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Harnessing the Anti-infective Activity of the Ocular Surface to Prevent Infection

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Harnessing the Anti-infective Activity of the Ocular Surface to Prevent Infection

by

James J. Mun

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University of California, Berkeley

Committee in charge:

Suzanne M.J. Fleiszig, Chair

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Lu Chen

Samuel Hawgood

Spring 2010

The dissertation of James J. Mun, titled, Harnessing the Anti-infective Activity of the Ocular Surface to Prevent Infection, is approved by:

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Abstract

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Approved:

Chair:_____ Date:_____

Abstract

Bacterial keratitis, a common infection in contact lens wearers, can lead to blindness caused by pathogenic and host factors. Seventy percent of bacterial keratitis cases are caused by the *Pseudomonas aeruginosa*, a gram-negative bacterium. Fortunately, a healthy cornea provides a defense system against bacterial invasion. Understanding the mechanism of how a healthy maintains its health would be beneficial in providing therapies for corneal infections and more importantly, in providing preventative measures.

My thesis project comprises of two parts: 1) To understand the role of surfactant protein-D (SP-D) in providing protection against *P. aeruginosa* colonization on the healthy eye *in vivo*; 2) To study the effect of tear fluid on corneal epithelial cells and to identify tear fluid-modulated corneal cell defenses.

We showed that healthy murine eyes clear both cytotoxic and invasive *P. aeruginosa* strains efficiently and that SP-D, present in human tear fluid, aided in this clearance. Our studies also revealed that protease mutants were more efficiently cleared from the healthy ocular surface as opposed to its parent strain. Experiments to study mechanisms for these differences revealed that purified elastase could degrade tear fluid SP-D *in vivo*. Together, these data showed that SP-D can contribute to the clearance of *P. aeruginosa* from the healthy ocular surface and that proteases can compromise that clearance. The data also suggested that SP-D degradation *in vivo* is a mechanism by which *P. aeruginosa* proteases could contribute to virulence.

Human tear fluid is known to increase transepithelial resistance of corneal epithelia *in vitro* and protects them against *P. aeruginosa* invasion, cytotoxicity and translocation but only retards the growth of some *P. aeruginosa* strains. However, we have also found that if a murine eye is removed and suspended in bacteria without tear fluid, the corneal epithelium remains resistant to *P. aeruginosa* translocation. This suggested the importance of tear fluid and corneal epithelial cells' interaction for protection. Through my thesis project, we discovered that pre-exposing the corneal epithelial cells to tear fluid is sufficient to induce protective activity against *P. aeruginosa* invasion and cytotoxicity. Our bacteriostatic study involving corneal cell lysates fractionated by size, showed that fraction < 3 kDa is bactericidal and that Histatin 5 (< 3 kDa), a potent antimicrobial whose expression is induced by tear fluid, may be a contributing bactericide. Furthermore, our microarray data revealed that RNase7, another potent antimicrobial heavily studied in human skin, and ST2, a member of the IL1R family, are upregulated with tear fluid, suggesting the protective role of tear fluid by modulating corneal cell defenses.

This thesis demonstrates using a novel "null-infection" model (in which the cornea is not damaged prior to inoculation with *P. aeruginosa*) that very large inoculation of *P. aeruginosa* is rapidly cleared from the healthy ocular surface *in vivo* and that SP-D plays a signification role in this protection from bacterial colonization. Furthermore, we demonstrate here that tear fluid provides protection by modulating corneal cell defenses. Understanding the roles of corneal cell defenses that provide protection against microbes in a healthy eye environment is essential in developing potential strategies for bacterial keratitis due to contact lens wear in the future.

for my wife Annie Mun and my future children if the Lord willing
&
my parents Hyung Dai and Eun Sook Mun

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How can I also forget my wonderfully caring parents. My parents moved to the United States for my sister's and my education. My parents' prayers, their hard work, and their efforts to look out for what's best for their children are truly unfathomable. They always thought of less of themselves for our sake, and I truly am grateful for their unconditional love towards me. Without their support and their upbringing, I would not be the man that I am today.

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Chapter 1: Introduction and Literature Review

1.1 Preface of Chapter

Bacterial keratitis (Figure 1), a common infection in contact lens wearers, can lead to blindness caused by pathogenic and host factors (51, 91). There are over 30 million contact lens wearers in the United States (5), and each year, 1 out of 2500 people, who wear soft, hydrogel contact lenses on a daily basis, and 1 out of 500 people, who wear contact lenses for extended periods develop bacterial keratitis (88). Seventy percent of bacterial keratitis cases are caused by *Pseudomonas aeruginosa*, a gram-negative bacterium (86). The ultimate goal of my research is to better understand how contact lens wear makes the patient more susceptible to developing bacterial keratitis. The following chapter provides the background from which the basic scientific investigation of this study was developed.

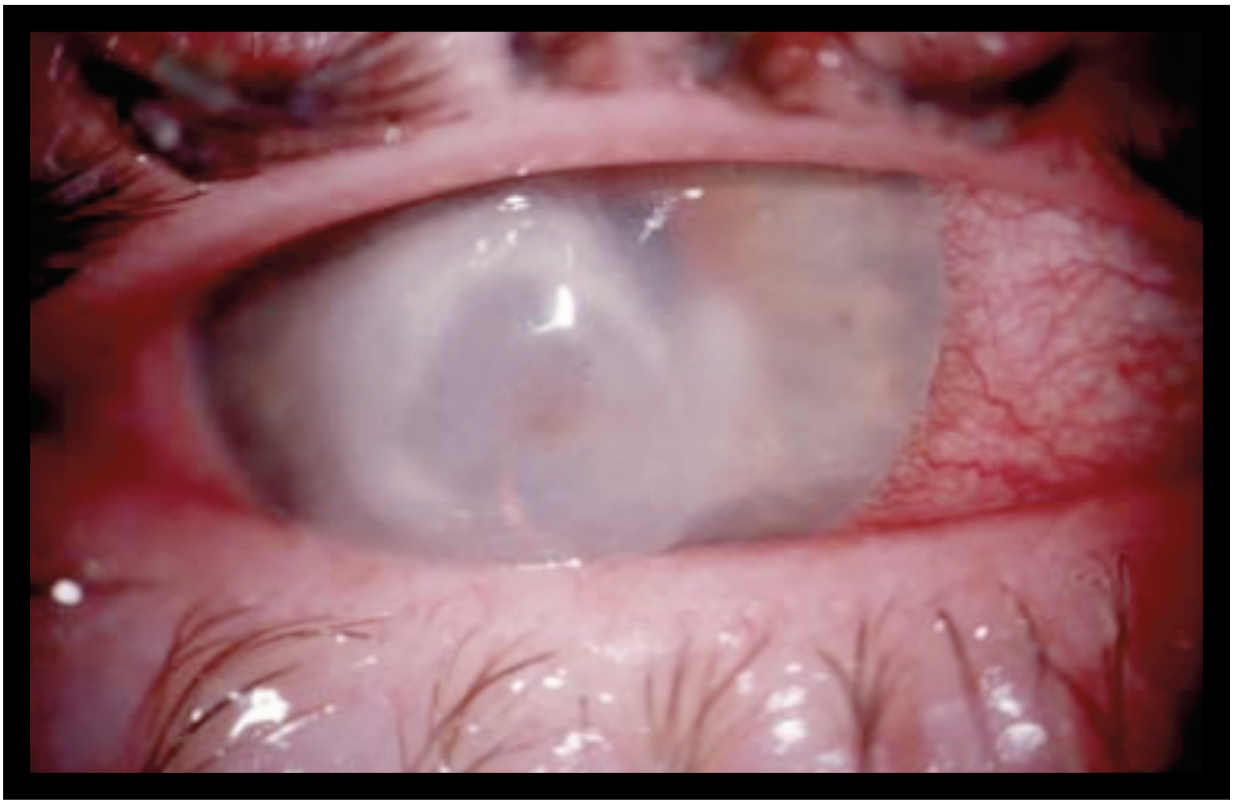


Figure 1. Severe case of *P. aeruginosa* keratitis.

1.2 Corneal Infection and *Pseudomonas aeruginosa*

P. aeruginosa keratitis can lead to destruction and opacity of the cornea caused by bacterial toxins and the immune response of the host (21, 45). Before soft contact lenses were introduced, gram-positive bacteria were the main cause of infectious keratitis followed by corneal injury or disease. However, with an increasing number of soft contact lens wearers, Gram-negative *P. aeruginosa* corneal infection became most common (15, 75, 92). Many healthy patients with no history of corneal injury or disease were showing contact lens related bacterial keratitis, and this correlation between soft contact lens wear and *P. aeruginosa* keratitis is still persistent (31, 74). Soft contact lenses create a favorable environment for opportunistic *P. aeruginosa* to take advantage of the ocular surface.

Since hypoxic factors were hypothesized to be the cause of infectious keratitis, silicone hydrogel lenses were developed, which allow physiological levels of oxygen to reach the ocular surface. However, such lenses have not significantly reduced the contact lens related bacterial keratitis (17, 87, 90). Also, Lin *et al.* have shown that the corneal epithelial barrier is significantly suppressed by both high and low oxygen permeable soft lenses, suggesting that the epithelial barrier may be affected by mechanical stress rather than hypoxic factors (59). This indicates that hypoxia is not required for infection, but it does not rule out hypoxia as a factor to the disease.

In addition, because many groups have attempted to fight *P. aeruginosa* corneal infection with various antibiotics, antibiotic resistant strains were isolated from the ocular surface (2, 8, 33). Clearly, different therapeutic methods are necessary to stop bacterial keratitis. By having a better understanding of the early events in the progression of this disease, we can provide therapeutic methods to prevent the onset of microbial keratitis.

1.3 Tear Film and the Cornea

The tear film comprised of a lipid layer, an aqueous layer and a mucus layer protects the outer surface of the cornea. The mucus layer, secreted by the goblet cells, is the largest component of the tear film and has glycoproteins called mucins. Interactions between hydrophobic and hydrophilic molecules in the mucus layer facilitate the association between the hydrophobic corneal surface with the aqueous layer of the tear film. The aqueous layer, produced mainly by the acinar cells of the lacrimal and other accessory glands, is comprised of various proteins and electrolytes, including immunoglobulin A, lactoferrin and lysozyme. The lipid layer made by the meibomian glands, helps to stabilize the tear film by lowering the surface tension and evenly spreading the tear film during blink cycles. This lipid layer also helps to prevent the evaporation of the aqueous layer of the tear film (32).

The cornea is comprised of five layers: epithelium, Bowman's membrane, stroma, Descemet's membrane and the endothelium (Figure 2). The epithelium is about 50 μm thick and usually has 5 to 7 layers of cells. The bottom layer of the epithelium, also known as the basal layer, has a single layer of columnar cells. The basal layer moves toward the superficial layer and are replaced by new cells from the limbus. The middle cell layers are composed of wing cells, which eventually become flatter as they move up towards the superficial layer. The superficial layers have large, nonkeratinized, flat cells. The surface layer has microprojections called microvilli covered by glycoproteins, called glycocalyx, which help to maintain the tear film on the corneal surface. The epithelial cells are held together by interdigitations and desmosomes and by zonula occludens, also known as tight junctions. Desomosomes lie between adjacent cell membranes, and tight junctions, where protein layers of cell membranes are fused together, are located at the superficial layer.

The basement membrane of the epithelium attaches the epithelium to Bowman's membrane. Fibrils from the basement membrane anchor the basement membrane to Bowman's membrane, and the epithelial cells are attached to the basement membrane by hemidesomosomes. Bowman's membrane is about 8 to 14 μm thick and is part of the stroma. This membrane has irregularly arranged collagen fibers and is acellular. When this layer is injured, it does not regenerate and a scar remains.

The thickest component of the cornea is the stroma comprised of fibroblasts and bands of parallel collagen fibrils called lamellae, that run from limbus to limbus. The stroma receives its nutrition mostly by diffusion from the aqueous humor and the limbus. Descemet's membrane, which is about 10 to 15 μm thick, is a modified basement membrane of the endothelium and creates some resistance to trauma due to its elasticity. Lastly, the endothelium, a monolayer of flattened cells connected by desmosomes and tight junctions, is about 5 μm thick and is attached to Descement's membrane by hemidesomosomes. When these cells are injured, they are replaced by the flattening of adjacent cells without cell regeneration (32).

Maintenance of corneal transparency is crucial for vision so the cornea has a unique and regular arrangement of collagen fibers in the stroma to decrease light scattering. The Benedek and Farrell model for transparency suggests that fibril density must be roughly uniform and that the distance between the fibrils is small and closely spaced. This arrangement reduces light scatter that happens if the interfibrillar distance is greater than 200 nm. In order to maintain its transparency, specific stroma thickness has to be maintained. Glycosylated glycoproteins called glycosaminoglycans (GAGs) create an osmotic force drawing in water from the tear film and the aqueous humor. GAGs also play an essential role of maintaining normal arrangement of collagen fibrils. However, if GAGs draw in too much water, the stroma thickness can increase, causing

increased distance between the fibrils, leading to corneal opacity. In order to ensure that the cornea does not become over-hydrated, tight junctions of the epithelium and endothelium prevent fluid influx. However, there are also active processes, such as cellular pumps, that constantly pump water out of the cornea to maintain proper hydration. Sodium/potassium pumps on the Descement's membrane remove sodium. Sodium simultaneously comes into the endothelium through a co-transporter that also brings in bicarbonate ions. As the concentration of bicarbonate increases, it leaks out on the aqueous side, causing water to leak out as well. The chloride channels on the superficial epithelial layer facing the tear film cause chloride ions, as well as water, to leave the epithelium (32).

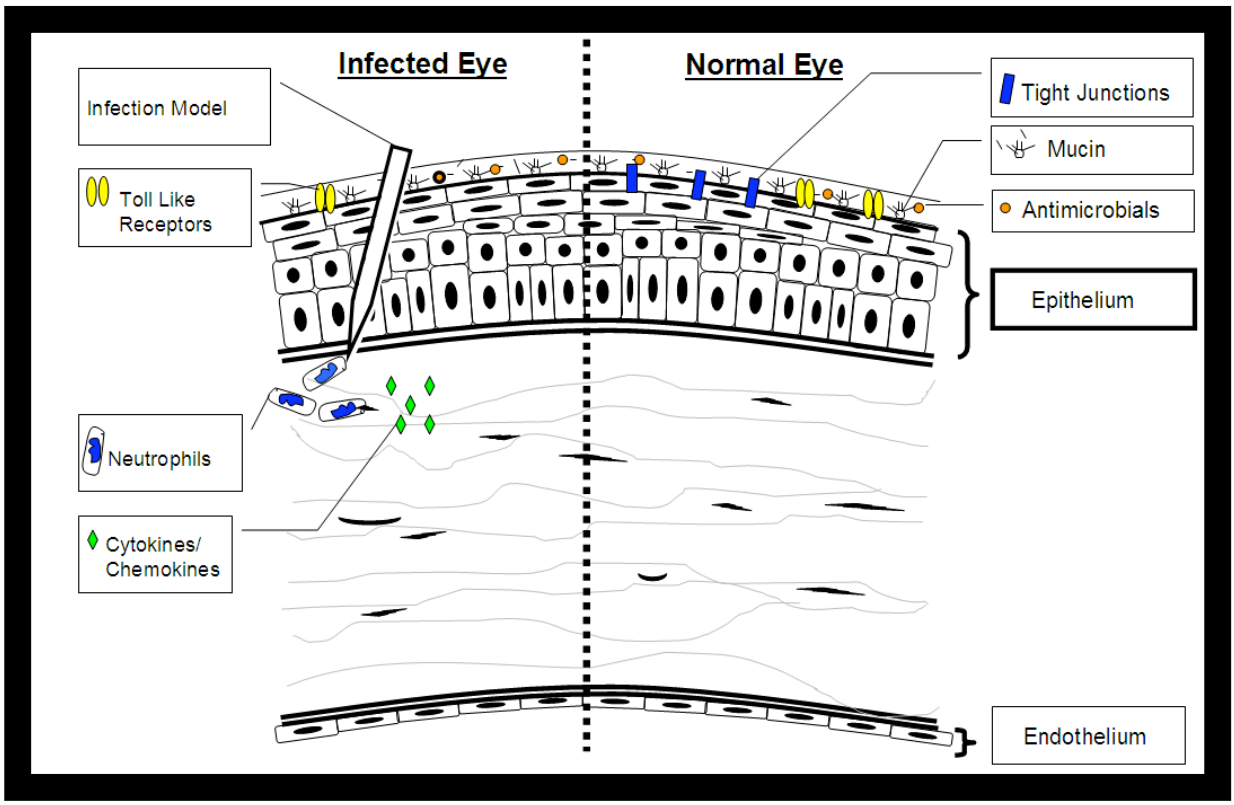


Figure 2. Anatomy of the ocular surface. This diagram shows host response of an infected eye and different components of a normal, healthy cornea.

1.4 Pathogenesis of *Pseudomonas aeruginosa*

Bacterial keratitis occurs only when bacteria penetrate through the six layers of the corneal epithelium and the Bowman's layer and into the stroma. There are two main mechanisms by which *P. aeruginosa* penetrate through the epithelium and into the stroma. Invasive strains of *P. aeruginosa* are engulfed by epithelial cells after binding to the cell membrane. Our lab reported that *P. aeruginosa* could be internalized by corneal epithelial cells *in vivo* (Figure 3) and *in vitro* (26, 28). The bacteria replicate in the cytoplasm at a rate of up to 10-fold per hour and eventually kill the infected cell by inducing apoptosis (29). Cytotoxic strains of *P. aeruginosa*, which have the ability to destroy corneal epithelial cells without invading them first, possess a needle apparatus on the surface that penetrates into cells and injects toxins, such as ExoU to cause cell death. This system is known as the type III secretion system (19). The invasive *P. aeruginosa* strains lack this toxin, and the cytotoxic *P. aeruginosa* strains also inject the ExoT toxin, which stops the cells from engulfing the bacteria (10, 18). The cytotoxic bacteria first aggregate around and damage the plasma membrane of host cells. This gives the bacteria access to the basal side of cells. After entering the epithelial cells, they slowly travel outwardly along the basal lamina in a surface-associated bacterial movement called twitching motility, eventually killing not only the host cells but also the adjacent cells (20). Both invasive and cytotoxic strains are capable of corneal epithelial cell invasion, and epithelial cell invasion has been shown to be crucial in pathogenesis of *P. aeruginosa* (18, 25, 96). *P. aeruginosa* can also traverse through the corneal epithelial cells intercellularly into the stroma, which is suggested by our preliminary data that invasive *P. aeruginosa* can cross corneal epithelia without decreasing trans-epithelial resistance (TER). The mechanism by which a healthy cornea inhibits *P. aeruginosa* invasion and how contact lens wear weakens the immune defense leading to *P. aeruginosa* entry into the cornea remain unknown.

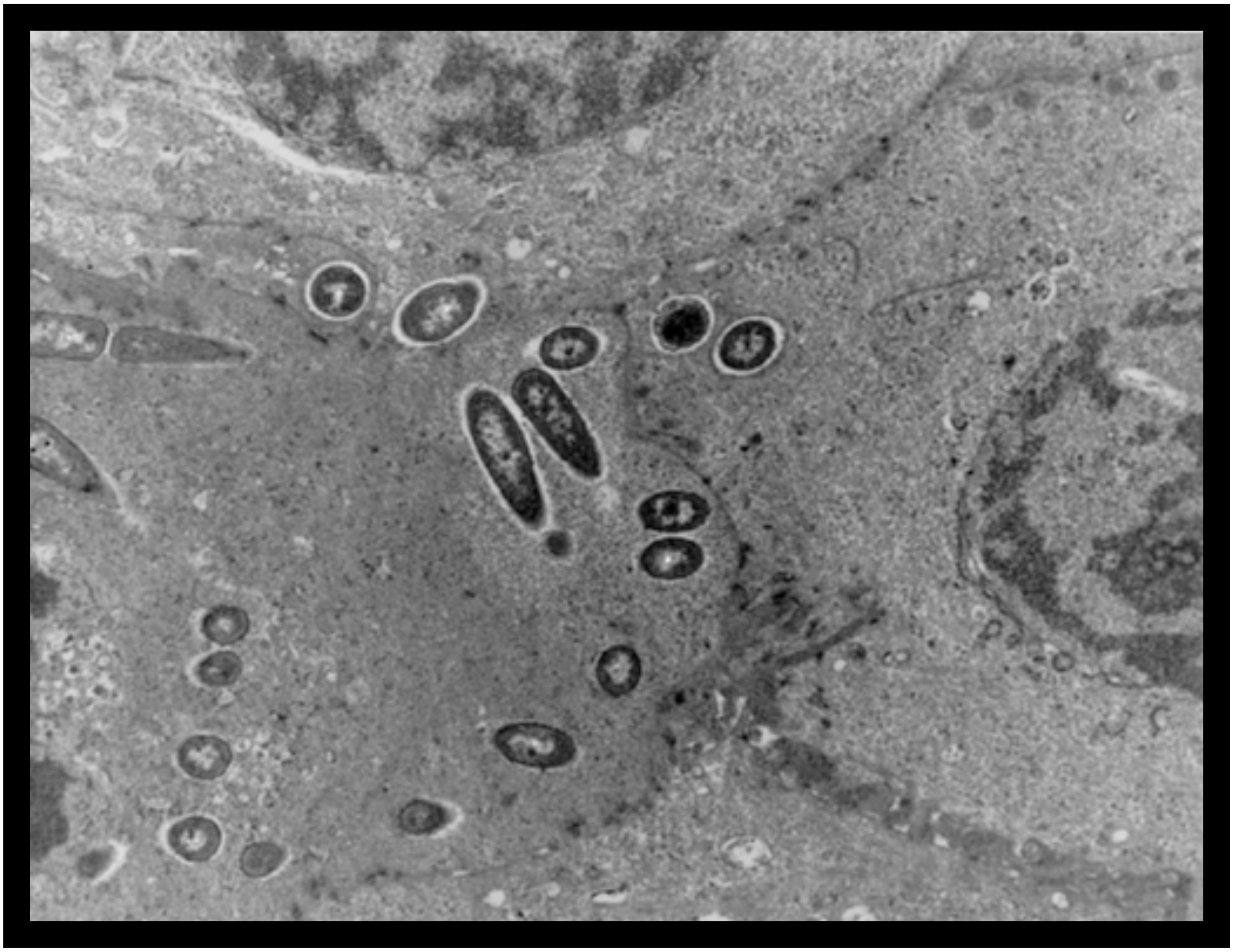


Figure 3. *P. aeruginosa* are internalized by corneal epithelial cells *in vivo*. Transmission electron micrographs of C57BL/6 mouse corneas after infection for 24 h with *P. aeruginosa* 6294. The peripheral epithelium (adjacent to the central infected area) was intact, and bacteria were present within these epithelial cells.

1.5 Innate Immunity of the Cornea

Healthy corneas are not susceptible to *P. aeruginosa* invasion, and it is important to note that although *P. aeruginosa* can infect epithelial cells *in vitro*, healthy corneas almost never develop infections even though the surface of the cornea constantly encounters various pathogens in the environment. Animal models reveal that it is very difficult to infect healthy corneas with *P. aeruginosa* or injury alone and that both *P. aeruginosa* and scratch injury are necessary for infection.

The corneal epithelium defense mechanisms can be categorized as "passive" or "active." One level of passive innate immunity is the tight junctions in the corneal epithelium, which prevent bacteria from traversing between cells (22). Tight junctions block the passage of bacteria to the basal side of the cornea, which is very susceptible to infection. Animal studies involving epithelial damage using filter paper and inoculation of bacteria have shown no corneal infection, suggesting that the corneal epithelial barrier is not the only barrier against infection. The layer underneath the corneal epithelium, the basal lamina, may play a protective role against bacterial infection. The pores of the basal lamina are much smaller than bacteria, working like a filter to prevent bacteria from entering the stroma (20). Furthermore, sloughing of the epithelium to remove bacteria from the surface provides a second line of passage defense (28). Eyelid movement can create shear stress that can kill *P. aeruginosa* and assist in tear exchange, which is important for the removal of pathogens in the eye or pathogens trapped under a contact lens. Human tears prevent *P. aeruginosa* from invading or destroying corneal epithelial cells *in vitro* (23) and protect mouse eyes against *P. aeruginosa* infection *in vivo* through mucin and surfactant protein D (SP-D) (27, 69, 70, 76). Other tear film components that contribute to protection comprise of bactericidal lysozymes, iron-binding protein lactoferrin (89), bacteria-binding immunoglobulin A (IgA) antibodies (65) and antimicrobial proteins including defensins and cathelicidin (LL-37) (43, 68).

The corneal epithelial cells also create defensins. Defensins are naturally occurring peptides that are considered to be among the earliest developed molecular effectors of innate immunity. They have antimicrobial activity against Gram-positive bacteria, Gram-negative bacteria, fungi and viruses (43, 68).

Active immunity in the corneal epithelial cells involves the upregulation of antimicrobial factors that inhibit bacterial growth and destroy bacteria and cytokines that recruit immune cells in response to recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (12, 71). Due to this multi-level defense system at the ocular surface, the normal cornea without contact lens wear or injury could remain healthy despite constant exposure to opportunistic pathogens that easily invade epithelial cells *in vitro*.

1.6 Surfactant Protein D

SP-D is a member of the collagenous carbohydrate binding proteins and is also known as a collectin. This group of proteins has a carbohydrate recognition domain (CRD) that can bind to carbohydrate residues, such as mannose, glucose, L-fucose and N-acetylglucosamine, in the presence of calcium. For self versus non-self distinction, these collectins do not recognize or bind to sialic acid, a common mammalian carbohydrate (14, 46).

The SP-D monomer is 43kDa. Three of these monomers twist together to form a trimer, which then aggregate to form multimers, most commonly dodecamer (Figure 4). Each trimer consists of a N-terminal domain, where the disulfide bond between two cysteine residues is found, followed by a collagen domain consisting of 59 amino acid triplets Gly-X-Y, then a trimeric neck and a carboxy-terminal, where the CRD is located (13). Experiments with truncated SP-D have shown that multiple domains and not just the CRD are necessary for proper biological activities (38, 42, 79, 93). Animals deficient in SP-D are more prone to lung infections by *Haemophilus influenzae*, a Gram-negative bacterium (57), and show slower clearance of *Pneumocystis carinii* (3). The CRD domain of SP-D can bind to *P. aeruginosa* and the lipopolyssacharide (LPS) of Gram-negative bacteria in the presence of calcium (58). SP-D also aggregates some *P. aeruginosa* strains and facilitates the engulfing but not destruction of *P. aeruginosa* by alveolar macrophages (7, 83). Furthermore, with certain bacteria, pulmonary SP-D can enhance bacterial clearance by increasing neutrophil uptake of bacteria (41). Wild-type mice clear *P. aeruginosa* more effectively in the lung compared to SP-D knockout mice, and knockouts have higher infiltration of immune cells such as neutrophils (35). 90% of cystic fibrosis patients with *P. aeruginosa* respiratory infections have significant reductions in SPD (80). In addition to all the studies done in the lung, Ni *et al.* have shown the presence of SP-D in human tear fluid and cultured corneal epithelial cells and that these epithelial cells secrete significant amounts of SP-D, which inhibits *P. aeruginosa* invasion (76). Others have shown that SP-D-deficient mice lose their capacity to recover from *P. aeruginosa* keratitis when the eye is made susceptible using an injury model (67).

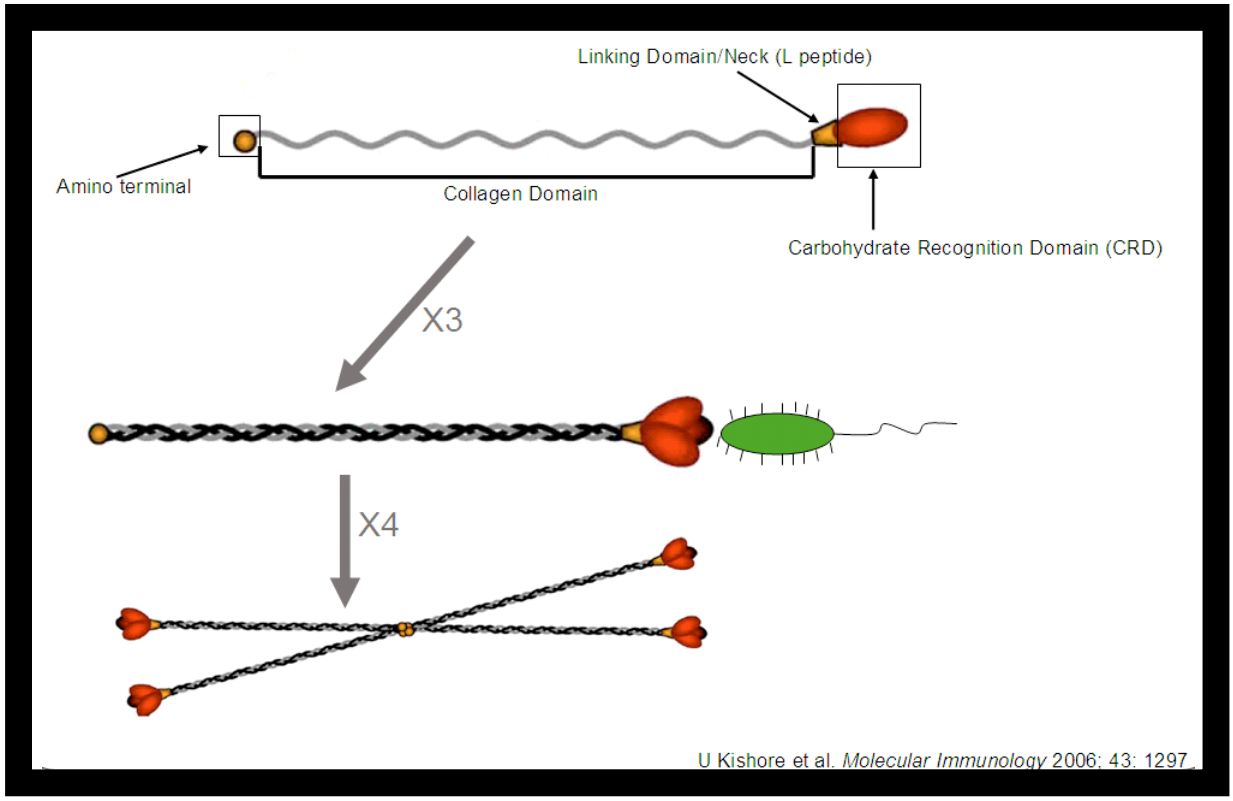


Figure 4. Structure of SP-D. Three monomers of SP-D form trimers and four of these trimers form dodecamers, most abundantly and biologically active form of SP-D.

Chapter 2: Clearance of *Pseudomonas aeruginosa* from a Healthy Ocular Surface Involves Surfactant Protein-D and is Compromised by Bacterial Elastase in a Murine "Null-Infection" Model.

2.1 Preface of Chapter

Our previous studies showed that surfactant protein-D (SP-D) is present in human tear fluid, and that it can protect corneal epithelial cells against bacterial invasion. Here we developed a novel "null-infection" model to test the hypothesis that SP-D contributes to the clearance of viable *P. aeruginosa* from the healthy ocular surface *in vivo*. Healthy corneas of Black Swiss mice were inoculated with 10^7 or 10^9 cfu of invasive (PAO1) or cytotoxic (6206) *P. aeruginosa*. Viable counts were performed on tear fluid collected at time points ranging from 3 to 14 h post-inoculation. Healthy ocular surfaces cleared both *P. aeruginosa* strains efficiently, even when 10^9 cfu was used; e.g. <0.01% of the original inoculum was recoverable after 3 h. Preexposure of eyes to bacteria did not enhance clearance. Clearance of strain 6206 (low protease producer), but not strain PAO1 (high protease producer), was delayed in SP-D gene-targeted (-/-) knockout mice. A protease mutant of PAO1 (PAO1*lasAlasBaprA*) was cleared more efficiently than wild-type PAO1, but this difference was negligible in SP-D (-/-) mice, which were less able to clear the protease mutant. Experiments to study mechanisms for these differences revealed that purified elastase could degrade tear fluid SP-D *in vivo*. Together, these data show that SP-D can contribute to the clearance of *P. aeruginosa* from the healthy ocular surface and that proteases can compromise that clearance. The data also suggest that SP-D degradation *in vivo* is a mechanism by which *P. aeruginosa* proteases could contribute to virulence.

2.2 Introduction

Pseudomonas aeruginosa, an opportunistic pathogen, is a leading cause of bacterial keratitis. While normal, healthy human corneas remain resistant to infection, contact lens wear or corneal injury/surgery can enable susceptibility (9, 50, 72). The mechanisms by which these factors predispose to infection are not yet well understood.

A murine scarification model has been used exclusively to study the pathogenesis of *P. aeruginosa* corneal infection (6, 34, 81). That model involves scratching the cornea with a sterile needle prior to adding bacteria, which enables bacteria to directly access the exposed stroma. The resulting disease resembles *P. aeruginosa* infection in people. More recently, we used a healing model of murine corneal infection to show that 6 hours after scratching, the mouse cornea remains susceptible to infection, but by 12 hours, it regains resistance to infection despite loss of barrier function to fluorescein staining (56). These injury models that enable *P. aeruginosa* to infect the cornea have led to a wealth of information about how infection develops and resulting pathology. Yet, the mechanisms by which the normal ocular surface remains healthy under normal circumstances have not been explored *in vivo*. This cannot be studied using a scratch model. The corneas' ability to resist disease despite constant daily exposure to potential pathogens is remarkable and learning about the mechanisms involved could help us to develop new therapies for disease of the eye and possibly other sites.

Results from the 12-hour healing situation suggested that defense systems other than barrier function can protect the ocular surface against infection. These defenses could involve biochemical factors constitutively expressed or upregulated in response to injury or bacterial exposure. Candidate factors could include defensin or other antimicrobial peptides, secretory IgA and mucin glycoproteins (36, 44, 65).

In this study we focused on SP-D which we have previously shown is present at the ocular surface, is upregulated by *P. aeruginosa* or its antigens, and can protect corneal epithelial cells against invasion (76, 77). Others have shown that SP-D deficient mice lose their capacity to recover from *P. aeruginosa* keratitis when it is made susceptible using an injury model (67). Here our aim was to explore the role of SP-D in protecting the healthy eye against bacterial colonization. Thus, we developed a novel "null-infection" model in which the cornea is not damaged prior to inoculation with *P. aeruginosa*. The objective was to allow bacteria to interact with the healthy ocular surface (intact cornea) to enable us to study host factors that normally protect the eye from developing infection when it is not susceptible, and the potential role that bacterial factors might play in compromising those defenses. This new model was then used to test the hypothesis that SP-D contributes to the clearance of *P. aeruginosa* from the healthy ocular surface and to explore the role of *P. aeruginosa* proteases in promoting ocular colonization *in vivo*.

2.3 Methodology

2.3.1 Bacterial Strains.

Cytotoxic strain 6206 and invasive strain PAO1 expressing GFP on pSMC2 plasmid (PAO1-GFP) were used for experiments comparing clearance of a cytotoxic and an invasive strain (Figs. 1, 2). PAO1-GFP was provided by Dr. Gerald B. Pier (Harvard Medical School, Boston, MA) and grown on Trypticase soy agar (TSA) supplemented with carbenicillin (300 µg/ml). Wild-type PAO1 and its isogenic mutant PAO1*lasAlasBaprA* (11) without GFP were used in other experiments (Figs. 3, 4). Inocula were prepared from overnight cultures grown on TSA plates at 37 °C for ~16 h before suspension in Dulbecco's Modified Eagle's Medium (DMEM, Sigma-Aldrich, St. Louis, MO) to a concentration of 10^9 or 10^{11} cfu/ml. Bacterial concentrations were confirmed by viable count.

2.3.2 Murine Null-Infection Model.

Wild-type Black Swiss mice (6-8 weeks old) and age-matched transgenic SP-D (-/-) mice were used. SP-D (-/-) Black Swiss mice were obtained from Dr. Jeffrey Whitsett (Children's Hospital Medical Center, Cincinatti, OH) (53). Wild-type Black Swiss mice with a matching genetic background to that of the SP-D (-/-) mice were purchased from Taconic (Seattle, WA).

After induction of anesthesia (IP injection with 21 mg/ml ketamine, 2.4 mg/ml xylazine, 0.3 mg/ml acepromazine), 5 µl of bacterial inoculum containing ~10^7 or 10^9 cfu was applied to the healthy ocular surface. At 3, 6, 12 and 14 h post-inoculation, tear fluid was collected from the ocular surface and the number of viable bacteria within was determined. Tear fluid was collected by capillary action using a 10 µl volume glass capillary tube (Drummond Scientific Co., Broomall, PA) from the lateral canthus after adding 4 µl of PBS to the ocular surface. In some experiments, whole eyes were enucleated after collection of tears and homogenized in 1 ml of PBS with 0.25% Triton X-100 for viable counts. In other experiments, eyes were preexposed to bacteria for ~16 h prior to inoculation in order to potentially stimulate innate defenses. Ocular health was monitored throughout the experiment to ensure the absence of disease pathology. All experiments involved between three and ten animals per group and were repeated at least twice. All procedures were carried out in accordance with the protocol established by the Association for the Research in Vision and Ophthalmology and were approved by the Animal Care and Use Committee, University of California, Berkeley.

2.3.3 Bacterial Elastolytic Activity Assay.

Bacterial inocula were prepared in DMEM at a concentration of 10^{11} cfu/ml. Inocula were centrifuged for 5 min at 14,000 x *g* and 50 µl of the supernatant was added to eppendorf tubes containing 10 mg of elastin Congo red (Elastin Products Company, Owensville, MO) in 1 ml of sodium phosphate buffer (Na_2HPO_4, 10 mM, pH 7.0). Known concentrations of purified elastase (Elastin Products Company, Owensville, MO) (Calbiochem, Gibbstown, NJ) were included to form a standard curve. Samples were incubated at 37 ^oC for 2 h with constant shaking before centrifuging for 5 min at 5,000 x *g* to remove insoluble substrate. Absorbance of the supernatants was measured at OD_{495} and elastase activity determined by reference to the standard curve.

2.3.4 Measurement of Elastase-Mediated SP-D Degradation *in vivo* and *in vitro.*

For *in vivo* studies, both ocular surfaces of 5 anesthetized wild-type Black Swiss mice were inoculated with 80 μg/ml of purified elastase in a vehicle of DMEM containing 4% v/v glycerol for 1 h. A control group of 5 anesthetized mice were inoculated with vehicle only (DMEM with 4% v/v glycerol). After 1 h, tear fluid was collected as described previously. The total protein concentration of each sample was measured using the DC Protein Assay (BioRad, Hercules, CA) and equivalent amount of each sample was resolved on a 10% SDS-PAGE precast Tris-HCl polyacrylamide gels (BioRad, Hercules, CA) under reducing conditions (100V, 1.5 h). SP-D was then detected by Western immunoblot as previously described using rabbit anti-mouse SP-D antiserum diluted 1:750. For *in vitro* studies, recombinant SP-D, murine or human tear fluid were mixed with either DMEM (vehicle) or 20 μg/ml of purified elastase suspended in DMEM for 3 h at 37 ^{o}C and SP-D detected as described above. Human tears were collected from a healthy human volunteer using a 30 μl volume capillary tube under a protocol approved by the Committee for the Protection of Human Subjects, University of California, Berkeley.

2.3.5 Statistical Analysis.

The statistical significance in number of viable bacteria was compared between two groups non-parametrically using the Mann-Whitney U Test. Accordingly, the number of bacteria recovered is expressed as the median and upper and lower quartiles. The asterisk markers show outliers within 3 interquartile ranges below the first quartile or above the third quartile, and the circles show outliers beyond 3 interquartile ranges of the first and third quartiles. Each of the figures presented (2A, 2B, 3A, 3B, 4A, 4B) are representative of independent experiments conducted on different days. In each instance, data sets were analyzed using the Brown-Forsythe Test which confirmed that there were no significant differences in the variance between the two groups compared, thereby allowing use of the Mann-Whitney U Test. $P < 0.05$ was considered statistically significant.

2.4 Results

2.4.1 *P. aeruginosa* is Efficiently Cleared from the Healthy Ocular Surface.

Healthy eyes of wild-type mice cleared both strains of *P. aeruginosa* (6206 and PAO1-GFP) efficiently in a time dependent manner. After inoculation with 10^7 cfu of invasive strain PAO1-GFP, only 0.00012% of the original inoculum was detected in the tear fluid after 6 h, and by 12 h no viable bacteria were recovered from the tears (Fig. 5A). Similar results were obtained using a larger inoculum of this strain (10^9 cfu) with only 0.0018% and 0.000038% of the original inoculum recovered from the tears after 3 h and 14 h respectively. Even more efficient clearance was noted for the cytotoxic strain 6206 with only 0.000093% recovered after 3 h (~20-fold lower than that of PAO1-GFP at the same time point), and no viable bacteria recovered after 14 h (Figure 5A). Similar low percentages of bacteria were recovered from ocular homogenates 14 h after inoculation using 10^9 cfu of either strain, showing that differences in clearance from the tear fluid did not result from bacterial relocation to other ocular sites. Fluorescein staining 14 h after inoculation showed that 6206 caused more superficial damage to the cornea than PAO1-GFP (Figure 5B). Nevertheless, 6206 bacteria were cleared more efficiently from the ocular surface than were PAO1-GFP bacteria and neither caused disease.

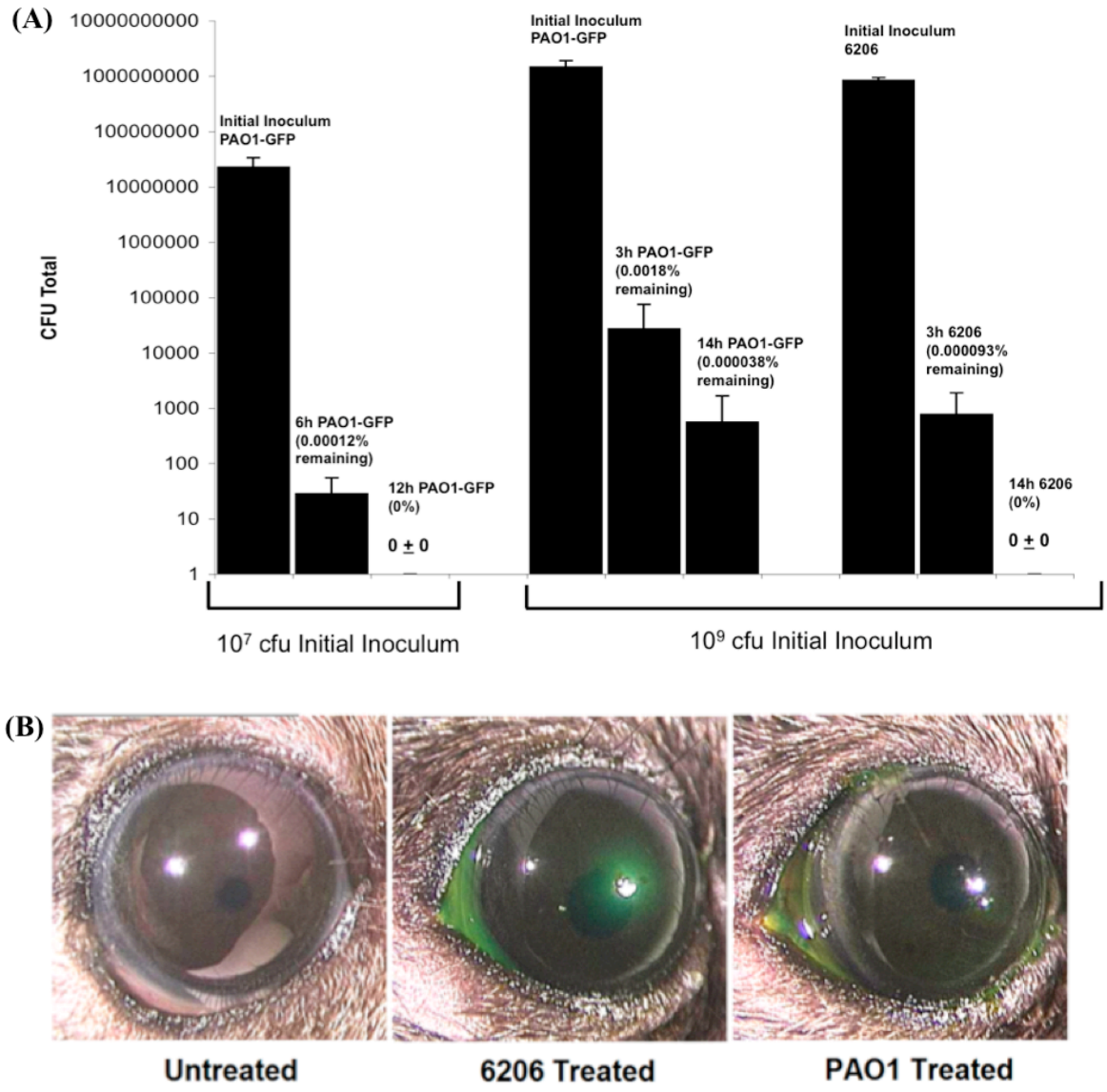


Figure 5. *P. aeruginosa* is efficiently cleared from the healthy ocular surface. (A) Detection of invasive PAO1-GFP or cytotoxic 6206 *P. aeruginosa* in the tear fluid of Black Swiss mice after inoculation of healthy eyes with 10^7 or 10^9 cfu bacteria. Data were quantified as the mean $\pm$ SD of viable bacteria recovered from the tear fluid at 3, 6, 12 or 14 h post-inoculation and expressed as a percentage of the initial inoculum. (B) Photographs of representative eyes of Black Swiss mice stained with fluorescein to observe any superficial disruption of the corneal epithelium 14 h after inoculation with 10^9 cfu of 6206 or PAO1-GFP. 6206 (but not PAO1-GFP) treated ocular surface shows superficial epithelial damage.

2.4.2 SP-D Deficient Mice Demonstrate Delayed Clearance of Cytotoxic Strain 6206.

SP-D deficient mice were used to study the role of SP-D in clearance of *P. aeruginosa* from the healthy ocular surface. Healthy eyes of wild-type and SP-D (-/-) Black Swiss mice were inoculated with 10^9 cfu of either 6206 or PAO1-GFP for ~16 h and were then rechallenged for 3 h with same number of bacteria. Preexposure with bacteria was used as a potential stimulant to upregulate potential innate defense factors (54, 63, 77), although it is not known if this occurs in this model. At 3 h post-inoculation with 10^9 cfu of 6206, significantly more bacteria (~12-fold) were isolated from the tear fluid of the SP-D (-/-) mice compared to wild-type ($p = 0.025$, Mann-Whitney Test) (Figure 6A). However, no significant differences were found for invasive strain PAO1-GFP (Figure 6B).

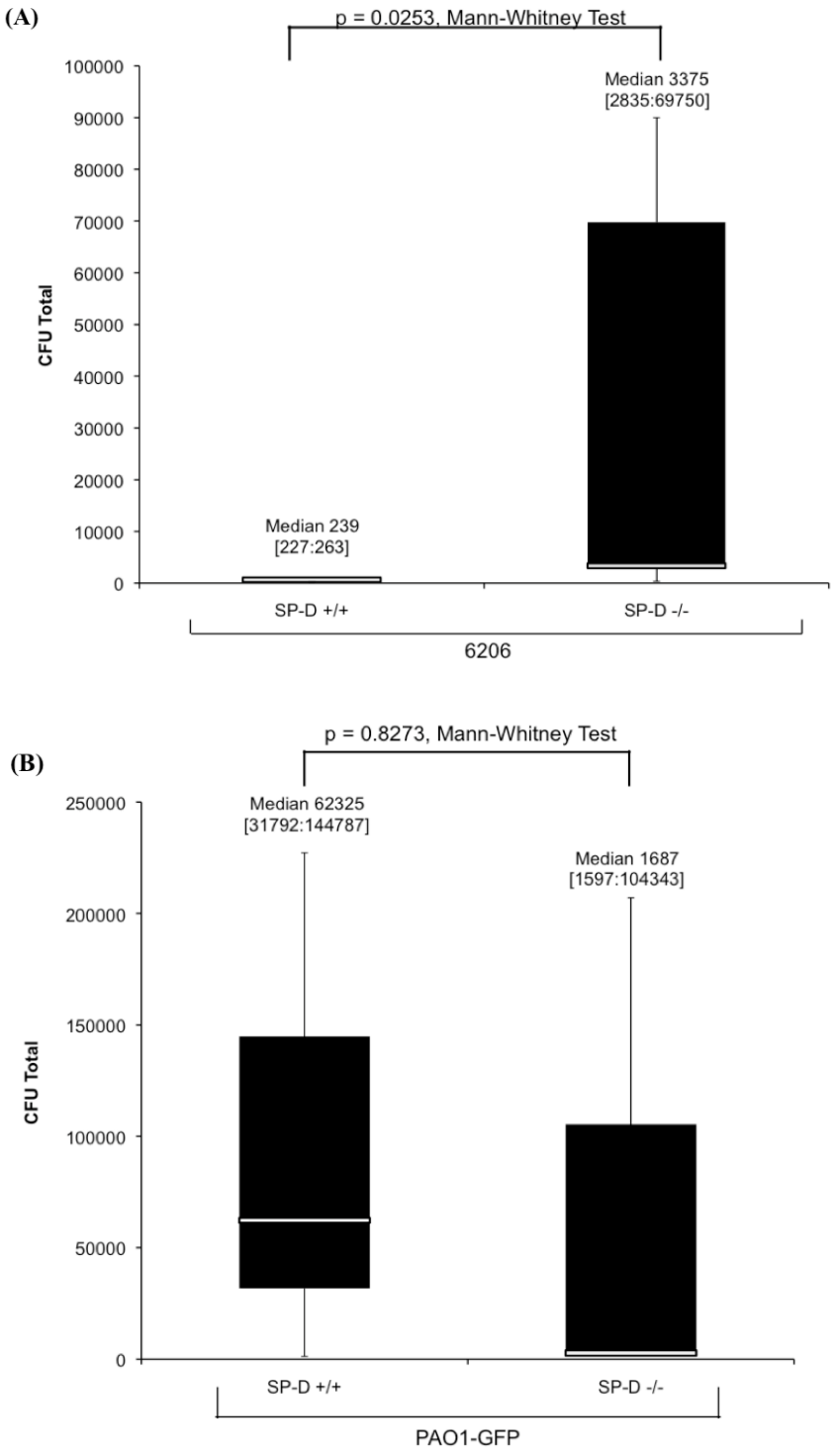


Figure 6. SP-D Deficient Mice Demonstrate Delayed Clearance of Cytotoxic Strain 6206. Clearance of 6206 (A) or PAO1-GFP (B) from the healthy ocular surface of wild-type versus SP-D (-/-) mice at 3 h post-inoculation with 10^9 cfu bacteria. Eyes were pre-exposed to the similar inoculum for 16 h prior to the assay. Data are expressed as the median [lower quartile:upper quartile] number of viable bacteria recovered from the tear fluid. Data represent one of three independent experiments (n = 3 to 5 mice per group).

2.4.3 SP-D Deficient Mice Show Delayed Clearance of the Protease Mutant of Strain PAO1.
Since the data indicated that 6206 cleared more efficiently from the ocular surface compared to PAO1-GFP, and 6206 is known to express low protease activity compared to PAO1 (1, 78), we hypothesized that proteases may compromise clearance. Thus, clearance of PAO1 was compared to an isogenic protease mutant in both wild-type and SP-D (-/-) mice. The data showed that significantly more protease mutant bacteria remained in the tear fluid of the SP-D (-/-) mice compared to wild-type mice ($p = 0.009$, Mann-Whitney Test) (Figure 7A). No such differences were found for PAO1 ($p = 0.4662$, Mann-Whitney Test) (Figure 7B). In wild-type mice, significantly lower numbers of protease mutant bacteria were recovered from the tear fluid compared to protease competent PAO1 (~ 43 fold, $p = 0.016$, Mann-Whitney Test) (Figure 8A). Differences between PAO1 and protease mutant bacteria were not statistically significant in SP-D (-/-) mice ($p = 0.4057$, Mann-Whitney Test) (Figure 8B).

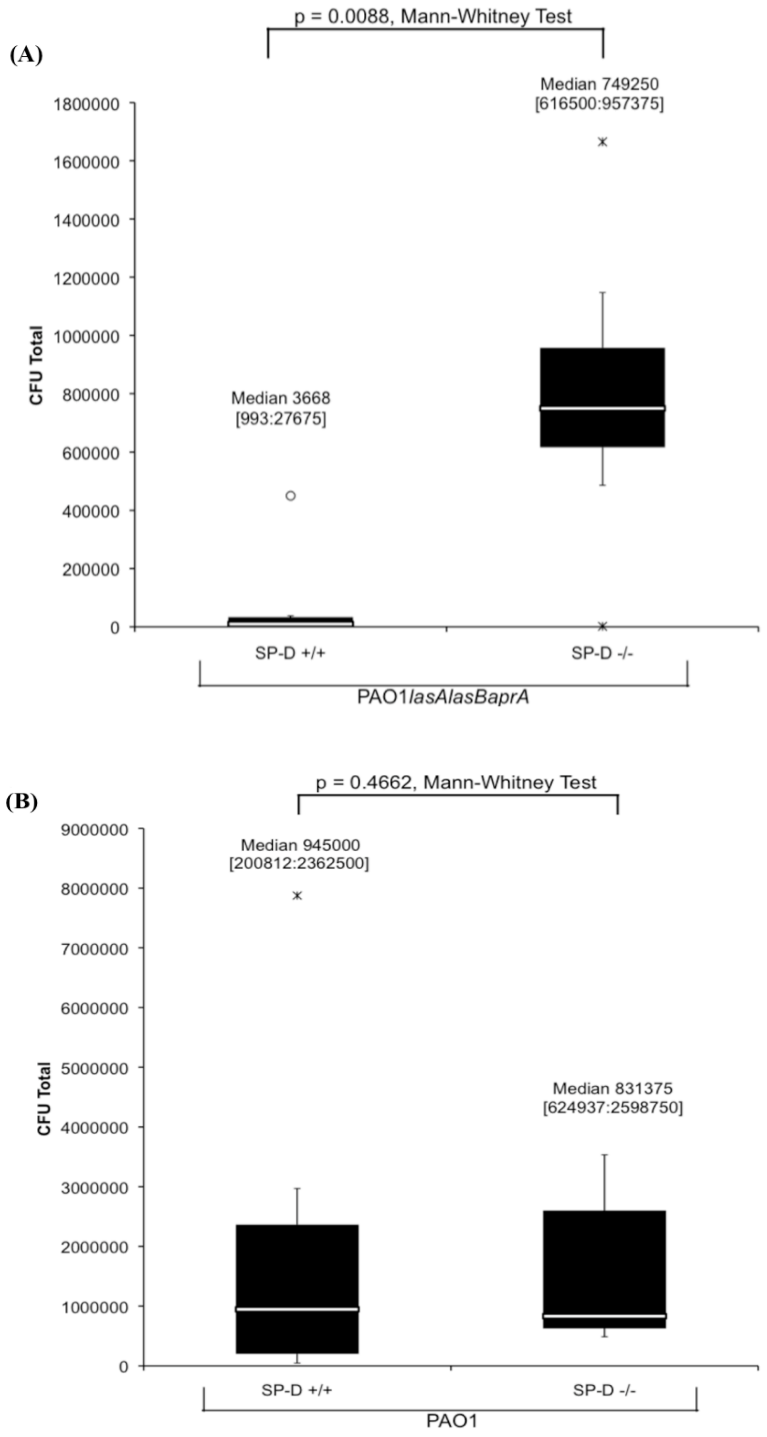


Figure 7. SP-D Deficient Mice Show Delayed Clearance of the Protease Mutant of Strain PAO1. Detection of the *P. aeruginosa* protease mutant PAO1*lasAlasBaprA* (A) or its parent PAO1 (B) in the tear fluid of wild-type or SP-D (-/-) mice at 3h after inoculation with 10^9 cfu bacteria. Eyes were pre-exposed to the same inoculum for 16 h prior to the assay. Data are expressed as the median [lower quartile:upper quartile] number of viable bacteria recovered from the tear fluid. Data represent one of three independent experiments (n = 7 to 8 mice per group).

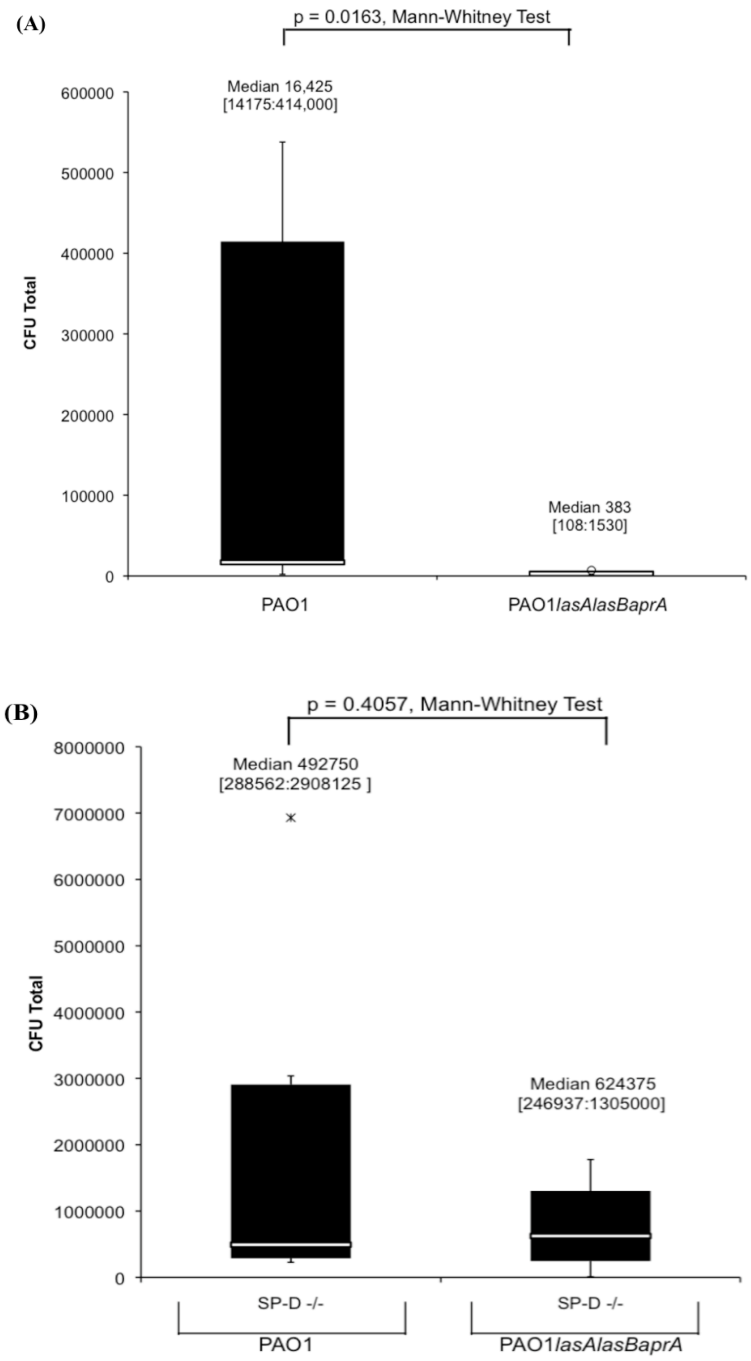


Figure 8. Protease Mutant of Strain PAO1 Clear More Efficiently. (A) Detection of viable *P. aeruginosa* strain PAO1 or its isogenic mutant PAO1*lasAlasBaprA* in wild-type murine tear fluid 3 h after inoculation with 10^9 cfu bacteria. Eyes were pre-exposed to the same inoculum for 16 h prior to the assay. (B) Clearance of PAO1 and its isogenic mutant were also compared in SP-D (-/-) mice. Data are expressed as the median [lower quartile:upper quartile] number of viable bacteria recovered in the tear fluid. Data represent one of three independent experiments (n = 5 to 10 mice per group).

2.4.4 Purified *P. aeruginosa* Elastase Degrades Tear Fluid SP-D *in vitro* and *in vivo.*

SP-D is known to be cleaved by *P. aeruginosa* proteases *in vitro* and in rat and human bronchoalveolar lavage fluid into an inactive 35 kDa form (1, 62, 64). Since the data showed that proteases delayed bacterial clearance and that SP-D expedites this clearance process in the absence of proteases, we next explored whether degradation of SP-D by *P. aeruginosa* proteases could occur in tear fluid which could provide a potential mechanism for that effect. *In vitro* elastolytic assays with typical inocula used in this study (10^{11} cfu/ml) revealed that PAO1 expressed ~70.4 μg/ml of elastase while 6206 expressed only ~5.6 μg/ml. To avoid other inoculum related confounding factors (i.e. toxins that might interfere with the assay), purified elastase, rather than bacteria, was used in this study. Tear fluid collected from wild-type mice was treated with 20 μg/ml of purified elastase for 3 h at 37 ^{o}C. The same treatment was applied to tear fluid collected from healthy human volunteers. Control samples were treated with vehicle (DMEM). Western immunoblot showed the presence of monomeric SP-D (~43 kDa) in mouse tears and a slightly larger (~50 kDa) form of SP-D in the human tears (Figure 9A, lanes 5 and 7 respectively), which may relate to a form of SP-D of similar size reported previously by others in human respiratory lavage and amniotic fluid (66). A degraded SP-D fragment of ~35 kDa was observed only in elastase-treated mouse and human tear fluid samples (Figure 9A, lanes 6 and 8 respectively) and in elastase-treated recombinant SP-D samples (Figure 9A, lane 4). This data showed that in the context of tear fluid, SP-D is susceptible to cleavage by *P. aeruginosa* elastase. To determine if tear SP-D was also degraded *in vivo*, purified elastase (80 μg/ml), at levels similar to those present in our experiments using bacteria, was added to the healthy ocular surface of wild-type mice for 1 h. Control eyes were inoculated with vehicle only. Western immunoblot of collected tears showed the presence of monomeric SP-D (~43 kDa) in control eyes (Figure 9B, lane 3). In contrast, elastase-treated eyes showed both monomeric SP-D and a ~35 kDa band of degraded SP-D (Figure 9B, lane 4).

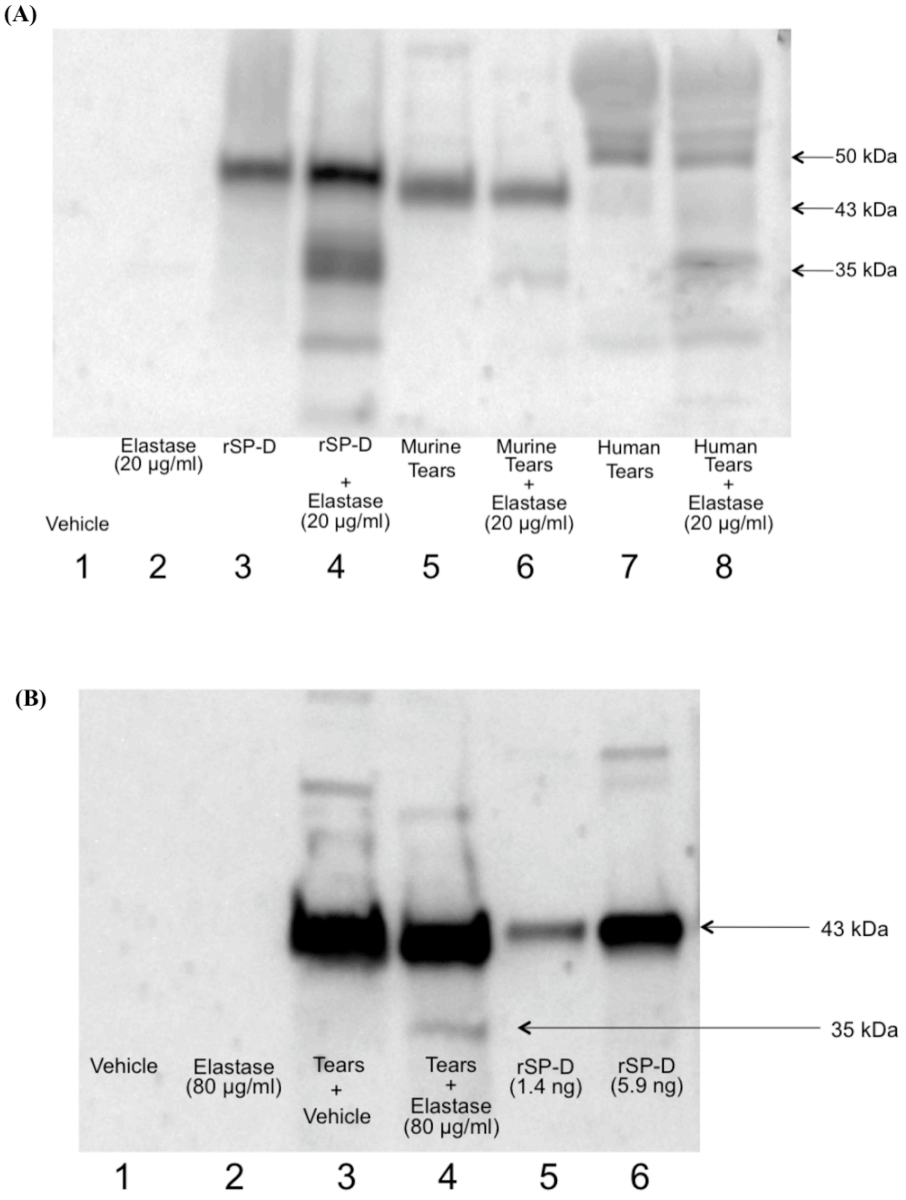


Figure 9. Purified *P. aeruginosa* Elastase Degrades Tear Fluid SP-D *in vitro* and *in vivo.*
(A) Western immunoblot showing the effects of purified elastase treatment (20 µg/ml, 3 h) on murine or human tears and recombinant SP-D *in vitro*. Under reducing conditions, the SP-D antibody detected degraded 35 kDa product in each of the elastase-treated samples but not in vehicle (DMEM) controls (lanes 4, 6, 8). (B) Western immuoblot of murine tear fluid collected from healthy eyes of wild-type Black Swiss mice 1 h after inoculation with purified elastase (80 µg/ml) *in vivo* (tears were pooled from 5 mice, see methods). Under reducing conditions, mononeric SP-D (~43 kDa) was detected in the murine tear fluid (lanes 3, 4). SP-D degradation product at 35 kDa was observed in eyes treated with elastase (lane 4) but not in eyes treated with vehicle (DMEM with 4% glycerol) (lane 3). The rabbit anti-mouse SP-D antibody did not react with the vehicle (lane 1) or purified elastase (80 µg/ml) (lane 2) when used alone. Lanes 5 and 6 were loaded with recombinant murine SP-D at different concentrations (positive control).

2.5 Discussion

2.5.1 Summary

In this study, we developed a novel null-infection model and used it to show that *P. aeruginosa* is rapidly cleared from the healthy ocular surface of mice. We found that SP-D can contribute to this process and that it can be delayed by the expression of bacterial proteases. Suggesting a connection between those two findings, is the result that SP-D within tear fluid can be degraded by *P. aeruginosa* elastase *in vitro* and *in vivo*, that SP-D (-/-) animals clear wild-type and protease mutant bacteria similarly, and that protease mutant bacteria (or strains producing less elastase) are cleared more effectively from wild-type animals compared to SP-D (-/-).

2.5.2 Implications of Experimental Results

The ocular surface is constantly exposed to a diverse array of potentially pathogenic microbes. The efficient clearance of microorganisms entering the eye is likely to be important in the maintenance of ocular health. Factors that are likely to contribute include blinking and tear exchange for physical removal of bacteria in addition to tear biochemical factors that bind, aggregate and/or inactivate microorganisms. Known activities of SP-D include binding and aggregation of *P. aeruginosa* (and other microbial pathogens) (7), direct antimicrobial activity (93), and a role in limiting *P. aeruginosa* induced corneal pathology in an injury model of corneal infection (67) and during infection of the respiratory tract (35). SP-D is also known to facilitate phagocytosis by macrophages and to modulate activity of phagocytes (83). It is known that there are resident dendritic cells within the cornea (39). The upregulation of corneal epithelial cell SP-D in response to bacterial antigens, that we previously reported (77), could be important in the mechanism by which SP-D contributes to clearance from the healthy ocular surface.

The data showing a relationship between protease expression and retention of *P. aeruginosa* at the ocular surface could involve SP-D degradation *in vivo* by proteases. While *P. aeruginosa* elastase and protease IV had already been shown to degrade purified SP-D into an inactive form (1, 62), those previous studies were done *in vitro*. In this study, we showed that elastase can also degrade SP-D when it is within tear fluid either *in vitro* or *in vivo*. The data also showed that differences in clearance between wild-type and protease mutant bacteria seen in wild-type animals are no longer statistically significant in SP-D (-/-) mice. Taken together, these data suggest that SP-D degradation provides a possible mechanism for the delayed clearance of protease competent bacteria as compared to protease mutants. However, the relationship between bacterial proteases, SP-D expression and ocular clearance of bacteria will require further study to determine the contribtion of elastase (and other *P. aeruginosa* proteases) towards the *in vivo* degradation of SP-D, and the biological significance of this finding given the continued renewal of this innate defense protein by the lacrimal apparatus and ocular surface epithelia (27, 28). In addition, proteases could also promote ocular colonization through other mechanisms. For example, *P. aeruginosa* proteases are known to degrade tear immunoglobulins (60) which could compromise their known ocular defense against infection (65). Whether previously demonstrated roles for elastase and other proteases in increasing bacterial adherence to the mouse cornea (37), or in invasion and penetration through epithelia (4, 11), relate to the role of proteases in colonization of the healthy cornea is yet to be determined.

Cytotoxic *P. aeruginosa* (6206) was found to be cleared more rapidly than the invasive strain (PAO1). Interestingly, 6206 encodes and expresses a powerful cytotoxin ExoU (absent in PAO1) which can repress phagocyte infiltration of infected corneas *in vivo* (99), injure and kill corneal epithelial cells *in vitro* (29) and can also damage the intact corneal epithelium *ex vivo* (24). Indeed, our data showed that at the inoculum used in this study, cytotoxic strain 6206 did damage corneal barrier function *in vivo*, as indicated by fluorescein staining. The rapid clearance of this cytotoxic strain (relative to PAO1), despite its capacity to cause superficial damage *in vivo*, may reflect its low level or lack of protease activity as previously reported (78), and confirmed in the present study.

2.5.3 Future Directions

Traditional models for studying bacterial keratitis in which disease is induced do not allow normal resistance factors to be directly examined. In this study, we developed, and demonstrated, the usefulness of a new "null-infection" model for this purpose. While we have shown SP-D to be involved in clearance and that *P. aeruginosa* proteases can compromise it, there are likely to be an array of host factor that protect the eye under normal circumstances and there are also likely to be bacterial factors with the potential to compromise clearance. Further studies using this model could facilitate our understanding of the circumstances surrounding resistance and susceptibility to infection, and could eventually lead to new approaches for treatment or prevention of infection of the eye and of other sites.

Chapter 3: Tear Fluid Modification of Corneal Epithelial Cell Defenses Against *Pseudomonas aeruginosa*

3.1 Preface of Chapter

This chapter investigates the effect of tear fluid on human corneal epithelial cells and possible tear fluid-regulated corneal cell defenses that provide protection for corneal epithelial cells against *P. aeruginosa* virulence mechanisms.

Cytotoxicity and invasion assays were used to study the protective effect of human tear fluid on corneal epithelial cells. Bacteriostatic activity of the corneal cell lysates fractionated by size with and without exposure to tear fluid were used to narrow down the possible corneal cell defense candidates that are providing protection. Semi-quantitative RT-PCR and microarrays were utilized to identify these potentially protective cell defenses.

Our data showed that pre-exposing the corneal epithelial cells to tear fluid is sufficient to provide protection against *P. aerugionsa* virulence mechanisms. Our reporter array assays also showed that tear fluid magnifies activation of NFkB and AP-1. Corneal cell lysates < 3 kDa showed significant bactericidal activity and that Histatin 5 (< 3 kDa) may be a potential antimicrobial that is providing protection for tear-exposed cells. Lastly, our microarray data showed that RNase7, an antimicrobial and ST2, a member of IL1R family, are upregulated with tear fluid and may play a protective role as well.

3.2 Introduction

Pseudomonas aeruginosa is a gram-negative opportunistic bacterium that can cause bacterial keratitis, resulting in vision loss. Contact lens wear, corneal injury or surgery can predispose patients to infection by this pathogen (9, 61, 72). In contrast, healthy cornea *in vivo* is resistant to infection (82), while *P. aeruginosa* can attack surface cells on cultured corneal epithelial cells and intact corneas *in vitro* (24, 28). One protective factor contributing to this resistance *in vivo* and absent in *in vitro* system is the tear fluid.

We have previously shown that human tear fluid provides protection against *P. aeruginosa* invasion and cytotoxicity *in vitro* and that this protection is independent of bacteriostatic activity (23). In an *in vivo* scratch injury model, we showed that tear fluid significantly reduced the severity of the disease in murine corneas infected with *P. aeruginosa* (55). Tear film components contributing to this protection comprise of bactericidal lysozymes, iron-binding protein lactoferrin (89), bacteria-binding immunoglobulin A (IgA) antibodies (65) and mucin glycoproteins (30) and antimicrobial proteins including defensins and cathelicidin (LL-37) (43, 68). Surfactant protein-D (SP-D), which has been shown to protect corneal epithelial cells against *P. aeruginosa* invasion, is also present in both murine and human tear fluid as we have reported previously (73, 76).

While these defense factors of the tear fluid are essential in *in vivo* defense against ocular surface infection, murine eye suspended in bacteria in the absence of tear fluid retains its resistance to *P. aeruginosa* translocation. Thus, in this study, we tested the hypothesis that corneal epithelial cells pre-exposed to human tear fluid continue to be protected against *P. aeruginosa* invasion and cytotoxicity even after tears are removed due to tear-mediated modulation of corneal epithelial cell defenses. As to which genes (corneal epithelial defenses) are involved in tear-induced ocular defense against infection were also investigated.

3.3 Methodology

3.3.1 Cell Culture.

Human telomerase-immortalized corneal epithelial cells (hTCEpi) were maintained in 10 cm tissue culture treated petri dishes (Becton Dickison, Franklin Lake, NJ) in serum-free KGM-2 medium (Lonza, Walkersville, MD) until confluent as previously described (84). Cells were then seeded onto 96-well tissue culture plate (Becton Dickinson, Franklin Lake, NJ) and grown to ~80-90% confluency. 2 days prior to experiment, the cells were incubated in high calcium KGM-2 medium containing 1 mM Ca^{2+} for 16 h, then washed with sterile phosphate-buffered saline (PBS; Sigma, St. Louis, MO) before being incubated in either 40 µl of fresh human tear fluid or high calcium KGM-2 without antibiotics either for 6 h or 16 h depending on the experiment. All cells were incubated at 37°C with 5% CO_2 while culturing and during experiments.

3.3.2 Preparation of Bacteria.

All bacteria were grown on trypticase soy agar plates overnight at 37°C. Bacteria were suspended in minimal cell culture KGM-2 media to a spectrophotometer optical density of 0.1 at OD_{650} which is approximately equivalent to $1x10^8$ CFU/ml and diluted to ~$1x10^6$ CFU/ml for invasion and cytotoxicity experiments. *P. aeruginosa* clinical isolates 6294 and 6206 were used for invasion and cytotoxicity assays, respectively.

3.3.3 *P. aeruginosa* Supernatant.

P. aeruginosa strain PAO1 was grown as single colonies on trypticase soy agar at 37°C overnight. Single colony at 37°C overnight with aeration was allowed to grow to late log phase in 5 ml of trypticase soy broth. The culture broth was then centrifuged at 14,000 rpm for 30 min and supernatant collected in a syringe, sterilized with a 0.22 µm polymer filter (Corning Star Corporation, Cambridge, MA), aliquoted and stored at -80°C until use. Either bacterial supernatant diluted 1:5 with minimal cell culture KGM-2 or equivalent amount of minimal cell culture media was used to stimulate the corneal epithelial cells for experiments.

3.3.4 Invasion and Cytotoxicity Assays.

Corneal epithelial cells either pre-exposed to high Ca^{2+} media or human tear fluid for 16 h (for effect of tear fluid pre-exposure experiments) were washed with 100 µl of pre-warmed PBS and 40 µl of 6294 or 6206 diluted in either cell culture media or lysate fractions (for effect of lysate fraction experiments) to a concentration of ~$1x10^6$ CFU/ml was inoculated onto each well in triplicates for 3 h at 37°C, 5.0% CO_2. The cells were then washed with PBS and incubated with 100 µl of gentamicin (0.4% [vol/vol]; 200 µg/ml; BioWhittaker, Walkersville, MD). After 1 h of incubation at 37°C, 100 µl of Trypan blue (10% [vol/vol]) in minimal KGM-2 was added, incubated for 15 min and Trypan blue replaced with 100 µl of minimal KGM-2 media. Brightfield and phase contrast images were taken on an inverted microscope (Olympus IX-70, Olympus America Inc., Center Valley, PA) attached to a video camera (Optronics, Goleta, CA) at 100X magnification. The cells were considered vulnerable to cytotoxicity if they exhibited Trypan blue staining. Number of stained cells were quantified for statistical analysis. To quantify intracellular bacteria, the cells were lysed in Triton X-100 (0.25% [vol/vol]; LabChem Inc., Pittsburgh, PA) in PBS for 15 minutes. The cells were then scraped for complete lysis and undiluted and 10^{-1} dilutions were plated onto MacConkey agar plates for viable count.

3.3.5 Lysate Fractionation.

Corneal epithelial cells seeded on 96 well plates pre-exposed to either cell culture media or human tear fluid for 16 h were washed with PBS and suspended in 30 µl of minimal KGM-2 media. Cells were lysed by repeatedly freezing on dry ice and thawing at 37°C. The lysates were collected and centrifuged through Microcon column filters of various sizes (100 kDa, 10 kDa, 3 kDa, Millipore, Billerica, MA) at 12,000 rpm at 4°C. Media that ran through 100 kDa filter would comprise of proteins < 100 kDa in size and this fraction would be run through 10 kDa filter to obtain proteins < 10 kDa in size. > 100 kDa, 10-100 kDa and 3-10 kDa fractions were obtained by reversing the column filter and adding equal amount of media as initial lysate and centrifuging.

3.3.6 Bacterial Growth Assay.

The effect of corneal cell lysate fractions on bacterial growth and viability were tested by adding 50 µl of bacteria suspended in either cell culture media or normal and conditioned crude lysates or fractionated lysates in empty eppendorf tubes. These suspensions were incubated at 37°C for 3 h and quantified by viable count. Initial suspensions were also quantified to study the bacteriostatic or bactericidial effect of corneal cell lysates.

3.3.7 Transfection and Luciferase Array Assay.

Human corneal epithelial cells were reverse transfected using SureFECT (SABiosciences, Frederick, MD) onto Cancer 10-Pathway Reporter Array (SABiosciences, Ferderick, MD). Each reporter consists of a mixture of an inducible transcription factor responsive firefly luciferase reporter and a constitutively expressing Renilla luciferase construct (20:1). Firefly and Renilla luciferase activities of cells incubated either in media or tear fluid for 16 h followed by 3 h incubation with *P. aeruginosa* supernatant were measured. The change in the activity of each signaling pathway was calculated by comparing the normalized luciferase activities of the reporter of each group.

3.3.8 Microarray.

For each sample, 1-2 µg of RNA was amplified and applied to Affymetrix Human Genome U133 Plus 2.0 Arrays at the University of California, Berkeley Functional Genomics Laboratory according to protocols recommended by Affymetrix (Santa Clara, CA). CEL files obtained via MAS 5.0 (Affymetrix) were analyzed in GeneTraffic 3.2 (Stratagene, La Jolla, CA) using the robust multichip analysis (RMA) method to obtain individual probe set expression values (48). The given background-corrected and normalized $\log_2$ values were anti-logged and fold expression level changes were determined by dividing the experimental group by the control. Any gene showing differential expression by at least five fold was subjected to Affymetrix Annotation Files. Functional gene classifications were derived from Gene Ontology information found in National Center for Biotechnology Information (NCBI) database, Onto-tools (Intelligent Systems and Bioinformatics Laboratory website, http://vortex.cs.wayne.edu/Projects.html) and from independent literature searches.

3.3.9 RNA Purification.

Total RNA of corneal epithelial cells incubated in either cell culture media or human tear fluid for 6 h and cells either pre-exposed to media or tear fluid for 16 h followed by 3 h incubation with *P. aeruginosa* supernatant were extracted with the RNeasy Kit (Qiagen, Valenica, CA) using Qiashredder columns for cell lysis and inserting Qiagen on-column DNase steps to remove any contaminating genomic DNA.

3.3.10 Semi-quantitative RT-PCR.

1 µg of total purified RNA was converted to cDNA using RETROscript Kit (Ambion, Austin, Texas). The expression of hBD-1, -2, -3, LL-37, Histatin 3, ST2 and RNase7 mRNAs were assessed for each condition mentioned previously using intron spanning primers. As an internal loading control, primers specific for ß-actin was used (Table 1). Polymerase chain reaction (PCR) products were separated on 2% agarose gel and visualized by ethidium bromide. Band intensities were quantified using the FluorChem Q SA software (Alpha Innotech, Santa Clara, CA) and normalized with those of ß-actin and medium treated controls. All experiments were repeated at least three times.

Gene	Accession No.	Sequence	Cycle no.
hBD-1	NM 005218	F-TCA TTA CAA TTG CGT CAG CAG R-TTG CAG CAC TTG GCC TTC	35
hBD-2	AF 040153	F-ATC AGC CAT GAG GGT CTT GT R-GAG ACC ACA GGT GCC AAT TT	35
hBD-3	NM 018661	F-AGC CTA GCA GCT ATG AGG ATC R-CTT CGG CAG CAT TTT CGG CCA	35
LL-37	NM 004345	F-AGG ATT GTG ACT TCA AGA AGG ACG R-GTT TAT TTC TCA GAG CCC AGA AGC	35
Histatin-3	NM_000200	F-GCA AAG AGA CAT CAT GGG TA R-GCC AGT CAA ACC TCC ATA ATC	35
ST2	NM 003856	F-CTT GAT TGA TAA ACA GAA TG R-CTG ATC CAG ATA CTG TTG AA	35
RNase7	NM_032572	F-GGA GTC ACA GCA CGA AGA CCA R-CAT GGC TGA GTT GCA TGC TTG A	25
ß-actin	NM001101	F-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA R-CTA GAA GCA TTT GCG GTG GAC GAT GGA GGG	25

Table 1. Primer Sequences for Semi-quantitative RT-PCR.

3.4 Results

3.4.1 Corneal Epithelial Cells Pre-exposed to Human Tear Fluid is Protected Against *P. aeruginosa* Invasion and Cytotoxicity.

Human corneal epithelial cells pre-exposed to human tear fluid for 16 h were protected against invasion and cytotoxicity by a highly invasive *P. aeruginosa* clinical isolate 6294 (Fig. 10A) and cytotoxic 6206 (Fig. 10B), respectively. Cells pre-exposed to tear fluid had significant reduction in 6294 invasion (~5 fold, $P = 0.007$, t-test) and number of cells damaged by 6206 (~ 3 fold, $P = 0.028$, t-test) as indicated by trypan blue staining.

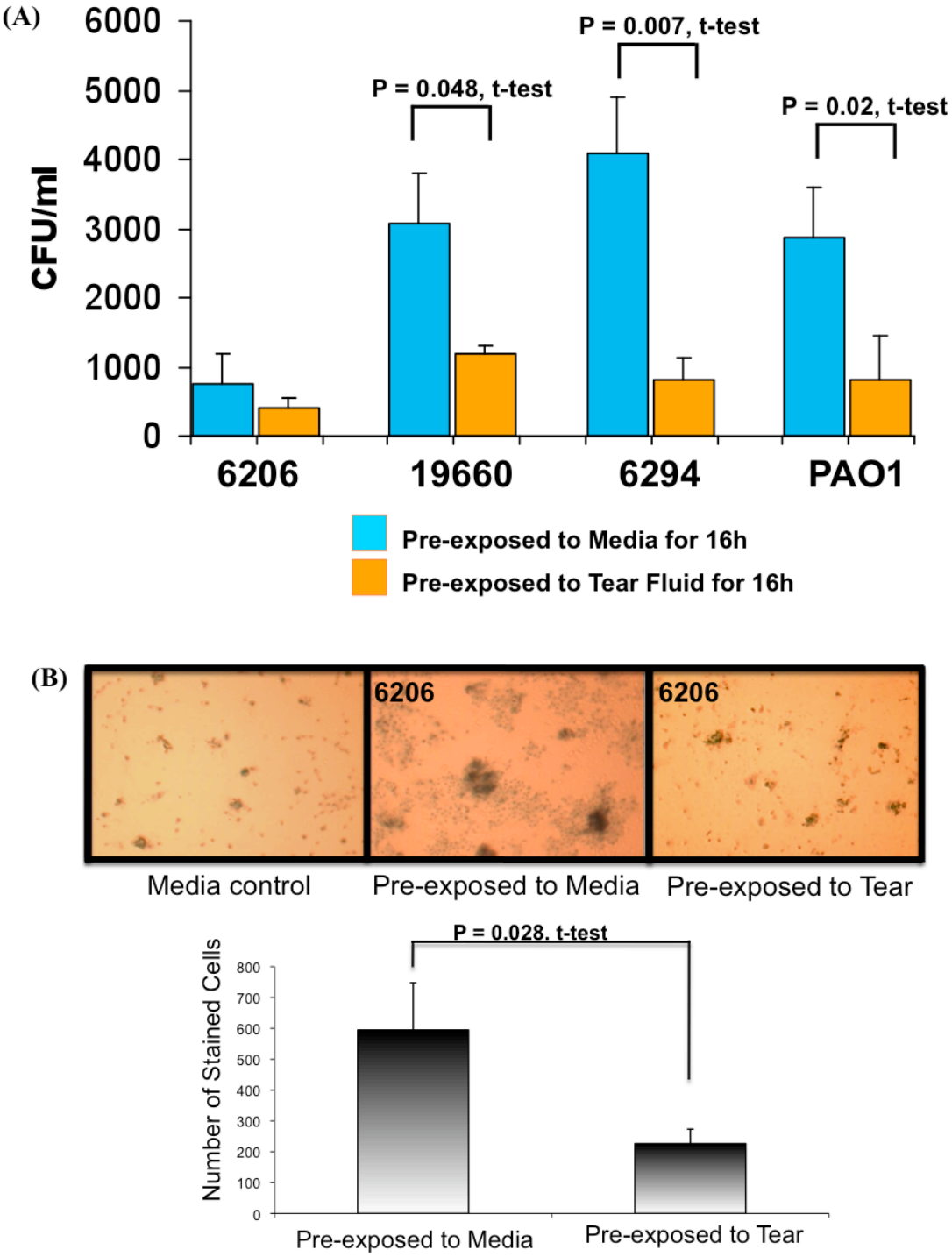


Figure 10. Corneal Epithelial Cells Pre-exposed to Human Tear Fluid is Protected Against *P. aeruginosa* Invasion and Cytotoxicity. Human corneal epithelial cells were either exposed to human tear fluid or cell culture media for 16 h before inoculation with ~104 cfu of *P.* a*eruginosa* for 3 h. Pre-exposing the cells to tear fluid was sufficient to provide protection against invasion by 19660, 6294 and PAO1 (A) and cytotoxicity by 6206 (B).

3.4.2 Pre-exposing the Human Tear Fluid to Human Corneal Epithelial Cells Does Not Increase Its Bacteriostatic Activity.

Human tear fluid has been shown to have bacteriostatic activity (23). To examine if pre-exposing the human tear fluid to corneal epithelial cells would increase its bacteriostatic activity, *P. aeruginosa* strains 6206 and 6294 were suspended in either cell culture media, fresh tears or tears pre-exposed to corneal epithelial cells for 16 h and incubated for 3 h at 37°C (Figure 11). Although both fresh tears and tears pre-exposed to cells retarded growth of *P. aeruginosa*, no difference in bacteriostatic activity was seen between the two different tears with 6206 or 6294.

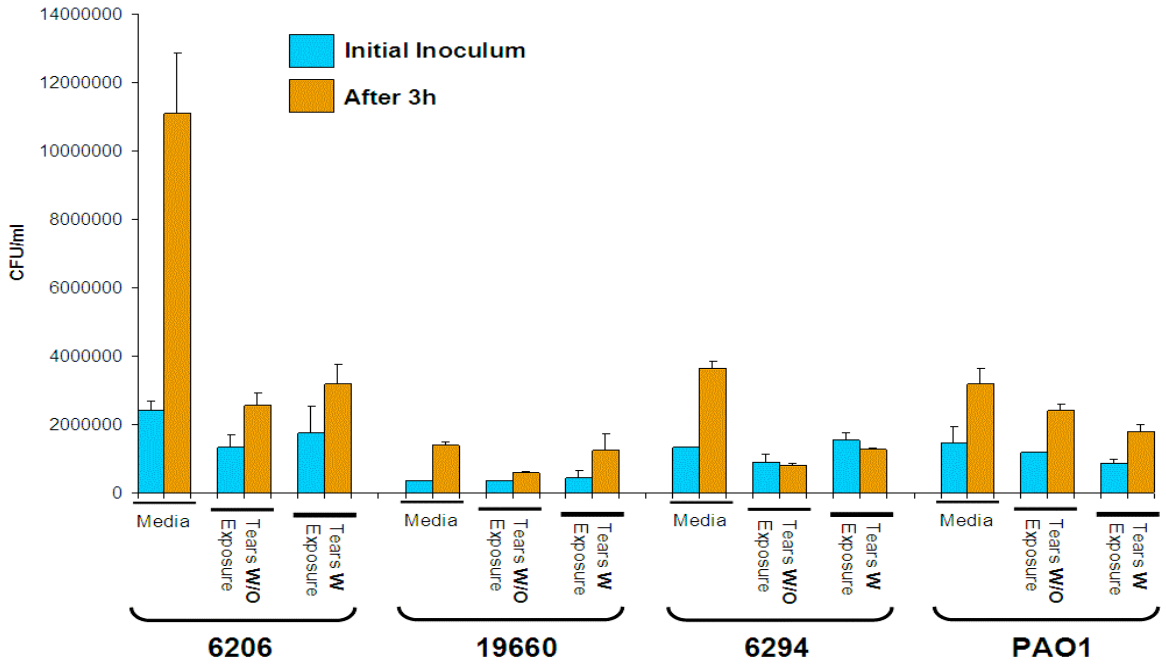
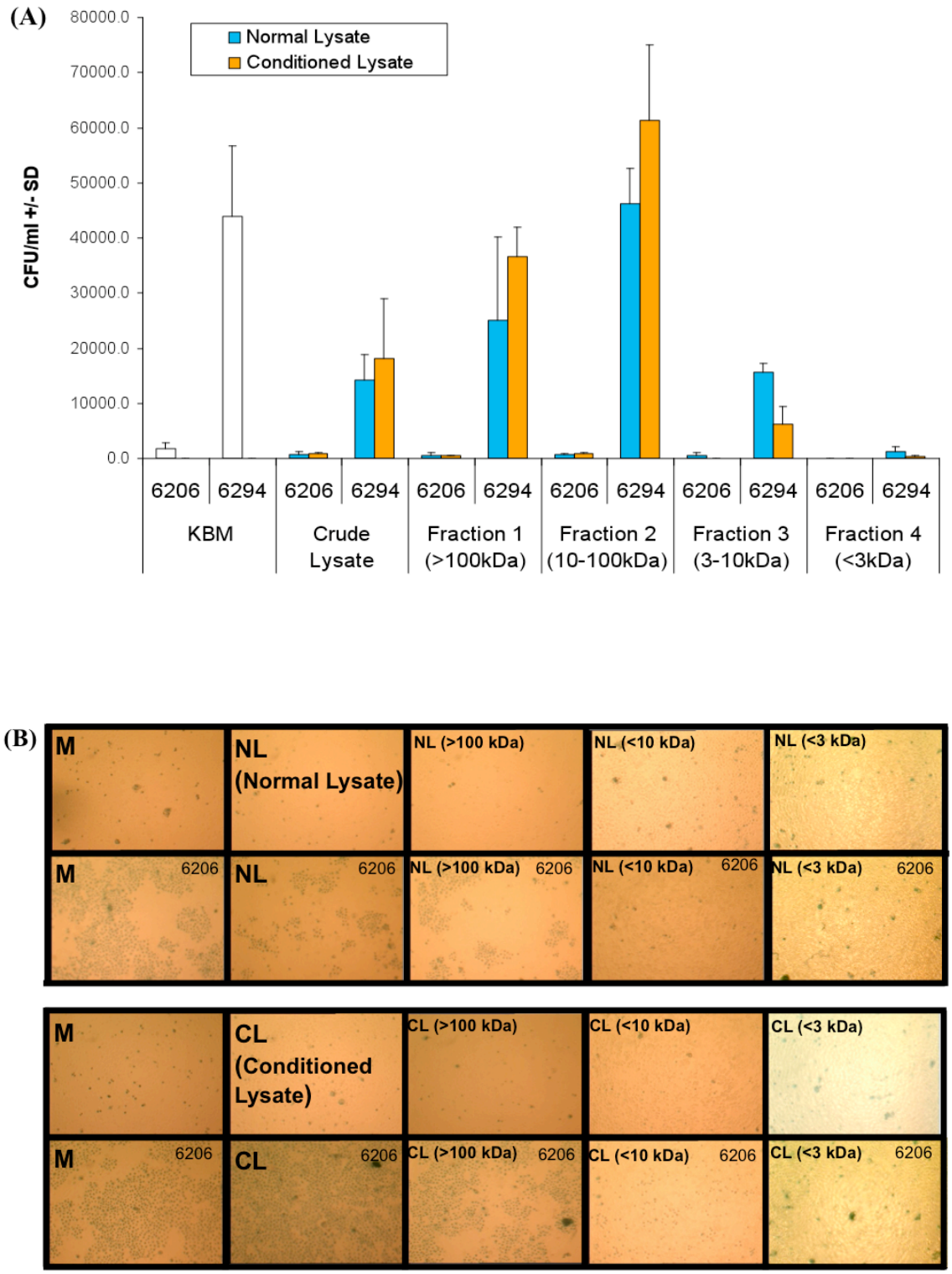


Figure 11. Pre-exposing the Human Tear Fluid to Human Corneal Epithelial Cells Does Not Increase Its Bacteriostatic Activity. To examine if pre-exposing human tear fluid to corneal epithelial cells would increase its bacteriostatic activity, human tear fluid was exposed to corneal epithelial cells for 16 h. Human corneal epithelial cells were inoculated with ~104 cfu of *P. aeruginosa* strains 6206, 19660, 6294 or PAO1 suspended either in cell culture media, fresh tears, or tears pre-exposed to cells.

3.4.3 Corneal Epithelial Cell Factors < 3 kDa Provide Protection Against *P. aeruginosa* Invasion, Cytotoxicity and are Bactericidal.

Our data showing that pre-exposing the corneal epithelial cells to human tear fluid is sufficient to provide protection against *P. aeruginosa* virulence mechanisms and that pre-exposing the tear fluid to corneal epithelial cells does not increase its bacteriostatic activity suggested that corneal cell defenses play an important role in protection. Therefore, protective effects of corneal epithelial cell-expressed molecular defenses, especially those modulated by tear fluid, were examined. Strain 6206 or 6294 suspended in either cell culture media or various lysate fractions (>100 kDa, 10-100 kDa, 3-10 kDa, < 3 kDa) of cells pre-exposed to cell culture media (normal lysate) or human tear fluid for 16 h (conditioned lysate) was inoculated onto naïve corneal epithelial cells. Invasion data showed that crude lysate, 3-10 kDa and < 3 kDa fractions of both normal and conditioned lysates provided significant protection against invasion by 6294 compared to media (Figure 12A). Interestingly, conditioned lysate fractions 3-10 kDa and < 3 kDa provided even greater protection against invasion. Cytotoxicity assay, assessed by trypan blue staining, showed that all normal and conditioned lysate fractions provided protection against 6206 (Figure 12B). Trypan blue exclusion assay controls in each experiment confirmed that each lysate fraction was not toxic to the cultured corneal epithelial cells. Surprisingly, while normal crude lysate provided protection against 6206, conditioned crude lysate had more cell injury compared to media control. Fractions containing factors < 3 kDa showed enhanced protective/bactericidal effects suggesting that these factors are likely to be functionally regulated by other lysate components of higher molecular weights.

Since the lysate fractions provided protection against invasion and cytotoxicity, its effect on bacterial survival was examined. Strains 6206 and 6294 were suspended in either media, normal or conditioned crude lysate or its fractions for 3 h at 37^{o}C. Normal and conditioned crude lysates, fractions >100 kDa and 10-100 kDa retarded growth of 6206 and 6294 with an exception of normal lysate fraction 10-100 kDa. Normal and conditioned fractions 3-10 kDa and < 3 kDa actually proved to be bactericidal with conditioned < 3 kDa fraction showing greater bactericidal activity compared to normal fraction < 3 kDa (Figure 12C). This result suggested that protection was provided by bacteriostatic or bactericidal activity of the lysates.


(A)
Normal Lysate
Conditioned Lysate
CFU/ml +/- SD
80000.0
70000.0
60000.0
50000.0
40000.0
30000.0
20000.0
10000.0
0.0
6206
6294
KBM
Crude Lysate
Fraction 1 (>100kDa)
Fraction 2 (10-100kDa)
Fraction 3 (3-10kDa)
Fraction 4 (<3kDa)
(B)
M
NL (Normal Lysate)
NL (>100 kDa)
NL (<10 kDa)
NL (<3 kDa)
6206
CL (Conditioned Lysate)
CL (>100 kDa)
CL (<10 kDa)
CL (<3 kDa)

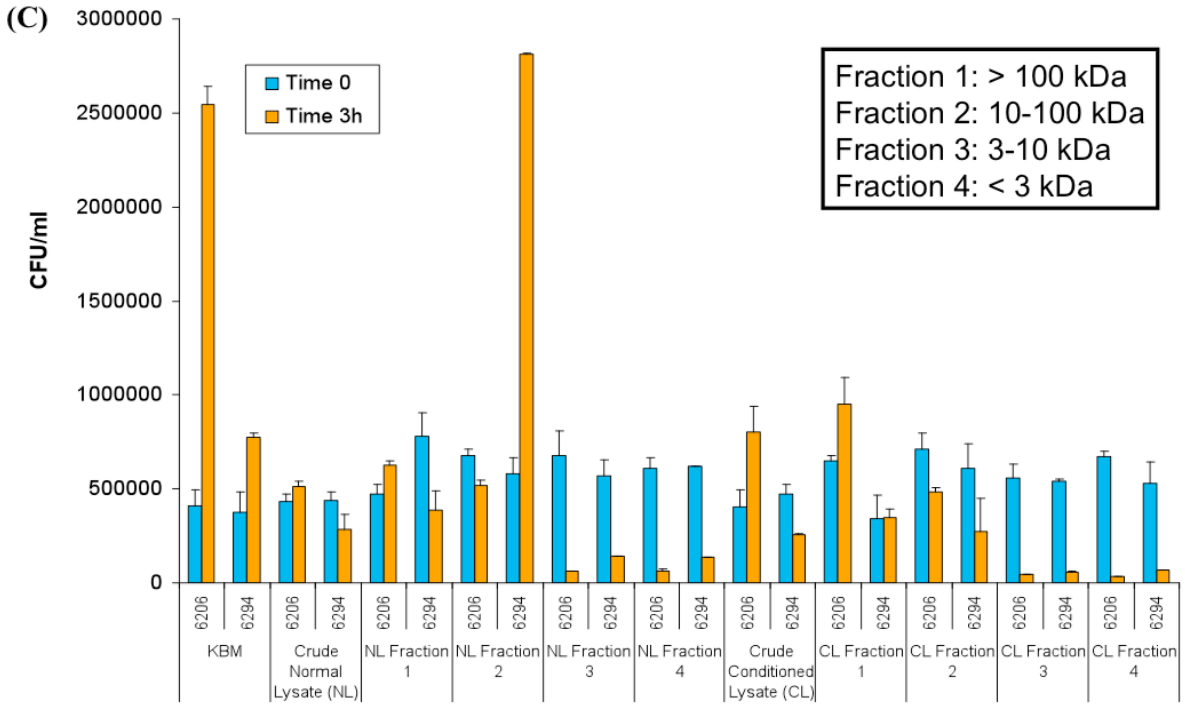


Figure 12. Corneal Epithelial Cell Factors < 3 kDa Provide Protection Against *P. aeruginosa* Invasion, Cytotoxicity and are Bactericidal. Tear fluid upregulated corneal epithelial cell factors < 3 kDa provide protection against *P. aeruginosa* invasion (A) cytotoxicity (B) and are bactericidal (C). To determine if corneal epithelial cell expressed molecular factors provide protection against *P. aeruginosa* virulence mechanisms and to examine if this protection is enhanced by pre-exposure to tear fluid, corneal cells exposed to either cell culture media or human tear fluid for 16 h were lysed and fractionated by size. Naïve corneal epithelial cells were then inoculated with ~10^4 cfu of 6206 or 6294 suspended in crude lysate or specific lysate fractions. Bacteriostatic activity and invasion were quantified by viable count. Cytotoxicity was assessed by trypan blue staining. Fractions containing factors < 3 kDa showed enhanced protective/bactericidal effects suggesting that these factors are likely to be functionally regulated by other lysate components of higher molecular weights.

3.4.4 Tear Fluid Magnifies Bacterial-Induced Increases in Luciferase Expression Controlled by NFkB and AP-1 Transcription Factors.

Corneal epithelial cells (transfected with a mixture of a pathway-focused transcription factor-responsive firefly luciferase construct and a constitutively expressing *Renilla* luciferase construct) were either incubated with tears or media for 16 h followed by 6 h incubation with *P. aeruginosa* supernatant. Tear fluid magnified bacterial-induced increases in luciferase expression controlled by NFkB and AP-1 transcription factors by ~5- and 20-fold, respectively (Figure 13).

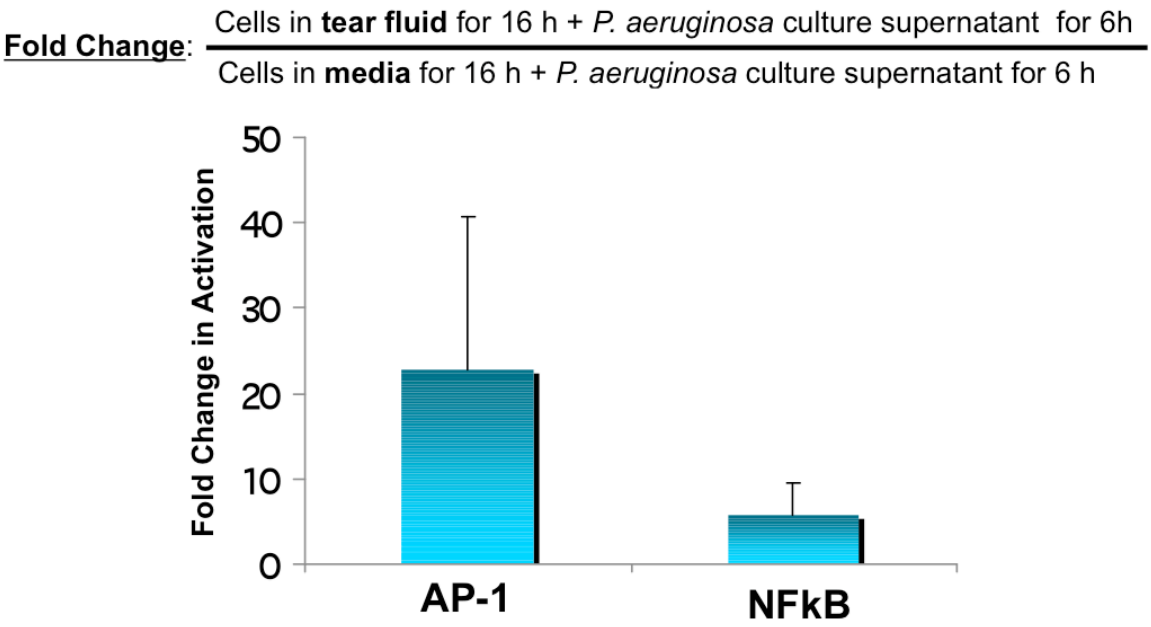


Figure 13. Tear Fluid Magnifies Bacterial-Induced Increases in Luciferase Expression Controlled by NFkB and AP-1 Transcription Factors. Corneal epithelial cells (transfected with a mixture of a pathway-focused transcription factor-responsive firefly luciferase construct and a constitutively expressing *Renilla* luciferase construct) were either incubated with tears or media for 16 h followed by 6 h incubation with *P. aeruginosa* supernatant.

3.4.5 Tear Fluid Induces Expression of Histatin-3 and Magnifies Bacterial-Induced Expression of Histatin-3 mRNA Expression.

To examine if protection provided by lysate fractions was due to upregulation of antimicrobials known to play a role against *P. aeruginosa*, mRNA expression of antimicrobials hBD-2, hBD-3, LL-37 and Histatin-3 were examined in corneal epithelial cells either exposed to media or tear fluid for 6 h (Fig. 14A) and in cells pre-exposed to media or tear fluid for 16 h followed by 3 h inoculation with *P. aeruginosa* supernatant (Fig. 14B). Antimicrobials hBD-2, hBD-3, LL-37 were chosen for study because they lie within 3-10 kDa fraction and are known to play a role in ocular defense (68), while Histatin-3 has been shown to be an important oral antimicrobial (16, 49, 85) and no other antimicrobial less than 3 kDa has been shown to be expressed in the corneal epithelial cells. There was no increase in expression of hBD-2, Histatin 3 and LL-37 with exposure to tear fluid, while Histatin-3 expression was induced with tear fluid. Furthermore, for cells that were pre-exposed to tear fluid and then inoculated with *P. aeruginosa* supernatant, there was about a 2- and 3-fold increase in expression of hBD-3 and Histatin 3, respectively.

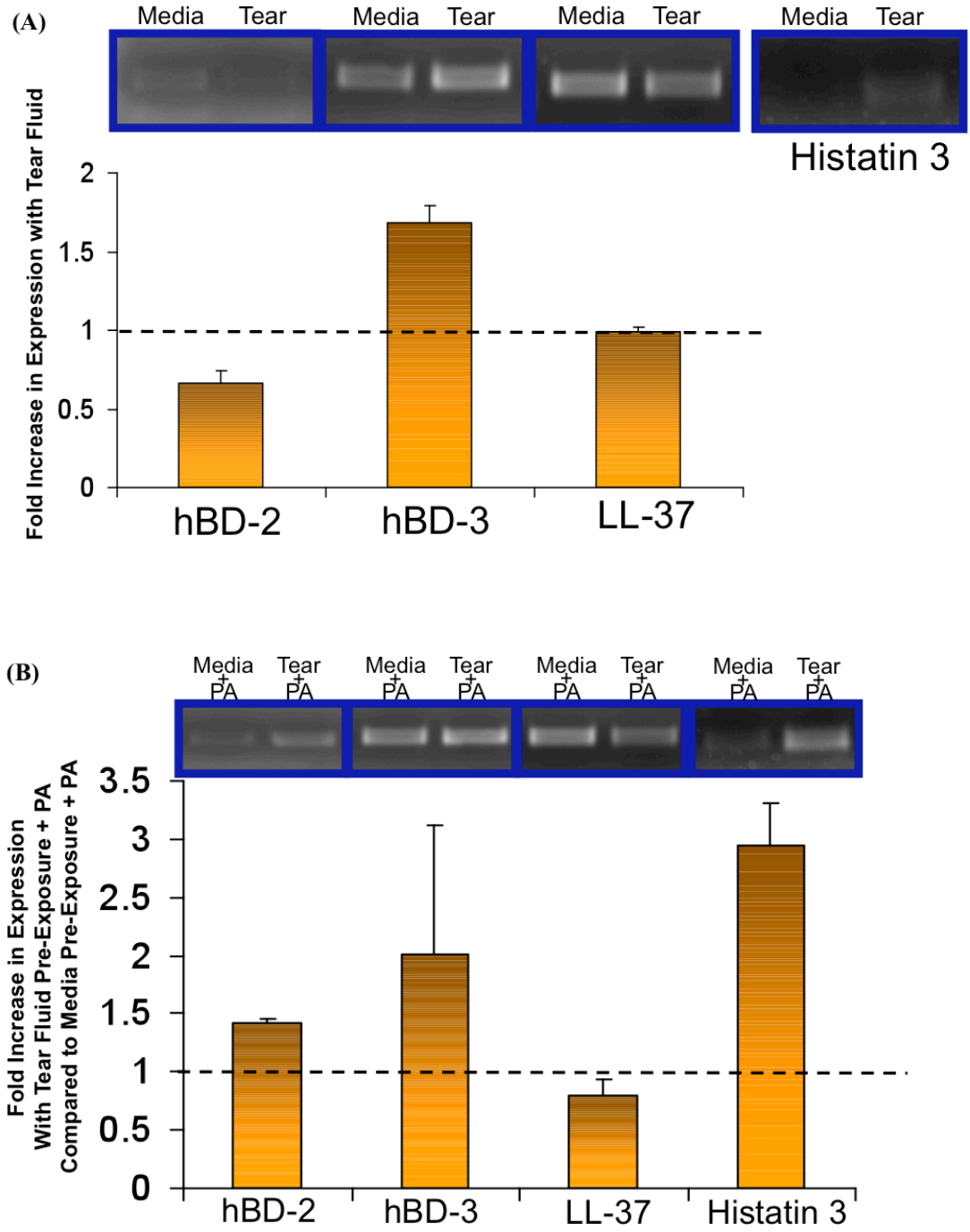


Figure 14. Tear Fluid Induces Expression of Histatin-3 and Magnifies Bacterial-Induced Expression of Histatin-3 mRNA Expression. Corneal cells exposed to cell culture media or human tear fluid for 6h were compared for expressions of hBD-2, hBD-3, LL-37 and Histatin-3 antimicrobials (A). Corneal cells pre-exposed to cell culture media or human tear fluid for 16h, washed, and then inoculated with *P. aeruginosa* supernatant for 3h were compared for expressions of antimicrobials mentioned above (B).

3.4.6 Differential Regulation of Genes in Human Corneal Epithelial Cells by Tear Fluid.

Tear fluid alone (6 h) upregulated 184 genes (Table 2) and downregulated 29 genes (Table 3). Pre-exposure to tear fluid alone (16 h) followed by exposure to *P. aeruginosa* antigens upregulated 168 genes (Table 4) and downregulated 102 genes (Table 5). Some of the upregulated genes were classified as encoding kinases, kinase regulators, enzymes, transcriptional regulators, tight junction proteins, cytokines and their receptors, proteases and protease inhibitors, an antimicrobial and a microRNA. Downregulated genes included kinases, kinase regulators, enzymes, transcriptional regulators, Ran/Ras and hypoxia- and cell cycle-related proteins.

Biological Functions Media vs. Tears 5 Fold Increase	# of Genes	Examples
Transcriptional Regulators/Factors	37	early growth response, zinc fingers, activating transcription factors, activator of NFKB, repressor of BIFN, Fos, FosB, tumor repressors
Cytokine Related	22	TNFalpha, gene induced by TNFalpha, IL8, IL1R1 , IL13 R, IL6R. Trib1 (negative regulator of NF-IL6), SOCS3 (induced by IL6, IL10, IFN-gamma) , chemokines that attracts neurtrophils, interferon, IL3, IL2 repressor.
Apotosis/Anti-Apoptosis	5	pleckstrin homology-like domain, family A, member 1
Other	30	Unknown functions, chromosome open reading frames
Response to Stress Stimuli	2	transcript levels are increased following stressful growth arrest conditions and treatment with DNA-damaging agents
Kinases/Kinase Regulators	12	Phosphatases in MAPK pathway, inositol 1,4,5-trisphosphate 3-kinase C, negative regulator of MAPK, IRAK2 (involved in IL-1 induced upregulation of NFKB), activator of MAPK/NFKB,
Protease/Protease Inhibitors	7	MMP1, MMP3, serine peptidase inhibitor/trypsin inhibitor,
Structural	7	hyaluronic acid, keratin associated protein
Immune cell Promoters / Related	2	Macrophage activation
Transmembrane Proteins/Cell-Cell Adhesion/ Junctional	7	Tight junction proteins (zona occludens), cell surface proteins or functionally unknown
TGFB/Smad Pathway	6	Down reg of MMP-1 and MMP-13 via TGFbeta dependent pathway, TGFB family proteins,
Cancer Related	6	-
Antimicrobial	2	ribonuclease (has antimicrobial activity 14.5 kDa in skin)
Regulatory Signal Transduction/Adaptor proteins	4	GTP binding protein, adaptor protein for B cells,
Enzyme/Catalysts	12	
Receptors/Receptor Interacting	8	
Development	3	
Post-transcriptional regulator/microRNA	1	MicroRNA21
Neural	7	
Protein Transport	4	Golgin-45

Table 2. Biological Functions of Genes Upregulated by 5-fold or More with 6 h Exposure to Tear Fluid.

Biological Functions Media vs. Tears 5 Fold Decrease	# of Genes	Examples
Transcriptional Regulators	1	
Transcription/Nuclear Factors	2	
Other/Unknown hypothetical proteins	7	Unknown functions, chromosome open reading frames
Kinases / Regulators of Kinases	2	
Stuctural	1	
Transmembrane Proteins/Cell-Cell Adhesion/Junctional	1	
Tumor related	2	
Regulatory Signal Transduction/Adaptor proteins	3	
Neural	1	
Enzyme	2	
Receptors/Receptor Interacting	1	
Development	1	
RAN/Ras protein related	4	
Hypoxia	1	Hypoxia inducible

Table 3. Biological Functions of Genes Downregulated by 5-fold or More with 6 h Exposure to Tear Fluid.

Biological Functions Media +PA vs. Tears +PA 5 Fold Increase	# of Genes	Examples
Transcriptional Regulators	4	
Cytokine Related	3	IL1RL1, IL1R2, IL13RA2
Transcription/Nuclear Factors	3	Histones
Other	38	Hypothetical, RAS oncogene, unknown etc.
Response To Stress Stimuli	1	
Kinases / Regulators of Kinases	9	Cyclins, cAMP dependent kinase inhibitors
Proteases/inhibitor	13	Cysteine protease inhibitor, MMP1, MMP10, trypsin inhibitor, serine proteases
Stuctural	13	keratin, hyaluronan, comeodesmosomes in epidermis, fibronectin
Immune Cell Related/Response Related	1	
Transmembrane Proteins/Cell-Cell Adhesion/ Junctional/Integrins	12	Cadherin, integrin, disintegrins, cingulin (interacts with ZO-1 for epithelial tight junction), connexin.
TGFB/Smad Pathway	3	
Tumor related	13	
Antimicrobial	1	ribonuclease (antimicrobial activity)
Regulatory Signal Transduction/Adaptor proteins	2	
Protein Transport	2	Non-clathrin coated vesicular coat proteins,
Mucin	1	Mucin 20
Apoptosis	3	Autophagy, engulfment of apoptotic cells, protector of apoptosis.
Neural	5	GABA
Enzyme	19	catalysts for signal transduction in retina
Receptors/Receptor Interacting	10	integrins, oxytocin receptor, acetylcholine receptor, anthrax toxin receptor, receptor for hyaluronic acid/MMPs,
Development	10	Embryonic development, cell growth, muscle atrophy, cell growth arrest.
Post-transcriptional regulator/ microRNA	2	RNA editing enyzmes, regulator of RNA splicing/export of target RNAs from the nucleus/ translation of proteins/RNA stability.

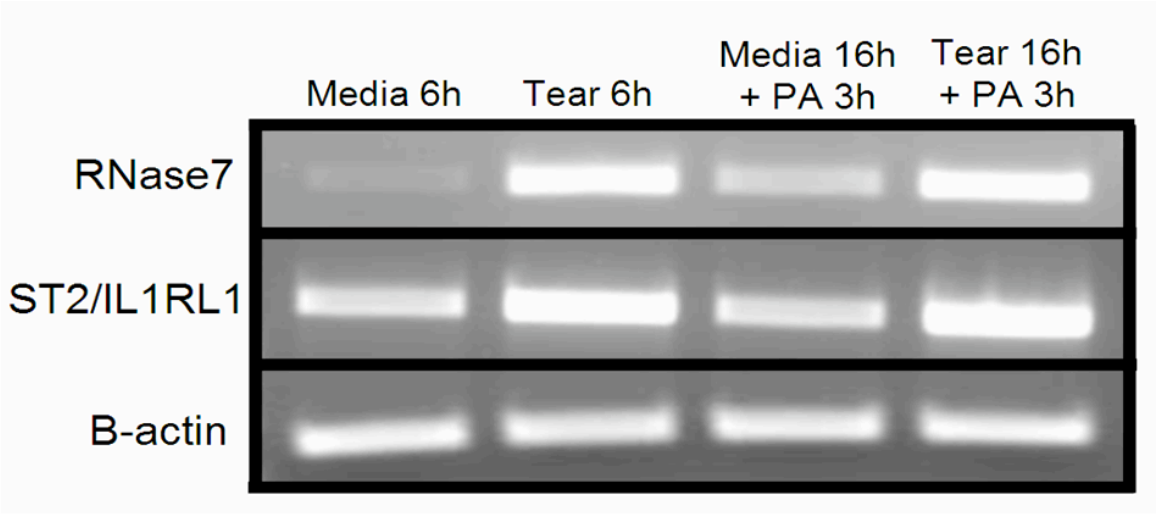
Table 4. Biological Functions of Genes Upregulated by 5-fold or More with Pre-exposure to Tear Fluid Followed by 3 h Incubation with *P. aeruginosa* Supernatant.

Biological Functions Media +PA vs. Tears +PA 5 Fold Decrease	# of Genes	Examples
Transcriptional Regulators/Factors	5	Zinc finger, nuclear proteins/repressors.
Cytokine Related	3	IL20R, biomarker for Th1 response in carcinoma
Other	13	Unknown functions, chromosome open reading frames
Kinases / Regulators of Kinases	2	Regulators of CDK/cyclin kinases.
Proteases/inhibitor	2	Calpains, serine peptidase inhibitor
Stuctural	4	Extracellular matrix glycoproteins, oxidase for biogenesis of connective tissue.
Transmembrane Proteins/Cell-Cell Adhesion/Junctional	2	Nuclear pore protein complex
Tumor related	6	
Protein Transport/Trafficking	2	Proteins involved in protein folding, cationic amino acid transporters (by keratinocytes),
Apoptosis Related	3	Caspase-1
Enzyme	17	Reductase, acetyltransferase, lipoxygenase,
Receptors/Receptor Interacting	2	
Development	1	
Mitosis/Meiosis/Cell Cycle	40	Cyclins, CDKs, segregation checkpoint proteins, etc.

Table 5. Biological Functions of Genes Downregulated by 5-fold or More with Pre-exposure to Tear Fluid Followed by 3 h Incubation with *P. aeruginosa* Supernatant.

3.4.7 RT-PCR Validation of mRNA Expressions of RNase7 and ST2.

The microarray data showed a significant increase in expression of RNase7, an antimicrobial, and ST2, also known as IL-1 like receptor 1 (IL1RL1). To confirm these upregulations in expression with tear fluid, semi-quantitative RT-PCR was carried out. Tear alone increased expressions of RNase7 and ST2 by ~4.5 and ~1.5-fold, respectively. Corneal epithelial cells pre-exposed to tear fluid followed by exposure to *P. aeruginosa* supernatant increased expressions of RNase7 and ST2 by ~2.5 and ~1.7-fold, respectively (Figure 15).



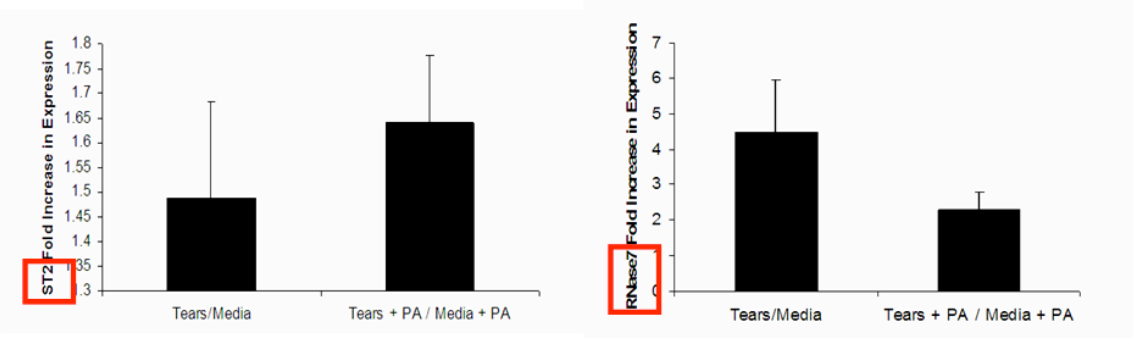


Figure 15. RT-PCR Validation of mRNA Expressions of RNase7 and ST2. 6 h tear exposure and pre-exposure to tear fluid followed by 3 h incubation with *P. aeruginosa* supernatant showed significant increases in expressions of RNase7 and ST2.

3.5 Discussion

3.5.1 Summary

In this study, we showed that pre-exposing the human corneal epithelial cells to tear fluid is sufficient to provide protection against *P. aerguinosa* virulence mechanisms but that pre-exposing the tear fluid to corneal epithelial cells does not increase its bacteriostatic activity. These data suggest that the protective effects of tear fluid against *P. aeruginosa* invasion and cytotoxicity involve the modulation of corneal epithelial cell defenses, some of which are known to be regulated by NFkB and AP-1. Magnified increases in luciferase expression controlled by these transcription factors also suggest that enhanced upregulation of innate defense molecules occurs in corneal epithelial cells in response to bacteria when cells are exposed to tear fluid. To investigate which defense factors may be playing this defensive role, the cell lysates fractionated by size, showed that corneal epithelial cell factors < 3 kDa (smaller than known epithelial antimicrobial peptides) can protect against *P. aeruginosa* invasion and cytotoxicity and that tear-mediated modulation of corneal epithelial cell defenses may enhance this protective effect. We showed that expression of histatin (< 3 kDa in size) is induced in the human corneal epithelia by tear fluid and may be a candidate explaining this protective mechanism. We have also shown that tear fluid has profound effects on gene expression by human corneal epithelial cells, which include altering their response to *P. aeruginosa* antigens. Among the differentially regulated genes, RNase7 and ST2 showed significant increase in expression with tear fluid.

3.5.2 Implications of Experimental Results

Our lab has previously shown that tear fluid retards the growth of certain strains of *P. aeruginosa* but does not kill them. Our current study showed that in the absence of tear fluid and merely pre-exposing the corneal epithelial cells to tear fluid is sufficient to provide protection, suggesting that the corneal epithelial cells play a greater role in terms of providing protection. This indicates that perhaps the tear fluid provides bacteriostatic activity because many of the defenses made by the corneal epithelial cells are secreted into the tear film including SP-D and LL-37 (68, 76, 77).

Although corneal lysates < 3 kDa showed bactericidal activity, tear fluid exposed cells did not show significantly more bactericidal activity or protection against virulence mechanisms. This may suggest that many of these defenses are constitutively expressed and that tear fluid only increases its production, and hence, providing greater protection for the cells. This is supported by the increases in activations of NFkB and AP-1, which regulate hBD-2 (63).

Our semi-quantitative RT-PCR and/or microarray data suggested that inducing or increasing expression of certain genes by tear fluid may attribute to its protective mechanism. Histatin 1, 3 and 5 are potent antimicrobials produced by parotid and submandibular glands and have been heavily studied in oral research (16). Furthermore, P113, a peptide of its parent molecule, Histatin 5, has been shown to have potent antibacterial activity against *P. aeruginosa* (85). Here, we showed that expression of Histatin 3, precursor to Histatin 5, is induced by tear fluid and/or *P. aeruginosa*. Histatin 5 and its peptide P113, which are both less than 3 kDa in size, may be one of the key contributing bactericidal factors in the < 3 kDa corneal cell lysate. RNase7, another antimicrobial (~14.5 kDa) heavily studied in human skin, showed increased expression with tear fluid (40, 97, 98). Lastly, expression of ST2, a member of the IL1R family, increased with tear fluid. ST2 is a member of the IL1R family and either comes in soluble (sST2) or transmembrane form (ST2L) (95). Huang et al. showed that ST2L is important in resistance to *P. aeruginosa* keratitis and in reducing corneal infection, bacterial load and inflammation (47).

3.5.3 Future Directions

In summary, we exhibited that tear fluid provides protection for corneal epithelial cells by modulating the expression of cell defenses and that these defenses may be regulated by NFkB and AP-1. Some of these tear fluid-regulated cell defenses may include Histatin (< 3 kDa), RNase7 and/or ST2, which all were upregulated with tear fluid exposure. However, we have found that the expression of many more genes is impacted by tear fluid exposure. Determining which other gene products play a role in tear-induced defense against infection need to be investigated further.

Chapter 5: The Healthy Eye and the Tear Fluid

This thesis work indicates that the surface of the healthy eye is protected against colonization by *P. aeruginosa* by maintaining antimicrobial activity through the corneal epithelial cells' interaction with the tear fluid. Our data supports this conclusion: bacteria added to the ocular surface lose viability, yet tear fluid removed from the eye supports bacterial growth. This data also suggests involvement of surface corneal cells in the mechanism for protection at the tear film/ocular surface interface *in vivo*.

This thesis provides a lead to understanding as to why soft contact lens wearers are more susceptible to bacterial keratitis. Soft contact lenses as opposed to hard lenses have been shown to deter tear flow and exchange underneath the lens (52, 94). We have shown that tear fluid not only provides protection by retarding the growth of bacteria but also by upregulating the expressions of antimicrobials (Histatin 3 and RNase7) and a receptor (ST2) shown to decrease bacterial load in murine corneal infection model (47). Therefore, with lack of tear accessing the corneal epithelial cells, this expression and/or upregulation of important antimicrobials may not occur, increasing the ocular surface's susceptibility to infection.

In this thesis work, we developed, and demonstrated, the usefulness of a new "null-infection" model because traditional models for studying bacterial keratitis in which disease is induced do not allow normal resistance factors to be directly examined. With this model, we have shown SP-D to be involved in clearance and that *P. aeruginosa* proteases can compromise it. There are likely to be an array of host factors (in addition to the ones discussed in this thesis) that protect the eye under normal circumstances and there are also likely to be bacterial factors with the potential to compromise clearance. Further studies using this model could facilitate our understanding of the circumstances surrounding resistance and susceptibility to infection, and could eventually lead to new approaches for treatment or prevention of infection of the eye and of other sites.

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