996) after 5 h exposure to ~10^6 cfu of the plasmid-complemented PA103ΔUT mutants of *P. aeruginosa* strain PA103. For cytotoxicity assays bacteria were suspended in KBM cell culture medium (Robertson et al., 2005).

2.3. Murine corneal infection and P. aeruginosa colonization

The murine scarification model of infectious keratitis was used (Preston et al., 1995). C57/BL6 mice (5–12 weeks old) were anesthetized by intraperitoneal infection with an anesthetic cocktail (21 mg/mL ketamine, 2.4 mg/mL xylazine and 0.3 mg/mL acepromazine). Eyes were checked for corneal clarity using a stereomicroscope prior to the initiation of experiments. Three parallel scratches (~1mm in length) were made on the right cornea of each animal using a sterile 25 5/8-guage needle, and eyes topically inoculated with a bacterial suspension containing ~10^6 cfu in 5 μl of DMEM/Hams F12. Four mice were assigned to each treatment group. Animals were observed daily, and overall disease severity was graded in a masked fashion after 24 h and 48 h using the following scoring system (Beisel et al., 1983); Grade 0, eye macroscopically identical to the uninfected contralateral control eye; Grade 1, faint opacity partially covering the pupil; Grade 2, dense opacity covering the pupil; Grade 3, dense opacity covering the entire anterior segment; Grade 4, perforation of cornea and /or phthisis bulbi (shrinkage of the eyeball following inflammatory disease). Eyes were also graded using another 5-point grading system (grade 0 = no infection to grade 4 = severe infection) that assessed three different characteristics of the disease (Cowell et al., 1999). Thus, scores were assigned to the area and density of the opacity and the epithelial surface quality. The calculated sum of scores for these three characteristics ranged from a possible 0 (clear, normal) to a maximum of 12. Eyes were photodocumented using an Optronics 3-chip cooled camera (Goleta, CA) attached to a Zeiss Stemi 2000-C dissecting microscope (Jena, Germany).

To quantify bacterial colonization, mice that had been infected for 48 h were euthanatized, and infected eyes enucleated and homogenized in PBS (1 ml) containing Triton X-100 (0.25 % vol/vol). Viable counts were performed on homogenates using TSA containing carbenicillin 300ug/ml to select plasmid-bearing bacteria. We have previously observed that this plasmid is retained by *P. aeruginosa in vivo* over 48 h (Lee et al., 2003). In other experiments, mice were sacrificed at 6 h after infection to explore the role of ExoU and its phospholipase activity on early bacterial colonization at a point prior to significant immune cell infiltration. All procedures were conducted in accordance with the policies established by the Association for the Research in Vision and Ophthalmology, and were approved by the University of California, Berkeley Animal Care and Use Committee.

2.4 Statistical analysis

Data were expressed as a mean with standard deviation, and differences between groups compared for statistical significance using ANOVA with Fisher PLSD and Scheffe F-test post-hoc analysis. Differences in disease severity scores were compared using the Kruskal-Wallis and Mann-Whitney non-parametric tests. P values < 0.05 were considered significant. All experiments were repeated at least once.

3. Results

3.1 Phospholipase-inactive ExoU is not cytotoxic towards HCEC in vitro

Trypan blue staining confirmed that the ΔUT mutant of *P. aeruginosa* strain PA103 complemented with vector control (pUCP18) was not cytotoxic towards cultured human corneal epithelial cells (Fig. 1A). As expected, complementation with pUCP*exoU*, but not the phospholipase-inactive form of *exoU*, restored normal cytotoxic activity to the ΔUT double mutant (Fig. 1B and C respectively).

3.2 The Phospholipase domain of ExoU is required for effects on disease severity

At both 24 and 48 h, disease caused by bacteria complemented with *exoU* was of significantly increased severity compared to that involving the phospholipase-inactive form of *exoU* (pUCP*exoU*D344A) or the vector control (pUCP18) ($p = 0.04$ at 24 h, $p = 0.004$ at 48 h, Kruskal-Wallis Test) (Tables 1 and 2, Fig. 2). Indeed, disease involving the phospholipase-inactive form of *exoU* did not differ from that caused by bacteria complemented with the plasmid control at both time points ($p > 0.05$, Mann-Whitney test). These results were confirmed with both the 4-point overall grading system (Table 1) and the multiplex scoring system (Table 2). Representative photographs of infected eyes at 24 and 48 h (Fig. 2) illustrate differences in disease severity caused by bacteria expressing phospholipase-active or –inactive forms of *exoU*.

A peripheral ring infiltrate, which we have previously reported to require ExoU, was observed only in those eyes infected with bacteria expressing intact *exoU* (Zolfaghar et al., 2006).

3.3 Loss of phospholipase activity abolishes ExoU-mediated corneal colonization at 48 h, but does not affect early (6 h) colonization

Mutation of the phospholipase active region of *exoU* was associated with a significant (~1000-fold) reduction in ocular colonization at 48 h ($p < 0.05$, ANOVA, Fig. 3). Indeed, there was no significant difference between the phospholipase-inactive form of *exoU* and the vector (plasmid) control at this time. In stark contrast, at 6 h post-inoculation, there was no significant difference in ocular colonization between bacteria complemented with vector control and either the phospholipase-active or the inactive forms of *exoU* ($p > 0.05$, ANOVA). In the absence of ExoU phospholipase activity, and its associated promotion of colonization noted at 48 h, there was little difference between colonization levels at 6 and 48 h post-infection (Fig. 3).

4. Discussion

We have previously shown that ExoU is involved in the ability of *P. aeruginosa* to colonize the cornea *in vivo* and to cause pathology (Lee et al., 2003) which involves inhibition of phagocyte infiltration of the central cornea (Zolfaghar et al., 2006). This followed our earlier work showing that ExoU was responsible for the acute cytotoxicity of cytotoxic strains of *P. aeruginosa* towards mammalian cells *in vitro* (Finck-Barbancon et al., 1997; Fleiszig et al., 1997). In this study we show that the mechanism for ExoU-mediated cytotoxicity towards

corneal epithelial cells *in vitro*, and its promotion of ocular colonization and corneal disease pathology by 48 h *in vivo*, requires the phospholipase activity of this toxin.

ExoU has been shown to cause acute cell death of numerous types of mammalian cell including epithelial cells, fibroblasts, and even professional phagocytes *in vitro* (Finck-Barbancon et al., 1997; Fleiszig et al., 1997; Garrity-Ryan et al., 2000; Finck-Barbancon and Frank 2001; Evans et al., 2002). Thus, the *in-vivo* role of ExoU may involve its ability to kill host cells. For example, killing of corneal epithelial cells might increase bacterial binding to damaged epithelia, or it could increase the ability of bacteria to translocate the epithelium *in vivo*. Killing of phagocytes would reduce the capacity of the host to clear bacteria.

Considering that cytotoxic strains of *P. aeruginosa* can rapidly kill corneal epithelial cells *in vitro* (Fleiszig et al., 1996) and also when they are on the surface of intact corneas *ex vivo* (Fleiszig et al., 1998), it was interesting that we were not able to demonstrate a role for ExoU in early bacterial colonization (6 h). It is possible that epithelial cytotoxicity of *P. aeruginosa* is not required in this particular model of keratitis in which the epithelium is already damaged/breached by needle injury. It is also possible that in the absence of alterations to the ocular surface biochemistry, that likely occur in both contact lens- and dry eye-related infection, the cytotoxic effects of ExoU towards corneal epithelial cells are inhibited by natural *in vivo* factor(s). Indeed, we have demonstrated that human tear fluid can protect corneal epithelial cells from ExoU-mediated killing *in vitro* (Fleiszig et al., 2003), and protect both injured and healing murine corneas from cytotoxic *P. aeruginosa* infection *in vivo* (Kwong et al., 2007).

We previously described the inflammatory infiltrate induced by PA103ΔUT, wild-type PA103 and mutants in known TTSS effectors a murine model of infectious keratitis (Zolfaghar et al., 2006). In that study, ExoU was responsible for repression of phagocyte infiltration of infected corneas at 48 h *in vivo*, correlating with promotion of bacterial colonization, and with disease severity scores and appearance. Eyes infected with bacteria encoding *exoU* showed a distinctive “ring” infiltrate (also seen with some human corneal infections) that was consistent with the accumulation of phagocytes at the peripheral regions of the cornea and their complete absence from the central cornea. In contrast, eyes infected with *exoU* mutants showed dense phagocyte infiltration of the central cornea. In the present study, we found that the phospholipase activity of ExoU was required for enabling efficient colonization by 48 h. In addition, the “ring” infiltrate was only observed in eyes infected with bacteria complemented with phospholipase-active *exoU*. The loss of colonization, reduction in disease severity, and loss of ring infiltrate with phospholipase mutant ExoU to a level similar to background PA103ΔUT suggested that the use of more animals to do further histological experiments was not justifiable. Considering that ExoU’s phospholipase activity is required for cytotoxic activity, and for colonization at 48 h but not at 6 h time points, these data suggest that the *in vivo* contribution of ExoU towards corneal virulence involves cytotoxic effects on infiltrating neutrophils and monocytes.

It is also possible, however, that the effects of ExoU on later (48 h) colonization are also a consequence of earlier events. For example, cytotoxic effects of ExoU on epithelial cells at earlier time points, if they do occur, could hinder the production of proinflammatory cytokines that contribute to the chemotactic “driving force” for phagocyte infiltration and persistence in the infected murine cornea at later time points (Rudner et al., 2000; Thakur et al., 2002; Xue et al., 2003; Hazlett 2007; Willcox 2007).

In conclusion, our earlier work established a role for ExoU as an important contributor to corneal disease involving cytotoxic strains of *P. aeruginosa*. Here we show that the colonization and disease promoting activities of this toxin require the domain encoding its

phospholipase activity. Inhibiting this activity could form the basis for a novel therapeutic intervention in *P. aeruginosa* keratitis albeit in conjunction with other therapeutic interventions which target the contributions of ExoU-independent virulence mechanisms, e.g. proteases, towards the pathogenesis of *P. aeruginosa* keratitis.

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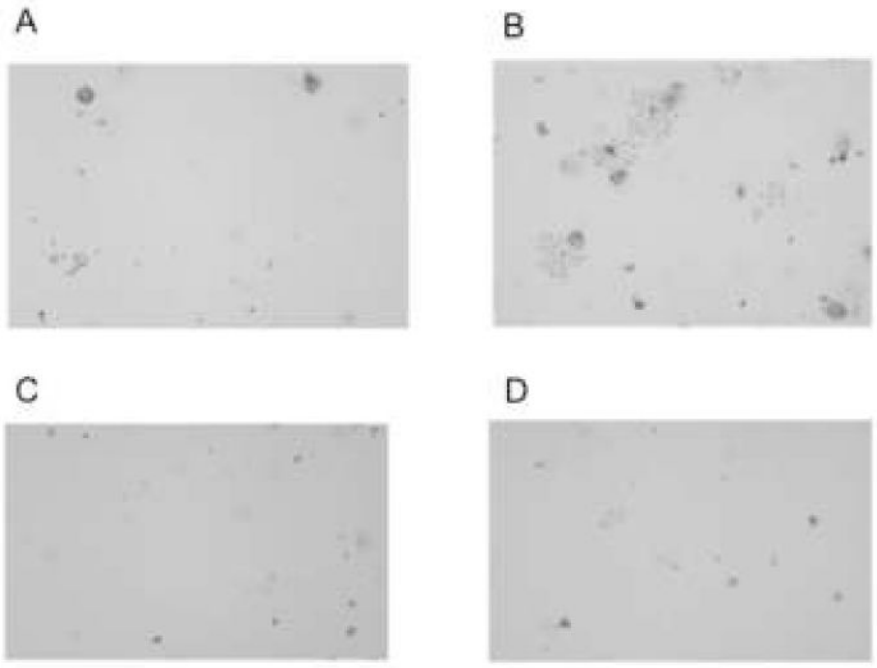

Figure 1.
Trypan blue staining (dead or dying cells) of cultured HCEC after 5 h exposure to ~1 × 10^6 cfu strain PA103Δ*exoUexoT*::Tc (PA103ΔUT) complemented with empty vector pUCP18 (A), pUCP*exoU* (B), the phospholipase-inactive mutant pUCP*exoU*D344A (C) or media (KBM) only (D). ExoU-complemented bacteria caused a typical pattern of cell death. Bacteria complemented with phospholipase-inactive ExoU caused little or no cell death similar to vector and media only controls. Images were adjusted using Adobe Photoshop to equate brightness and contrast.

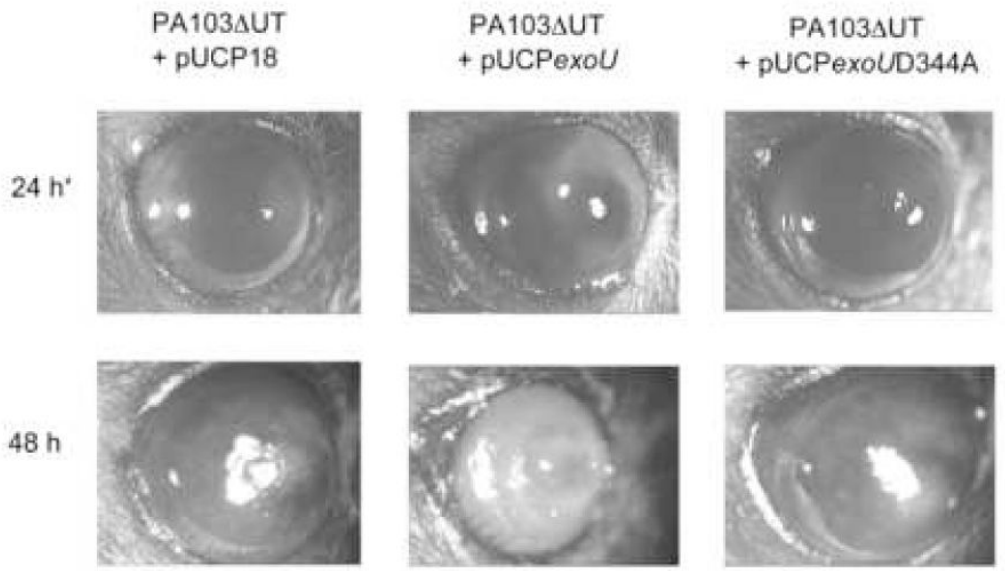

Figure 2.

Overall disease pathology in the murine scarification model of *P. aeruginosa* keratitis at 24 h and 48 h after inoculation with 5 μl of cell culture medium containing ~1×10^6 cfu strain PA103Δ*exoUexoT*::Tc (PA103ΔUT) complemented with pUCP18, pUCP*exoU*, or the phospholipase mutant pUCP*exoU*D344A. Note the ring infiltrate of the eyes infected with bacteria expressing *exoU* (Zolfaghar et al., 2006). *24 h images were equally adjusted for brightness and contrast using Adobe Photoshop to match backgrounds of 48 h data.

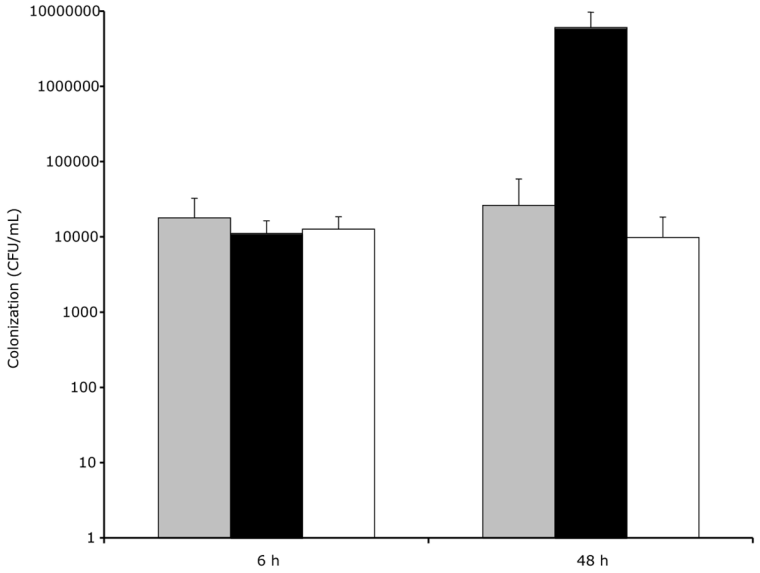

Figure 3.

Ocular colonization in the murine scarification model of *P. aeruginosa* keratitis at 6 h and 48 h (separate experiments) post-inoculation with 5 μl of medium containing ~1×10^6 cfu strain PA103Δ*exoUexoT*::Tc (PA103ΔUT) complemented with pUCP18 (gray), pUCP*exoU* (black), or pUCP*exoU*D344A (white). At 48 h, bacteria complemented with phospholipase-inactive *exoU* showed significantly reduced colonization compared to *exoU*-complemented bacteria ($p < 0.05$, ANOVA and Fisher PLSD and Scheffe F-test post-hoc analysis). At 6 h, there were no significant differences between the groups ($p > 0.05$, ANOVA and by Fisher PLSD and Scheffe F-test post-hoc analysis)

Table 1

Overall disease severity scores (see methods) in the murine scarification model of *P. aeruginosa* keratitis after inoculation with 5 μl of cell culture medium containing ~1 × 10^6 cfu strain PA103Δ*exoU**exoT*::Tc (PA103ΔUT) complemented with pUCP18 (control), pUCP*exoU*, or the phospholipase mutant pUCP*exoU*D344A.

Time	PA103ΔUT + pUCP18	PA103ΔUT + pUCP*exoU*	PA103ΔUT + pUCP*exoU*D344A
24 h*	1, 1, 1, 0	1, 2, 2, 2	0, 1, 1, 0
48 h*	2, 2, 2, 2	3, 3, 3, 3	2, 2, 2, 2

* Disease involving pUCP*exoU* was of significantly increased severity compared to both vector control and phospholipase catalytic mutant at each time point (p = 0.04 at 24 h; p = 0.004 at 48 h, Kruskal-Wallis Test).

Table 2

Scoring individual characteristics of ocular disease severity (see methods) in the murine scarification model of *P. aeruginosa* keratitis after inoculation with 5 μl of cell culture medium containing ~1×10^6 cfu strain PA103Δ*exoUexoT*::Tc (PA103ΔUT) complemented with pUCP18, pUCP*exoU*, or pUCP*exoU*D344A.

	Total Disease Scores at 48 h [= Area + Density + Epithelial Quality] Each Scored From 0 to 4, Possible Maximum = 12		
	PA103ΔUT + pUCP18	**PA103ΔUT + pUCP*exoU****	**PA103ΔUT + pUCP*exoU*D344A**
Mouse 1	8 [4+2+2]	10 [4+3+3]	9 [4+3+2]
Mouse 2	8 [4+2+2]	10 [4+4+2]	8 [4+3+1]
Mouse 3	9 [4+3+2]	10 [4+4+2]	7 [3+3+1]
Mouse 4	8 [4+2+2]	11 [4+4+3]	7 [3+3+1]

* Disease involving pUCP*exoU* was of increased overall severity compared to the phospholipase catalytic mutant ($p = 0.017$, Mann-Whitney Test) which was not different from control ($p = 0.350$, Mann-Whitney Test).