Title

Transient Receptor Potential (TRP) Ion Channels and Corneal Defense Against Bacterial Adhesion

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Publication Date

2024

Peer reviewed|Thesis/dissertation

Transient Receptor Potential (TRP) Ion Channels and Corneal Defense Against Bacterial Adhesion

By

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A dissertation submitted in partial satisfaction of the

requirements for the degree of

Doctor of Philosophy

in

Vision Science

in the

Graduate Division

of the

University of California, Berkeley

Committee in charge:

Professor Suzanne Fleiszig, Chair

Professor Diana Bautista

Professor Teresa Puthussery

Spring 2024

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Abstract

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Professor Suzanne Fleiszig, Chair

While the eye's surface is constantly exposed to microbes in the environment, healthy corneas lack a microbiome and have an unusual ability to resist colonization and infection by opportunistic pathogens *Pseudomonas aeruginosa* (Gram-negative) and *Staphylococcus aureus* (Gram-positive). However, extended contact lens (CL) wear and overt injury can result in the development of sight-threatening bacterial keratitis. Essential to understanding the pathogenesis of bacterial keratitis are the defenses that normally prevent corneal colonization and infection. The cornea has a high density of sensory nerve endings expressing the polymodal ion channels TRPV1 (Vanilloid) and TRPA1 (Ankyrin) whose main function is detecting noxious stimuli and modulating pain and itch. Some recent studies have shown that in some circumstances they or the nerves expressing them can detect bacterial ligands and modulate inflammatory responses. In the healthy cornea, our lab previously showed that TRPA1 can counter the adhesion of deliberately inoculated *P. aeruginosa* in large numbers. Experiments that I contributed to in that publication also showed TRPV1 can counter environmental colonization (likely commensal bacteria in fewer numbers), suggesting specificity in their protective roles in the healthy cornea. Here, I tested the hypothesis that the specificity of TRPA1 and TRPV1 in contributing to corneal defense against adhesion is influenced by bacterial status as a pathogen versus a commensal, respectively, or bacterial Gram-type. Otherwise, specificity relates to bacterial inoculum size. Using a Gram-positive pathogen of the eye, *Staphylococcus aureus S33* (a human, ocular clinical isolate), I found that TRPV1, not TRPA1, prevented the adhesion of this pathogen (Chapter 2). Contrasting with the TRPA1-mediated defense against *P. aeruginosa*, TRPV1-mediated defense against *S. aureus* did not require sensory nerve-firing mechanisms, suggesting a local mechanism.

I further observed different TRPV1/A1 and nerve-dependent immune cell responses. *P. aeruginosa* increased the number of CD45+ and CD11c+ cells, the latter involving sensory nerve-mediated expression of inflammatory cytokines and chemokines including IL-6, IL-1β, CCL7, and CXCL5 (Chapter 3). Conversely, *S. aureus* triggered a smaller CD45+ cell response with no increase in CD11c+ cell numbers. Further immune cell analysis revealed distinct morphologies and migration changes induced by *S. aureus* and *P. aeruginosa* inoculation. While both pathogens decreased CD11c+ cell sphericity

(cells appeared more dendriform), their migration patterns differed. Following *S. aureus* inoculation, CD11c+ cells migrated further from the corneal surface while *P. aeruginosa* induced their migration toward the corneal surface. Additionally, CD45+ cells became more spherical in response to *S. aureus* rather than less spherical as observed for *P. aeruginosa*. Lyz2+ cell numbers were not impacted by either pathogen; however, cells were more spherical in response to *S. aureus* only and moved closer to the surface for *P. aeruginosa* only.

Since *P. aeruginosa* is Gram-negative, while *S. aureus* is Gram-positive as are most environmental bacteria that colonize TRPV1 mutated corneas, Chapter 4 investigated the hypothesis that bacterial Gram-type, rather than pathogen versus commensal, differentiates their involvement in corneal defense. This was tested using a Gram-positive mouse eyelid commensal *Macrococcus epidermidis*, and *Corynebacterium mastitidis*, a Gram-positive conjunctival commensal. The results showed that neither TRPA1 nor TRPV1 were involved for corneal defense against *C. mastitidis*. However, both receptors participated in corneal defense against *M. epidermidis*. In this instance, TRPA1-V1-mediated defenses were not dependent on sensory nerve firing suggesting local mechanisms as observed for the Gram-positive pathogen *S. aureus*. Using quantitative mass spectrometry analysis of corneal surface eye washes following *M. epidermidis* challenge (cultured supernatant), I discovered significant differences in the abundance of tear-fluid associated proteins mediated by TRPV1, some with known antimicrobial functions. These included the anterior gradient protein 2 (AGR2) an important mucin-producing factor, Polymeric Immunoglobulin Receptor (pIgR) for SIgA, Lipocalin 11, and S100 A11 calcium-binding protein.

A mouse model of CL wear developed by our lab was previously used to show that a parainflammatory response (subclinical inflammation) occurs after 24 h and 6 days of lens wear as observed during human lens wear. This parainflammatory response was found to be driven by microbes at the ocular surface during contact lens wear, with an antibiotic inhibiting the response, and restoration after the addition of various commensal bacteria including *C. mastitidis* and a coagulase-negative *spp* (later identified as *M. epidermidis*). Thus, there are parallels between the phenotype I have studied and contact lens-mediated immune cell responses. As part of my dissertation research, I contributed data to a subsequent publication that showed contact lens-related parainflammation is modulated by both TRPA1 and TRPV1. Further, unpublished data that I contributed to collecting showed that the TRPA1/V1 mediated CL-induced parainflammatory response is associated with increased corneal defense against the commensal *M. epidermidis*, suggesting it can protect against commensal bacteria adhesion.

Taken together, the findings presented in this dissertation revealed differences in the healthy cornea's ability to recognize, respond, and initiate defense against bacteria, involving TRPA1/V1-sensory nerves. These findings also advance our understanding of the local and immune cell responses that can occur within the healthy infection-resistant cornea upon bacterial inoculation and the relative roles of sensory nerves/TRP receptors in modulating these. Which aspects of those cellular responses contribute to defense against bacterial adhesion, and the molecular factors that prevent adhesion at the corneal surface, remain to be determined.

Dedication

To the loving memories of my grandma Joycelyn Clarke and grandad Winston Folks

To my amazing mom Rosemarie Clarke

To the most supportive friends Avriel and Adriana

Acknowledgments

This journey would not have been possible without the people who have guided and supported me along the way. I was fortunate to participate in the **Passport to College** program that afford students in Jamaica the opportunity to obtain an undergraduate degree in the US. Through this program, a host of opportunities presented themselves and I was able to pursue my PhD, the first in my family to do so. This journey was by no means easy and plagued with challenges including a global pandemic so I would like to highlight the people who have been instrumental in my growth and development over the last 5 years.

As mentors**, Suzi and David** were extremely kind, and supportive, and always knew what was best for me to grow as a Scientist. Suzi's knack for the big picture and David's love for the finer details taught me a lot about developing questions, thinking through problems, and being detail oriented. I feel very fortunate to have been mentored by two of the most amazing people who I will never forget and always have the utmost love and respect for. I would also like to acknowledge my committee members **Dr. Diana Bautista and Dr. Teresa Puthussery** for their invaluable support. As successful women in academia, they have been truly inspirational.

I was also fortunate to spend time with some of the smartest and most supportive **labmates** who started to feel like home. When I joined the lab, **Vince and Abby** were extremely nice and helped make the transition from undergrad to PhD very smooth. They taught me all the basic skills I needed to start doing experiments, which were fundamental to my dissertation project. **Ananya** and I joined the lab together and were inseparable. We laughed, cried, and explored the Bay area together. She was a personal and professional mentor who believed in me every step of the way, even after leaving the lab. **Naren** has been like a third PI. We worked together on some of the most foundational experiments of my dissertation. His ability to think critically, his excitement for science, and his out-of-the-box ideas inspired and challenged me in ways that were crucial for my growth. **Eric** was the most comforting energy to be around. He never took himself too seriously and was always down to teach us. I am still in awe at the depth of his general knowledge, and I could not ask for a better graduate student to share this journey with. **Yujia** (aka the scruff master) has been a true friend. I looked forward to Thursdays and Fridays when she would be in our lab part-time. We would go for walks and lunch breaks which brought me a lot of peace and comfort regardless of how stressful things were. Those were the moments that truly helped me to reset, and I will never forget just how kind and amazing she is as a Scientist and friend. **Daniel** is fairly new to our lab, but it feels like I have known him forever. Whenever I wanted a mental break, I would sneak in the breakroom to chat with him, and he always has the best stories. I will walk into that room upset and leave with the biggest smile on my face after distracting him from work. He also took very good care of my emotional support lab plants and is always willing to get lab supplies from the very high shelves for me. **Sarah** is also new but has quickly become a part of our lab culture. She is kind, sweet, and has a very peaceful energy to be around in the lab. Consequently, Yujia and I have started taking her on our lunch breaks/walks. There are so many great things I can say about all these amazing people and more. If they ever read this, I hope they will understand just how much they mean to me and how fundamental they were to my journey. My forever family.

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Chapter 1

Introduction

1.1. Bacterial keratitis

The healthy cornea is exceptionally resistant to virtually all pathogens. However, factors such as contact lens wear and ocular injury/trauma can predispose the cornea to bacterial keratitis. Bacterial keratitis is a prevalent sight-threatening infection characterized by inflammation of the cornea most often caused by opportunistic Gram-negative pathogen *Pseudomonas aeruginosa* and Gram-positive pathogen *Staphylococcus aureus*. Other causes include Gram-positive *Streptococcus pneumoniae*, and Gram-negative *Serratia marcescens* (Dart *et al.*, 1991; Keay *et al.*, 2006; Otri *et al.*, 2013; Bouhenni *et al.*, 2015; Pifer *et al.*, 2023). Corneal infections pose a significant clinical challenge due to their potential to cause severe vision loss if not quickly diagnosed and treated. Furthermore, the challenge of bacterial keratitis is complicated by the increase of multidrug-resistant strains of *P. aeruginosa* and *S. aureus,* rendering traditional antibiotic therapies less effective, resulting in poor treatment outcomes and prolonged infections (Egrilmez and Yildirim-Theveny, 2020). Even successful antibiotic treatments do not always prevent lasting damage such as corneal scarring, which can further impair vision. Therefore, there is an urgent need to understand how contact lens predisposes the healthy, infection-resistant cornea to keratitis is crucial in preventing its occurrence in the first place.

Previous research related to keratitis has relied on infection models that circumvent the cornea's intrinsic defenses (Girgis *et al.*, 2003; Marquart, 2011), leaving a critical gap in our understanding of the intrinsic defenses that normally protect against bacteria. As a result, our lab has directed its efforts toward understanding the constitutive corneal defenses against bacteria during health to inform how predisposing factors such as contact lens wear and injury may set up conducive conditions for infection. Our research has been guided by questions to elucidate both host defenses and bacterial virulence, with an emphasis on characterizing the intrinsic factors that typically protect against infection, which is the focus of this dissertation.

1.2. How Does the Healthy Cornea Normally Resist Infection?

Central to comprehending the cornea during pathology is a complete understanding of the factors that enable this unique resistance. Indeed, the healthy corneal epithelium demonstrates remarkable resistance to microbiome formation and pathogen adhesion (Wan *et al.*, 2018), the latter being an important step in infection pathogenesis (Ramphal, McNiece and Polack, 1981). Importantly, this resistance extends to the adhesion of pathogens, including *P. aeruginosa* and *S. aureus*. Using a null infection model ((challenging healthy corneas that do not become infected), our lab has shown murine corneas can swiftly clear either of these pathogens inoculated in large concentrations *in vivo* (~10^{11} Colony-Forming Units (CFU)) (Mun *et al.*, 2009; Augustin *et al.*, 2011; Wan *et al.*, 2018). Interestingly, clearance occurs without the induction of pathology, even in enucleated eyes (Tam *et al.*, 2011; Metruccio *et al.*, 2017). However, corneal epithelial cells grown in cell culture are more susceptible to pathogenic bacterial insults, even at lower concentrations (~10^6 CFU) (Fleiszig S M, Zaidi T S, and Pier G B, 1995; Jett Bradley D. and Gilmore Michael S., 2002), suggesting factors present in and/or around the intact cornea are critical for mediating epithelial defense. Our lab has identified many factors that lend themselves to the tissue's incredible defense resistance during health (Fleiszig *et al.*, 2020). Key ones absent in cell culture include tear fluid, basal lamina, immune cells, and sensory nerves. These factors will be discussed in this section, shedding light on some of the foundations of the corneal epithelium's resilience *in vivo*, and the gaps this dissertation will fill.

1.2.1. Tear Fluid

An integral part of the defense at mucosal sites is the mucosal fluid which serves as a physical barrier to prevent pathogens from accessing and infecting cells beneath. At the ocular surface, this is achieved through the tear fluid/film that bathes the cornea and neighboring tissues. The tear fluid consists of three important layers (Mucin, aqueous, and lipid) that all work in concert to protect the ocular surface (Figure 1.1). Defense is achieved through an elaborate system of antimicrobial factors that can modulate both epithelial defense and virulence mechanisms of prominent ocular pathogens including *P. aeruginosa and S. aureus* (Fleiszig, McNamara and Evans, 2002; Fleiszig, Kwong and Evans, 2003; Mudgil, 2014; Wu *et al.*, 2017).

Our lab has shown a significant role for the tear fluid glycoprotein Deleted in Malignant Brain Tumors 1 (DMBT-1) in reducing *P. aeruginosa* virulence, thereby preventing infection in murine corneas *in vivo*, and inhibiting epithelial traversal of the pathogen when supplemented *in vitro* (Li *et al.*, 2019). Additionally, our previous studies have demonstrated the effectiveness of surfactant protein D, found in human tear fluid, in preventing *P. aeruginosa* binding to murine corneas *in vivo* (Mun *et al.*, 2009). Other proteins within tear fluid contribute to the defense against bacterial colonization by breaking down bacterial cell walls, inhibiting iron availability, and suppressing bacterial growth. These include lysozyme, lysozyme-like proteins, lactoferrin, secretory Immunoglobulin A (sIgA), and lipocalin (Fleming, Alexander, 1922; Oram and Reiter, 1968; Selsted and Martinez, 1982; Alizadeh *et al.*, 2001; Singh *et al.*, 2002; Williams, Schneider and Willcox, 2003; Fluckinger *et al.*, 2004; McDermott, 2013). Some of these can also be impacted during lens wear. For example, lysozyme was shown to be less

abundant in contact lens wearers (Kramann *et al.*, 2011). Additionally, lens wearers demonstrated reduced tear sIgA, potentially impacting ocular surface defense versus *P. aeruginosa* (Willcox Dr and Lan, 1999).

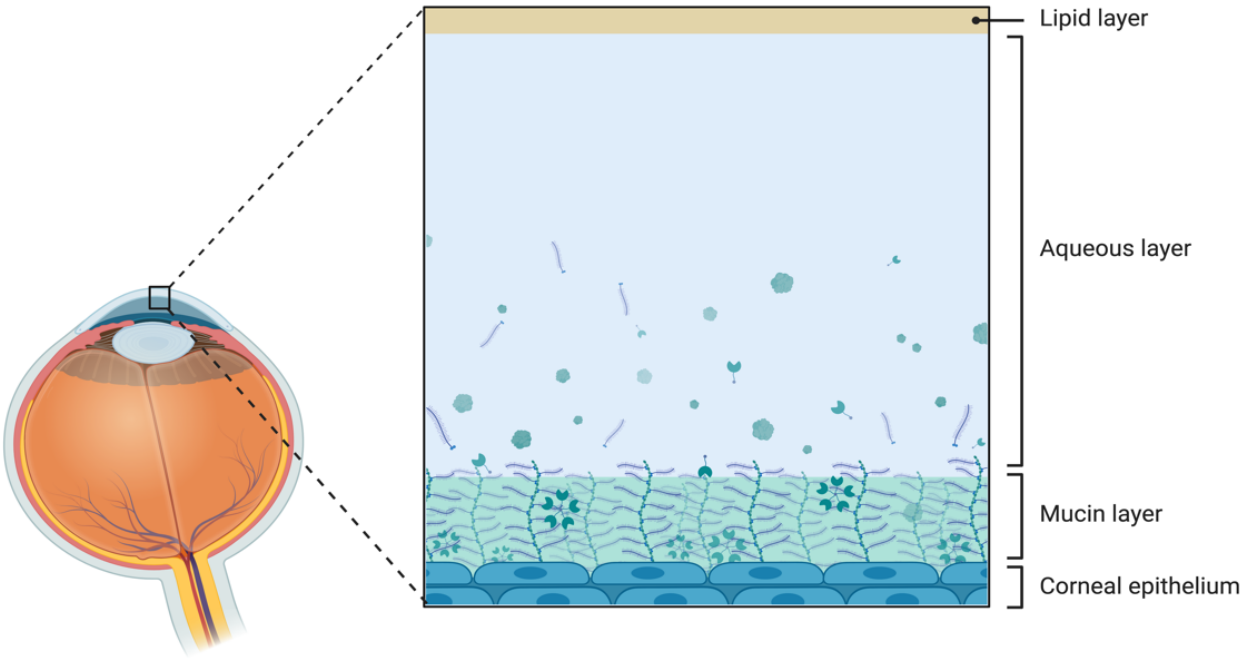


***Figure 1. 1**: Schematic of the structure of the tear fluid that bathes the ocular surface*

1.2.2. Corneal Epithelium and Basal Lamina

It is critical that the cornea remain transparent to enable optimal vision (Gonzalez-Andrades, Argüeso and Gipson, 2019). Consequently, in concert with the tear fluid, the cornea is tasked with fending off microbes and various environmental elements it routinely encounters that may disrupt transparency. This is achieved particularly through the mucin layer of the tear fluid that interfaces with the corneal epithelium which can prevent adhesion of bacteria and other harmful debris (Fleiszig *et al.*, 1994; Davidson and Kuonen, 2004). However, if bacteria manage to bypass the defenses provided by the tear fluid, the corneal epithelium *in vivo* serves as a formidable barrier, exerting antimicrobial roles both molecularly and functionally to deter adhesion and penetration.

Molecularly, the expression of antimicrobial peptides such as defensins, cytokeratin 6A, and RNase7 at the epithelium also contribute to pathogenic defense during health as demonstrated in our previous studies (McNamara *et al.*, 1999; Augustin *et al.*, 2011; Tam *et al.*, 2012; Mun *et al.*, 2013). Bound epithelial mucins/glycoproteins have also been shown to participate in epithelial defense by inhibiting bacterial adhesion to the corneal surface (Fleiszig *et al.*, 1994; McNamara, Sack and Fleiszig, 2000).

Functionally, intact corneal epithelial cells can prevent the invasion of microorganisms into intracellular spaces, owing to the tight junctions between adjacent cells. Additionally, the polarized nature of the epithelial cells have been shown to contribute to defense against *P. aeruginosa* invasion and cytotoxicity (Fleiszig *et al.*, 1997; Fleiszig *et al.*, 1998). The underlying basal lamina also functionally contributes to the epithelium's formidable defense by acting as a selective filter with pore sizes of ~1μm, less than that of bacteria. Our lab has shown that this layer acts as a physical barrier to protect against *P. aeruginosa* infections and can influence epithelial barrier function (Alarcon *et al.*, 2009, 2011). During circumstances that compromise the epithelial barrier, such as lens wear or ocular injury/trauma, pathogens can traverse the corneal epithelium. Once traversal occurs, the otherwise impenetrable basal lamina can be breached, permitting bacteria to enter the stroma. The presence of bacteria in the stroma initiates the characteristic inflammation and vision loss associated with keratitis.

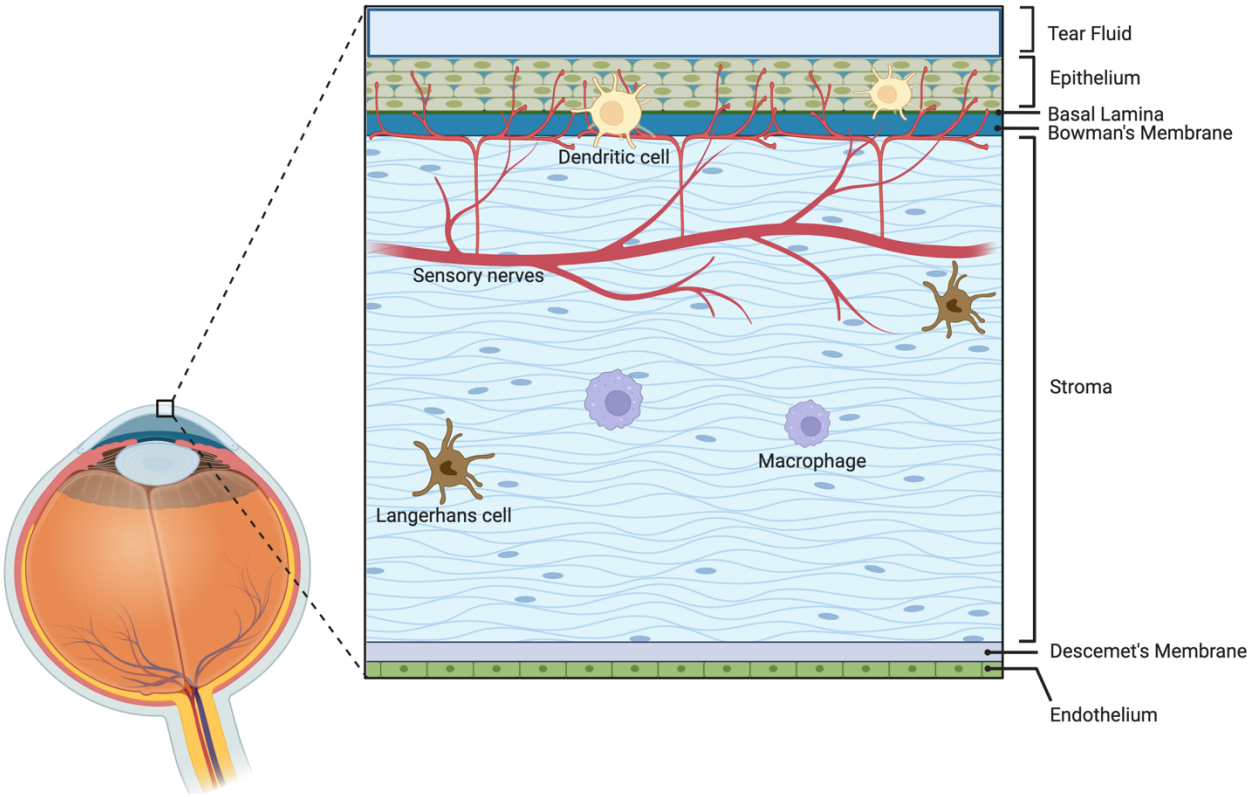


***Figure 1. 2**: Schematic of corneal anatomy and resident cell types*

1.2.3. Resident Immune Cells

Another important aspect of the corneal defense system is the resident immune cells, also absent *in vitro*. Once believed to be void of immune cells due to its immune privilege, the cornea contains resident immune cells that indeed contribute to immune surveillance and host defense. These include antigen-presenting CD11c+ cells, which our lab has previously shown can counter the adhesion of inoculated *P. aeruginosa* during

superficial injury in the absence of outright inflammation or pathology. In addition, the innate immunity receptors and signaling molecules MyD88, IL-1R, and TLR4, all play roles in this defense and can contribute to preventing a corneal microbiome (Tam *et al.*, 2011; Metruccio *et al.*, 2017; Wan *et al.*, 2018). We further reported antibiotic treatment reduces baseline CD11c+ cell numbers, complemented by the addition of commensal bacteria suggesting an important role for bacteria/ bacterial ligands (potentially from neighboring tissues) in maintaining this baseline population (Datta *et al.*, 2022). Other resident immune cells include antigen-presenting Langerhans cells and Macrophages (Figure 1.2). They possess phagocytic and antigen-presenting capabilities as well as the ability to secrete inflammatory cytokines and chemokines (IL-1β, TNF-α, IL-8, IL-10, and CCL5) that can recruit immune cells such as neutrophils to sites of trauma or infection (Brissette-Storkus *et al.*, 2002; Chinnery *et al.*, 2008; Sokol and Luster, 2015; Jeang, Margo and Espana, 2021). Similarly, our lab has also shown that Ly6G+ neutrophils are recruited during extended contact lens wear mediated by the IL-17 cytokine (Metruccio *et al.*, 2019; Datta, Truong, *et al.*, 2023). However, in those studies, the mouse corneas showed no signs of visible pathology despite infiltrating immune cells. This is indicative of a subclinical immune response, previously termed parainflammation (Medzhitov, 2008). Interestingly, we have also shown that an antibiotic can suppress parainflammation during lens wear, suggesting microbes play a role in mediating it (Datta *et al.*, 2022).

1.2.4. Corneal Sensory Nerves

While the factors discussed previously, among others, contribute significantly to corneal epithelial defense *in vivo*, the defense system remains incomplete. Supporting this, the neighboring conjunctiva also has access to tear fluid and epithelial defenses and has access to an even larger population of immune cells via its blood supply (absent in the healthy cornea), but it still harbors a microbiome and is much more susceptible to routine bacterial infections.

The corneal surface houses two primary cell types: epithelial cells, which we know are more susceptible to bacterial adhesion when grown in cell culture, and a large number of sensory nerve endings, the latter not extensively explored in the cornea's intrinsic defense against bacteria. This dense innervation is also a salient difference between the cornea and other mucosal surfaces such as the conjunctiva, making it exquisitely sensitive to a host of noxious stimuli (Oliveira-Soto and Efron, 2001). Roughly 50–450 sensory trigeminal neurons transmit nerve fibers via the ophthalmic division of the trigeminal nerve to the cornea where they branch and terminate as free nerve endings in the corneal epithelium (Figure 1.2). In addition to enabling sensation, these sensory nerve endings play important roles in modulating homeostasis and wound healing in the cornea via their well-established neurochemicals calcitonin gene-relate peptide (CGRP) and Substance P (SP) (Labetoulle *et al.*, 2019). When produced, these neurochemicals can bind to human corneal epithelial cells to induce IL-18 and IL-8 synthesis, the latter resulting in neutrophil influx (Tran *et al.*, 2000; Tran, Lausch and Oakes, 2000), and in this way can play roles in modulating corneal epithelial defense.

1.2.4.1. Transient Receptor Potential (TRP) Ion Channels

The capacity of the corneal sensory nerve endings to detect a wide range of stimuli involves the calcium-permeable polymodal Transient Receptor Potential (TRP) Ion

channels. These are mostly TRPV1 (Subfamily, Vanilloid), with TRPA1 (Subfamily, Ankyrin) present only on a subset of TRPV1-expressing nerves (Belmonte, Carmen Acosta and Gallar, 2004; González-González *et al.*, 2017) (Figure 1.3). In the cornea, TRPA1, and TRPV1 are expressed on unmyelinated c-fibers or Aδ fibers at different layers of the corneal epithelium with TRPV1 colocalizing with CGRP and/or glial cell line-derived neurotrophic factor family receptor alpha3 (GFRα3) (Alamri *et al.*, 2015; Schecterson *et al.*, 2020; Jiao *et al.*, 2021). Some corneal nerve endings also express TRPM8 (Melastatin) which mostly lacks the characteristics of nociceptors but can modulate basal tearing at the ocular surface (Parra *et al.*, 2010).

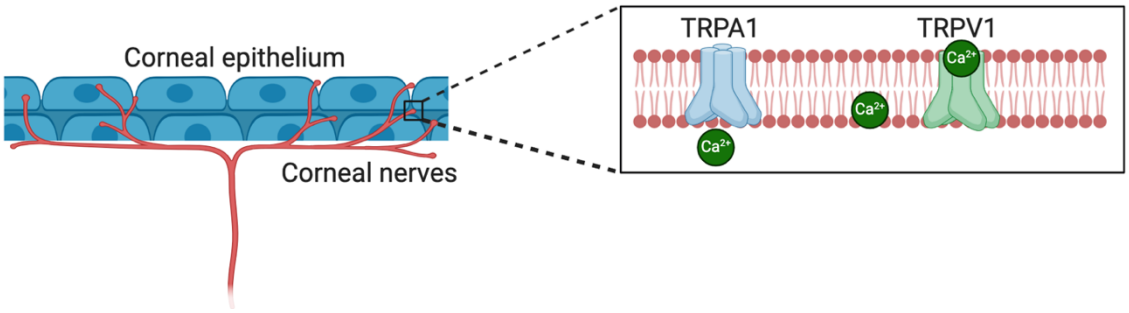


Figure 1. 3: *Schematic of the calcium-permeable Transient Receptor Potential (TRP) Ion Channels, Vanilloid 1 (TRPV1) and Ankyrin 1 (TRPA1) on corneal sensory nerve endings*

1.2.4.2. Transient Receptor Potential (TRP) Ion Channels <u>During Infection</u>

TRP receptors have been extensively studied during infection (bacterial and viral), and inflammatory diseases (Engel *et al.*, 2011; Kun *et al.*, 2014; Lapointe *et al.*, 2015; Gouin *et al.*, 2017; Omar *et al.*, 2017; Csekő *et al.*, 2019; Xu *et al.*, 2019; Jain *et al.*, 2020; Bousquet *et al.*, 2021; Cremin *et al.*, 2023). Moreover, evidence suggests that lipopolysaccharide (LPS) from gram-negative bacteria can directly activate sensory nerves to release neuromodulators that drive inflammation and pain via TRPA1 (Meseguer *et al.*, 2014). TRPV1+ neurons have also been identified in protecting against *Salmonella* infection through CGRP-mediated mechanisms (Lai *et al.*, 2020). Additionally, the supernatant of gram-positive pathogen *S. aureus* culture can induce CGRP release from both TRPA1 and TRPV1-expressing neurons *in vitro* (Blake *et al.*, 2018). At the ocular surface, *P. aeruginosa* virulence factors lead to inflammatory responses that depend on TRPV1 in a keratitis murine model (Lin *et al.*, 2021).

While these studies suggest an important relationship between sensory nerves and microbial defense mechanisms, this has only been studied during outright infection and inflammation. Investigations into the role of sensory nerves during health remain limited, presenting a significant gap in our understanding of their protective role(s), especially in the cornea where maintaining transparency is essential.

1.2.4.3. Transient Receptor Potential (TRP) Ion Channels <u>During Health</u>

The proximity of the sensory nerve endings to the corneal epithelial cells, and the speed with which they can detect and respond to noxious stimuli provided compelling evidence that TRPA1 and TRPV1 sensory nerves are an ideal first line of defense against bacteria in the healthy cornea. In support of this, research to which I contributed during my first year in the lab showed that corneal nerves and TRPA1 both play roles in the intrinsic defense against deliberately inoculated *P. aeruginosa* (~10^{11} CFU/mL) during health and that this correlates with sensory-nerve mediated CD11c+ cell recruitment (Wan *et al.*, 2021). This was discovered using three different methods: 1) blocking corneal sensory nerve function with bupivacaine, which increased *P. aeruginosa* adhesion to healthy and superficially injured mouse corneas; 2) ablation of TRPA1 and TRPV1 using RTX, resulting in increased *P. aeruginosa* adhesion to WT corneas; and 3) utilizing gene knockout mice, where TRPA1 (-/-) mouse corneas exhibited increased *P. aeruginosa* adhesion to healthy and superficially injured mouse corneas. In contrast, the study revealed that TRPV1 can prevent colonization by environmental contaminants, which can include commensal bacteria at adjacent tissues.

1.2.4.3.1. Gaps

While our previous study highlights the important contributions of sensory nerves, TRPA1, and TRPV1 in the corneal resistance to distinct bacterial types, the factors that dictate their specificity require further characterization. Indeed, *P. aeruginosa* is a Gram-negative pathogen inoculated in large numbers, while the bacteria TRPV1 counter would mostly be Gram-positive commensal, and in fewer numbers. Therefore, their specificity may rely on bacterial status as a pathogen or commensal, bacterial Gram type, and/or inoculum size to distinguish threats to corneal health. In addition, although the study identifies TRPA1 and TRPV1 as key players in corneal defense against bacterial adhesion, the mechanisms by which they exert their protective roles are not fully understood. Given their differential involvement, it is likely that the mechanisms differ. For example, TRPA1-mediated defense against *P. aeruginosa* correlated with a corneal CD11c+ cell response above baseline without outright inflammation, while TRPV1-mediated corneal defense would need to be consistently present to prevent environmental colonization. Moreover, it remains unclear whether TRPA1 and TRPV1 can also play roles in the mechanisms underlying the parainflammatory response during lens wear, and the significance of this response in terms of corneal defense or susceptibility to infection.

1.2.4.3.2. Hypotheses and General Approaches

In Chapters 2-4 of this dissertation, I tested the hypothesis that the specificity of TRPA1 and TRPV1 in contributing to corneal defense against adhesion is influenced by bacterial status as a pathogen versus a commensal, or Gram type. The alternative hypothesis was that specificity was determined by bacterial inoculum size. To test these, C57BL/6J wild type (WT) or gene knockout mice in TRPA1 or TRPV1 were inoculated with Gram-positive (a pathogen and commensal) or Gram-negative bacteria (a pathogen). In some experiments, before inoculation, WT mice were treated with bupivacaine to block sensory nerve function, resiniferatoxin (RTX) to ablate TRPV1-expressing sensory nerves, or a TRPV1 antagonist and compared to controls.

I began testing the hypothesis that TRPA1 and TRPV1-mediated protective mechanisms against bacterial adhesion differed. In some inoculation experiments,

corneas were assessed for Lyz2+, CD11c+, CD45+ cells or cytokine/chemokine responses to explore the role of immune cells in the TRPA1/V1-mediated defense against pathogens (Chapters 2 and 3). I also explored whether TRPV1-mediated defense against commensal/Gram-positive bacteria was related to antimicrobial factors secreted at the ocular surface/tear fluid interface (Chapter 4). WT or TRPV1 (-/-) eyewashes were collected after bacterial supernatant (*M. epidermidis*) challenge and then analyzed using quantitative mass spectrometry for differentially expressed proteins.

Exploring a link between TRPA1/V1-mediated corneal defense and contact lens-induced parainflammation published work that I contributed to, tested the hypothesis that TRPA1/V1 sensory nerves can play a role in modulating lens-induced parainflammation. To test this, WT mice were subjected to our murine model of lens wear and compared to lens-wearing TRPA1 (-/-), TRPV1 (-/-), or RTX-treated mice. Corneas were then assessed for MHC-II+, CD45+, γδ T, or TNF-α+ cell responses after 24h or 6 days of lens wear. Subsequent unpublished work that I contributed to, tested the hypothesis that this parainflammation associated with lens wear protects the otherwise sterile cornea against commensal bacteria colonization. Therefore, after 4-6 days of continuous lens wear, corneas were challenged with *M. epidermidis*.

These experiments revealed differences in the TRPA1/V1-mediated corneal defense against bacteria, including sensory nerve firing requirements, parainflammatory responses, and modulation of tear fluid-associated proteins. These findings and their significance will be detailed in Chapters 2-5 and further summarized in Chapter 6.

Chapter 2

TRP-dependent Corneal Defense Varies by Ocular Pathogen and Mediates Specific Parainflammatory Responses

This Chapter includes some findings and reprint from Wan et al., *FASEB J* (2021), (Figure 2.10 A-F) in which I contributed to experiments that discovered environmental colonization of TRPV1 (-/-) mice corneas versus WT and TRPA1 (-/-) (Figure 2. 10 E, F). This finding laid the foundation for my dissertation project.

Relevant Publication: Wan, SJ, Datta A, Flandrin O, Metruccio MME, Ma S, Nieto V, Kroken AR, Hill RA, Bautista D, Evans DJ, Fleiszig SMJ (2021) Nerve-associated transient receptor potential ion channels can contribute to intrinsic resistance to bacterial adhesion in vivo. *FASEB Journal*, 35 (10). https://doi.org/10.1096/fj.202100874R

Chapter 2: TRP-dependent Corneal Defense Varies by Ocular Pathogen and Mediates Specific Parainflammatory Responses

2.1. Abstract

We have previously shown Transient Receptor Potential (TRP) ion channels Ankyrin 1 (TRPA1) and Vanilloid 1 (TRPV1) located on sensory nerves can prevent corneal colonization by the potential pathogen *P. aeruginosa* and environmental (commensal) colonization, respectively. Here, we investigated whether they play specific roles in preventing adhesion depending on bacterial status as a pathogen versus commensal, with TRPA1 being essential for preventing pathogen binding in the healthy cornea. Corneas of anesthetized C57BL/6J wild-type (WT), TRPA1 (-/-), and TRPV1 (-/-) gene knockout mice were inoculated with *Staphylococcus aureus* (Gram-positive pathogen, and human ocular clinical isolate). In some experiments, before inoculation, pharmacological methods were employed to determine TRP-sensory nerve involvement. CD11c+, Lyz2+, and CD45+ immune cell responses were assessed, and adhered bacteria were identified using a universal bacterial 16S rRNA FISH probe. Differing from *P. aeruginosa*, murine corneas deficient in TRPV1 were more susceptible to *S. aureus* adhesion. This phenotype was mirrored by RTX (resiniferatoxin) ablation of TRPV1-expressing sensory nerves and further confirmed by local TRPV1 antagonism. *Ex vivo*, the TRPV1-dependent defense for *S. aureus* was also abrogated, and in contrast to *P. aeruginosa*, corneal defense against *S. aureus* persisted after bupivacaine anesthesia to block sensory nerve firing. *S. aureus* and *P. aeruginosa* inoculation did not increase Lyz2+ immune cell numbers while CD11c+ cell numbers increased for *P. aeruginosa* only. However, both pathogens increased CD45+ cell numbers which was inhibited by RTX. These findings suggest a crucial difference in the requirement for corneal sensory nerve firing in TRP-mediated defense against pathogens, aligning with a different immune cell response for *S. aureus* versus *P. aeruginosa*.

2.2. Introduction

Pseudomonas aeruginosa and *Staphylococcus aureus* are implicated in a range of ocular diseases, from mild infections to severe complications that can lead to vision impairment. Intriguingly, unlike the adjacent conjunctiva which hosts a viable bacterial community and is susceptible to pathogenic infections, healthy corneas are mostly resistant to bacterial colonization (Willcox, 2013; St. Leger *et al.*, 2017; Wan *et al.*, 2018). Even intentionally challenging corneas with *S. aureus* or *P. aeruginosa,* results in rapid clearance (Mun *et al.*, 2009; Wan *et al.*, 2018), underscoring the tissue's unusual ability to also resist colonization, an important step in the infection process. Consequently, investigating why the cornea does not permit bacterial colonization in the first place is key to understanding the factors that may be exploited by *P. aeruginosa* and *S. aureus* to initiate sight-threatening infections.

Previous work by our lab and others highlighted multiple facilitators of this unique defense against corneal colonization by the potential pathogen *P. aeruginosa* and, to a lesser extent, *S. aureus* during health. These include the tear fluid, mucin glycoproteins, corneal epithelial tight junctions, antimicrobial peptides, and resident immune cells (Fleiszig *et al.*, 1994, 2020; McNamara *et al.*, 1999; Brissette-Storkus *et al.*, 2002; McDermott, 2009, 2013; Augustin *et al.*, 2011; Mun *et al.*, 2011; Metruccio *et al.*, 2017;

Foulsham *et al.*, 2018; Shannon *et al.*, 2022). While these factors underscore an elaborate defense system, they are not exclusive to the cornea. The adjacent conjunctiva, with its access to the tear fluid, shares many of these defense mechanisms. Moreover, it has access to a greater number of immune cells, a rich blood supply, and is replete with goblet cells compared to the cornea. Despite this, the conjunctiva is more prone to colonization by non-pathogens and infection by pathogens (Fleiszig and Efron, 1992; Willcox, 2013; Ozkan and Willcox, 2019; Mohamed *et al.*, 2021). Thus, our knowledge of corneal defense mechanisms, although extensive, remains incomplete.

One of the unique features of the cornea compared to the conjunctiva and other mucosal sites, is the dense innervation of pain-transducing sensory nerves (nociceptors) (Müller *et al.*, 2003; Marfurt *et al.*, 2010; Belmonte *et al.*, 2015). These sensory nerve endings, primarily located in the corneal epithelium, are equipped with polymodal Transient Receptor Potential (TRP) ion channels that enable the detection of harmful stimuli. Given the continuous exposure of the cornea to potential pathogens, rapid detection and discrimination are crucial for initiating an appropriate defense to maintain ocular health. While previous studies have demonstrated the ability of TRPA1 (ankyrin) and TRPV1 (vanilloid) to detect bacterial virulence factors and induce inflammation, (Bautista, Pellegrino and Tsunozaki, 2013; Chiu *et al.*, 2013; Pinho-Ribeiro, Verri and Chiu, 2017; Lai *et al.*, 2020; Lin *et al.*, 2021), these and other investigations have primarily utilized infection models. Consequently, there remains a significant gap in understanding the defense functions of TRP receptors at mucosal sites under healthy conditions.

Our lab has shown there is a rapid corneal CD11c+ cell response induced by *P. aeruginosa* that is absent *ex vivo* in enucleated eyes and correlates with reduced *P. aeruginosa* binding (Metruccio *et al.*, 2017). Related, we reported that TRPA1 and TRPV1 associated with sensory nerves can participate in the corneal defense during health (Wan *et al.*, 2021). In that study, TRPA1 prevented *P. aeruginosa* (~10^{11} CFU/mL) binding to healthy and superficially injured (blotted) corneas, dependent on sensory nerve firing that correlated with CD11c+ cell recruitment. Meanwhile, TRPV1 prevented environmental bacteria from colonizing the cornea suggesting specificity in their protective roles.

Therefore, in this study, we tested the hypothesis that these receptors can confer defenses based on bacterial status as a pathogen versus a commensal, with TRPA1 preventing pathogenic bacterial adhesion. We discovered that WT murine corneas deliberately inoculated with *S. aureus S33* (~10^{11} CFU/mL), a human clinical ocular isolate, required TPRV1, not TRPA1 for clearance during health. Moreover, while TPRV1 sensory nerves were required for clearing this ocular pathogen, there were distinctions in the requirement for sensory nerve firing and parainflammatory responses involving CD11c+, Lyz2+, and CD45+ cells compared to *P. aeruginosa*.

2.3. Methods

2.3.1. Mice

Six- to twelve-week-old male or female C57BL/6J wild-type (WT) mice (Jackson Laboratory) and gene knockouts in TRPV1 (-/-) or TRPA1 (-/-) (kindly provided by Dr. Diana Bautista, University of California, Berkeley, CA USA) were used for bacterial

clearance experiments. In some experiments, transgenic mice with Lysozyme M-Cre (Lyz2-positive immune cells, myeloid-derived, green) or CD11c+-YFP (all CD11c+ immune cells, yellow) were used to assess immune cell responses to inoculated bacteria. All procedures were carried out per standards established by the Association for the Research in Vision and Ophthalmology (ARVO), under the protocol AUP-2019-06-12322 approved by the Animal Care and Use Committee, the University of California Berkeley (an AAALAC-accredited institution). The protocol adheres to PHS policy on the humane care and use of laboratory animals, and the guide for the care and use of laboratory animals.[1]

2.3.2. Bacterial Identification

A clinical isolate (*Staphylococcus spp*) was sequenced for strain identification using previously described methods (Wan *et al.*, 2018). Briefly, bacterial colonies were isolated followed by PCR amplification. Samples were then examined by electrophoresis in 1% agarose gels in 1× TBE buffer. Amplicons were extracted and purified using PureLinkTM PCR Purification Kit (Invitrogen) and sequenced at the UC Berkeley DNA Sequencing Facility. Sequences obtained were identified using the NIH BLAST Search Tool.

2.3.3. Bacterial Strains

Staphylococcus aureus S33 (identified from sequencing, Figure 2.9) clinical isolate from a human ocular infection) was used for bacterial clearance experiments. In some experiments, *Pseudomonas aeruginosa* (PAO1) was used for comparison to *S. aureus*. Strains were prepared by growing on a TSA plate overnight for ~16 hours at 37 °C, followed by suspension in phosphate-buffered saline (PBS) to a concentration of ~10^{11} colony-forming units (CFU)/mL.

2.3.4. Bacterial Clearance Assay

The *in vivo* model of bacterial clearance was used as described previously (Mun *et al.*, 2009; Wan *et al.*, 2021). Mice were anesthetized by intraperitoneal injection of ketamine (80 – 100 mg/Kg) and dexmedetomidine (0.25 – 0.5 mg/Kg). Corneas were inoculated with 5 µl of bacteria once every hour for 4 h. Contralateral corneas were sham inoculated with PBS. After 4 h, animals were euthanized by intraperitoneal injection of ketamine (80–100 mg/Kg) and xylazine (5 – 10 mg/Kg), or isoflurane overdose followed by cervical dislocation. Eyes were enucleated, rinsed with PBS, and fixed in 2% paraformaldehyde (PFA) overnight at 4°C. For *ex vivo* experiments, freshly enucleated eyes were submerged in 200 µL bacterial suspension for 6 h at 37°C.

[1]Throughout my Ph.D., I managed the lab's mouse colonies to ensure mice availability for all projects included in this dissertation. Responsibilities included breeding, weaning, genotyping, recording, personnel training, and adherence to regulatory guidelines.

2.3.5. Fluorescence *in Situ* Hybridization (FISH)

After fixation, whole eyes were labeled for adhered bacteria using a universal 16S rRNA-targeted FISH probe as previously described (Wan *et al.*, 2018, 2021). Briefly, fixed eyes were washed in PBS, 80% ethanol, and 95% ethanol for 10 min each at room temperature (RT). Eyes were then placed in a hybridization buffer solution (0.9 M NaCl, 20 mM Tris-HCl, and 0.01% SDS). This was followed by incubation at 55°C for 30 min. The 16S rRNA-targeted gene probe [Alexa488]-GCTGCCTCCCGTAGGAGT- [Alexa488] (Eurofins Genomics) was added to eyes at a final concentration of 100nM for overnight incubation at 55°C.

2.3.6. TRPA1V1 Nociceptor Ablation and Corneal Sensory Nerve Block

TRPV1-expressing corneal nerves were selectively ablated using resiniferatoxin (RTX) (AdipoGen: AG-CN2-0534-MC05) as previously described (Bates *et al.*, 2010; Muñoz-Carrillo *et al.*, 2017; Wan *et al.*, 2021). Briefly, mice were lightly anesthetized with 3% isoflurane, and 100 µL RTX solution was injected subcutaneously in the scruff of the neck at a final concentration of 30 µM for 3 consecutive days. To inhibit corneal sensory nerve firing, 0.5% bupivacaine hydrochloride solution was injected into the subconjunctival sac (5 µl) and added topically (5µl) in anesthetized mice for 20 minutes[19]. Following RTX ablation or sensory nerve block, bacterial clearance assays and FISH were performed as described above.

2.3.7. TRPV1 Ion Channel Inhibition and Fluorescein Staining

The JNJ-17203212 (Cayman Chemical; #30930) antagonist was used to selectively inhibit TRPV1 channel activity as previously described (Ghilardi *et al.*, 2005; Meents *et al.*, 2015). Briefly, mice were anesthetized and 5µl antagonist was injected into the subconjunctival sac at a final concentration of 500 µM. Additionally, 5µl was immediately added topically. After 20 minutes, corneas were washed with PBS followed by a bacterial clearance assay. In a prior experiment, without bacterial inoculation, a capsaicin eye wipe test was performed after antagonist treatment to confirm antagonist efficacy. At 4 h after the TRPV1 antagonist, 5 µL of fluorescein solution (0.02%) was added to the ocular surface, and corneal epithelial integrity was examined using a slit lamp.

2.3.8. Immunohistochemistry

In some experiments, enucleated eyes were fixed overnight in 2% PFA and then washed for 10 minutes with rotation as previously done (Wan *et al.*, 2021; Datta, Lee, *et al.*, 2023). Corneal dissections were performed using a dissecting microscope and then transferred to a blocking solution (3% bovine serum albumin [BSA] with 0.3% Triton X-100 in PBS) for 1 hour at room temperature. Corneas were then incubated in primary antibody (rat anti-mouse CD45+ [1:500; BD Pharmingen: #550539]) overnight at 4°C with rotation. Corneas were moved to secondary antibody (anti-rat antibody [Life Technologies: #A21434]) diluted in DAPI (4,6-diamidino-2-phenylindole dihydrochloride; 12.5 µg/mL; Thermo Fisher: #D1306) for 2 h at room temperature with rotation and covered with foil. Corneas were then transferred to fresh PBS and washed 3

times for 10 minutes with rotation at room temperature and flat-mounted with Prolong Gold (Thermo Fisher: #P36970) before confocal imaging.

2.3.9. Confocal Microscopy and Image Analysis

Samples were imaged using an Olympus FV1000 confocal microscope (Olympus BX615Wi upright microscope with Olympus FluoView 1000 detection system equipped with Laser Diodes 405, 440, 559, 635 and an Argon Laser 488/515 with a 20×/ 1.0 NA water-dipping objective. The 488 nm laser was used for the detection of bacteria labeled with the FISH probe, fluorescein stain, and Lyz2+ cells. The 515 nm laser was used for CD11c+- YFP cells and the 635 nm laser was used to visualize corneal surface reflectance (excitation and emission at the same wavelength) and corneas labeled with anti-CD45+ antibody. Z stacks were acquired in the central cornea at a 0.4 µm step size and an aspect ratio of 1024 µm x 1024 µm for bacteria detection. For immune cells, a 1.25 µm step size and 512 µm x 512 µm aspect ratio were used to image a central field and 2 random peripheral fields. Acquired Z stacks were reconstructed as 3-D images using Imaris Software. Adhered bacteria were identified and quantified using Imaris spot detection.

2.3.10. Statistical Analysis

Statistical analysis was performed using Prism (GraphPad Software, Inc.). Data were expressed as mean ± standard deviation (SD). Unpaired Student's t-test was used for two group comparisons. Comparisons between three or more groups were performed using One-way or Two-way ANOVA tests with Tukey's multiple comparisons. P-values < 0.05 were considered significant. All experiments were repeated at least twice.

2.4. Results

2.4.1. TRPV1 is Required for Corneal Defenses Versus *S. aureus*

Our previous work demonstrated a role for TRPA1 in preventing adhesion of *P. aeruginosa*, and TRPV1 in preventing environmental (commensal) bacterial colonization (Wan *et al.*, 2021) (See supplemental Figure 2.10 A-D). To extend our understanding of TRPA1 and TRPV1 in the cornea's intrinsic resistance to bacterial adhesion, we explored whether there is a distinction in their protective mechanism related to bacterial status as a pathogen versus a commensal. Corneas of gene knockout mice in TRPA1 or TRPV1 were inoculated with ~1x10^{11} CFU/mL *S. aureus* and compared to WT. TRPV1 (-/-) corneas showed increased FISH labeling (~ 4-fold) compared to WT corneas (Figure 2.1 A-C). Consistent with our previous findings, FISH labeling revealed a significant increase in background environmental bacteria on sham-inoculated TRPV1 (-/-) corneas compared to WT (P = 0.0224, not shown on the figure). Together, these findings support the involvement of TRPA1 and TRPV1 in preventing the adhesion of potential pathogens to healthy corneas.

2.4.2. WT Corneal Defense Versus *S. aureus* is Inhibited by RTX, Eliminated *Ex vivo,* and Retained with Bupivacaine

Next, we assessed the role of sensory nerves in corneal TRPV1-mediated defense against *S. aureus*. Three methods of nerve manipulation were employed as done by us previously to show sensory nerve involvement for defense against *P. aeruginosa* (Wan *et al.*, 2021). The first method involved the use of RTX, a potent, long-lasting TRPV1 activator that can ablate TRPV1-expressing neurons, and by extension co-expressed TRPA1 (Pecze *et al.*, 2009; Malin *et al.*, 2011; Patil *et al.*, 2023). WT mice were treated with RTX for 3 days followed by inoculation with *S. aureus* (~1x10^{11} CFU/mL) *in vivo*. RTX treatment inhibited WT defense against *S. aureus* as demonstrated by the increased FISH labeling compared to sham-treated corneas (~2.7-fold) (Figure 2.2 A-C). This finding mirrors the results using TRPV1 gene knockout mice in Figure 2.1. The second method of nerve perturbation involved our *ex vivo* inoculation model (Metruccio *et al.*, 2017; Wan *et al.*, 2021), which requires enucleating the whole eye, thereby ablating sensory function. After enucleation, eyes were placed in *S. aureus* inoculum (~1x10^{11} CFU/mL) for 6 h. WT corneas showed increased FISH labeling following *S. aureus* inoculation, similar to TRPV1 (-/-) mice suggesting a loss of TRPV1-dependent defense (Figure 2.2 D). The third method involved the use of bupivacaine hydrochloride as a short-term, local anesthesia to block sodium channels on sensory nerves, thereby preventing nerve firing and signal transmission *in vivo*. Surprisingly, corneas were still able to clear inoculated *S. aureus* (Figure 2.2E). To validate the observed phenotype, we performed a positive control experiment using *P. aeruginosa*. As anticipated, inoculation with *P. aeruginosa* showed significantly increased FISH labeling in bupivacaine-treated WT corneas compared to sham controls (~4-fold) (Figure 2.3). Together, these findings demonstrate sensory nerve involvement in the TRPV1-mediated protective mechanisms versus *S. aureus* challenge. Notably, differences in the requirement for nerve firing were observed for defenses against *P. aeruginosa* versus *S. aureus*.

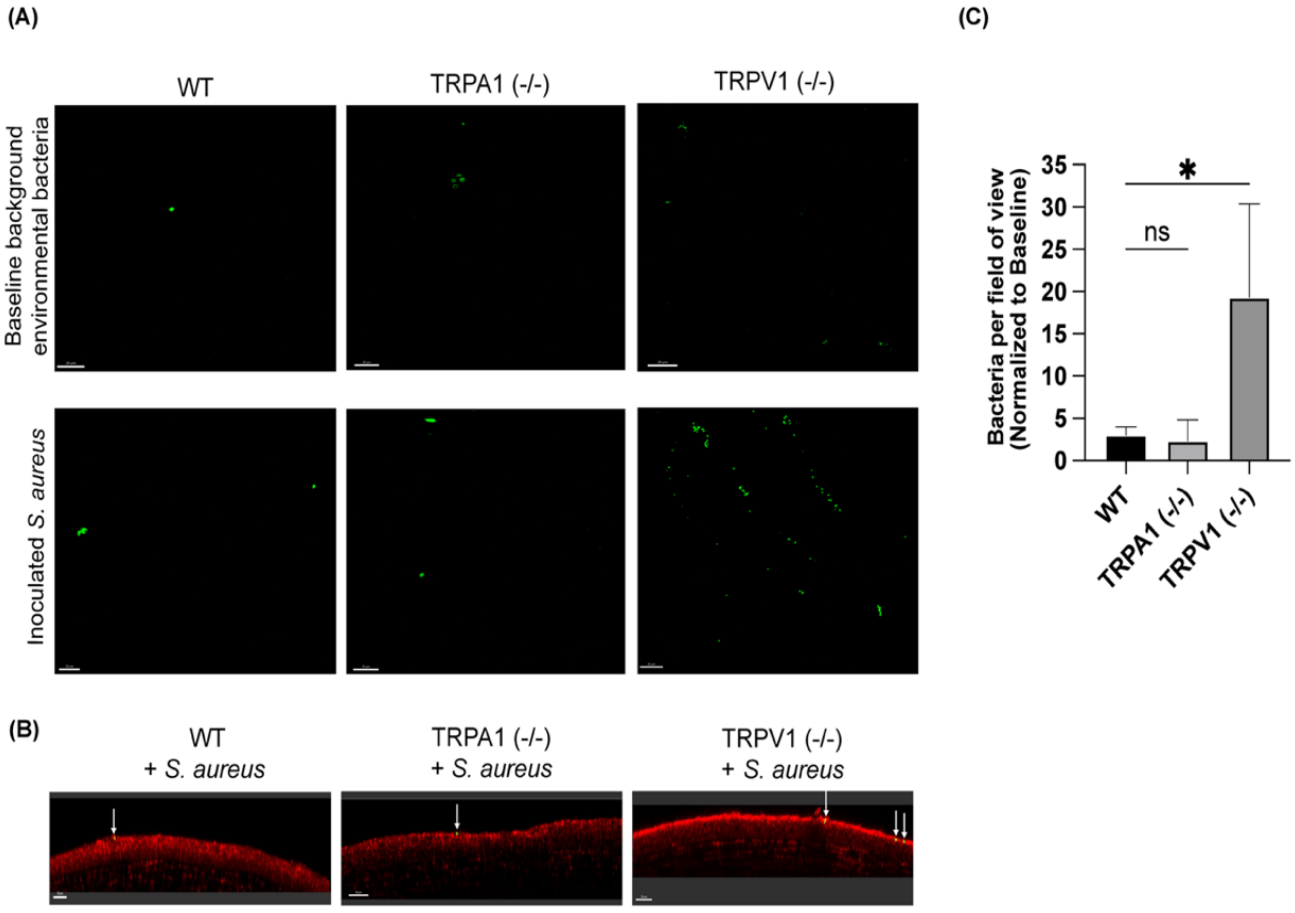


Figure 2. 1: *Corneal defense against pathogen S. aureus requires TRPV1 ion channels.* ***(A)*** *Corneas were labeled using a universal 16S rRNA- targeted FISH probe. Representative images show baseline background environmental bacteria (green) adhering to healthy WT, TRPA1 (−/−), and TRPV1 (−/−) control corneas (top panel) and corneas inoculated with ~1x10^{11} CFU/mL S. aureus for 4 h (bottom panel). 20X Objective. Scale bars = 10 µm, bacterial channel only.* ***(B)*** *XZ optical slices showing bacteria were only surfaced-attached following S. aureus inoculation (green, arrows) and did not penetrate the corneal epithelium (red). Scale bars = 30 µm* ***(C)*** *Quantification shows significantly greater FISH-labeled bacteria on TRPV1 (−/−) corneas inoculated with S. aureus (~4-fold) compared to WT or TRPA1 (−/−). Data were normalized to baseline bacteria and expressed as the mean ± SD of bacteria per field of view. * P <0.05 , ns= no significant difference (Two-way ANOVA with Tukey's multiple comparisons test).*

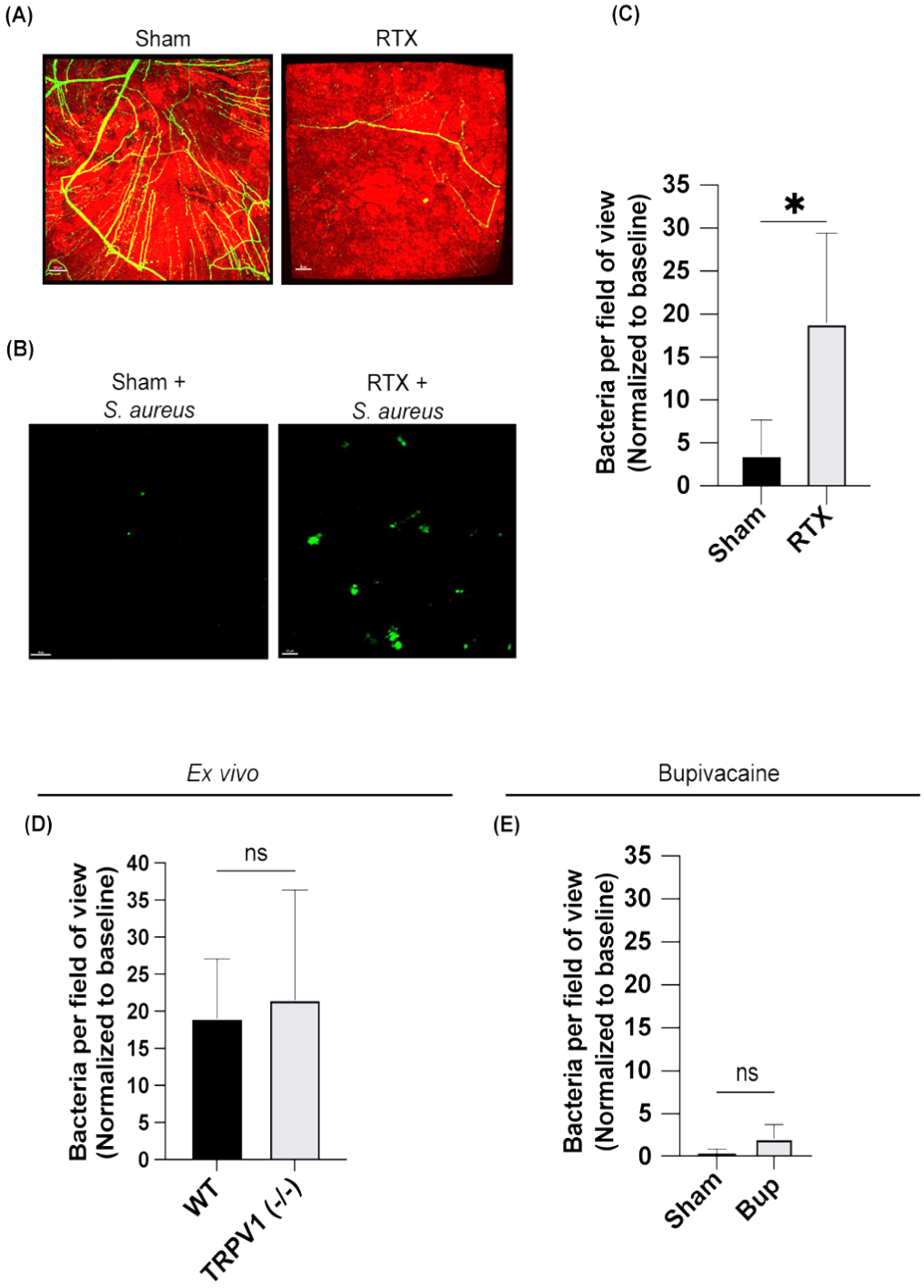


Figure 2. 2: *WT defense against S. aureus was inhibited by RTX, inhibited ex vivo, but retained with bupivacaine sensory nerve block.* ***(A)*** *Corneal sensory nerve density decreases following RTX (30 μM) ablation, as shown by representative images (corneal nerves in green, corneal epithelium in red). Scale bars = 50 μm* ***(B)*** *Corneas were labeled using a universal 16S rRNA-targeted FISH probe to detect background environmental bacteria in controls and also S. aureus in the inoculated group. Representative images show bacterial adhesion to WT corneas post-RTX treatment compared to sham*

treatment following hourly inoculation of ~1x10^{11} CFU/mL S. aureus for 4 h. 20X Objective. Scale bars = 30 µm, bacterial channel only. ***(C)*** *Quantification shows significantly greater FISH labeling to WT corneas inoculated with S. aureus post-RTX compared to sham-treated controls (~2.7-fold). Data are expressed as the mean ± SD of bacteria per field of view. * P <0.05, ns= no significant difference (Two-way ANOVA with Tukey's multiple comparisons test).* ***(D)*** *TRPV1 defense against S. aureus is also inhibited ex vivo. Quantification of adhered bacteria following ex vivo inoculation of ~1x10^{11} CFU/mL S. aureus for 6 h. Inoculated WT corneas showed increased adhered FISH-labeled bacteria compared to controls (~6-fold), which was similar to that of TRPV1 (-/-) inoculated corneas. Data are expressed as the mean ± SD of bacteria per field of view., ns= no significant difference (Two-way ANOVA with Tukey's multiple comparisons test).* ***(E)*** *TRPV1 sensory nerves are important for corneal defense against S. aureus, but sensory nerve firing/activation is not. Quantification of bacterial adhesion reveals similar numbers in FISH-labeled bacteria for both sham-treated and bupivacaine-treated corneas inoculated with S. aureus. Data were normalized to baseline bacteria and are expressed as the mean ± SD of bacteria per field of view. ns= no significant difference (Two-way ANOVA with Tukey's multiple comparison test).*

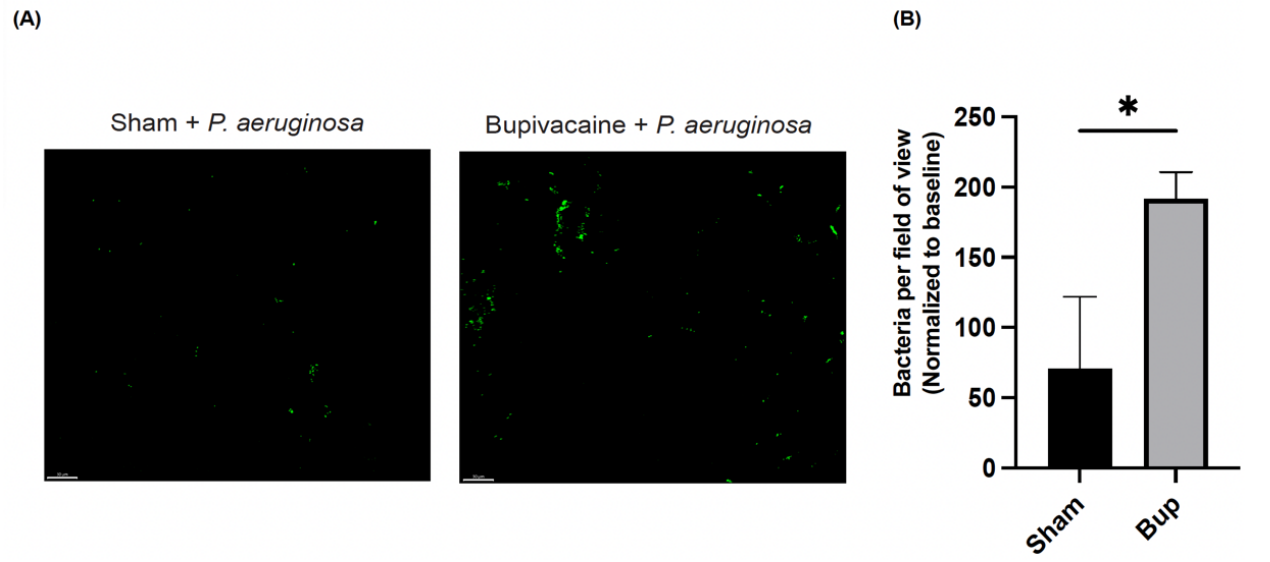


Figure 2. 3: *Increased P. aeruginosa adherence to WT corneas following bupivacaine-mediated sensory nerve block.* ***(A)*** *Corneas were labeled using a universal 16S rRNA FISH probe to detect background environmental bacteria in controls and also P. aeruginosa in the inoculated group. Representative images show increased bacterial adhesion to WT corneas post bupivacaine (0.5%) sensory nerve block compared to sham controls following hourly inoculation of ~1x10^{11} CFU/mL P. aeruginosa for 4 h. 20X Objective. Scale bars = 30 µm, bacterial channel only.* ***(B)*** *Quantification showing bupivacaine significantly increased FISH-labeled bacteria to WT corneas inoculated with P. aeruginosa compared to sham-treated controls (~4-fold). Data were normalized to baseline bacteria and are expressed as the mean ± SD of bacteria per field of view. * P < 0.05 (One-way ANOVA with Tukey's multiple comparisons).*

2.4.3. Antagonist Supports a Local Protective Role in TRPV1-dependent Corneal Defense Versus *S. aureus*

The observation that inoculated *S. aureus* was unable to colonize corneas treated with bupivacaine indicated a role for TRPV1 independent of sensory nerve firing and potentially mediated locally. To examine whether a local TRPV1-mediated response was sufficient to inhibit *S. aureus* adhesion, we inhibited corneal TRPV1 channel activity using the antagonist JNJ-17203212. JNJ-17203212 selectively blocks neuronal TRPV1 activity both *in vivo* and *in vitro* by competing for the capsaicin binding site, rendering the channel inactive to other noxious stimuli (Ghilardi *et al.*, 2005; Meents *et al.*, 2015). Local injection and topical application of JNJ-17203212 to WT corneas showed antagonistic effects lasting up to 3 h in the murine cornea, as demonstrated by decreased defensive wipes in the capsaicin eye wipe test (Figure 2.4 A). Corneas were stained with fluorescein in a separate experiment to investigate potential changes to epithelial integrity, which can promote bacterial colonization (Augustin *et al.*, 2011; Tam *et al.*, 2011; Wan *et al.*, 2018). Both sham and JNJ-17203212 corneas had little to no staining confirming normal epithelial integrity (Figure 2.4 B). JNJ-17203212 treatment increased FISH labeling in

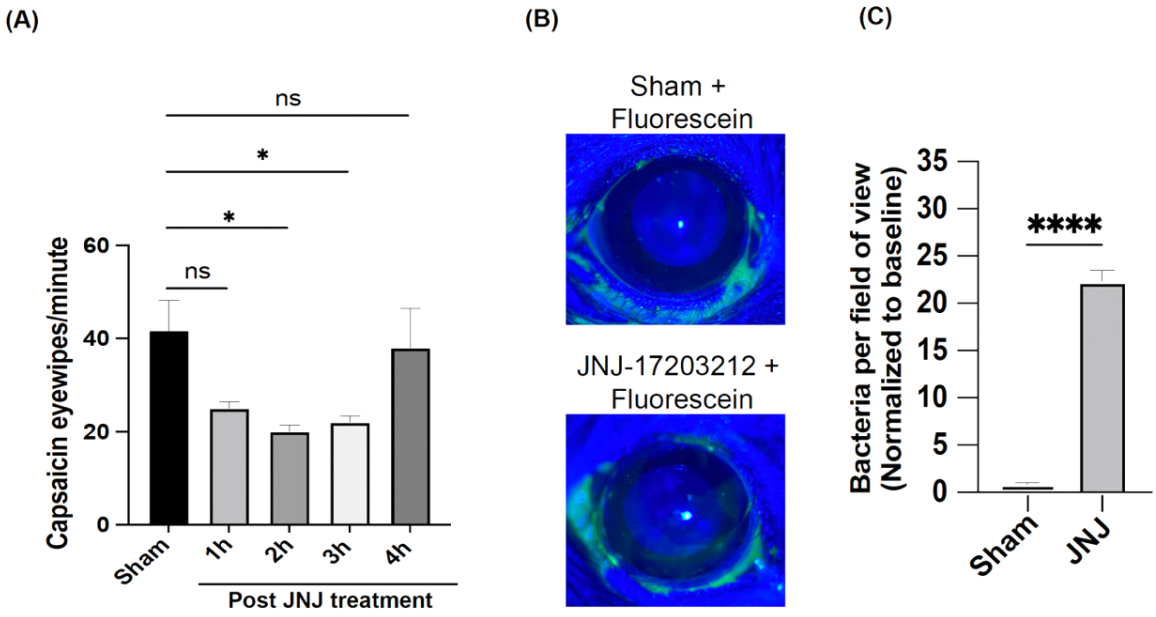


Figure 2. 4: *TRPV1 antagonist reveals a local protective mechanism versus S. aureus.* ***(A)*** *WT corneas treated with the TRPV1 antagonist JNJ-17203212 (500 µM) exhibited significantly reduced sensitivity to capsaicin (100 µM) compared to sham controls, lasting up to 3 h. * P <0.05, ns= no significant difference (One-way ANOVA with Tukey's multiple comparisons test).* ***(B)*** *Slit lamp fluorescein images confirmed normal epithelial integrity in both healthy control and antagonist-treated WT corneas.* ***(C)*** *Quantification of adherent bacteria showed the antagonist significantly increased FISH-labeled bacteria to WT corneas inoculated with S. aureus (~4-fold) compared to sham-treated controls, data were normalized to baseline bacteria. Data are expressed as the mean ± SD of bacteria per field of view. * P <0.05, *** P < 0.001, ns= no significant difference (Two-way ANOVA with Tukey's multiple comparisons test).*

corneas inoculated with *S. aureus* (~4-fold) compared to sham controls, supporting a local protective mechanism for TRPV1-mediated defense against this potential pathogen (Figure 2.4 C, D).

2.4.4. Corneal CD11c+ Immune Cell Responses differ for *P. aeruginosa* versus *S. aureus* challenge

It is well appreciated that sensory nerve activation can enable rapid local inflammatory responses (Bautista, Pellegrino and Tsunozaki, 2013; Pinho-Ribeiro, Verri and Chiu, 2017). Related, our prior work reported CD11c+ cells with distinct morphological changes are recruited in response to *P. aeruginosa* challenge and we have established that TRP-sensory nerves play a role in this recruitment (Metruccio *et al.*, 2017; Wan *et al.*, 2021). Therefore, we next assessed the involvement of CD11c+ cells in corneal defense versus *S. aureus*. CD11c+-YFP mice were inoculated with *S. aureus* or *P. aeruginosa*. Intriguingly, differing from *P. aeruginosa*, there was no increase in corneal CD11c+ cells from the baseline following *S. aureus* challenge (Figure 2.5 A, B). Next, applying our established morphology analysis methods (Metruccio *et al.*, 2019), we performed a quantitative analysis of three arbitrarily divided sub-groups (Figure 2.6). This revealed *S. aureus*-mediated changes in CD11c+ cells with cells appearing significantly less spherical (more dendriform), and farther away from the corneal epithelium but with no change in overall size (Figure 2.5 C-E). Aligning with our previous findings, *P. aeruginosa*-induced CD11c+ cells were significantly less spherical, but closer to the corneal surface, and larger in size (Figure 2.6 H-J). These findings suggest that there is a distinction between the *S. aureus* and *P. aeruginosa* CD11c+ cell response, and for *P. aeruginosa* this involves increased cell numbers and migration to the corneal epithelium which we have previously shown correlated with decreased *P. aeruginosa* binding (Metruccio *et al.*, 2017).

2.4.5. *S. aureus* and *P. aeruginosa* Differentially Impact Baseline Corneal Lyz2+ Immune Cell Morphologies

Next, we assessed the involvement of a broader population of immune cell types expressing Lyz2 (expressed on macrophages, neutrophils, and some dendritic cells). Lysozyme M (Lyz2)-Cre GFP mice (expressing all myeloid-derived immune cells, green) were inoculated with *S. aureus, P. aeruginosa* (since we have not previously studied their involvement) or PBS (control). Interestingly, baseline Lyz2+ cell numbers remained unchanged in response to either pathogen (Figure 2.7). However, morphological analysis revealed *S. aureus* inoculation significantly increased Lyz2+ cell sphericity (cells more rounded) and size (Figure 2.7 C-E). Alternately, *P. aeruginosa* inoculation had no impact on Lyz2+ cell sphericity or size, but the cells moved closer to the epithelium as observed in the CD11c+ cell response (Figure 2.7 H-J).

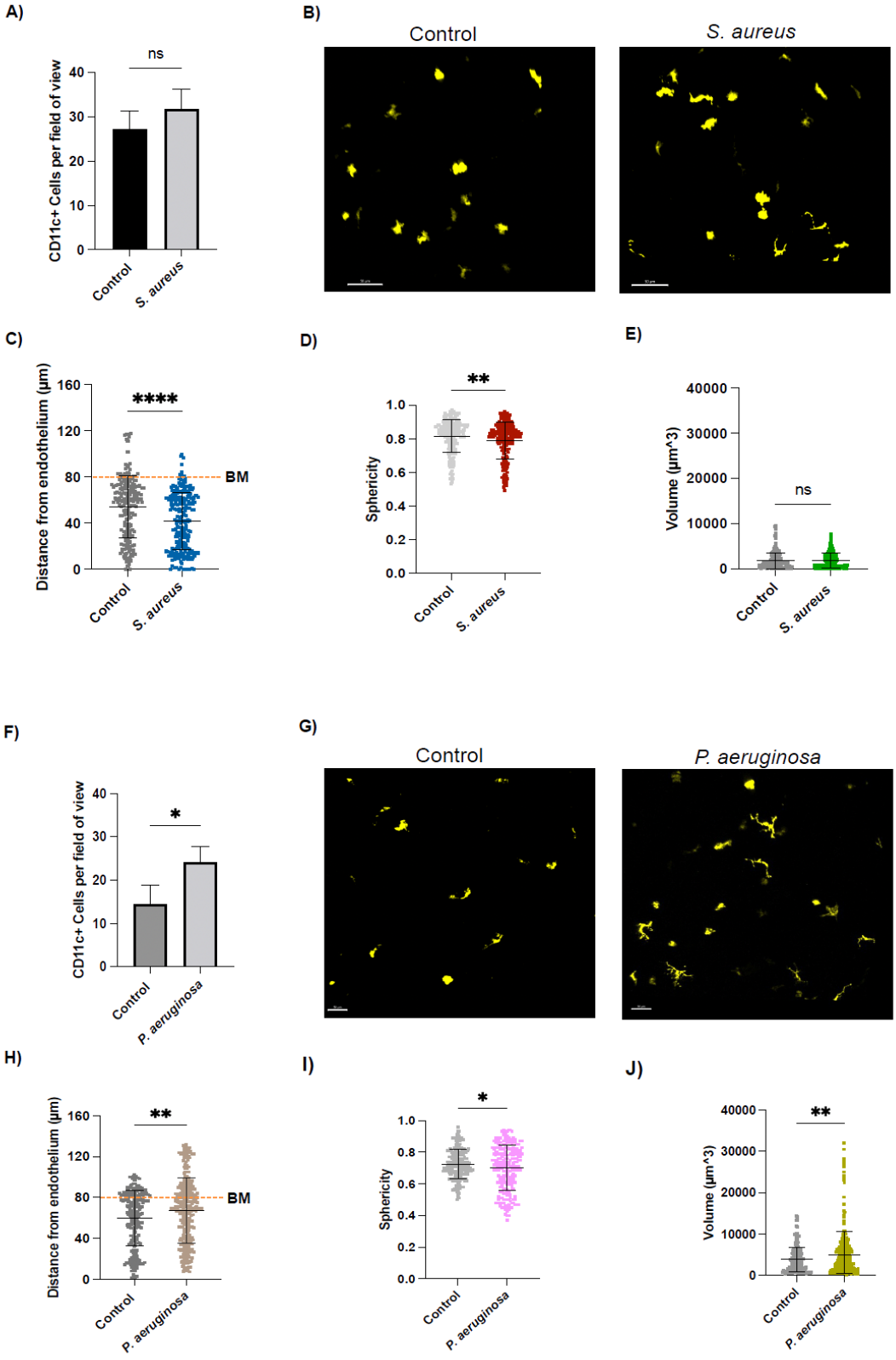

A)
B)
Control
S. aureus
ns
CD11c+ Cells per field of view
Control
S. aureus
C)
D)
E)
****
**
ns
BM
Distance from endothelium (µm)
Sphericity
Volume (µm^3)
F)
G)
Control
P. aeruginosa
*
H)
I)
J)
**
*
**
BM
P. aeruginosa

Figure 2. 5: *Distinct CD11c+ immune cell responses were observed in corneas challenged with P. aeruginosa and S. aureus.* ***(A)*** *Quantification of CD11c+ cells in healthy WT corneas at baseline and 4 h post-S. aureus challenge reveals no significant increase in CD11c+ cells after inoculation (P > 0.05).* ***(B)*** *Z-projections of the YFP channel (all CD11c+ cells, yellow, projected into one plane). Scale bars = 50 μm. 20X Objective.* ***(C)*** *Morphological analysis of baseline CD11c+ cells indicates cells were significantly further away from the corneal epithelium following S. aureus inoculation, (P < 0.0001). The dashed line denotes the basement membrane (BM), with areas above representing the corneal epithelium, below the stroma, and zero indicating the endothelium.* ***(D)*** *Graph demonstrating that baseline CD11c+ cells were significantly less spherical after S. aureus inoculation (P <0.01).* ***(E)*** *Baseline CD11c+ cells were similar in size to those in control corneas following S. aureus inoculation (P > 0.05). Data are expressed as the mean ± SD cells per field of view. * P <0.05, ** P < 0.01, **** P < 0.0001, ns = No significant difference (Student's t-Test).* ***(F)*** *Quantification of CD11c+ cells in healthy WT corneas at baseline and 4 h post-P. aeruginosa challenge shows a significant increase in CD11c+ cells after inoculation (P <0.05).* ***(G)*** *Z-projections of the YFP channel (all CD11c+ cells, yellow, projected into one plane). Scale bars = 50 μm. 20X Objective.* ***(H)*** *Morphological analysis of CD11c+ cells reveals that cells were significantly closer to the corneal epithelium following P. aeruginosa inoculation (P < 0.01). The dashed line denotes the basement membrane (BM), with areas above representing the corneal epithelium, below the stroma, and zero indicating the endothelium.* ***(I)*** *Graph showing CD11c+ cells had significantly reduced sphericity following P. aeruginosa inoculation (P <0.05).* ***(J)*** *Graph showing infiltrating CD11c+ cells were significantly larger following P. aeruginosa inoculation (P <0.01). Data are expressed as the mean ± SD cells per field of view. * P <0.05, ** P < 0.01, **** P < 0.0001, ns = no significant difference (Student's t-Test).*

2.4.6. Corneal CD45+ Immune Cell Recruitment after *S. aureus* or *P. aeruginosa* Challenge is TRP/nerve Dependent

We have also shown that CD45+ (a pan-immune cell marker) cell recruitment correlates with TRP-mediated defense versus *P. aeruginosa* in healthy corneas (Wan *et al.*, 2021). Here we explored whether these cell types were involved in *S. aureus*-induced TRP defense. WT mice were inoculated with *S. aureus* or PBS control followed by CD45 antibody labeling. The results showed a small but significant increase in corneal CD45+ immune cells from the baseline following *S. aureus* inoculation (~1.7-fold) (Figure 2.8 A, B). Next, we determined whether the observed CD45+ immune cell response was sensory nerve-dependent. WT mice were treated with RTX before *S. aureus* inoculation. RTX inhibited the CD45+ cell response to *S. aureus* (Figure 2.8 A, B). Furthermore, CD45+ cells were significantly more spherical and smaller following *S. aureus* inoculation while RTX treatment reversed these phenotypes (Figure 2.8 C, D). While our prior work demonstrated a reduced corneal CD45+ response to *P. aeruginosa* in TRPA1 (-/-) /TRPV1 (-/-) mice versus WT, the involvement of sensory nerves was not established. As expected, there was a more robust increase in corneal CD45+ cells in WT corneas inoculated with *P. aeruginosa* (~2.5-fold) which was reduced by RTX (Figure 2.8 E, F). Morphological analysis indicated that recruited cells were smaller and less spherical post- *P. aeruginosa* challenge which remained unchanged with RTX treatment (Figure 2.8 G, H). These findings showed TRPA1/V1-sensory nerve involvement in corneal CD45+ cell recruitment mediated by *S. aureus* and *P. aeruginosa*.

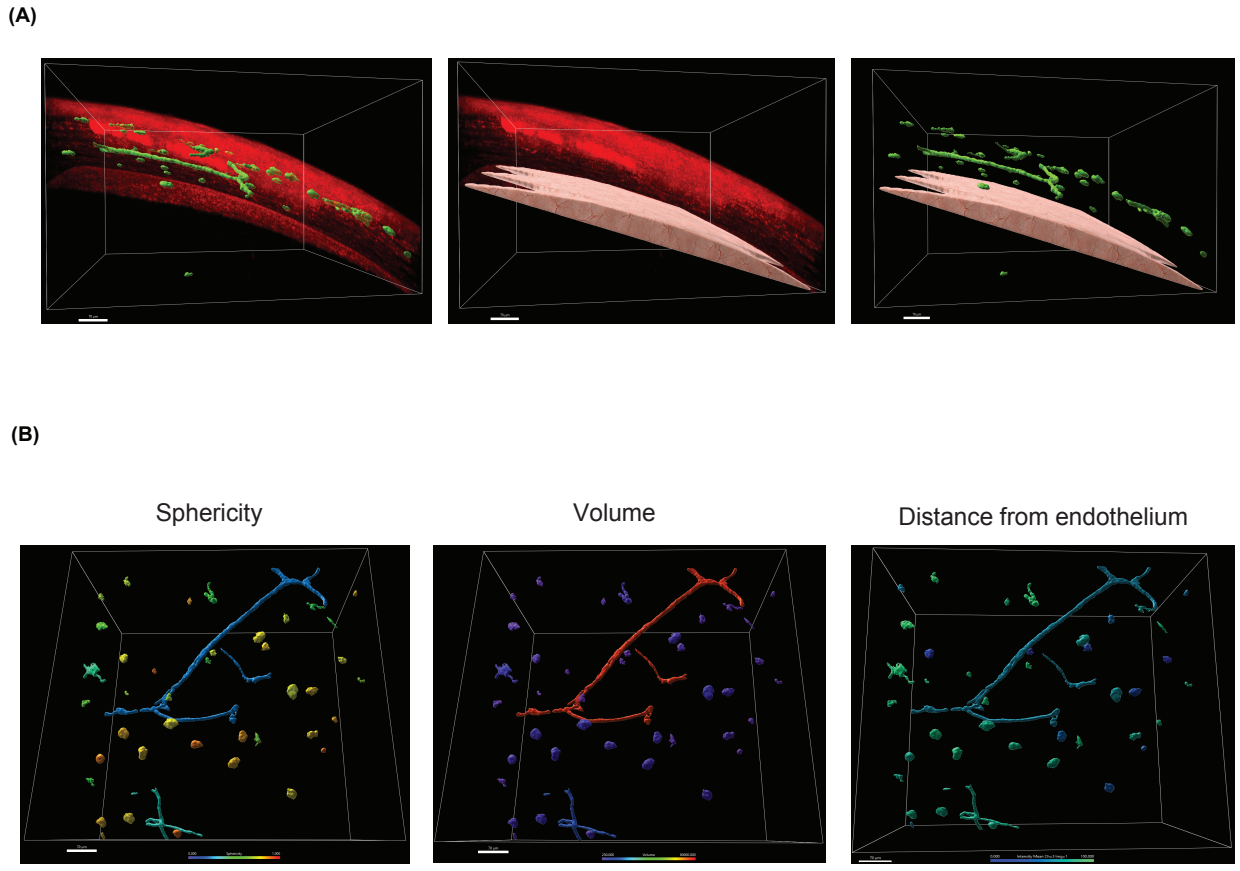


Figure 2. 6: *Morphological analysis of corneal immune cells using Imaris.* ***(A) Left image****: A "surface object" of each immune cell (representative image shows Lyz2+ immune cells, green) was created using an XY size of 10 µm.* ***Center image:*** *Another surface object was manually drawn using the red channel (or reflectance for CD11c-YFP mice) to select the total immune cells signal above the corneal endothelium (pink).* ***Right image****: From the created endothelium, a distance transformation was performed. This created a new channel (channel 3, not shown in the image) to determine the distance of surface 1 (immune cells) from surface 2 (endothelium).* ***(B) Left image****: Immune cells were statistically color-coded to reflect sphericity ranges: Purple ≤ 0.3 = most dendriform, 0.3< >0.8, and Red ≥ 8 = most circular.* ***Center image****: Immune cells were statistically color-coded to reflect ranges in volumes/sizes.* ***Right image:*** *Immune cells were assessed for their location in the cornea based on their mean intensity (distance) from the endothelium. Cells were statistically coded to reflect ranges in distance: Blue, 0 µm= at the endothelium, 0< >80 µm= stroma, and Green ≥ 80 µm = epithelium.*

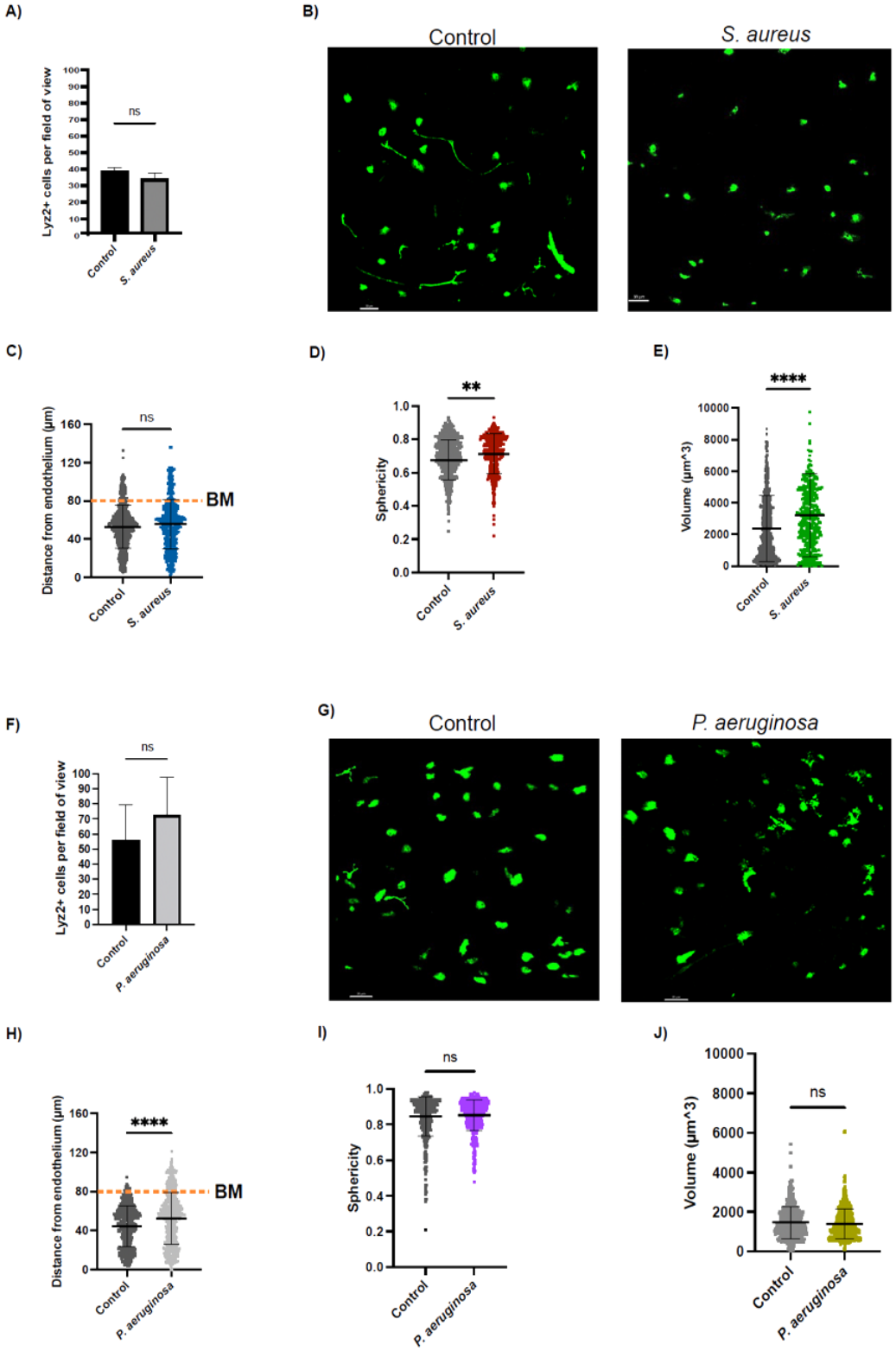
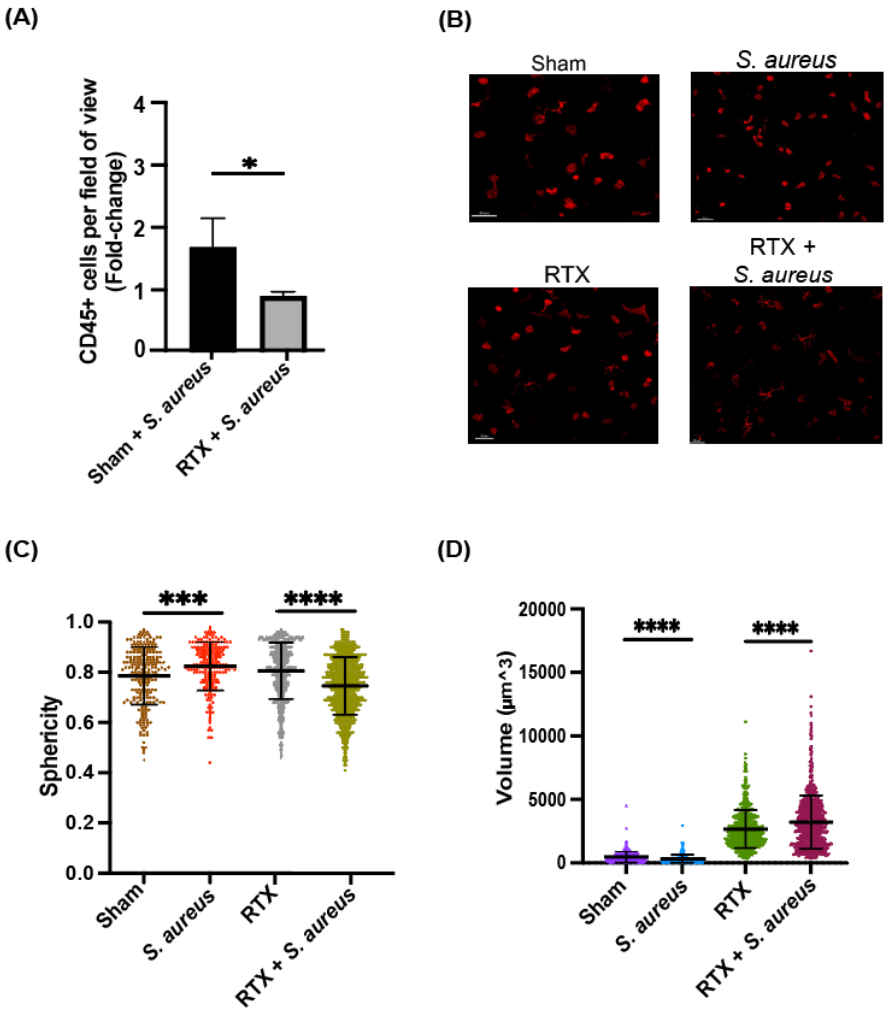


Figure 2. 7: *Quantitative and morphological analysis of Lyz2+ cells in healthy WT corneas following pathogenic bacteria challenges.* ***(A)*** *Quantification of Lyz2+ cells in healthy WT corneas at baseline and 4 h post-S. aureus*

challenge reveals no significant increase in Lyz2+ cells after inoculation (P > 0.05) ***(B)*** *Z-projections of the GFP channel (all Lyz2+ cells, green, projected into one plane). Scale bars = 50 µm. 20X Objective.* ***(C)*** *Morphological analysis of baseline Lyz2+ cells demonstrates that following S. aureus inoculation, Lyz2+ cells maintain similar distances from the corneal epithelium compared to controls (P > 0.05). The dashed line denotes the basement membrane (BM), with areas above representing the corneal epithelium, below the stroma, and zero indicating the endothelium.* ***(D)*** *Graph showing baseline Lyz2+ cells were significantly more spherical following S. aureus inoculation (P < 0.01).* ***(E)*** *Graph showing baseline Lyz2+ cells are significantly larger than controls following S. aureus inoculation (P < 0.0001). Data are expressed as the mean ± SD cells per field of view. ** P < 0.01,**** P < 0.0001, ns = no significant difference (Student's t-Test).* ***(F)*** *Quantification of Lyz2+ cells in healthy WT corneas at baseline and 4 h post-P. aeruginosa challenge demonstrates no significant increase in Lyz2+ cells after inoculation (P > 0.05).* ***(G)*** *Z-projections of the GFP channel (all Lyz2+ cells projected into one plane). Scale bars = 50 µm. 20X Objective.* ***(H)*** *Morphological analysis shows that baseline Lyz2+ cells following P. aeruginosa inoculation are significantly closer to the corneal epithelium compared to controls (P <0.0001). The dashed line denotes the basement membrane (BM), with areas above representing the corneal epithelium, below the stroma, and zero indicating the endothelium.* ***(I)*** *Graph depicting no significant change in sphericity of baseline Lyz2+ cells after P. aeruginosa inoculation (P > 0.05).* ***(J)*** *Baseline Lyz2+ cells exhibit no significant change in size compared to controls following P. aeruginosa inoculation (P > 0.05). Data are expressed as the mean ± SD cells per field of view. * P < 0.01,**** P < 0.0001, ns = no significant difference (Student's t-Test).*



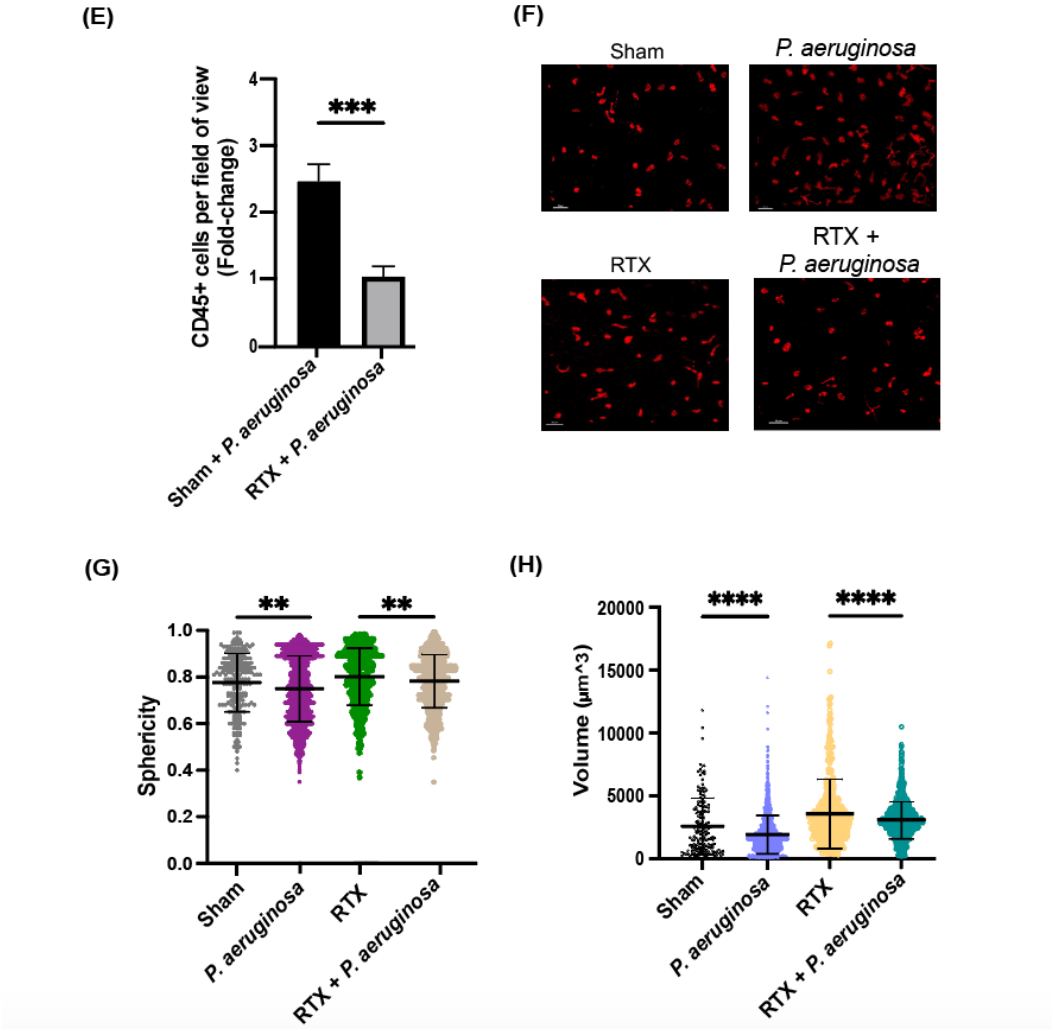


Figure 2. 8: *WT corneas show increased CD45+ cell infiltration post-S. aureus and P. aeruginosa inoculation that was inhibited by RTX.* ***(A)*** *Quantification of CD45+ cells reveals a significant increase in CD45+ cells from the baseline post-S. aureus inoculation (~1.7-fold) compared to RTX-treated corneas. Data normalized to baseline CD45+ cells. * P <0.05 (Student t-Test).* ***(B)*** *Representative maximum intensity projections of CD45+ cells (red) in the healthy murine cornea at baseline and post-S. aureus inoculation in sham and RTX-treated corneas. Scale bars = 50 µm. 20X Objective.* ***(C)*** *Morphological analysis of CD45+ cells shows cells were more spherical post-S. aureus inoculation (P < 0.01) but decreased in sphericity following RTX treatment (P < 0.0001).* ***(D)*** *CD45+ cells responding to S. aureus were smaller than in sham controls (P < 0.0001) and larger after RTX treatment (P < 0.0001). Data are expressed as the mean ± SD cells per field of view. * P <0.05, *** P < 0.001, **** P < 0.0001, ns = no significant difference (Student t-Test). CD45+ cells present post- S. aureus inoculation in RTX-treated corneas were larger than uninoculated controls (P < 0.0001).* ***(E)*** *Quantification of CD45+ cells reveals a significant increase in CD45+ cells from the baseline post-P. aeruginosa inoculation (~2.5-fold) compared to RTX-treated corneas. Data normalized to baseline CD45+ cells.* ***(F)*** *Representative maximum intensity projections of CD45+ cells (red) in the healthy murine cornea at baseline and post-P. aeruginosa inoculation in sham and RTX-treated corneas. Scale bars = 50 µm. 20X Objective. Data are expressed as the mean ± SD cells per field of view. *** P < 0.001 (Student t-Test).* ***(G)*** *Morphological analysis of CD45+ cells indicates cells were less spherical following P. aeruginosa inoculation (P < 0.01) and remained less spherical after RTX treatment (****P < 0.0001).* ***(H)*** *CD45+ cells responding to P. aeruginosa were smaller than uninoculated controls for both sham and RTX-treated corneas (P < 0.0001). Data are expressed as the mean ± SD cells per field of view. ** P < 0.01, *** P < 0.001, ns = No significant difference (Student t-Test).*

2.5. Discussion

The results in this chapter provide insights into the novel role of TRPA1 and TRPV1 in preventing bacterial colonization by pathogens *Staphylococcus aureus* and *Pseudomonas aeruginosa,* respectively. Experiments involving RTX to ablate TRPA1/V1-associated nerves, and enucleated eyes confirmed a role for sensory nerve involvement in TRPA1/V1-mediated corneal defense. However, in contrast to the previously reported TRPA1-mediated defense for *P. aeruginosa*, results using bupivacaine anesthesia show that the mechanism(s) for TRPV1 defense against *S. aureus* does not necessarily require sensory nerve firing. Also differing from *P. aeruginosa*, CD11c+ cell numbers in the cornea did not change in response to *S. aureus*. Further, *S. aureus* and *P. aeruginosa* inoculation did not increase corneal Lyz2+ cell numbers but increased CD45+ immune cells, with the latter showing a greater increase post-*P. aeruginosa* challenge. Analysis of cell morphology and location revealed other differences with RTX abrogating the CD45+ cell increase for *P. aeruginosa* and *S. aureus*.

Staphylococcus aureus and *P. aeruginosa* can pose a significant threat to ocular health, particularly in the context of corneal infections (keratitis), which can lead to vision loss. In our previous study, corneal adhesion of *P. aeruginosa* (PAO1, ~10^{11} CFU/mL) was countered by TRPA1, while adhesion of environmental commensals (present in far fewer numbers) was countered by TRPV1. In this study, corneal adhesion of *S. aureus* (~10^{11} CFU/mL) was countered by TRPV1, not TRPA1. These findings suggest distinct involvement of TRPA1 versus TRPV1 in corneal defense against bacterial adhesion that does not necessarily relate to bacterial status as a pathogen versus commensal. This finding also effectively rules out the alternative hypothesis that inoculum size is involved. Instead, the data suggests a role for bacteria-specific factors in the distinct TRP-mediated corneal defense that may relate to pattern recognition. Indeed, previous studies reported that *P.aeruginosa* can activate TRPA1 through LPS modifications (Meseguer *et al.*, 2014), whereas *S. aureus* can trigger TRPV1-expressing neurons via pore-forming toxins (Blake *et al.*, 2018). The mechanism for TRPV1 in recognizing and/or modulating *S. aureus* adhesion may also involve associated previously reported virulence factors, such as the *Staphylococcal* enterotoxin Q (SEQ) associated with the S33 R strain (Indrawattana *et al.*, 2013; Hu *et al.*, 2017). Further, both TRPA1 and TRPV1 can serve as receptors for quorum-sensing molecules (Tobita *et al.*, 2021) providing additional bacterial factors that could selectively trigger them.

Although it is widely considered that TRPA1 and TRPV1 are primarily expressed in neurons, some studies suggest their expression in non-neuronal cell types (Nassini *et al.*, 2012; Mergler *et al.*, 2014; Reinach *et al.*, 2015; Khalil *et al.*, 2018). This necessitates establishing a nerve-specific role for the TRPV1-mediated defense. Further, we previously established a role for sensory nerves in the TRPA1 defense against the potential pathogen *P. aeruginosa*. Results from resiniferatoxin (RTX) experiments mirrored TRPV1 gene knockout outcomes, establishing a role for TRP-associated sensory nerves in corneal defense versus *S. aureus* (Figure 2.1 A-C). Disrupting TRP-sensory nerve function and signaling *ex vivo* abolished TRPV1-dependent corneal defense versus *S. aureus* (Figure 2.1 D). However, experiments using bupivacaine sensory nerve block revealed differences in the requirement for sensory nerve firing mechanisms in TRP-mediated corneal defense

versus these potential pathogens (Figure 2.2E) (Figure 2.3). Of note, while RTX ablates all sensory nerves expressing TRPV1, bupivacaine is a sodium channel blocker that can inhibit the generation of an action potential by all sensory nerves. Therefore, we hypothesized that localized antimicrobial activity could still occur by virtue of TRPV1 unless the channel is specifically inhibited. Corroborating this hypothesis, administering a local antagonist for TRPV1 promoted *S. aureus* adhesion to WT corneas without disrupting corneal integrity (Figure 2.4) suggesting defense versus *S. aureus* involves an antimicrobial mechanism locally modulated under TRPV1 activation.

Also supporting the observations of this study, when TRP receptors bind bacterial ligands, two main events can occur; (1) the local release of neuromodulators at the sensory nerve terminal and (2) an action potential is generated. Therefore, while the activation of TRP receptors can lead to changes in membrane potential, it does not necessarily result in the generation of an action potential. Instead, the response of a sensory neuron to TRP receptor activation depends on factors such as the specific type of TRP channel, the sensory modality it is associated with, the tissue type, and the strength of the stimulus (Chiu *et al.*, 2013; Jardín *et al.*, 2017; Chiu, 2018). Possibly related, *P. aeruginosa* infections can often be sight-threatening and systemic infections. Thus, the need for nerve firing may suggest a more comprehensive defense strategy that includes alerting tissues beyond the impacted cornea. This could differ from the defenses required for *S. aureus* which is often found colonizing the skin. Moreover, the failure of TRPA1 to protect against *S. aureus* adhesion could be because it lacks the ligands to trigger this defense. Responding only when certain ligands are present could be a safeguard to preserve immune privilege when a lesser response is sufficient.

It would also be of interest to elucidate whether a local mechanism for TRPV1 defense is through antimicrobial factors secreted directly at the corneal surface via the TRPV1 channel (i.e. neuropeptides or other molecular factors). Given their size, charge, and how rapidly they can be disseminated, neuropeptides would be an ideal candidate for local TRP-mediated defense against bacteria. Indeed, several neuropeptides have been shown to have direct antimicrobial activity against a large range of bacteria including pathogens *S. aureus* and *P. aeruginosa,* thus, extending their functions beyond driving inflammation (El Karim *et al.*, 2008; Aresti Sanz and El Aidy, 2019). While it remains possible that neuropeptides can exhibit direct antimicrobial activity at the ocular surface, these or other TRP-mediated molecular factors may also indirectly modulate antimicrobial activity via the tear fluid, mucin-producing glands, and/or immune cells.

TRP receptor activation on sensory nerves can recruit immune cells, which can release or influence the release of various antimicrobial peptides (Marischen *et al.*, 2009; Biswas *et al.*, 2023). Evidence also suggests nociceptor activation can influence Substance P release, leading to shifts in dendritic cell activation states and migration (Perner *et al.*, 2020). Corroborating this and our published findings (Wan *et al.*, 2021), *P. aeruginosa* inoculation increased CD11c+ cell numbers resulting in a response characterized by cells being less spherical, larger, and closer to the corneal epithelium, phenotypes that correlated with reduced *P. aeruginosa* binding in murine corneas (Metruccio *et al.*, 2017). However, a different CD11c+ cell response was observed for *S. aureus* (Figure 2.5). There was no increase in CD11c+ cell numbers, but cells also exhibited a less spherical morphology. However, we also observed *S. aureus* inoculation resulted in CD11c+ cells

that were further away from the epithelium. This observation may also be attributed to bacteria producing immunosuppressive signals that modulate the behavior of immune cells. *S. aureus*, for example, produces various toxins, that can modulate and evade immune responses (Thammavongsa *et al.*, 2015; de Jong, van Kessel and van Strijp, 2019), a phenotype also observed during their activation of TRPV1 neurons (Huang *et al.*, 2023).

To further explore the involvement of immune cell types in the TRP-mediated defense against pathogens, Lyz2+ cells were assessed. This lysozyme protein is a marker for all myeloid-derived immune cells including monocytes, neutrophils, macrophages, and some dendritic cells. While our study found no increase in Lyz2+ immune cell numbers from the baseline influenced by *P. aeruginosa* or *S. aureus* inoculation, we found changes in cell morphologies and locations in the healthy murine cornea (Figure 2.7). It was noted that Lyz2+ cells became more spherical and larger following *S. aureus* inoculation only. However, cells migrated closer to the epithelium following *P. aeruginosa* inoculation, mirroring the pattern observed for the CD11c+ cell response in Figure 2.5. Since CD11c+ cells partially overlap with Lyz2+ cells, it is plausible that the other observed CD11c+ cell responses to *P. aeruginosa* might be obscured within the broader population of Lyz2+ immune cells present.

Previous morphological assessments have revealed two distinct morphologies of CD11c+ cells within the cornea: dendriform and round-shaped, the former associated with the CD86 marker for maturation or activation (Hamrah *et al.*, 2003). Dendriform types are situated at the sub-basal space where the nerve plexus resides and extend their process across nerve endings. However, the round-shaped types, are closely associated with nerve fiber branching points further into the stroma (Gao, Lee and Yu, 2016). Although the proliferation and recruitment of CD11c+ cells are important components of the corneal immune response, the overall process is complex and involves different cellular and molecular interactions that can influence morphological changes. Determining the significance of these morphological changes and nerve dependence in the overall TRPA1- and TRPV1-mediated corneal defense against pathogens warrants further investigation involving more specific molecular markers.

We also demonstrated that the absence of TRPA1 and TRPV1 ion channels is associated with a reduction in cells expressing CD45 (a pan-immune cell marker) in the corneal defense versus *P. aeruginosa* (Wan *et al.*, 2021). Building upon this, our current findings using RTX establish that the *P. aeruginosa*-induced CD45+ cell response is dependent on TRPA1 and TRPV1 sensory nerves (Figure 2.8). Moreover, this was also associated with morphological changes suggesting different types, or activation states of cells being recruited. A similar, albeit less robust, result was found for *S. aureus*. Interestingly, we observed an increase in the CD45+ cells at baseline, as well as their overall size following RTX treatment. This finding suggests ablation of TRPV1 neurons may disrupt the normal crosstalk between sensory neurons, affecting the recruitment and behavior of immune cells. Furthermore, the absence of TRPV1-expressing sensory nerves may alter the local microenvironment, impacting factors such as cytokine levels, or other signaling molecules that can regulate immune cell recruitment and morphologies. Related to this hypothesis and our observations, it has been reported that nociceptor

depletion can lead to exaggerated immune responses during infection (Chiu *et al.*, 2013; Yu *et al.*, 2024).

Although the CD45 marker represents a diverse and overlapping population of immune cells, the results presented in Figures 2.5 and 2.7 may effectively exclude cell types that are Lyz2+ and CD11c+ in the defense versus *S. aureus*, and the latter involved in defense versus *P. aeruginosa* only. Other important immune cell types that may be involved include T cells. A recent study showed that a substantial population of immune cells in the cornea are T cells, some of which may have been previously classified as dendritic cells (Downie *et al.*, 2023). Furthermore, activated T cells can undergo clonal expansion (Zhan *et al.*, 2017; Huang *et al.*, 2019), which could contribute to an increase in cell size and numbers, possibly relating to the CD45+ findings of our study. Nevertheless, further investigations are warranted to elucidate the specific cell types involved in the TRP-dependent parainflammatory response driven by *P. aeruginosa* and *S. aureus,* and their contribution to defense against adhesion.

Considering these findings, the relationship between TRP receptor activation on sensory nerves and the subsequent immune cell responses emphasizes the complexity of host-microbe interactions at the ocular surface. Our observations of distinct CD11c+ cell responses to *P. aeruginosa* and *S. aureus* inoculation present an interesting challenge regarding the mechanisms mediated by sensory nerves in shaping the corneal defense. This gap in our understanding highlights the need for further investigation, which will be addressed in Chapter 3. Importantly, *P. aeruginosa* and *S. aureus* are Gram-negative and Gram-positive pathogens, respectively. However, the specific and direct involvement of TRPA1 and TRPV1 in modulating antimicrobial activity based on the bacterial Gram type is an area that requires further characterization and will be explored in Chapter 4. Further, if bacterial-induced parainflammation and defense against bacterial adhesion are linked, parainflammation during prolonged contact lens wear may be protective and/or relate to the pathogenesis of lens-related infection, a hypothesis that we will explore in Chapter 5.

2.6. Limitations

Our study was limited to two potential pathogens. Additional studies to expand the range of bacterial species used could provide a more comprehensive understanding of the roles of TRPA1 and TRPV1 ion channels in corneal defense. Another limitation lies in the use of murine models. While mice are anatomically similar, they may have unique immune responses and microbiome compositions compared to human corneas. Therefore, correlating the findings from our murine models to human corneal defense mechanisms requires caution. Further studies in humans if possible, or more complex animal models will be required to validate and extend our observations for clinical applications. While widely used, another limitation of this study lies in using the universal 16S rRNA-targeted FISH probe. This presents the challenge of distinguishing between inoculated and environmental/transiently present bacteria. To address this limitation and increase the accuracy of bacterial quantification, future studies could explore the use of fluorescently labeled bacterial strains.

2.7. Conclusion

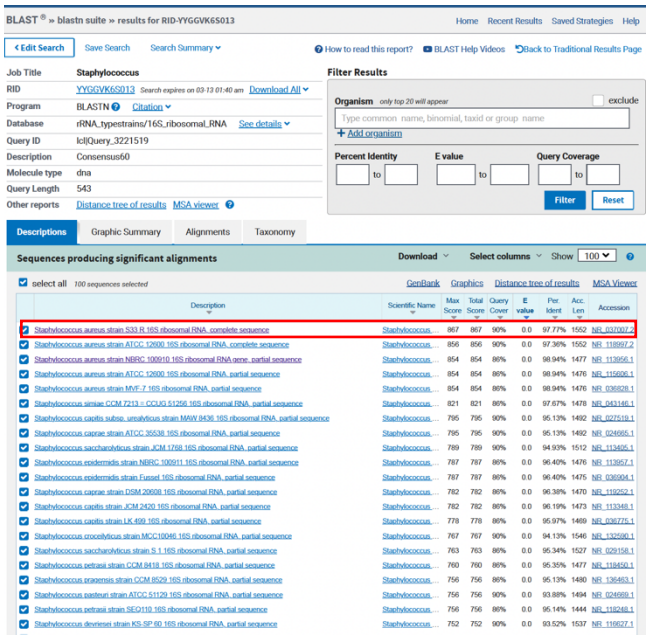
Together, the findings of this study revealed differences in the healthy cornea's response to, and intrinsic defense against two significant bacterial pathogens, involving sensory nerves and TRPA1/V1. Moreover, the role of these ion channels in preventing bacterial adhesion appears to involve corneal sensory nerve firing or TRP-dependent local activity that can modulate distinct parainflammatory responses in the absence of pathology. These discoveries further our understanding of the mechanisms that protect healthy corneas against bacterial colonization, with potential clinical implications where the use of topical medications or invasive surgeries affect corneal nerve function may increase susceptibility to corneal colonization and risk of sight-threatening infections.

2.8. Acknowledgements

This work was supported by the National Institutes of Health; EY011211(SF)

2.9. Supplementary Materials

A)



B)

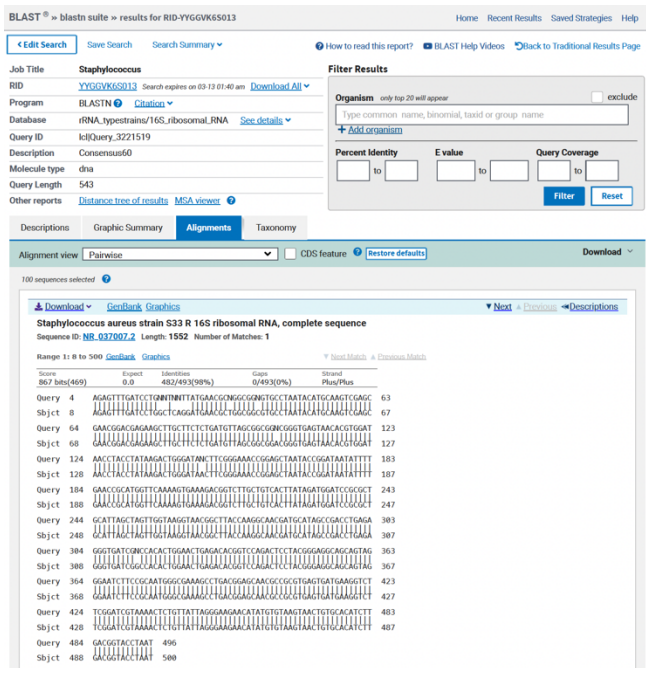


Figure 2. 9: *NCBI Blast results for sequenced ocular clinical isolate confirm strain as* Staphylococcus *aureus S33 R.* ***(A)*** *sequence description and top percentage identity.* ***(B)*** *Complete sequence alignment*

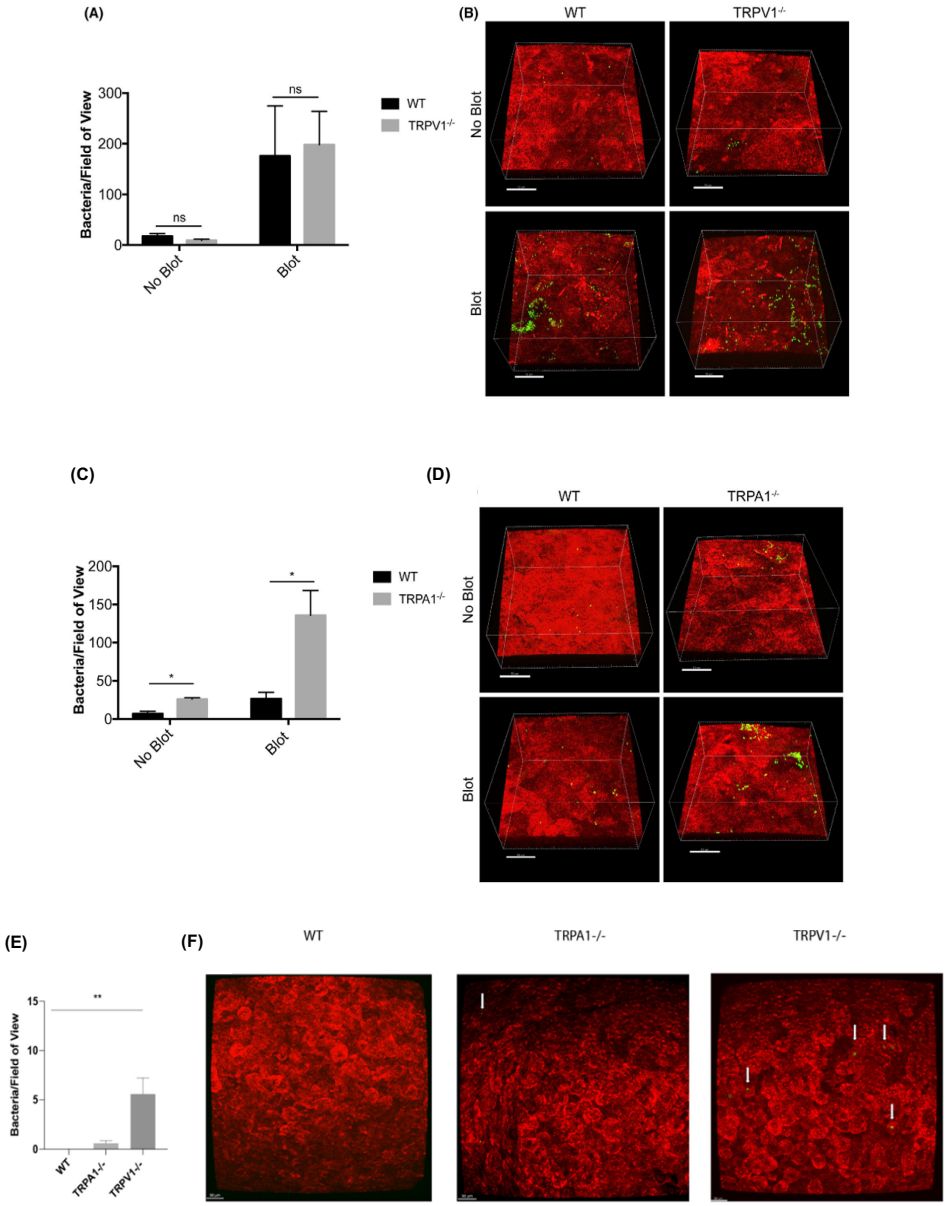


Figure 2. 10: *(from Wan et al, 2021) TRPA1 and TRPV1 play distinct roles in preventing adhesion* ***(A)*** *Quantification of Pseudomonas aeruginosa adhesion to healthy and superficially injured murine corneas after inoculation of ~1 × 10^{11} CFU/ml every hour for 4h. Under each condition, there is no significant difference in bacterial adhesion between TRPV1-/- corneas and WT. As expected, more bacteria adhere to blotted corneas. ns = Not Significant (Two- way ANOVA).* ***(B)*** *Representative images of P. aeruginosa (green) adhering to the murine cornea (red) in each condition. Scale bar = 50 μm. TRPA1-/- corneas show significantly greater bacterial adhesion compared to WT in vivo.* ***(C)*** *Quantification of bacterial adhesion to healthy and blotted murine corneas after inoculation of ~1 × 10^{11} CFU/ml every hour for 4h comparing TRPA1-/- mice to WT. Healthy TRPA1-/- corneas show ~3.8-fold greater adhesion than WT, and blotted TRPA1-/- corneas show a ~5.1-fold increase over WT. *p < .05 (Two-way ANOVA).* ***(D)*** *Representative images of P. aeruginosa (green) adhering to the murine cornea (red) in each*

Chapter 3

The Role of Corneal Sensory nerves in Modulating Corneal Cytokine/Chemokine Response to *Pseudomonas aeruginosa*

The preliminary experiment in this chapter was conducted by Dr. Tyler Spillman, a former T-35 student, and Dr. Abby Kroken, a former Postdoctoral Fellow in our lab. I analyzed the data in collaboration with Dr. Naren G Kumar, a current Postdoctoral Fellow in our lab (Figures 3.2 and 3.3) and validated interesting findings (Figure 3.4).

This chapter also includes supplemental figures from previous publications using our contact lens model, authored by our former Postdoctoral Fellow, Dr. Ananya Datta, to which I contributed as a co-author (Figures 3.6 and 3.7). Please refer to Chapter 5 for details on the contact lens model.

Relevant publications:

1. Datta A, Truong T, Lee JH, Horneman H, Flandrin O, Lee J, Kumar NG, Caspi R, Evans DJ, Fleiszig SMJ (2023) Contact lens-induced corneal parainflammation involving Ly6G+ cell infiltration requires IL-17A and γδ T cells. The Ocular Surface. 28;79-89. Doi: https://doi.org/10.1016/j.jtos.2023.02.004

2. Datta A, Lee JH, Troung T, Flandrin O, Yang Y, Evans DJ, Fleiszig SMJ (2024) Persistence of Contact Lens-Induced Corneal Parainflammation Following Lens Removal. IOVS. 65:8. Doi: https://doi.org/10.1167/iovs.65.3.8

3.1. Abstract

The pathogen *Pseudomonas aeruginosa* poses a significant threat to corneal health, particularly in cases of corneal injury. Our previous research demonstrated an important role for TRPA1 and corneal sensory nerves in defending against *P. aeruginosa* adhesion, with implications for both healthy and superficially-injured corneas. During injury, the defense mechanism involved the recruitment of CD11c+ cells, known to counter *P. aeruginosa* adhesion, and was associated with elevated cytokine and chemokine gene expression. Here, we explored whether corneal sensory nerves modulate cytokine and chemokine expression. CD11c-YFP BL/6 mice were administered subconjunctival injections of bupivacaine to block all sensory nerve firing, or saline (sham-treated control). Corneas were then superficially-injured before inoculation with *P. aeruginosa* (strain PAO1) expressing a d-Tomato fluorescent protein. Mice were sacrificed after 4 h and corneal epithelia were collected and homogenized in TRIzol to purify RNA. A custom RT² Profiler Polymerase Chain Reaction (PCR) array was used to determine changes in corneal gene expression of cytokines and chemokines. Select genes of interest were further verified by conducting RT-PCR with additional primers. Uninoculated corneas showed altered expression of 23 cytokine/chemokine genes after bupivacaine treatment. Following *P. aeruginosa* inoculation, the expression of 20 genes was altered and was dependent on sensory nerve firing. These included; IL-6, IL-1β, CXCL5, and CCL7. Additional RT-PCR validation of genes of interest confirmed altered expression of CCL7 in bupivacaine-treated corneas inoculated with *P. aeruginosa*. These findings show that the modulation of some corneal pro-inflammatory factors driven by *P. aeruginosa* challenge is sensory nerve-dependent.

3.2. Introduction

Pseudomonas aeruginosa is well known for its virulence and ability to resist many of the current antimicrobial therapeutic agents. Despite this, *P. aeruginosa* rarely colonizes the cornea, even after deliberate inoculation, unless the tissue is rendered susceptible through epithelial injury or manipulation of intrinsic defenses such as CD11c+ depletion and sensory nerve perturbations (Alarcon *et al.*, 2011; Metruccio *et al.*, 2017; Wan *et al.*, 2021). However, contact-lens-related infections with *P. aeruginosa* can trigger damaging immune responses in the cornea that can lead to sight-threatening damage. Therefore, understanding the mechanisms underlying corneal defense against *P. aeruginosa* is critical to developing strategies for preventing infection.

In Chapter 2 we showed that TRPA1-mediated defense versus *P. aeruginosa* in healthy and superficially-injured corneas correlated with corneal CD11c+ cell recruitment, and morphology changes, facilitated by corneal sensory nerves/nerve firing (Wan *et al.*, 2021). Further, our lab has previously shown that recruited CD11c+ cells can inhibit *P. aeruginosa* adhesion to superficially-injured corneas, and that their depletion was associated with decreased expression of inflammatory cytokines/chemokines in the *P. aeruginosa* response (Metruccio *et al.*, 2017). These included the inflammatory factors IL-6, IL-1β, CXCL1, CXCL2, and CXCL10, some of which have been extensively studied in *P. aeruginosa* infections. Other chemokines such as CCL2, CCL3, CCL7, and CCL20 can specifically recruit immune cells (including CD11c+ cells) to sites of infection or injury

contributing to overall immune responses. Therefore, investigating the roles of these signaling molecules can provide insights into the mechanisms underlying corneal host defense against pathogens such as *P. aeruginosa*.

The influence of CD11c+ cells in corneal defense is an evolving field of study with current evidence strongly supporting the concept that corneal immune and sensory nerves are closely associated and functionally dependent (Gao, Lee and Yu, 2016; Frutos-Rincón *et al.*, 2022). Moreover, the involvement of cytokines/chemokines and sensory nerves in recruiting immune cells depending on the neuropeptide released, as previously reported in studies related to pain,(Ramesh, MacLean and Philipp, 2013), may provide a potentially similar mechanism in the injured cornea during *P. aeruginosa* challenge and infection. However, the specific mechanisms by which sensory nerves modulate DC responses in the cornea are unclear.

Here, we investigated immune signatures as a mechanism by which corneal sensory nerves regulate the recruitment of immune cells in response to *P. aeruginosa* in superficially-injured corneas. A custom RT2 Profiler cytokine/chemokine array was used to assess transcriptional changes induced by *P. aeruginosa* challenge and modulated by corneal sensory nerves. The data revealed altered gene expression of 20 pro-and anti-inflammatory factors following bupivacaine nerve block with *P. aeruginosa* inoculation (including IL-6, IL-1β, CXCL5, CCL7) and 23 without inoculation (including IL-3, IL-1β, and IL-12b). Additional validation confirmed the altered expression of CCL7 in bupivacaine-treated corneas inoculated with *P. aeruginosa*. These findings suggest an important role for sensory nerves in driving the expression of pro-inflammatory genes that relate to CD11c+ cell-mediated corneal defense against *P. aeruginosa*.

3.3. Methods

3.3.1. Bacteria

Pseudomonas aeruginosa, PAO1 expressing d-Tomato on plasmid p67T1 (PAO1-dtom) was used for bacterial challenge. Bacteria were grown on TSA supplemented with carbenicillin (400 μg/mL) overnight for ~16 hours at 37 °C, followed by suspension in phosphate-buffered saline (PBS) to a concentration of ~10^{11} colony-forming units (CFU)/mL.

3.3.2. Bacterial Challenge

All procedures were carried out per standards established by the Association for the Research in Vision and Ophthalmology (ARVO), under the protocol AUP-2019-06-12322 approved by the Animal Care and Use Committee, the University of California Berkeley, an AAALAC-accredited institution, adhering to PHS policy on the humane care and use of laboratory animals, for the care and use of laboratory animals. Six- to twelve-week-old male or female CD11c-YFP transgenic wild-type mice were used for all experiments. Mice were anesthetized by intraperitoneal injection of ketamine (80–100 mg/Kg) and dexmedetomidine (0.25–0.5 mg/Kg). Corneas were lightly superficially-injured (blotted) using a kimwipe™ tissue paper as previously described (Alarcon *et al.*,

2011; Tam *et al.*, 2011; Metruccio *et al.*, 2017; Wan *et al.*, 2021) before inoculation with PAO1 (5µL) once every hour for 4h.

3.3.3. Corneal Sensory Nerve Block

Before superficial injury and inoculation, anesthetized mice were subjected to sensory nerve block. To inhibit corneal sensory nerve firing, 0.5% bupivacaine hydrochloride solution was injected into the subconjunctival sac (5µL) and added topically (5µL) in anesthetized mice for 20 minutes as previously described (Wan *et al.*, 2021).

3.3.4. Real-Time Quantitative Polymerase Chain Reaction (qPCR)

Following bacterial inoculation experiments, corneal epithelia (four per condition) were carefully removed using an Algerbrush. In a separate experiment, fluorescein staining was performed (as described in Chapter 2.3.8) to visualize corneal epithelial cell removal with the Algerbrush (Figure 4.1). Corneal samples were suspended in Trizol (Thermo Fisher Scientific) and then disrupted using a hand-held tissue homogenizer (Kinematica Polytron, Thermo Fisher Scientific). RNA was extracted from the homogenate in Trizol using Direct-zol™ RNA Miniprep (Zymo Research). RNA concentration was examined using a spectrophotometer and then prepared using an RT² Profiler™ PCR Array (Mouse Cytokines & Chemokines, Qiagen: PAMM-150Z) per the manufacturer's instructions. For validation experiments, RNA was extracted before cDNA synthesis using iScript (Bio-Rad) and RT-qPCR was performed using Faststart Sybergreen (Roche) running on a Light Cycler 96 real-time PCR machine (Roche).

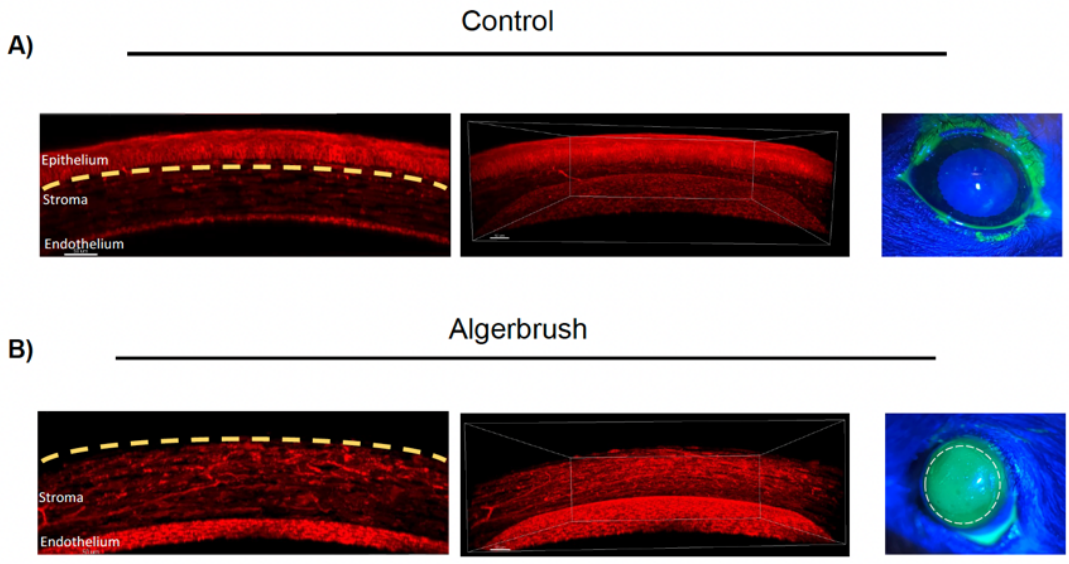


Figure 3. 1: *Corneal epithelial cell collection. A) Control corneas showing intact epithelium, stroma, and endothelium. 50µm, 20X objective.* ***Upper right panel****: Slit lamp image showing no fluorescein staining confirming intact cornea.* ***B)*** *Cornea following Algerbrush removal of the epithelium. 50µm, 20X objective. Lower right Panel: Slit lamp image of fluorescein penetration confirming the absence of epithelium after algerbrush.*

Primers were designed to be separated by at least one intron to ensure selective amplification of cDNA and tested for efficiency, and specificity under the conditions used. Primers used are listed in Table 1. GAPDH was used as an internal control for normalization.

3.3.5. Immunohistochemistry

In some experiments, freshly enucleated eyes were fixed overnight in 2% PFA and prepared for antibody labeling as previously done (Wan *et al.*, 2021; Datta, Lee, *et al.*, 2023). Briefly, corneas were dissected and blocked (3% bovine serum albumin [BSA] with 0.3% Triton X-100 in PBS) for 1 hour at RT followed by overnight incubation in primary antibody (rat anti-mouse IL-6+ [1:500; BD Pharmingen: #550539]). Corneas were moved to secondary antibody (anti-rat antibody [Life Technologies: #A21434]) diluted in DAPI (4,6-diamidino-2-phenylindole dihydrochloride; 12.5 µg/mL; Thermo Fisher: #D1306) for 2 h at room temperature with rotation then washed with PBS 3 times. Corneas were then flat mounted with Prolong Gold (Thermo Fisher: #P36970) and imaged using a confocal microscope.

***Table 3. 1**: Primer Sequences*

CXCL5	Sino Biological	CCG CTG GCA TTT CTG TTG CTG T	CAG GGA TCA CCT CCA AAT TAG CG
CCL7	Sino Biological	CCA CAT GCT GCT ATG TCA AGA	ACA CCG ACT ACT GGT GAT CCT
IL-6	NCBI	CCT CTC TGC AAG AGA CTT CCA TC	CCA TTG CAC AAC TCT TTT CTC A
IL-1β	primerBank	CAA CCA ACA AGT GAT ATT CTC CAT G	GAT CCA CAC TCT CCA GCT GCA
GAPDH	NCBI	TGC GAC TTC AAC AGC AAC TC	GCC TCT CTT GCT CAG TGT CC

3.3.6. Confocal Microscopy

Immunohistochemistry samples were imaged as described in Chapter 2.3.10. Briefly, an Olympus FV1000 confocal microscope equipped with Laser Diodes 405, 440, 559, 635 and an Argon Laser 488/515 with a 20×/ 1.0 NA water-dipping objective was used. The 515 nm laser was used for detecting IL-6 + cells. Z stacks were acquired at a 1.25 µm step size and an aspect ratio of 512 µm x 512 µm. Acquired Z stacks were reconstructed as 3-D images using Imaris Software.

3.3.7. Quantification and Statistical Analysis

GraphPad Prism v9.0 was used for statistical analyses. For qPCR, the ratios of mRNA levels were calculated using the $-\Delta\Delta Ct$ method ($2^{-\Delta\Delta Ct}$), and statistical analysis was performed using One-way ANOVA with Tukey's multiple comparisons. *P* values less than 0.05 were considered significant. Validation experiments were repeated twice.

3.4. Results

3.4.1. Bupivacaine Altered Baseline and *P.aeruginosa*-induced Expression of Corneal Cytokines and Chemokines

Our previous work showed that CD11c+ cells are recruited in response to *P. aeruginosa* and that their depletion reduces the expression of cytokines and chemokines including IL-6, IL-1β, CXCL1, CXCL2, and CXCL10 (Metruccio *et al.*, 2017). Further, we also reported corneal sensory nerves can play a role in the recruitment of CD11c+ cells following *P. aeruginosa* inoculation of superficially-injured (blotted) corneas (Wan *et al.*, 2021). Here we investigated whether the cytokine and chemokine response are also dependent on sensory nerve firing. Before inoculation, corneas were blotted with a KimWipe which removes the epithelial surface cell layer and enables bacterial adhesion. Transcriptional analysis of 96 mouse cytokine/chemokine genes was assessed with or without bupivacaine treatment and with or without *P. aeruginosa* inoculation. Interestingly, in uninoculated corneas, we found that the baseline expression of 23 cytokines/chemokines was affected by bupivacaine with 17 being upregulated and 6 being downregulated (Figure 3.2). Upregulated genes (2-fold or more) included IL-3, Mstn, CCL24, and CXCL5, while downregulated genes (2-fold or more) included IL-10, IL-12b, CD70, and the neurotrophic growth factor CNTF. Results from inoculated corneas revealed IL-1β, IL-6, and CXCL10 expression were reduced or reversed by bupivacaine (2-fold or more) (Figure 3.3). Other similarly impacted cytokines included CCL2, CCL7, and CXCL5.

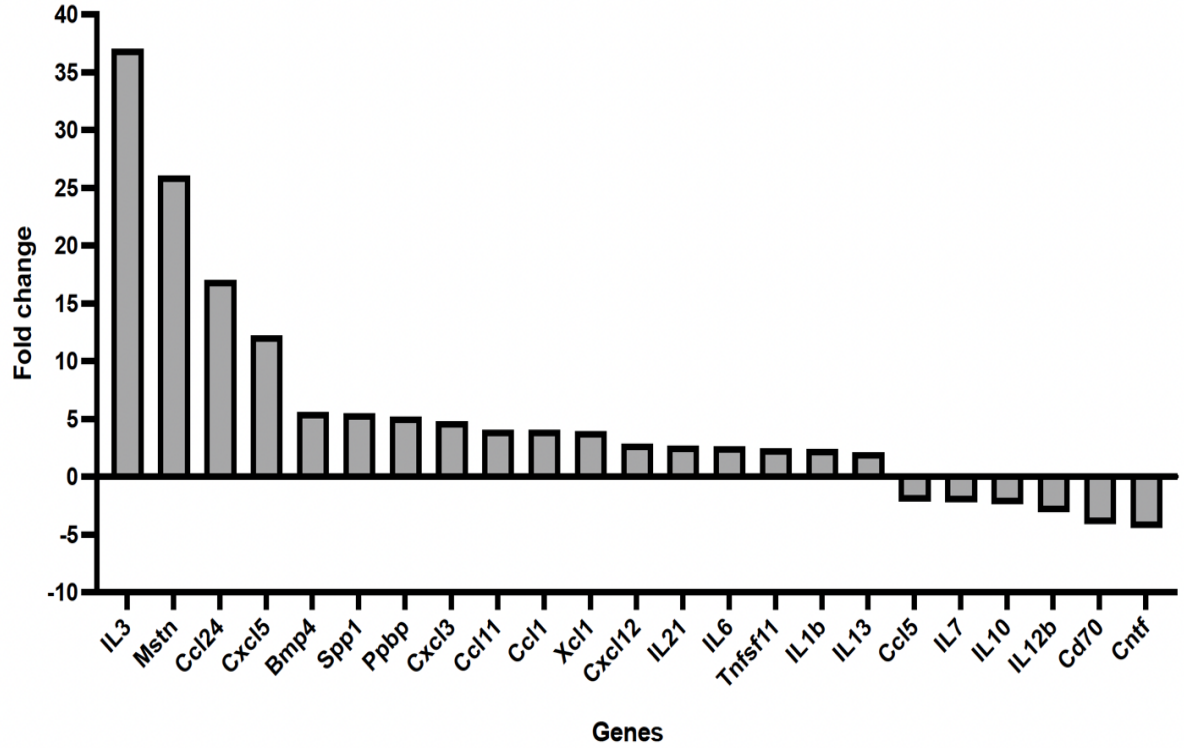


Figure 3. 2: *Gene expression analysis of cytokines and chemokines in superficially-injured control mouse corneas (without P. aeruginosa inoculation). Bupivacaine altered 23 cytokines/chemokines expression at baseline. Data were normalized to sham-treated (no bupivacaine) baseline.*

3.4.2. RT-qPCR Validation Confirms Impacted Cytokines and Chemokines

Next, we validated interesting changes in the cytokine/chemokine response to *P. aeruginosa* mediated by sensory nerves. Given its well-established involvement in *P. aeruginosa* infections (Cole *et al.*, 1999; Jäger *et al.*, 2021; Kheir *et al.*, 2022), we first sought to validate IL-6 results using protein and transcriptional analysis methods. Interestingly, transcriptional results were not replicated (Figure 3.4). Differing from the results in Figure 3.3, there was a small increase in expression across both groups. Similarly, protein validation using immunohistochemistry methods did not yield significant differences in expression across the groups (Figure 3.5). However, as observed in Figure 3.3, there was decreased expression in inoculated bupivacaine-treated corneas (green bars) for IL-1β (0.6-fold decrease) and CXCL5 (~39-fold decrease) compared to inoculated sham-treated controls, grey bars). However, a significant decrease in gene expression was only observed for CCL7 (127-fold decrease) compared to inoculated sham-treated corneas (Figure 3.4). These findings suggest an important role for corneal sensory nerves in the maintenance of the baseline immune molecular signatures and the induction of their expression during *P. aeruginosa* challenge.

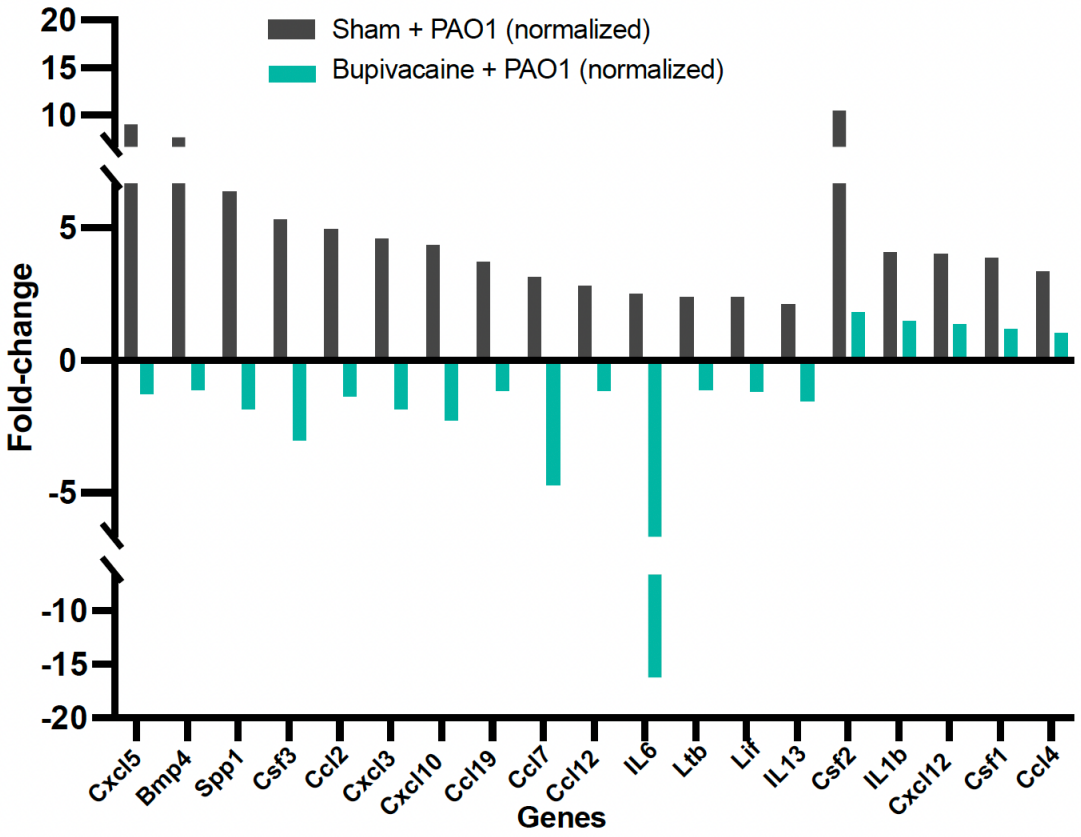


Figure 3. 3: *Gene expression analysis of cytokines and chemokines in mouse corneas inoculated with P. aeruginosa. Bupivacaine reduced or reversed 20 cytokines/chemokines expression induced by P. aeruginosa. Data in each group were normalized to their respective baseline.*

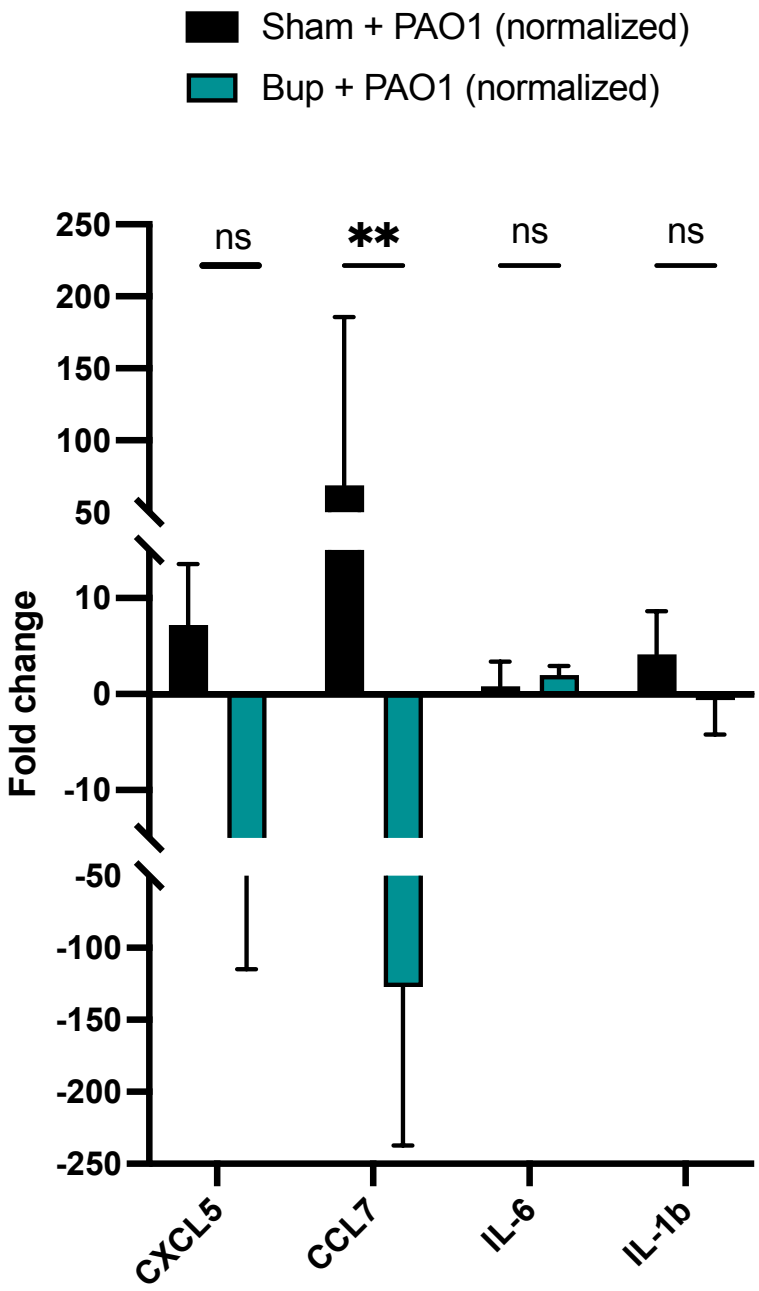


Figure 3. 4: *Validation of cytokines and chemokines in mouse corneas induced by P. aeruginosa. Bupivacaine reversed CXCL5 and IL-1β expression induced by P. aeruginosa, while significantly reversing CCL7 expression. Data in each group were normalized to their respective baseline. Ns, no significant difference, ** P < 0.01. One-way ANOVA.*

3. 5. Discussion

Although lens wear does not overtly injure the epithelium, it can increase susceptibility to keratitis by pathogens such as *P. aeruginosa,* which necessitates understanding the corneal defenses during health. In this Chapter, we investigated the role of corneal sensory nerves in modulating signaling molecules that are important drivers of immune cell recruitment and other parainflammatory responses during *P. aeruginosa* challenge. We found altered gene expression of cytokines and chemokines in the absence of sensory nerve firing, with or without *P. aeruginosa* inoculation. Validation experiments confirmed that CXCL5 and CCL7 expression induced by *P. aeruginosa* challenge is modulated by sensory nerve function/firing, with CCL7 results being more robust and statistically significant. These findings suggest that sensory nerve firing, likely induced by *P. aeruginosa* virulence factors, influences the expression of signaling molecules including cytokines and chemokines to modulate corneal immune defenses in the absence of infection.

Cytokines and chemokines, play an important role in regulating immune responses throughout the body. *P. aeruginosa* is known to trigger a robust immune response characterized by the release of various cytokines and chemokines at the ocular surface and other sites. For example, a previous study assessed the expression of proinflammatory cytokines such as IL-1β, IL-6, and TNF-α following ocular infection with different *P. aeruginosa* strains. In that study, they reported distinct patterns of cytokine and chemokine expression associated with each strain, where their overexpression correlated with the severity of inflammation and corneal damage by *P. aeruginosa* infection (Xue, Thakur and Willcox, 2002). Another study shows evidence of IL-12 and IFN-γ production that correlated with reduced bacterial load in a *P. aeruginosa* keratitis model (Hazlett *et al.*, 2002). While studies and others have highlighted the significance of pro-inflammatory cytokines and chemokines in combating *P. aeruginosa* at the ocular surface, our investigation examines the role of corneal sensory nerves in modulating these immune factors in the absence of pathology.

The cornea has a unique immune privilege that limits immune cell infiltration under homeostatic conditions. However, upon contact lens wear, infection, or injury, adaptive immune responses are mobilized. Often implicated in this immune response are resident dendritic cells, known for their antigen-presenting function. We have previously observed corneal sensory nerves can regulate *P. aeruginosa*-induced CD11c+ cell recruitment and that this recruitment is associated with increased expression of proinflammatory cytokines and chemokines (Metruccio *et al.*, 2017; Wan *et al.*, 2021). In the former study, morphology changes were also observed in dendritic cells, becoming less circular, migrating towards the corneal surface, and extending processes towards inoculated *P. aeruginosa* that correlated with reduced adhesion of the potential pathogen in injured corneas indicating they can sense and respond to bacterial presence. A similar finding was observed in Chapter 2 where CD11c+ cells moved anteriorly, had more dendriform process, and were larger. These findings indicate CD11c+ cell interaction with the epithelial surface and/or bacteria which can prompt the release of additional cytokines and chemokines to influence activation states and migration.

Sensory nerves and immune cells play a pivotal role in modulating mucosal defenses against pathogens. At the cornea, sensory nerves, including those that express TRPA1/V1, also play a role in maintaining homeostasis and contributing to wound healing (Pal-Ghosh, Tadvalkar and Stepp, 2017; Liu *et al.*, 2022; Feinberg *et al.*, 2023). Therefore, it is plausible that disruption of their function, in this case with bupivacaine anesthesia, may prompt compensatory changes in the molecular environment critical to maintaining homeostasis. The baseline changes in gene expressions of important cytokines and chemokines observed in uninoculated corneas may reflect this response. Also supporting this theory, studies have shown that local anesthetics like bupivacaine can reduce the production of certain pro-inflammatory cytokines and chemokines suggesting anti-inflammatory properties (Cassuto, Sinclair and Bonderovic, 2006; Chiang *et al.*, 2008). Interestingly, downregulated factors in the latter study include IL-13, one of the factors downregulated at baseline in our study (Figure 3.2).

In our current study, we observed a significant reduction in the expression of CCL7 in corneas treated with bupivacaine and inoculated with *P. aeruginosa*. This finding suggests corneal sensory nerves can modify CCL7 expression in response to the potential pathogen (Figures 3.3 and 3.4). CCL7 is a well-studied chemotactic factor primarily produced by monocytes, macrophages, and epithelial cells. Once released, CCL7 attracts monocytes, T cells, and dendritic cells, recruiting them to the site of inflammation or injury (Fioretti *et al.*, 1998; Zhang *et al.*, 2020; Chang, Chen and Chen, 2022). Therefore, our findings that it is downregulated suggest a potential impact on the recruitment of these immune cells to combat the pathogen, consistent with our previous observation of reduced CD11c+ cell recruitment in bupivacaine-treated corneas challenged with *P. aeruginosa* (Wan *et al.*, 2021).

Among the well-studied cytokines implicated in *P. aeruginosa* infections at the ocular surface are IL-6 and IL-1β (Cole Nerida *et al.*, 1999; Rudner *et al.*, 2000; Ghasemi, 2018). Interestingly, our study revealed an increase in both IL-6 and IL-1β levels during *P. aeruginosa* inoculation, even in the absence of pathology. We observed a robust decrease in IL-6 expression in bupivacaine-treated and inoculated corneas (~17-fold) (Figure 3.3). Conversely, a smaller reduction in IL-1β was observed. These increases were attenuated by bupivacaine treatment, indicating a potential regulatory role of corneal sensory nerves.

Moreover, the parallel increase in IL-6 and IL-1β levels observed by our lab during contact lens wear points to the multifaceted nature of ocular immune responses, where both bacterial challenge and lens wear can induce similar inflammatory pathways (Datta, Truong, *et al.*, 2023; Datta *et al.*, 2024) 2024) (Figures S3.6 and S3.7). Other studies also suggest contact lenses alter the expression of a similar cytokine/chemokine profile corroborating similarities in inflammatory responses (Insua Pereira, Sampaio and Lira, 2022), suggesting a potential link between sensory nerve mediated defenses during lens wear and pathogenic defense.

Furthermore, along with pathogenic challenge, injured tissues and activated immune cells produce a variety of cytokines and chemokines, including IL-1β and IL-6 that can further stimulate CCL7 production. While the IL-1β and IL-6 findings were not validated (Figures 3.4 and S3.5), it remains possible that subtle changes in their expression may also contribute to the observed reduced expression of CCL7. Since we

incorporated both tissue injury and pathogen challenge, various events may occur simultaneously to trigger cytokine/chemokine expression and subsequent CD11c+ cell recruitment for resolution. A possible sequence of events is proposed in our theoretical model (Figure 1.4). Following superficial injury and subsequent pathogen challenge, activated sensory nerves release molecular factors that modulate the expression of inflammatory mediators, regulating the recruitment of antigen-presenting cells like dendritic cells. Notably, sensory nerves expressing TRPA1 and TRPV1 receptors often co-localize with key inflammatory mediators such as CGRP and Substance P neuropeptides. Previous studies, including from our lab, have shown the involvement of TRPA1 receptors in defense against pathogens like *P. aeruginosa* or associated virulent factors (Meseguer *et al.*, 2014; Boonen *et al.*, 2018; Wan *et al.*, 2021). Moreover, research suggests that TRPA1 activation can influence sensitivity in the skin and dendritic cell trafficking (Shiba *et al.*, 2012). Also related, a study suggests TRPA1 is confined to Aδ fibers in the mouse cornea, and consequently not expressed in the epithelial layers (Schecterson *et al.*, 2020). It is possible that these may become exposed during superficial injury, potentially influencing the subsequent parainflammatory response. While these findings expand on the potential mechanisms underlying the observed responses, further studies are warranted to fully characterize the sequence of events and the precise role of TRPA1/V1 sensory nerves in mediating parainflammatory responses induced by potential pathogens during healthy and epithelial compromised states such as contact lens wear or injury.

3.6. Limitations

Although cytokines and chemokines play crucial roles in immune responses, they represent only a part of the complex network of immune mediators involved in the corneal host defense. Factors such as antimicrobial peptides and cell adhesion molecules may also contribute to the overall corneal CD11c+ cell response to *P. aeruginosa*. Additionally, our study primarily assessed changes in cytokine and chemokine expression at the transcriptional level, which may not fully reflect protein-level alterations or downstream functional effects. Further protein-level analyses such as Western blots, ELISA, and immunohistochemistry would provide a more comprehensive understanding of the sensory nerve-modulated dendritic cell response involved in corneal defense against *P. aeruginosa*.

3.7. Conclusion

In summary, corneal sensory nerves may contribute to *P. aeruginosa*-induced CD11c+ recruitment by influencing the expression of cytokines and chemokines. Importantly, this includes CCL7, a known chemoattractant for macrophages, monocytes, and dendritic cells. However, the mechanisms involved are potentially complicated and may involve signaling pathways that communicate with resident immune cells, to shape the local immune profile and parainflammatory responses. Thus, requiring further studies.

3.8. Acknowledgement

This work was supported by the National Institutes of Health; EY011211(SF)

3.9. Supplemental Material

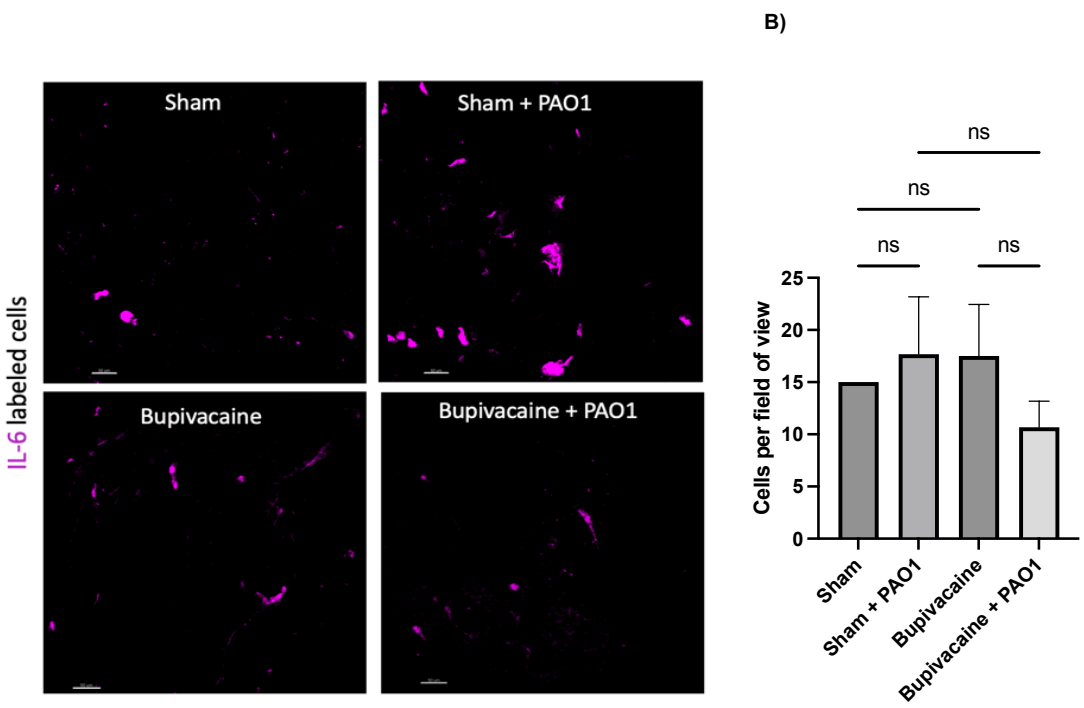


Figure 3. 5: *Protein validation revealed no changes in IL-6-expressing immune cell numbers induced by bupivacaine and/or P. aeruginosa.* ***(A)*** *Representative maximum intensity projections of IL-6+ antibody-labeled immune cells (pink) in the superficially injured corneas that were sham or bupivacaine-treated, with or without P. aeruginosa (PAO1) inoculation. Scale bars = 50 µm. 20X Objective* ***(B)*** *Quantification of IL-6+ cells revealed no significant difference in cell numbers in sham or bupivacaine-treated corneas, with or without P. aeruginosa (PAO1) inoculation. Data are expressed as the mean ± SD cells per field of view. ns = no significant difference (One-way ANOVA).*

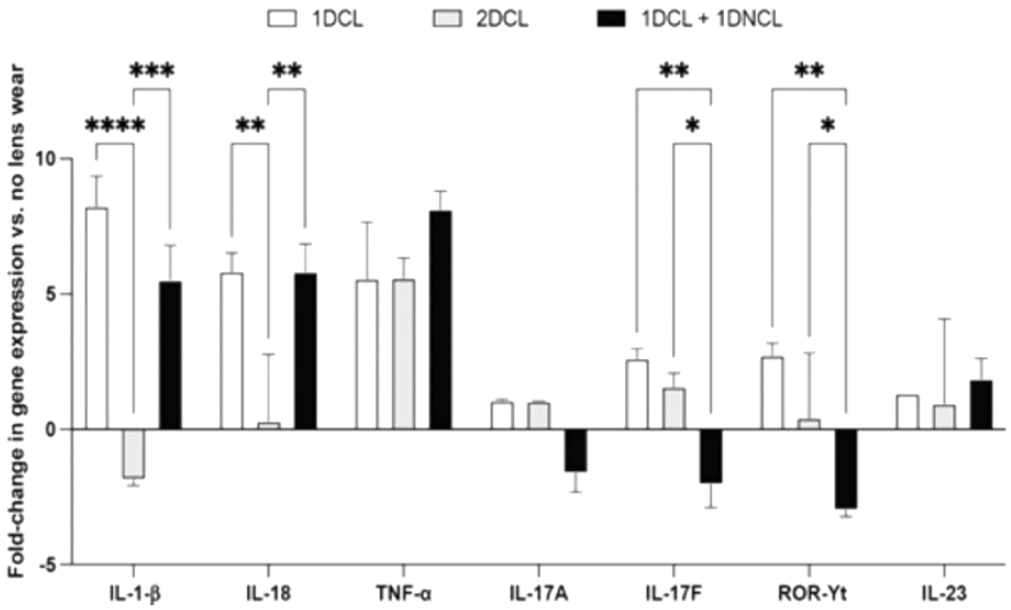


Figure 3. 6: *(from Datta et al 2024) qPCR analysis of corneal cytokine gene expression under different lens wearing conditions. Data expressed as fold-change in lens-wearing corneas relative to their respective contralateral eyes. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001 (two-way ANOVA with Tukey's multiple comparison test).*

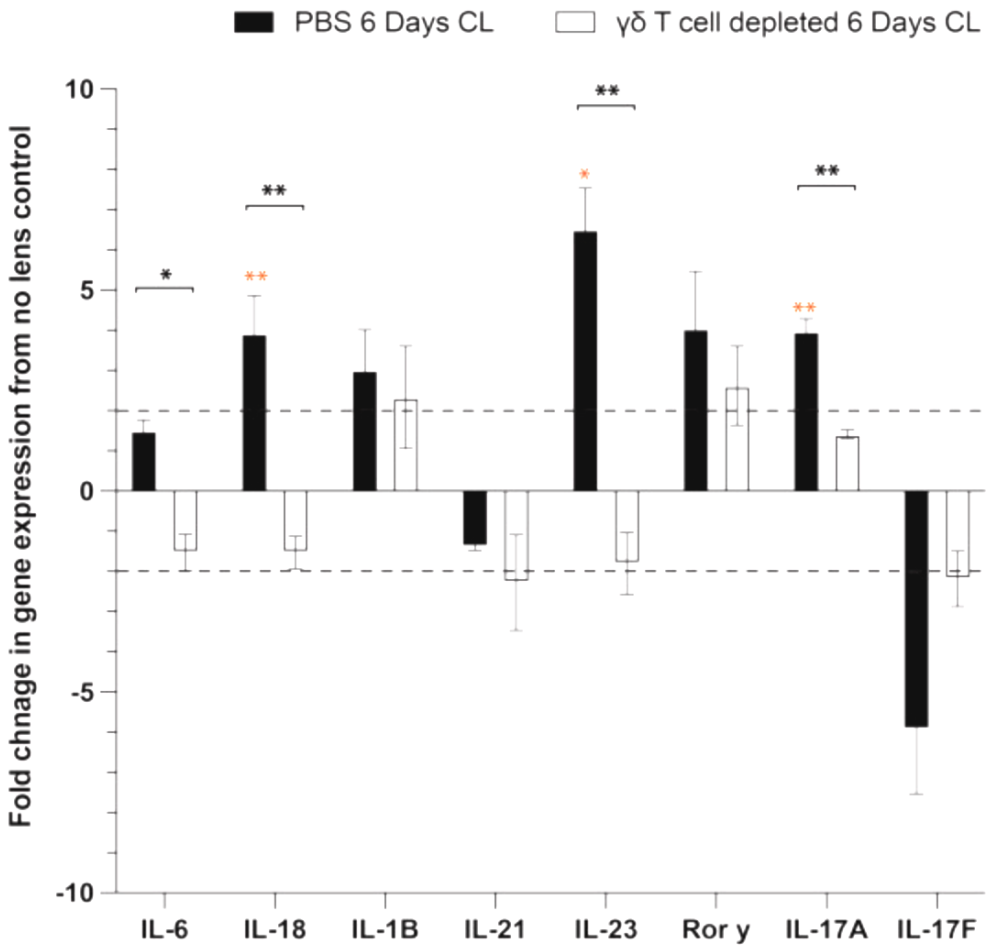


Figure 3. *7: (from Datta et al, 2022) qPCR analysis of cytokine gene expression in the mouse cornea after 6 days of contact lens wear in γδ T cell depleted mice versus PBS-treated controls. Data expressed as the fold-change in the lens wearing cornea relative to the contralateral control (i.e. normalized to zero) with the dashed lines indicating a 2-fold change. *P < 0.05, **P < 0.01 (Students t-Test). Lens-wearing PBS-treated control mice (black bars) were also compared to lens wearing γδ T cell depleted mice (white bars). *P < 0.05, **P < 0.01 (Student's t-Test). ns = Not Significant*

Chapter 4

TRPA1 and TRPV1 Modulate Corneal Defense Against an Eyelid Commensal Bacterium and Tear Fluid-Associated Proteins at the Ocular Surface

4.1. Abstract

Results from Chapter 2 show a novel role for TPRA1 and TRPV1 in the cornea's intrinsic defense extending to Gram-negative *Pseudomonas aeruginosa* (TRPA1-mediated), and Gram-positive *Staphylococcus aureus,* and environmental bacteria (both TRPV1-mediated). To further characterize their involvement in the cornea's intrinsic defense against colonization, this study tested the hypothesis that their specific roles relate to bacterial Gram type, i.e. Gram-positive versus Gram-negative. Corneal colonization experiments were performed using two relevant Gram-positive eye commensals; *Macrococcus epidermidis* (isolated from a mouse eyelid) and *Corynebacterium mastitidis*, a stable colonizer of the conjunctiva. Wild-type (WT) and TRPA1 (-/-), TRPV1 (-/-), or TRPA1V1 (-/-) mice were subjected to bacterial inoculation, and TRP-sensory nerve perturbation was done in WT corneas before some experiments. Eyes were harvested, fixed, and subjected to Fluorescence *in situ* Hybridization (FISH) for quantifying viable adherent bacteria. Mass spectrometry experiments were also performed on WT or TRPV1 (-/-) eyewashes following corneal inoculation with bacterial culture supernatant to determine if antimicrobial factors were modulated by TRPV1. In some experiments, CD11c+ and Lyz2+ immune cells were assessed to determine whether parainflammatory responses were involved. The results showed that TRPA1 (-/-), TRPV1 (-/-) and TRPA1V1 (-/-) corneas had a significant increase in *M. epidermidis* adhesion compared to WT. However, defenses against *C. mastitidis* did not require TRPA1 or TRPV1. Bupivacaine sensory nerve block had no significant effect on the cornea's ability to prevent *M. epidermidis* adhesion. Instead, defenses appeared to be modulated locally, with tear fluid-associated proteins (some antimicrobial) showing increased abundance in WT mice eyewash following supernatant challenge, some being TRPV1-dependent. These findings suggest that bacterial-specific factors distinctly activate TRPA1 and TRPV1 receptors, with defenses against *M. epidermidis* locally modulated without relying on sensory nerve firing and additionally independent of CD11c+ and Lyz2+ immune cell recruitment.

4.2. Introduction

Microbiomes play a crucial role in maintaining health and functionality throughout mucosal sites (Kumar and Chordia, 2017). However, the cornea of the eye stands out among exposed mucosal surfaces for its exceptional ability to avoid microbiome formation (Wan *et al.*, 2018). A complete understanding of the cornea's formidable barrier function is important to comprehend the interactions between bacteria and the ocular surface. This will provide insights into the mechanisms by which the healthy eye defends itself against external factors and whether various bacterial species play a role in maintaining the natural balance of the ocular surface, similar to their functions in other mucosal tissues.

So far, we have established that the cornea's ability to resist colonization is in part attributed to TRPV1 ion channels abundantly expressed on the corneal sensory nerve endings (Chapter 2) (Wan *et al.*, 2021). We further expanded on these findings which led to the discovery that TRPV1^{+} sensory nerves can also mediate corneal defense against the

Gram-positive ocular pathogen *Staphylococcus aureus*. This defense was locally modulated, correlated with CD45+ immune cell recruitment, and unlike defense versus the Gram-negative pathogen *P. aeruginosa,* was independent of sensory nerve firing. Moreover, sensory nerve firing mechanisms correlated with CD11c+ immune cell responses and cytokine/chemokine expression. These findings indicate differential involvement of TRPA1 and TRPV1 based on bacterial factors, irrespective of their status as a pathogen versus commensal. Indeed, this direct involvement of sensory nerves and TRP receptors against bacterial challenge underscores their role in initiating antimicrobial mechanisms, with only a few of the ligands/factors driving their involvement being identified (Boonen *et al.*, 2018). Therefore, it remains to be determined whether bacterial Gram-type is a factor that can influence the differential involvement we observed for TRPA1 and TRPV1 against bacterial colonization.

Central to protecting the ocular surface against bacterial insults (especially Gram-Positives/commensals) is the tear fluid. This is achieved through a combination of antimicrobial, physical, and chemical mechanisms that not only inhibit bacterial viability but also modulate both epithelial defense and bacterial virulence (Fleiszig, Kwong and Evans, 2003; Fleiszig *et al.*, 2020). Antimicrobial factors within tears including lysozyme and lactoferrin can inhibit bacterial growth and adherence to prevent infections. Immunoglobulins, particularly sIgA, are abundantly expressed in the tear fluid where they can neutralize and eliminate bacteria. Additionally, bound and unbound mucins act as a physical barrier to discourage bacterial adhesion (Friedman, Maureen G, 1990; Mantelli and Argüeso, 2008; McDermott, 2013). Despite this comprehensive defense, a relationship between TRPA1/V1 activation and the modulation of molecular factors in tear fluid or at the interface of the cornea and tear fluid to prevent bacterial adhesion has not been explored.

In this chapter, we explored two hypotheses: 1) the role of TRPA1 and TRPV1 in the corneal defense against bacteria requires a distinction between bacterial Gram types with TRPV1 being specific for Gram-positive commensal bacteria, and 2) TRPV1 locally modulates antimicrobial factors released at the ocular surface to prevent bacterial adhesion. Interestingly, our findings revealed that both TRPA1 and TRPV1 can prevent the adhesion of *Macrococcus epidermidis*, a murine eyelid commensal, to healthy murine corneas. Meanwhile, corneal defense versus the murine conjunctival commensal, *Corynebacterium mastitidis* was independent of TRPA1 and TRPV1. Defense versus *M. epidermidis* did not rely on CD11c+ and Lyz2+ immune cell recruitment but correlated with a differential local abundance of antimicrobial proteins at the ocular surface. These findings provide a better understanding of the TRP-dependent mechanisms that contribute to the cornea's exceptional ability, compared to other external body surfaces, to remain amicrobic.

4.3. Materials and Methods

4.3.1. Mice

All procedures were carried out per standards established by the Association for the Research in Vision and Ophthalmology (ARVO), under the protocol AUP-2019-06-12322 approved by the Animal Care and Use Committee, the University of California

Berkeley, an AAALAC-accredited institution. The protocol adheres to PHS policy on the humane care and use of laboratory animals, and the guide for the care and use of laboratory animals. Mice used were six- to twelve-week-old male or female C57BL/6 wild-type (WT) (from Jackson Laboratory) and gene knockouts in TRPA1 (-/-) or TRPV1 (-/-) (kindly provided by Dr. Diana Bautista, University of California, Berkeley, CA USA).

4.3.2. Bacterial Strains

A coagulase-negative murine eyelid commensal (*Macroccocus epidermidis*, isolated in this laboratory) and murine conjunctival commensal *Corynebacterium mastitidis* (kindly provided by Dr. Anthony St. Leger, University of Pittsburgh, PA, USA) were used. *M. epidermidis* was prepared by growing on a Tryptic Soy Agar (TSA) plate overnight for ~16 hours at 37 °C, followed by suspension in phosphate-buffered saline (PBS) to a concentration of ~10^{11} colony-forming units (CFU)/mL. *C. mastiditis* was grown on blood agar for 72 h before being suspended in PBS to a concentration of ~10^{11} CFU/mL.

4.3.3. Bacterial Clearance Assay

Our *in vivo* model of bacterial clearance was used as described previously in Chapter 2. Briefly, mice were anesthetized by intraperitoneal injection of ketamine (80–100 mg/Kg) and dexmedetomidine (0.25–0.5 mg/Kg). Corneas were inoculated with 5 µl *M. epidermidis* once every hour for 4 h. Contralateral corneas were sham inoculated with PBS. After 4 h, animals were euthanized by intraperitoneal injection of ketamine (80–100 mg/Kg) and xylazine (5–10 mg/Kg), or isoflurane overdose followed by cervical dislocation. Eyes were enucleated, rinsed with PBS, and fixed in 2% paraformaldehyde (PFA) overnight at 4°C. For *ex vivo* experiments, freshly enucleated eyes were submerged in 200 µl bacterial suspension (~10^{11} CFU/mL) for 6 h at 37°C.

4.3.4. Fluorescence *in Situ* Hybridization (FISH)

After fixation, whole eyes were labeled for adhered bacteria using a universal 16S rRNA-targeted FISH probe, following established procedures (Wan *et al.*, 2018, 2021). Briefly, fixed eyes were sequentially washed in PBS, 80% ethanol, and 95% ethanol for 10 minutes each at room temperature (RT). Subsequently, the eyes were immersed in a hybridization buffer solution (0.9 M NaCl, 20 mM Tris-HCl, and 0.01% SDS), followed by an incubation at 55°C for 30 minutes. The 16S rRNA-targeted gene probe [Alexa488]-GCTGCCTCCCGTAGGAGT- [Alexa488] (Eurofins Genomics) was added to the eyes at a final concentration of 100nM for an overnight incubation at 55°C.

4.3.5. Confocal Imaging

Samples were imaged as described in Chapter 2.3.10. Briefly, an Olympus FV1000 confocal microscope equipped with Laser Diodes 405, 440, 559, 635 nm and an Argon Laser 488/515 nm with a 20×/ 1.0 NA water-dipping objective was used. The 488 nm laser was used for the detection of bacteria labeled with the FISH probe and Lyz2+ cells. The 515 nm laser was used for imaging CD11c+- YFP cells and the 635 nm laser was used to visualize corneal surface reflectance (excitation and emission at the same wavelength).

Z stacks were acquired at 0.4 μm steps and an aspect ratio of 1024 μm x 1024 μm for bacteria and 512 μm x 512 μm for immune cells. Acquired Z stacks were reconstructed as 3-D images using Imaris Software. Adhered bacteria were identified and quantified using Imaris spot detection.

4.3.6. Quantitative Mass Spectrometry

4.3.6.1. *M. epidermidis* Culture Supernatant

Bacteria were cultured overnight on a TSA plate at 37°C. A single colony was then selected and propagated in 5 mL of Tryptic Soy Broth (TSB) under continuous aeration at 200 rpm and 37°C until the late log-phase. Following growth, the culture underwent centrifugation at 10,000 rpm and 4°C. The resulting supernatant, containing bacterial components, was subjected to filtration using a 0.22-μm membrane filter and stored at -80°C until required for experimental use.

4.3.6.2. Sample Collection and Preparation

Anesthetized WT, TRPA1 (-/-), or TRPV1 (-/-) mice corneas were inoculated with 5 μl of *M. epidermidis* supernatant (diluted to 20% in PBS), or TSB media (control) once every hour for 4 h. After 4 h, eyes were carefully rinsed with 10μl cold ProteaseMax detergent (0.1% in PBS, Promega: V2071) to recover tear fluid and corneal surface proteins. Animals were then euthanized by isoflurane overdose followed by cervical dislocation. Two eye washes were pooled per sample and stored immediately at -80°C. Samples were prepared for mass spectrometry using a 10plex Isobaric Mass Tag Kit (ThermoFisher: 90113) as previously described (Comai, Katz and Mallick, 2017). Briefly, samples were thawed, and the total protein content was determined with the Pierce™ BCA Protein Assay (ThermoFisher: 23227). Samples (1μg each) were reduced and alkylated before trypsin digestion overnight. Digested samples were labeled with individual peptide mass tags and pooled together.

4.3.6.3. LC-MS/MS analysis

Mass spectrometry was performed at the Proteomics/ Mass Spectrometry Laboratory at the University of California, Berkeley. A nano-LC column was packed in a 75-μm inner diameter glass capillary with an integrated pulled emitter tip. The column consisted of 25 cm of Dr. Maisch (GmbH) c18 1.9-μm packing material. The column was loaded and conditioned with a pressure bomb. The column was then coupled to an electrospray ionization ion source (Thermo Scientific™ Nano spray Flex™ ion source) and mounted on a Thermo Scientific Orbitrap Fusion Lumos Tribrid Mass Spectrometer. A Thermo Scientific™ EASY-nLC™ 1200 HPLC was used to deliver a flow rate of 300 nL/min. Peptides were eluted with a 180-minute gradient from 100% buffer A to 60% buffer B. Buffer A was 5% acetonitrile/0.02% heptafluorobutyric acid (HBFA); buffer B was 80% acetonitrile/0.02% HBFA. Higher-energy C-trap dissociation (HCD) spectra were acquired for each m/z. Protein identification, quantification, and analysis were done with Integrated Proteomics Pipeline-IP2 (Bruker Scientific LLC, Billerica, MA), using ProLuCID/Sequest (1,2), DTASelect2 and Census. Spectrum raw files were extracted into ms1 and ms2 files from raw files using RawExtract 1.9.9

(http://fields.scripps.edu/downloads.php) 10, and the mass spectra were searched against the mouse database (Eng, Mccormack and Yates, 1994; Tabb, McDonald and Yates, 2002).

4.3.7. Statistical Analysis

Statistical analysis was performed using Prism (GraphPad Software, Inc.). Data were expressed as mean ± standard deviation (SD). Unpaired Student's t-test was used for two group comparisons. Comparisons between three or more groups were performed using One-way or Two-way ANOVA tests with Tukey's multiple comparisons. All experiments were repeated at least twice. For mass spectrometry data, identified peptides were quantified based on reporter ion relative abundance. Data was normalized to the total intensity chromatograph (TIC) and the significance of differences in the relative abundance of peptides between samples was determined by Student t-test analysis. (GraphPad prism). Biological significance was set at a fold change of ≥ 2 between groups. P-values < 0.05 were considered significant.

4.4. Results

4.4.1. TRPA1 and TRPV1 Contribute to Corneal Defense Versus an Inoculated Murine Commensal

To extend our understanding of the protective roles of TRPA1 and TRPV1, we explored the hypothesis that a distinction between Gram-negative versus Gram-positive bacteria is important to modulate their defenses. Corneas of WT, TRPA1 (-/-) or TRPV1 (-/-) mice were inoculated with the Gram-positive commensals *Macrococcus epidermidis* or *Corynebacterium mastitidis* (10^{11} CFU/mL). Contralateral eyes were inoculated with PBS (control). Corneas were labeled with a universal 16S rRNA-targeted FISH probe to detect adhered bacteria. Results revealed increased *M. epidermidis* adhesion (~3-fold) to both TRPV1 (-/-) and TRPA1 (-/-) corneas compared to WT (Figure 4.1 A, B), showing both receptors are important for preventing *M. epidermidis* colonization of the murine cornea. A different result was found for *C. mastitidis*. WT corneas were able to clear this conjunctival commensal within 4h, however, the defense was independent of TRPA1 or TRPV1 (See Supplemental Figure 4.5).

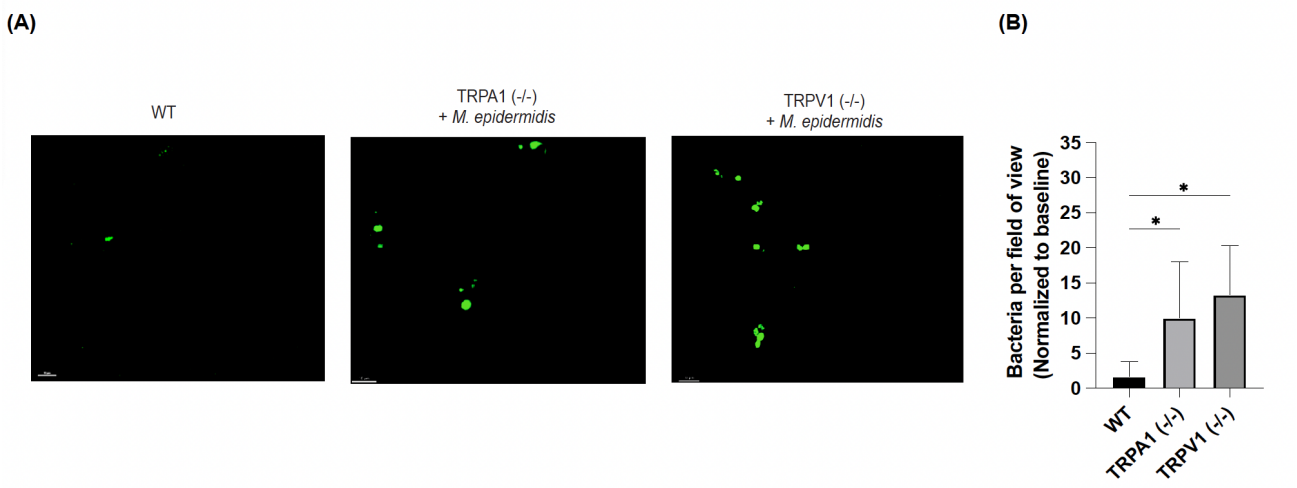


Figure 4. 1: *Corneal defense versus a murine eyelid commensal involves both TRPA1 and TRPV1 ion channels.* ***(A)*** *A universal 16S rRNA-targeted FISH probe to detect background environmental bacteria (green, upper panel) and M. epidermidis in the inoculated group (green, lower panel) comparing TRPA1V1 (–/–), TRPA1 (–/–), and TRPV1 (–/–) mice to WT. 20X Objective. Scale bars, 10 µm.* ***(B)*** *Quantification showed a significant increase in M. epidermidis adhesion in TRPA1 (–/–), and TRPV1 (–/–) mice compared to WT (~3-fold increase each). Data were normalized to baseline bacteria and expressed as the mean ± SD of bacteria per field of view. * P <0.05. Two-way ANOVA with Tukey's multiple comparisons.*

4.4.2. Corneal Defense Versus *M. epidermidis* is Reduced by RTX, Abrogated *Ex vivo*, Retained with Bupivacaine, and Inhibited by a TRPV1 antagonist

Building on our prior work demonstrating sensory nerve involvement in TRP-mediated corneal defense against *P. aeruginosa* and *S. aureus*, we investigated the role of TRPA1 and TRPV1 ion channels associated with sensory nerves in mediating corneal defense versus *M. epidermidis*. In WT mice, resiniferatoxin (RTX) was used to ablate TRPA1/V1-expressing nerve terminals. WT, TRPA1 (-/-), and TRPV1 (-/-) eyes were also inoculated *ex vivo* as previously described (Wan *et al.*, 2021) and also done in Chapter 2. Additionally, bupivacaine anesthesia was used to block corneal sensory nerve firing, and the TRPV1 antagonist JNJ-17203212 was used to locally inhibit TRPV1 function. As anticipated, RTX reduced WT defense versus *M. epidermidis* with a significant increase in FISH-labeled bacteria compared to sham-treated corneas (~3-fold) (Figure 4.2A). *Ex vivo*, WT corneas exhibited increased susceptibility to colonization by *M. epidermidis,* with the number of adherent bacteria similar to TRPA1 (-/-) and TRPV1 (-/-) corneas, suggesting a loss of WT defenses (Figure 4.2B). Results from bupivacaine experiments indicate sensory nerve activation/firing was not necessary for corneal defense against *M. epidermidis*, despite requiring TRPA1/TRPV1 sensory nerves (Figure 4.2C), a finding similar to that of *S. aureus* (Chapter 2, Figure 2.2). Also aligning with our findings for *S. aureus*, inhibiting TRPV1 channels *in vivo* using the antagonist JNJ-17203212

significantly increased *M. epidermidis* binding to WT corneas (~6-fold), supporting a local protective mechanism versus the murine commensal (Figure 4.2D).

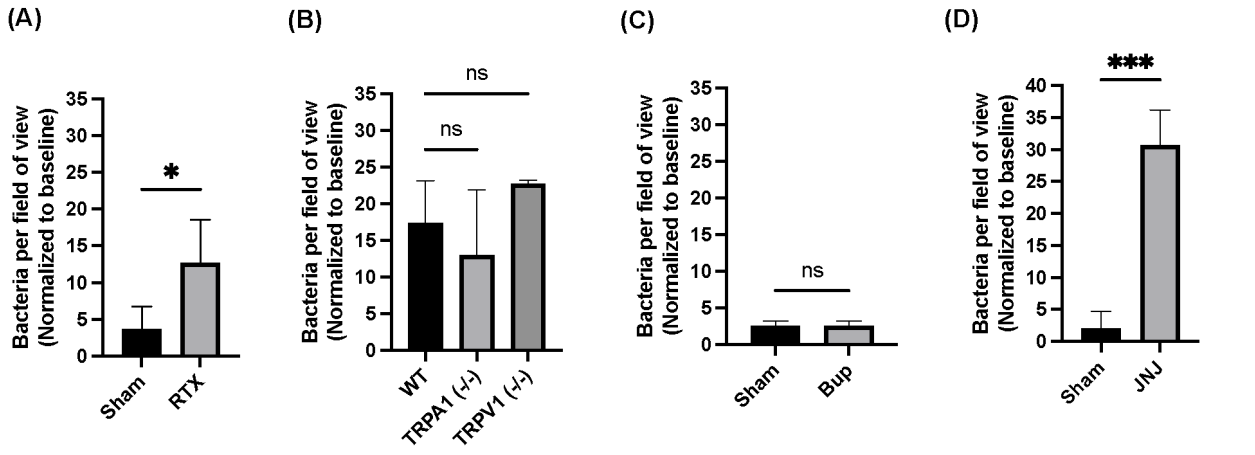


Figure 4. 2: *TRPA1 and TRPV1 sensory nerves are important for the corneal defense against M. epidermidis adhesion, but sensory nerve firing is not required.* ***(A)*** *Quantification of adhered bacteria after RTX treatment (30 μM) reveals a significant increase in FISH-labeled bacteria (~3-fold) compared to sham-treated controls.* ***(B)*** *Ex vivo inoculation of ~1x10^{11} CFU/mL M. epidermidis for 6 h abrogated TRPA1V1-mediated corneal defense, resulting in a similar increase in adhered FISH-labeled bacteria in WT, TRPA1(-/-), and TRPV1(-/-) corneas.* ***(C)*** *Bupivacaine (0.5%) does not affect WT corneal defense against M. epidermidis, with similar numbers of adhered bacteria compared to sham-treated corneas.* ***(D)*** *A TRPV1 antagonist resulted in a ~4-fold increase in adherent bacteria versus sham-treated WT corneas. Data were normalized to baseline bacteria and are expressed as the mean ± SD of bacteria per field of view. Ns, no significant difference, * P <0.05, *** P < 0.001. Two-way ANOVA with Tukey's multiple comparisons.*

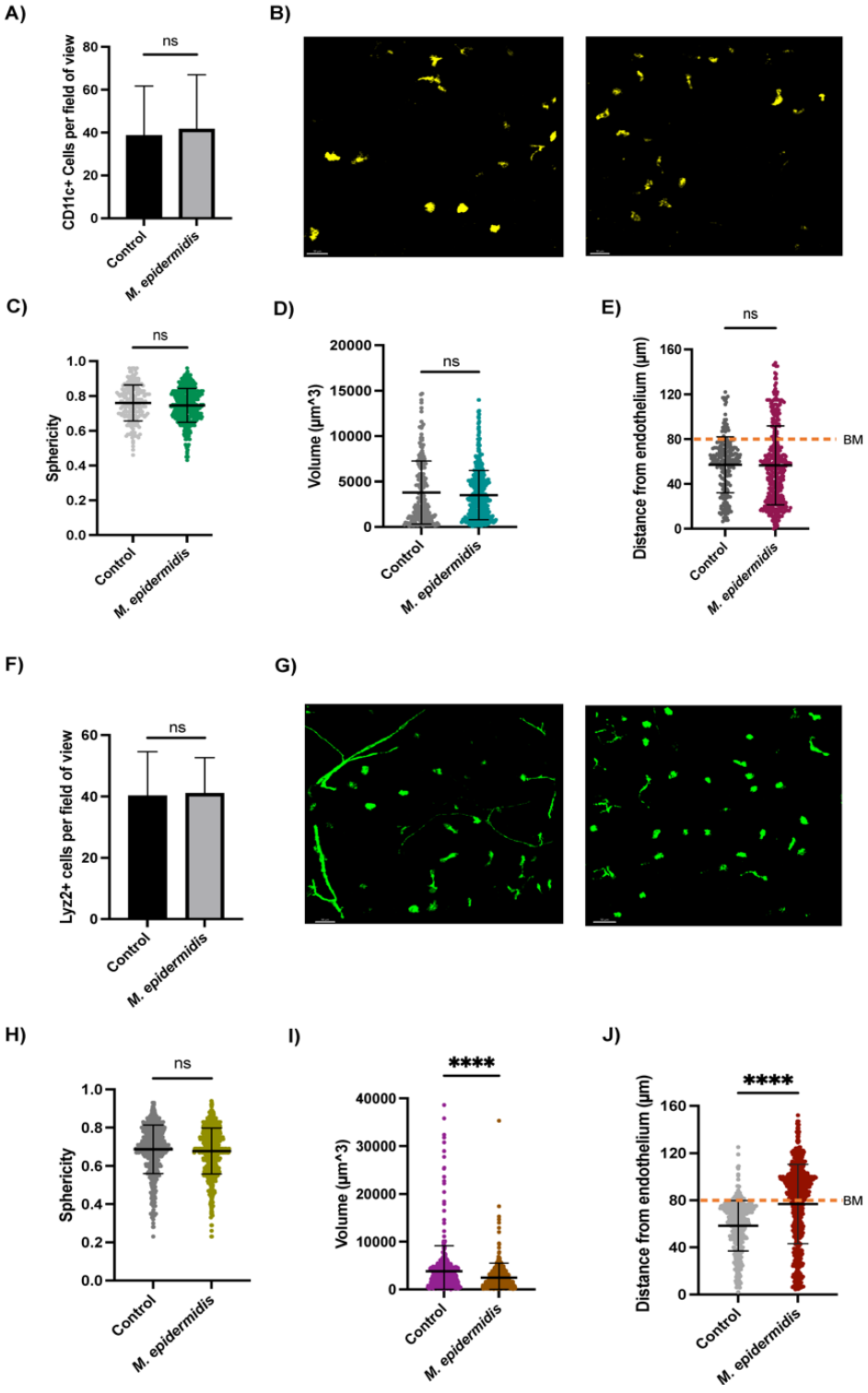


Figure 4. 3: *No corneal CD11c+ or Lyz2+ immune cell recruitment was observed for M. epidermidis.* ***(A)*** *Quantification of CD11c+ cells in healthy WT corneas at baseline and 4 h post-M. epidermidis challenge reveals no significant increase in CD11c+ cells after inoculation (P > 0.05).* ***(B)*** *Z-projections of the YFP channel (all CD11c+ cells, yellow, projected into one plane). Scale bars = 50 µm. 20X Objective.* ***(C)*** *Graph demonstrating no significant*

change in baseline CD11c+ cells sphericity following M. epidermidis challenge (P > 0.05). ***(D)*** *Baseline CD11c+ cells were similar in size to those in control corneas following M. epidermidis inoculation (P > 0.05).* ***(E)*** *Further morphological analysis of CD11c+ cells indicates cells were similarly positioned in the cornea at baseline and following S. aureus inoculation (P > 0.05). The dashed line denotes the basement membrane (BM), with areas above representing the corneal epithelium, below the stroma, and zero indicating the endothelium. Data are expressed as the mean ± SD cells per field of view. ns = No significant difference (Unpaired Student's t-Test).* ***(F)*** *Quantification of Lyz2+ cells in healthy WT corneas at baseline and 4 h post-M.epidermidis challenge no significant increase in Lyz2+ cells after inoculation (P > 0.05).* ***(G)*** *Z-projections of the GFP channel (all Lyz2+ cells, yellow, projected into one plane). Scale bars = 50 μm. 20X Objective.* ***(H)*** *Morphological analysis of Lyz2+ immune cells shows cells at baseline and post-M. epidermidis inoculation was similar in sphericity (P >0.05).* ***(I)*** *Graph showing Lyz2+ cells were significantly smaller following M. epidermidis inoculation (P < 0.0001).* ***(J)*** *Lyz2+ cells were significantly closer to or in the corneal epithelium following M. epidermidis inoculation (P < 0.0001). The dashed line denotes the basement membrane (BM), with areas above representing the corneal epithelium, below the stroma, and zero indicating the endothelium. Data are expressed as the mean ± SD cells per field of view. **** P < 0.0001, ns = no significant difference (Unpaired Student's t-Test).*

4.4.3. Inoculation of *M. epidermidis* is not associated with Corneal CD11c+ or Lyz2+ Immune Cell Recruitment

Next, we investigated whether the TRP-dependent corneal defense against *M. epidermidis* colonization could involve immune cell recruitment as observed for *P. aeruginosa* and *S. aureus*. CD11c+ immune cells were assessed at baseline and 4 hours after *M. epidermis* inoculation. Surprisingly, our results revealed no significant increase in CD11c+ cells post-inoculation. Moreover, analysis of the cells in both control and inoculated corneas showed no significant differences in shape, size, and location within the cornea (Figure 4A-E). Similarly, there was no significant difference in Lyz2+ cell numbers between control and inoculated corneas. However, Lyz2+ cells in the inoculated corneas were significantly smaller and migrated closer to and into the epithelium (Figure 4.3 H-J).

4.4.4. *M. epidermidis* Factors Differentially Modulate Tear Fluid Proteins in WT vs TRPV1 (-/-) Mice

Our results show that corneal defense versus the murine commensal; 1) involves both TRPA1 and TRPV1, 2) does not involve recruitment of CD11c+ or Lyz2+ immune cells, and 3) can be modulated locally by TRPV1. Therefore, we explored whether a different local molecular environment created by TRPV1 is required to mediate defense against *M. epidermidis*. WT and TRPV1 (-/-) corneas were challenged with *M. epidermidis* factors (present within culture supernatant) or TSB (media only control). Eyes were washed with 0.1% ProteaseMax detergent to collect surface-associated proteins after 4h. Eyewashes were prepared for high-resolution quantitative mass spectrometry. A total of 149 proteins were identified with 4 being unique to the control group (Figure 4.4 A). Of these, TRPV1 (-/-) eyewashes exhibited a significant reduction in Lipocalin 11 compared to WT (1.75-fold). Meanwhile, S100 calcium-binding protein A11 (S100A11) was not detected in TRPV1 (-/-) eyewashes (Table 4.1). Following *M. epidermidis* supernatant inoculation, 7 of 8 identified proteins were significantly reduced in TRPV1 (-/-) eyewashes compared to WT (Figure 4.4 B). Among the reduced proteins was the polymeric immunoglobulin receptor (pIgR) for IgA, a 2.4-fold decrease. Interestingly, the

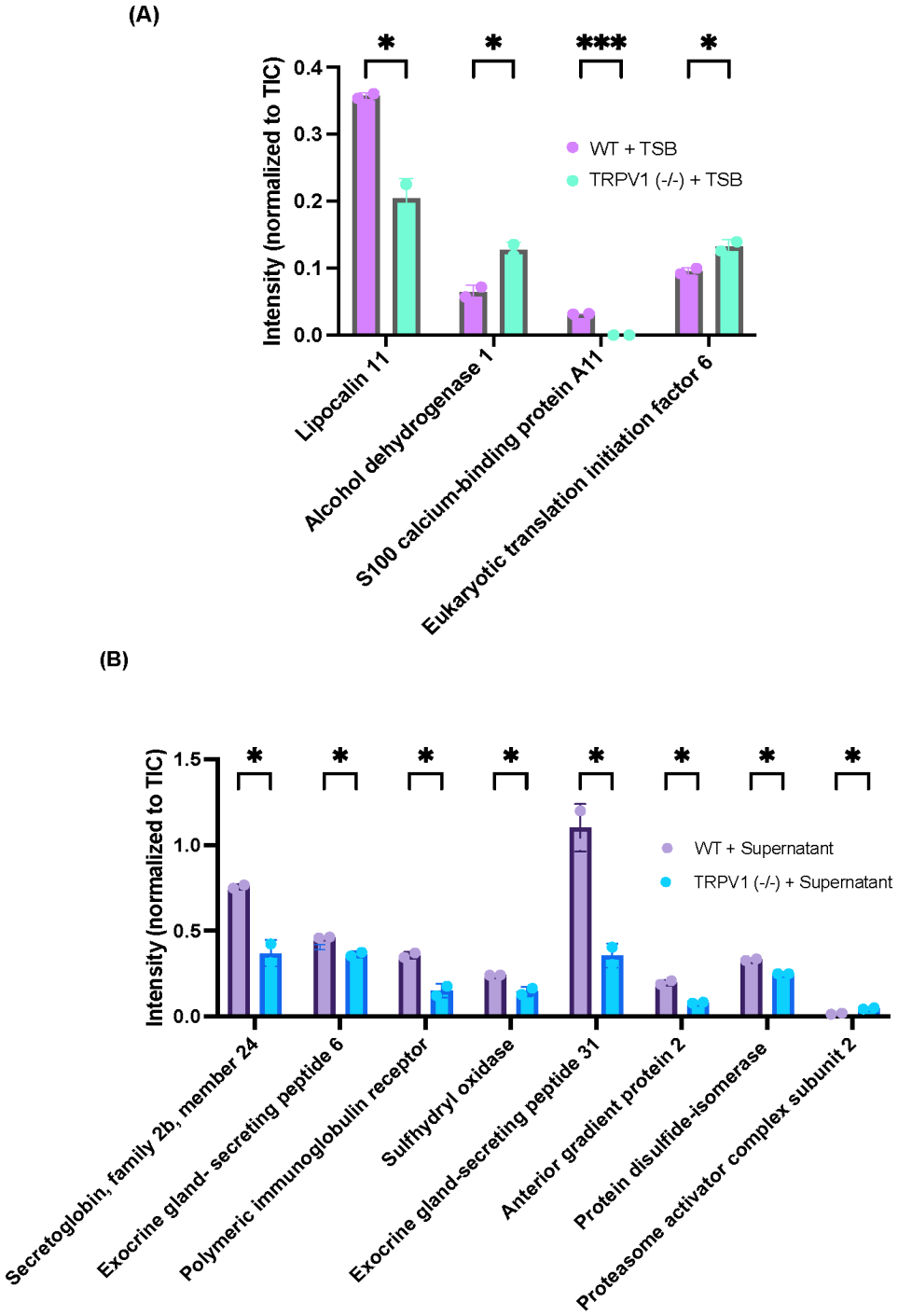


Figure 4. 4: *TRPV1 can modulate tear fluid-associated proteins at the corneal surface with or without commensal bacteria challenge.* ***(A)*** *Proteins identified after data normalization of TRPV1 (-/-) versus WT samples with significant differences in abundance without bacterial challenge, some known to be antimicrobial.* ***(B)*** *Proteins identified after data normalization of TRPV1 (-/-) versus WT samples with significant differences in abundance following bacterial challenge (5 µL cultured supernatant from Macrococcus epidermidis, eyelid commensal). Data are expressed as the mean ± SD cells per field of view. * P <0.05, *** P < 0.001, ns = no significant difference. Unpaired Student's t-Test.*

anterior gradient protein 2 (AGR2), required to produce soluble mucins at other mucosal sites (Bergström *et al.*, 2014), was also reduced 2.4-fold. Additionally, the pheromone exocrine gland-secreting peptide 31 (ESP31, absent in humans) was the most significantly reduced (3.1-fold) in TRPV1 (-/-) eyewashes. The Proteasome Activator Complex Subunit 2 (PSME2) was the only identified protein with increased abundance in TRPV1 (-/-) compared to WT (2.9-fold) (Table 4.2).

Table 4. 2: *Proteins identified with the most significant changes in their abundance levels in TRPV1 (-/-) mice eyewash compared to WT (uninoculated)*

Accession ID	Protein name	Average Protein Abundance % (n=2)		Fold-change Relative to WT	P- value
		WT	TRPV1 (-/-)		
A2BHR2	Lipocalin 11	0.36	0.21	-1.75	0.01862882
P50543	S100- calcium-binding protein A11	0.2	0.00	0.0	5.38174E-05
Q3UKA4	Alcohol dehydrogenase 1	0.06	0.18	1.98	0.028601686
Q545K4	Eukaryotic translation initiation factor 6	0.76	0.37	1.38	0.043639315

Table 4. 1: *Proteins identified with the most significant changes in their abundance levels in TRPV1 (-/-) mice eyewash compared to WT following bacterial challenge*

Accession ID	Protein name	Average Protein Abundance % (n=2)		Fold-change	P- value
		WT	TRPV1 (-/-)		
A8R0W3	Exocrine gland-secreting peptide 31	1.10	0.35	-3.1	0.020823
O88312	Anterior gradient protein 2	0.20	0.08	-2.5	0.008283
O70570	Polymeric immunoglobulin receptor	0.36	0.15	-2.4	0.021336
D2XZ39	Secretoglobin family 2B member 24	0.76	0.37	-2.1	0.01953
G3X9V0	Proteosome activator complex subunit 2	0.02	0.05	2.9	0.019989
A8R0U0	Exocrine gland-secreting peptide 6	0.46	0.36	-1.3	0.012296
Q8BND5	Sulfhydryl Oxidase	0.24	0.15	-1.7	0.035885
P09103	Protein disulfide isomerase	0.33	0.25	-1.3	0.003208

4.5. Discussion

Despite continuous exposure to environmental microbes, the healthy cornea exhibits a remarkable ability to avoid microbiome formation. A comprehensive understanding of the mechanisms critical to maintaining the cornea's amicrobic state under constant exposure to bacteria is imperative to elucidating how pathogens evade these protective mechanisms. We have shown novel roles for TRPV1 in preventing Gram-positive bacteria (both environmental and pathogenic *S. aureus*) and for TRPA1 in preventing corneal colonization by the Gram-negative pathogen *P. aeruginosa* in our previous studies. In this chapter, we explored whether the TRPA1 and TRPV1-mediated corneal defense requires a distinction between bacterial Gram type and the involvement of locally modulated antimicrobial factors. Results show that both TRPA1 and TRPV1 prevent corneal colonization by the Gram-positive commensal *Macroccocus epidermidis*.

Interestingly, the Gram-positive murine conjunctival commensal *C. mastitidis* was rapidly cleared from WT, TRPA1 (-/-), and TRPV1 (-/-) corneas suggesting corneal defense was independent of both ion channels. Moreover, defense versus *M. epidermidis* did not require sensory nerve firing mechanisms. Instead, a local protective mechanism independent of CD11c+ and Lyz2+ immune cell recruitment was required. Interestingly, defense correlated with the modulation of antimicrobial tear fluid proteins by TRPV1.

The direct and specific responses of TRPA1 and TRPV1 to bacteria based on Gram-type and their associated components have not been extensively studied. In this study, we observed both TRPA1 and TRPV1 can play an important role in the corneal defense against the Gram-positive commensal *M.* epidermidis, differing from the individual roles they play against *P. aeruginosa* and *S. aureus* adhesion, respectively. Possibly related to this finding, one study also suggests that TRPA1 and TRPV1 can maintain a diverse community of commensal bacteria within the gut (Nagpal *et al.*, 2020), supporting the notion that they can interact with different types of commensal-type bacteria regardless of Gram-type.

TRPA1 and TRPV1 are mostly co-expressed on nociceptors (Staruschenko, Jeske and Akopian, 2010; Lee *et al.*, 2015; Patil *et al.*, 2023). Therefore, they have roles that involve cooperation in detecting and responding to various noxious stimuli, potentially including specific bacterial factors. This would ensure a comprehensive system for detecting potential bacterial threats to trigger appropriate corneal defense(s) during health. A similar TRP-mediated defense shown in Chapter 2 for *S. aureus* was also observed for *M. epidermidis*. While TRPA1 and TRPV1 associated with sensory nerves were important in the corneal defense against the commensal, sensory nerve firing was not required, and defense was locally modulated (Figure 4.2). Of note, the *Macrococcus genus* is closely related to *Staphylococcus*. Consequently, they may have similarities in bacterial components that can mediate defenses independent of nerve-firing mechanisms as observed for *S. aureus* (Mašlanová *et al.*, 2018). Exploring further how TRP receptors collaborate, and the role bacterial factors play will be critical to understanding the sensory processes involved in identifying and/or responding to bacterial threats in the healthy cornea.

Corynebacterium mastitidis is a Gram-positive filamentous bacterium commonly found colonizing the conjunctiva and which can modulate host immunity via IL-17 to initiate protective immune responses against corneal *P. aeruginosa* and *C. albicans* infection (St. Leger *et al.*, 2017). While the cornea is largely inhospitable to bacteria, we have shown contact lenses can serve as a medium for bacterial colonization, with *Corynebacterium spp* being among the bacterial species capable of adhering to lens surfaces (Metruccio *et al.*, 2019). In this study, we explored whether TRPA1/V1 played a role in corneal defense against *C. mastitidis* and found that neither ion channels were involved. This result, together with our other findings, suggests that specific bacterial components, regardless of Gram-type or bacterial status, can engage signaling pathways that require either, or both ion channels and subsequent antimicrobial mechanisms. Further, since the healthy cornea would be routinely exposed to *Corynebacterium spp* colonizing the nearby ocular surface tissues, the cornea has likely evolved defense strategies beyond sensory nerves that can prevent *C. mastitidis* adhesion. Indeed, the microenvironment and defense mechanisms of cornea and conjunctiva are different in

other protective factors such as epithelial surface characteristics, and immune cells (Hamrah *et al.*, 2002; Turner *et al.*, 2007; Forrester *et al.*, 2016; de Paiva, St. Leger and Caspi, 2022).

Additionally, it is worth noting that TRPA1/V1 co-expression on cells can result in distinctive or diminished responses to different agonists (Sadofsky *et al.*, 2014), indicating complexities in their gating and associated signaling pathways. Therefore, antimicrobial mechanisms at the corneal surface may involve alternative pathways and receptors dedicated to bacterial sensing that could compensate for their absence.

Our previous work demonstrated other intrinsic factors such as TLRs, IL-1R, MyD88, and CD11c+ cells that can contribute to bacterial defense in healthy corneas (Fleiszig *et al.*, 2020). Furthermore, the CD11c+ response induced by *P. aeruginosa* correlated with TRPA1 and sensory nerve firing mechanisms (Metruccio *et al.*, 2017; Wan *et al.*, 2021). Building on this, we explored the role of immune cells in the TRPA1/V1-mediated defense against *M. epidermidis*. However, our data revealed no correlation between TRPA1/V1-mediated defenses and the recruitment of CD11c+ cells versus *M. epidermidis* (Figure 4.3), but the cells showed no change in morphologies, differing from *P. aeruginosa* and *S. aureus* findings in Chapter 2. A similar recruitment result was observed for Lyz2+ cells. Cell numbers remained unchanged in inoculated corneas compared to baseline. However, significant changes in the size and location of cells after inoculation were noted, indicating potential surveillance of the corneal surface promoted by *M.epidermidis* challenge. While the results of this study likely exclude CD11c+ and Lyz2+ cell recruitment in this TRP-mediated defense, we did not assess the larger population of CD45+ immune cells. Therefore, the potential involvement of other immune cell types cannot be ruled out, as observed in the response to *S. aureus* (Chapter 2, Figure 2.7). Additionally, it is worth noting that although TRPA1 is involved in defense against *M. epidermidis*, which may be influenced by bacterial factors, it's important to recognize that *M. epidermidis* is a commensal that might not necessitate the same level of defense mechanisms as observed for *P. aeruginosa* in Chapter 2.

TRPA1 and TRPV1 channels are found in sensory nerves across the body, including those at the ocular surface, however, their specific involvement in lacrimal gland innervation remains relatively underexplored compared to other tissues. Nonetheless, evidence suggests a potential role for TRPA1 and TRPV1 channels in regulating lacrimal gland function indirectly through afferent-efferent nerve interactions. The corneal afferent nerves expressing TRPA1 and TRPV1 can relay signals to efferent nerves innervating the lacrimal gland, prompting tear production and blink reflex (often called corneal reflex) to protect the ocular surface from harmful objects (Shaheen, Bakir and Jain, 2014; Peterson and Hamel, 2023). This suggests a potential mechanism by which corneal sensory nerves, possibly via TRPA1 and TRPV1 channels, could contribute directly or indirectly to the defense against bacterial adhesion. Furthermore, to effectively carry out their protective role against environmental colonization, TRPV1 sensory nerves would likely require constitutive mechanisms, like tear fluid which contains antimicrobial components. This is particularly relevant for frequently encountered bacteria such as *M. epidermidis* on the murine ocular surface.

Mass spectrometry analysis of eyewashes from TRPV1 (-/-) mice (sham-inoculated) revealed significant differences in the abundance of four proteins compared

to WT. There was a significant reduction in the abundance of a member of the Lipocalin family, Lipocalin 11. Although less is known about Lipocalin 11, other well-studied Lipocalins are known to bind and transport lipids, influence the composition of the tear film to maintain its stability, and importantly exhibit antimicrobial activity (Fluckinger *et al.*, 2004; Glasgow, 2021). Furthermore, they can impact cell morphologies of neutrophils and macrophages (both Lyz2+) (Wang *et al.*, 2019), potentially related to our Lyz2+ immune cell results shown in Figure 4.3. Another exciting baseline finding, was that the S100 A11 calcium-binding protein was absent in TRPV1 (-/-) eye washes compared to WT. S100 proteins in the tear fluid have been shown to regulate pro-inflammatory responses, with S100A11 playing complex immunological roles in diseases such as dry eye, cancers, and neurological disorders (Tong *et al.*, 2014; Zhang *et al.*, 2021). Another study has also reported a reduction in other S100 proteins in TRPV1 (-/-) mice, influencing inflammation outcomes during dermatitis (Zhou *et al.*, 2018). While the mechanisms involved are still under investigation, it is possible that S100A11 could sensitize or enhance TRPV1 responses to specific stimuli, including bacterial ligands. These findings may relate to why TRPV1 (-/-) mice corneas are more susceptible to the adhesion of environmental and inoculated Gram-positive bacteria and/or to TRPV1's role in maintaining baseline immune cell populations (Datta, Lee, *et al.*, 2023).

Following bacterial supernatant inoculation, 8 proteins were identified as significantly reduced in TRPV1 (-/-) eyewashes compared to WT. These included the polymeric immunoglobulin receptor (pIgR) (2.4-fold), the receptor that binds IgA to form secretory IgA (SIgA). SIgA is an important immune system component, particularly at mucosal sites including the ocular surface where it is abundantly expressed, and plays a crucial role in defending against pathogens including *P. aeruginosa* (Masinick *et al.*, 1997; Mantis, Rol and Corthésy, 2011). We also discovered reduction in the abundance of Anterior Gradient Protein 2 (AGR2) in TRPV1 (-/-) mice eyewashes following supernatant inoculation. AGR2 is essential for the production of soluble mucins by intestinal epithelial cells, which are critical components of the mucosal defense system (Park *et al.*, 2009; Bergström *et al.*, 2014). Mucins have been previously shown to aid in the corneal defense against bacterial binding and colonization (Fleiszig *et al.*, 1994).

Conversely, the proteasome activator complex subunit 2 (PSME2) was upregulated in the absence of TRPV1. PSM2 is known to be involved in the processing of antigens for presentation by major histocompatibility complex (MHC) class I molecules, a crucial step in many immune responses (Wu *et al.*, 2022). Moreover, TRPA1 is still present and functional in TRPV1 (-/-) mice, and as such, it remains possible that TRPA1 can also facilitate molecular responses to *M. epidermidis* since both are required to prevent its binding. A similar study using TRPA1 (-/-) mice would be required to test this hypothesis. Notably, there is increasing evidence to suggest that TRP receptors play a role in the regulation of mucosal immunity (Assas, Miyan and Pennock, 2014; de Jong *et al.*, 2015; Feng, Xie and Hu, 2022) Therefore, it is plausible that the activation of TRP receptors can alternately or in parallel, influence the release of neuropeptides and other immune factors that can potentiate SIgA-mediated and other immunomodulatory tear fluid factors discussed in the corneal defense. Our findings show a relationship between tear fluid and ocular surface antimicrobial factors and TRPV1, that provide the basis for further

exploration of the role of sensory nerves, immune cells, and the local environment in corneal/ocular defense.

While the cornea does not produce mucins directly it can indirectly benefit from the antimicrobial properties of mucins produced by neighboring mucosal tissues such as the conjunctiva. Additionally, it has recently been shown that nociceptors (which usually express TRPV1) activated by commensals can increase mucin production via goblet cells in the gut to improve barrier protection (Yang *et al.*, 2022). Although direct evidence linking TRPV1 to the regulation of mucin or tear production at the ocular surface is lacking, it is noteworthy that the conjunctiva is also innervated by TRPV1+ sensory nerves (Yu *et al.*, 2024). Additionally, the conjunctiva, with its numerous goblet cells (also innervated) and a microbiome, plays a key role in maintaining ocular surface homeostasis (Diebold *et al.*, 2001; St. Leger *et al.*, 2017; Ko *et al.*, 2018). Furthermore, the accessory lacrimal glands, such as the glands of Wolfring located in the palpebral conjunctiva, are known to contribute to tear fluid composition by secreting essential components like lysozyme, lactoferrin, tear lipocalin, and the polymeric immunoglobulin receptor (HUNT *et al.*, 1996; Ubels *et al.*, 2012). Given these pieces of evidence, it is plausible to hypothesize that TRPV1 sensory nerves may indirectly influence tear production/antimicrobial factors that mediate corneal defense. Future research directions could also focus on elucidating the impact of TRPV1 activation or inhibition on conjunctival function and lacrimal gland secretory activity.

4.6. Limitations

While the study evaluates CD11c+ and Lyz2+ immune cell recruitment in response to commensal bacteria challenge, these do not capture the full spectrum of the immune cell population in the cornea. Therefore, we cannot completely rule out the involvement of immune cells. Further characterization of other immune cell populations (such as CD45+ cells assessed in Chapter 2) would be required. Additionally, although the TRPV1 modulation of tear fluid-associated antimicrobial proteins in response to bacterial challenge is an important discovery, we must also consider contributions from other antimicrobial factors present in the cornea or surrounding tissues.

4.7. Conclusion

Our current study sheds light on the potential involvement of TRPV1 in ocular surface physiology and defense. We discovered a novel correlation between TRPV1-mediated corneal defenses and antimicrobial factors secreted at the ocular surface. Additionally, despite TRPA1 and TRPV1 receptors being able to respond to bacterial threats in the cornea, our study shows the responses may not be universally the same for all bacteria even ones with similar factors. Further investigation into whether TRPV1 modulation affects mucin or tear fluid production could provide valuable insights into ocular surface defenses against microbial pathogens and stressors such as contact lens wear.

4.8. Acknowledgements

This work was supported by the National Institutes of Health; EY011211(SF)

4.9. Supplemental Material

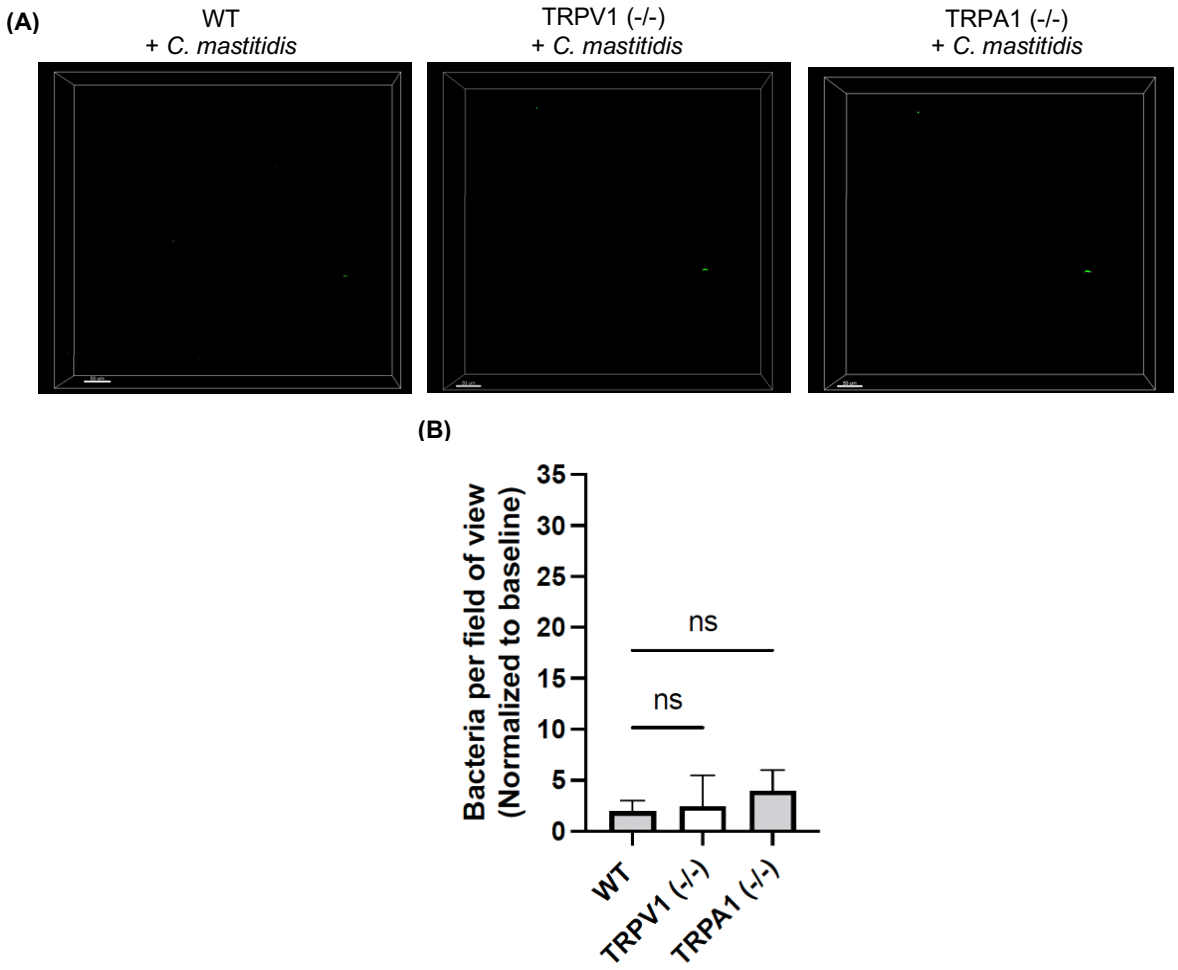


Figure 4. 5: *Corneal defense versus C. mastitidis was independent of TRPA1 and TRPV1 ion channels.* ***(A)*** *A universal 16S rRNA-targeted FISH probe to detect C. mastitidis in the inoculated groups comparing TRPA1 (-/-) and TRPV1 (-/-) mice to WT. 20X Objective. Scale bars, 50 µm.* ***(B)*** *Quantification of adhered bacteria showed no significant difference in FISH-labeled bacteria across the groups following C. mastitidis inoculation (~10^{11} CFU/mL). Data were normalized to baseline bacteria and are expressed as the mean ± SD of bacteria per field of view. Ns, no significant difference. Two-way ANOVA with Tukey's multiple comparisons.*

Chapter 5

Contact Lens-Induced Parainflammation can Prime Corneal Defense to Prevent Commensal Bacteria Adhesion

The work in this chapter was conducted in collaboration with Dr. Yujia Yang and incorporates some findings and reprints from previously published work done by Dr. Ananya Datta in which I contributed as a co-author (Figures 5. 6 and 5.7).

Relevant publications:

1. Datta A, Truong T, Lee JH, Horneman H, **Flandrin O**, Lee J, Kumar NG, Caspi R, Evans DJ, Fleiszig SMJ (2023) Contact lens-induced corneal parainflammation involving Ly6G+ cell infiltration requires IL-17A and γδ T cells. *The Ocular Surface*. 28;79-89. Doi: https://doi.org/10.1016/j.jtos.2023.02.004
2. Datta A, Lee JH, **Flandrin O**, Horneman H, Lee J, Metruccio ME, Bautista M, Evans DJ, Fleiszig SMJ (2023) TRPA1 and TPRV1 Ion Channels Are Required for Contact Lens-Induced Corneal Parainflammation and Can Modulate Levels of Resident Corneal Immune Cells. IOVS. 64:21. Doi:https://doi.org/10.1167/iovs.64.11.2

5.1. Abstract

The induction of corneal para-inflammation in mice due to contact lens wear, mirroring human conditions, involves diverse immune cell responses, including CD11c+, Lyz2+, γδ-T, and Ly6G+ cells. This study investigates the correlation between the Lyz2+ immune cell responses and corneal susceptibility to commensal bacterial colonization and the role of TRPA1/TRPV1 in mediating this response. Using LysMcre mice, gene knockouts in TRPA1 and TRPV1, and custom-made contact lenses, we conducted experiments involving superficially-injured corneas and *Macrococcus epidermidis*. inoculation following 6 days of continuous contact lens wear. A universal bacterial 16S rRNA-targeted FISH probe and confocal microscopy were used to quantitatively assess bacterial adhesion, Lyz2+ cell numbers, and morphological changes. The findings revealed an increase in Lyz2+ cells in inoculated corneas subjected to lens wear for 6 days. These Lyz2+ cells also exhibited migration toward the corneal surface and distinct morphological changes after contact lens wear. The changes in Lyz2+ cells correlated with fewer adherent bacteria to contact lens-wearing corneas. However, those present showed increased penetration into the corneal epithelium. Other experiments showed that TRPA1/V1 modulated lens-induced parainflammatory responses involving MHC-II+ cells and γδ T cells, as well as their baseline levels in the cornea. Thus, contact lens-related parainflammation can be modulated by TRPA1/V1 and can help to prime the cornea's defense against bacterial colonization.

5.2. Introduction

Contact lenses are widely used for vision correction and to manage certain eye conditions, yet their wear can lead to bacterial colonization and infection of the normally infection-resistant cornea. Understanding the mechanisms underlying contact lens-related infections is crucial. To facilitate further understanding, our lab established a murine model of lens wear (Metruccio *et al.*, 2019).

During contact lens wear, even in the absence of infection, a subclinical corneal immune response, known as parainflammation, occurs. In humans, this involves increased numbers of Langerhans cells (from 2 - 24 h) and in the mouse model this includes rapid responses from CD11c+ cells (after 24 h), Lyz2+ cells (24 h and 6 days), and Ly6G+ cells (neutrophils) (6 days) (Chao *et al.*, 2017; Efron, 2017; Metruccio *et al.*, 2019). Further, 24-hour responses can persist well beyond lens removal (Datta *et al.*, 2024). Despite these advances, the significance of parainflammation during lens wear remains unclear.

During contact lens wear, factors such as improper lens hygiene, extended wear schedules, and overnight use can contribute to the accumulation of microbes on the lens surface (Willcox *et al.*, 2001; Szczotka-Flynn *et al.*, 2009; Szczotka-Flynn, Pearlman and Ghannoum, 2010; Udomwech *et al.*, 2022). Corroborating this, our previous research using the murine model showed an accumulation of commensal bacteria on worn lenses (Metruccio *et al.*, 2019), some of which can elicit protective host immune responses during corneal infection (Kugadas *et al.*, 2016; St. Leger *et al.*, 2017). In the latter study, protective response during infection relied on IL-17 responses via γδ T cells, which we have also shown to mediate the lens-induced Ly6G+ parainflammatory responses after 6

days of wear (Datta *et al.*, 2023)[2]. These findings suggest that parainflammatory responses during lens wear may play a protective role, potentially driven by commensal bacteria. This protective role may help to defend against pathogens or prevent commensal colonization of the normally amicrobic and infection-resistant cornea.

Contact lens wear induces alterations in tear fluid pH, osmolarity, and temperature, which are stimuli known to activate the polymodal TRPA1 and TRPV1 receptors. In Chapter 2, we demonstrated that the TRP-mediated defenses against bacterial adhesion *in vivo* relied on sensory nerve-associated TRPA1/V1, aligning with TRP-dependent CD45+ and CD11c+ cell parainflammatory responses. Furthermore, Chapter 4 revealed that while there was no significant increase in CD11c+ or Lyz2+ cell numbers, defense against *M. epidermidis* in the healthy cornea was also reliant on both TRPA1 and TRPV1. *M. epidermidis* also induced morphology changes in corneal Lyz2+ cells, causing them to take on a more spherical shape and migrate closer to the epithelium.

Given this background, we investigated whether lens-induced parainflammation in mice protected against commensal bacteria like *M. epidermidis* and the role of TRPA1 and TRPV1 in mediating this response during lens wear. Our results show that extended lens wear enhanced corneal defense against *M. epidermidis* yet facilitated bacterial adhesion deeper in the corneal epithelia. Furthermore, this defense mechanism correlates with TRPA1/TRPV1-mediated parainflammatory responses during lens wear.

5.3. Methods

5.3.1. Mice

Six- to 8-week-old male and female mice F1 derived from the cross of mT/mG with LysMcre (Myeloid-derived immune cells green and all cell membranes red) were used for contact lens and bacterial clearance experiments. All procedures were carried out per standards established by the Association for the Research in Vision and Ophthalmology (ARVO), under the protocol AUP-2019-06-12322 approved by the Animal Care and Use Committee, the University of California Berkeley, an AAALAC-accredited institution. The protocol adheres to PHS policy on the humane care and use of laboratory animals, and the guide for the care and use of laboratory animals.

5.3.2. Contact Lens Fitting

Mice were lightly anesthetized with 1%- 3% isoflurane using a precision vaporizer (VetEquip Inc., Pleasanton, CA, USA). A custom-made silicone-hydrogel mouse contact lens was fitted onto the right eye of each mouse as previously described (Metruccio *et al.*, 2019; Datta, Lee, *et al.*, 2023; Datta *et al.*, 2023). Briefly, after administering anesthesia, an Elizabethan collar (Kent Scientific) was applied to each mouse and contact lenses were

[2] **Relevant Co-authored Publication:** Datta A, Truong T, Lee, J.H, Horneman H, **Flandrin O**. Lee J, Kumar, N.G, Caspi, R.R., *et al.* (2023) Contact lens-induced corneal parainflammation involving Ly6G+ cell infiltration requires IL-17A and γδ T cells', *The Ocular Surface*, 28, pp. 79–89. Available at: https://doi.org/10.1016/j.jtos.2023.02.004. **Contributed to Figure 3 in publication.**

fitted using a Handi-Vac suction pen (Edmund Optics, Barrington, NJ, USA). After lens insertion, mice were individually housed with Pure-o'Cel paper bedding (The Andersons Inc., Maumee, OH, USA), and contact lenses were worn for up to 6 days.

5.3.3. Bacteria

A coagulase-negative murine eyelid commensal (*Macroccocus epidermidis*) was used for bacterial clearance experiments. *M. epidermidis* was prepared by growing on a Tryptic Soy Agar (TSA) plate overnight for ~16 hours at 37 °C, followed by suspension in PBS to a concentration of ~10^{11} colony-forming units (CFU)/mL.

5.3.4. Bacterial Clearance Assay

Following contact lens wear, mice were anesthetized as described in Chapters 2-4. Corneas were lightly superficially-injured (blotted) using a kimwipe™ tissue paper. Blotting was followed by inoculation with *M. epidermidis* (5µL) once every hour for 4 h. Contralateral corneas were sham inoculated with PBS. After 4 h, animals were euthanized by intraperitoneal injection of ketamine (80–100 mg/Kg) and xylazine (5–10 mg/Kg), followed by cervical dislocation. Eyes were enucleated, rinsed with PBS, and fixed in 2% paraformaldehyde (PFA) overnight at 4°C.

5.3.5. FISH

FISH was conducted to label adhered bacteria on whole eyes following fixation, employing a universal 16S rRNA-targeted FISH probe as described in Chapters 2 and 4. Eyes were washed in PBS, 80% ethanol, and 95% ethanol for 10 minutes each at room temperature. This was followed by immersion in a hybridization buffer solution (0.9 M NaCl, 20 mM Tris-HCl, and 0.01% SDS) and incubation at 55°C for 30 minutes. The 16S rRNA-targeted gene probe [Alexa647]-GCTGCCTCCCGTAGGAGT-[Alexa647] (Eurofins Genomics) was added to eyes at a final concentration of 100nM before overnight incubation at 55°C.

5.3.6. Confocal Microscopy

Confocal Microscopy was performed as described in Chapter 2.3.10. Briefly, an Olympus FV1000 confocal microscope with a 488 nm laser was used for the detection of Lyz2- GFP cells. The 555 nm laser was used to visualize red corneal cell membranes. FISH-labeled bacteria were detected using the 635nm laser. Z stacks were acquired at 0.4 µm steps and an aspect ratio of 1024 µm x 1024 µm for bacteria and 640 µm x 640 µm for immune cells. Acquired Z stacks were reconstructed as 3-D images using Imaris Software. Adhered bacteria were identified and quantified using Imaris spot detection and Lyz2+ cells were quantified and assessed for morphology changes as described in Chapter 2 (Figure 2.6).

5.3.7. Statistical Analysis

Quantitative data were presented as mean ± standard error of the mean (SEM) using GraphPad Prism. Student's t-test was used for comparing two groups, while One-way or Two-way ANOVA was used for three or more groups. For non-parametric data,

either the Mann-Whitney U test or the Wilcoxon test was applied. $P < 0.05$ was considered significant.

5.4. Results

5.4.1. Contact Lens Wear Reduces Adherent Commensal Bacteria to Superficially-Injured Corneas

The role of parainflammation during lens wear is unclear but it has been hypothesized to be protective (Efron, 2017; Metruccio *et al.*, 2019). Therefore, we first sought to establish a role for parainflammation in protecting against commensal bacteria adhesion. We explored if prior lens wear for 4-6 days could prevent the adhesion of commensal-type bacteria. Following 6 days of lens wear, corneas were superficially-injured then inoculated with *M. epidermidis*, a murine eyelid commensal. Adhered bacteria were labeled using a universal 16S rRNA-targeted probe. Results revealed significantly fewer bacteria (white) adhered to the lens-wearing (CL) corneas (red) versus no lens-wearing (NCL) controls (~2-fold). This finding suggested increased host defenses mediated by contact lens-induced changes in the murine cornea (Figure 5.1 A, B).

5.4.2. Adherent Bacteria Penetrated Further into the Corneal Epithelium after Lens Wear

Alterations in the corneal surface environment, induced by contact lens wear, can promote bacterial traversal. Furthermore, dysregulated immune responses, as observed during lens-induced parainflammation, may compromise the corneal barrier function, and facilitate bacterial traversal. In line with this, our prior work showed lens wear can enable *P. aeruginosa* to start traversing the corneal epithelium, an important step in the initiation of sight-threatening infection (Metruccio *et al.*, 2019). Therefore, we next assessed whether prolonged contact lens wear can also promote traversal by a commensal bacterium in murine corneas. Interestingly, despite fewer bacteria adhering to lens-wearing corneas, adherent bacteria traversed deeper into the epithelial layer (Figure 5.2 A, B). These findings suggest contact lens wear can disrupt corneal barrier function making it more susceptible to bacterial penetration.

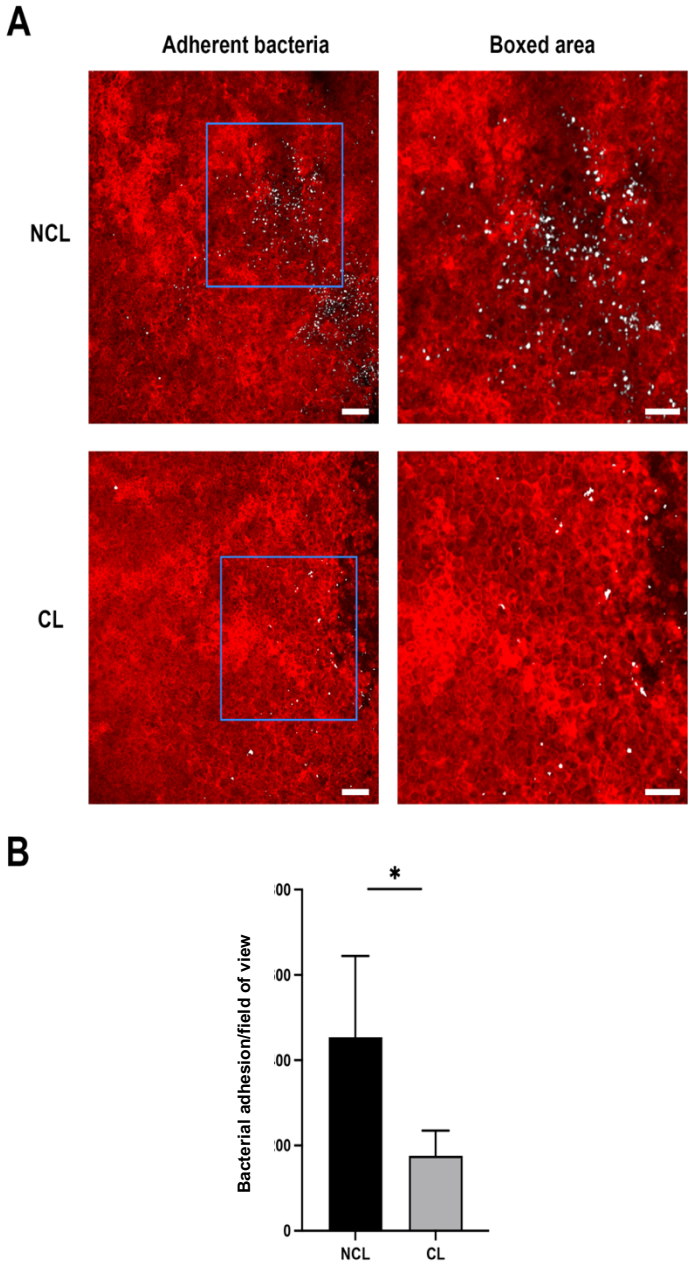


Figure 5. 1: *Lens-wearing corneas showed significantly fewer adherent bacteria than no lens wear.* ***(A)*** *Adherent Macrococcus spp. on superficially-injured corneas of LysMcre-mT/mG mice (red cell membrane) with (CL) or without (NCL) contact lens wear. Bacteria were detected by 16S rRNA-targeted FISH probe (white). Right panels show higher magnification of areas with adherent bacteria (Blue box). Scale bar: left: 50* ***µm****, right: 30* ***µm****.* ***(B)*** *Quantification of adherent bacteria per field of view, showing a significant reduction of bacterial adhesion. P = 0.0312, Wilcoxon test*

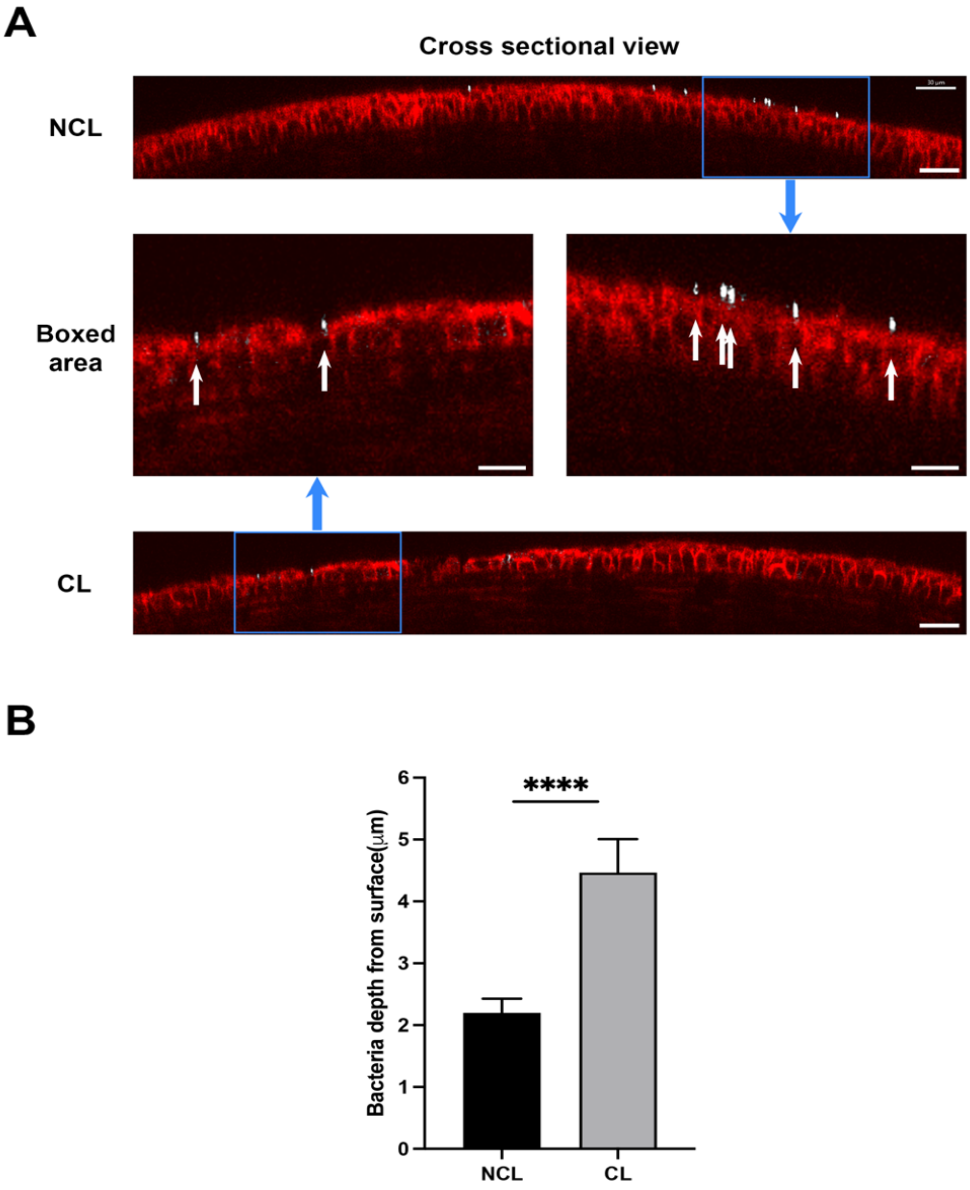


Figure 5. 2*:Contact lens wear induces deeper traversal of the adherent commensal bacteria.* ***(A)*** *Spatial positions of adherent bacteria (white) on the corneal epithelium (red) shown in a cross-sectional view. Middle panels showed blue boxed areas from upper and lower panels in a higher magnification, bacteria indicated by white arrows. Scale bar: upper and lower: 30* ***μm****, middle: 20* ***μm****.* ***(B)*** *Quantification of the depth of bacteria from the epithelial surface, showing a deeper traversal of bacteria into the cornea after contact lens wear. P < 0.0001, Student t test.*

5.4.3. Contact Lens Wear and Bacterial Inoculation Increased Corneal Lyz2+ Cell Numbers

We previously showed that Lyz2+ cells are involved in lens-induced corneal parainflammation after 24 h and 6 days (Metruccio *et al.*, 2019; Datta *et al.*, 2024). Here we investigated a correlation between this response and the corneal defense against commensal bacterial adhesion following 6-day lens wear. As expected, lens-wearing corneas showed a significant increase in Lyz2+ cell numbers compared to no lens-wear (~1.5-fold). We also observed a greater increase in Lyz2+ cells in the peripheral cornea versus the center in both groups. (Figure 5.3 A, B). Additionally, correlation analysis showed that increased Lyz2+ cells (green circle) aligned with decreased bacterial binding (red square) across the cornea (Figure 5.3 C). These results support the hypothesis that lens-induced parainflammation can be protective against bacterial adhesion.

5.4.4. Lyz2+ Cells Were Present Within the Corneal Epithelium or Extending Processes into the Epithelium Following Lens Wear and Inoculation

During parainflammation, immune cells become highly motile, often migrating anteriorly in the cornea (Metruccio *et al.*, 2019; Datta *et al.*, 2024). First, we assessed the migration of Lyz2+ cells. Aligning with previous observations, results showed Lyz2+ cells recruited in lens-wearing corneas were significantly more abundant in the epithelium or extending processes into the epithelium versus no lens-wearing corneas (~2-fold more) (Figure 5.4 A, B).

5.4.5. Morphological analysis revealed other Lyz2+ cell changes influenced by lens wear

In addition to being more motile, lens-wear can induce morphological changes in immune cells (Metruccio *et al.*, 2019; Datta *et al.*, 2022, 2024). Therefore, we assessed the phenotypic changes of Lyz2+ cells associated with lens wear and subsequent bacterial challenge. Lyz2+ cell migration into the epithelium following lens wear and commensal inoculation occurred in the peripheral cornea versus the center (Figure 5.4 A). However, cells were significantly smaller in volume in lens-wearing inoculated corneas in the central region and larger in volume in the peripheral cornea. Lyz2+ cells were more spherical following lens wear and commensal bacteria inoculation in the peripheral cornea only (Figure 5.4 C, D).

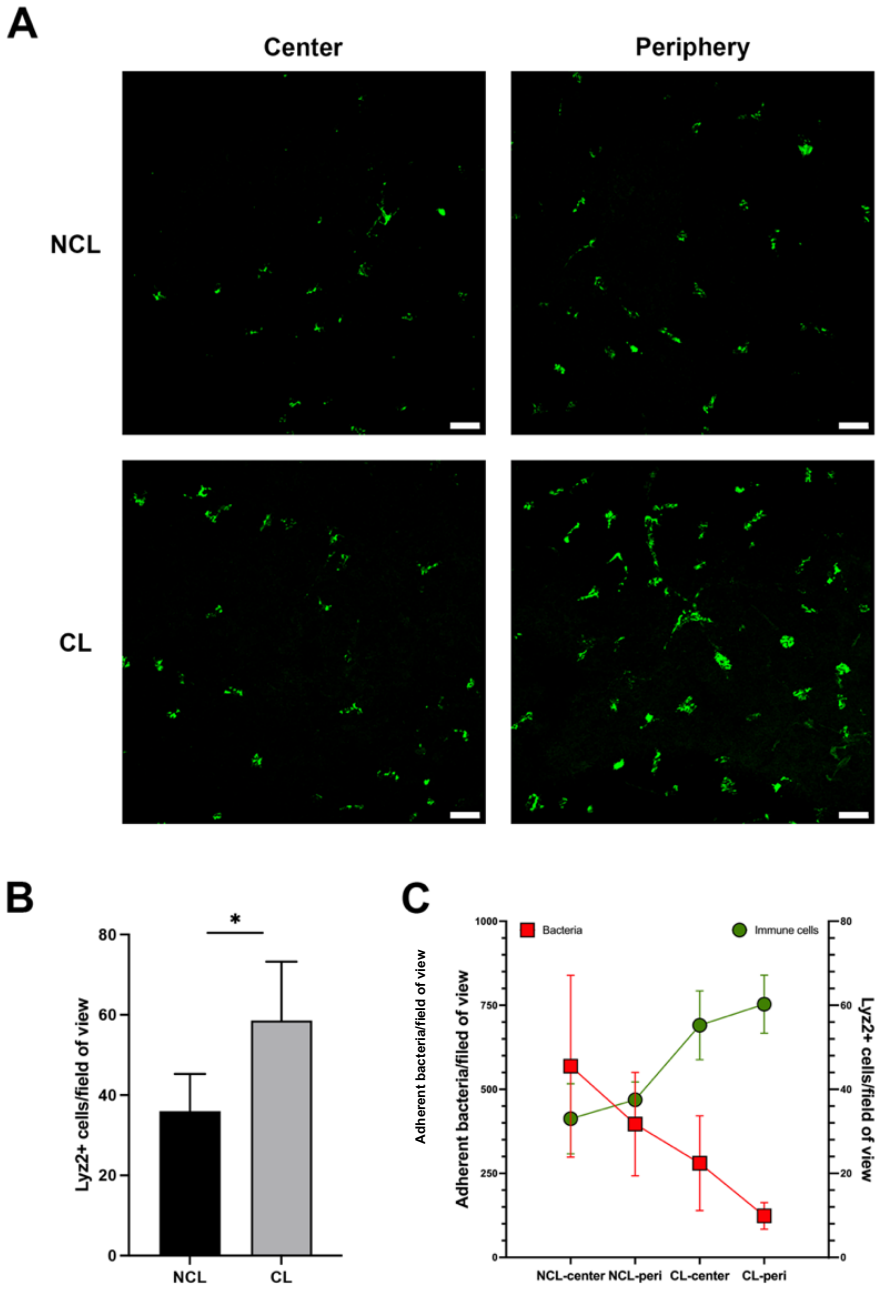


Figure 5. 3*: Contact lens wear induces Lyz2+ immune cell recruitment to the cornea and reduces bacterial adhesion.* ***(A)*** *Maximum intensity z-projection of GFP signal from Lyz2+ cells in LysMcre mice corneas. Differences in Lyz2+ cell distribution were shown in the central and peripheral areas of corneas with or without contact lens wear. Scale bar 10* ***µm.*** ***(B)*** *Quantification of averaged Lyz2+ cell number per field of view on corneas with or without contact lens wear, showing a significant increase of Lyz2+ cell recruitment after contact lens wear. P = 0.0404, Student t - Test.* ***(C)*** *Correlation analysis shows a negative correlation between bacterial adherences and Lyz2+ cell number in different corneal areas and conditions. P = 0.042, r = -1*

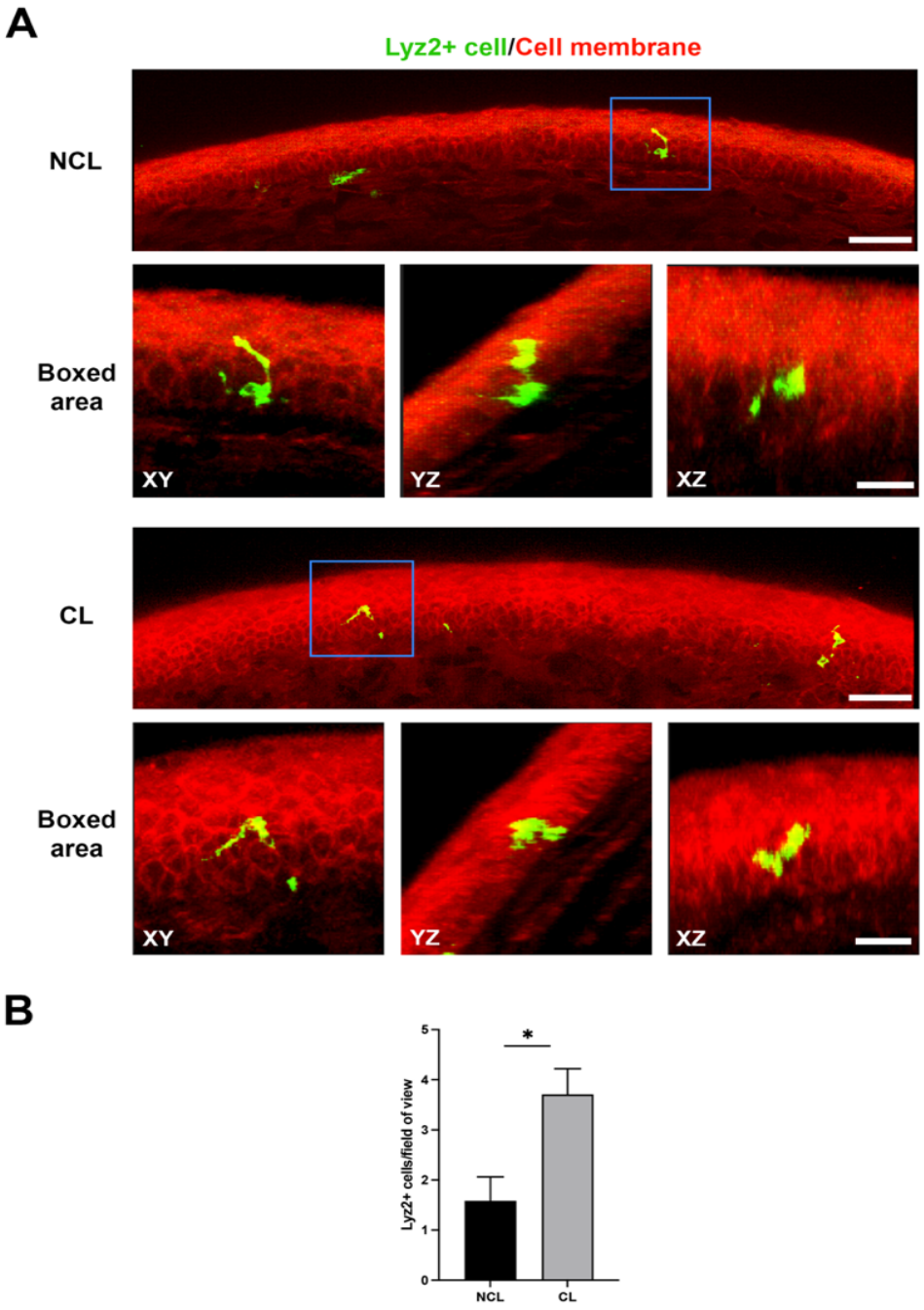


Figure 5. 4: *Contact lens wear induces Lyz2+ immune cell migration toward the corneal epithelium.* ***(A)*** *Extended sectional view (15* ***µm****) of the LysMcre-mT/mG mouse peripheral cornea showing Lyz2+ cells (green) inside or extended processes into the corneal epithelium (red). Representative three-dimensional images of Lyz2+ cells in the corneal epithelium, indicated by blue boxes and displayed in XY, YZ, and XZ planes in higher magnification. Scale bar: 50* ***µm****, boxed area: 20* ***µm****.* ***(B)*** *Quantification of averaged Lyz2+ cell number per field of view in corneal epithelium, showing a significant increase of Lyz2+ cells inside or extended processes into corneal epithelium after contact lens wear. P = 0.023, Student t - Test*

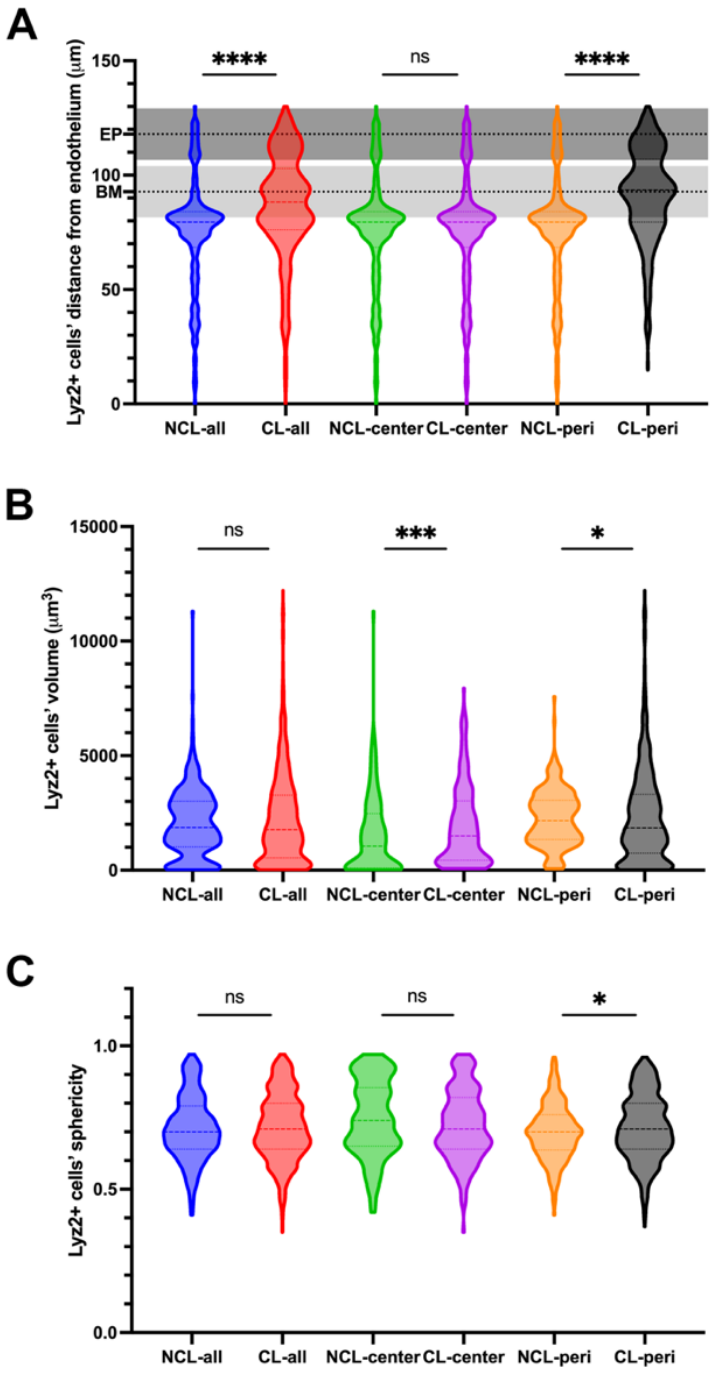


Figure 5. 5: *Distributional and morphological changes of Lyz2+ corneal immune cells following lens wear and commensal bacteria inoculation.* ***(A)*** *Depth distribution of Lyz2+ cells showed significant migration towards corneal epithelium (EP) through the basal membrane (BM) after contact lens wear in the corneal periphery (peri). ****P < 0.0001, ns= not significant, Mann-Whitney U test.* ***(B)*** *After Surface rendering, Lyz2+ cell volume was measured, and differences in NCL and CL groups observed in the central and peripheral cornea, respectively. * P < 0.05, *** P < 0.001, ns= not significant, Mann-Whitney U test.* ***(C)*** *Lyz2+ cells were more spherical at the peripheral cornea after contact lens wear (1 being most spherical). * P < 0.05, ns= not significant Mann-Whitney test.*

5.4.6. TRPA1 and TRPV1 can Modulate Parainflammation During Lens Wear and Baseline Resident Immune Cells

Because our previous observations suggested corneal nerves expressing TRPA1 and TRPV1 are important for CD11c+ and other immune cell defenses to combat bacterial challenge, we investigated whether TRPA1 and/or TRPV1 are required for initiating the protective corneal parainflammatory responses following lens wear. Lens-wearing WT mice were compared to lens-wearing gene knockout mice in TRPA1 (−/−) or TRPV1 (−/−) for corneal Major Histocompatibility Complex class II expressing (MHC-II) cells (typically these include antigen-presenting dendritic cells and macrophages that can also be Lyz2+) after 24 h. Results show that lens-induced MHC class II+ cell responses in the murine cornea after 24 hours of wear were present in both WT and TRPA1 (−/−) mice, but not TRPV1 (−/−) mice. Surprisingly, baseline numbers of resident MHC class II+ cells in contralateral corneas (i.e. no lens wear), were significantly reduced in both TRPA1 (−/−) and TRPV1 (−/−) mice, the latter expressing five or fewer cells per cornea (Datta, Lee, *et al.*, 2023)(Figure 5.6). Further, in that study, TRPA1 and TRPV1 were also involved in maintaining γδ T cell numbers in the murine cornea as well as their responses to lens wear (Figure 5.7). These findings suggest that TRPA1/V1 ion channels are not only

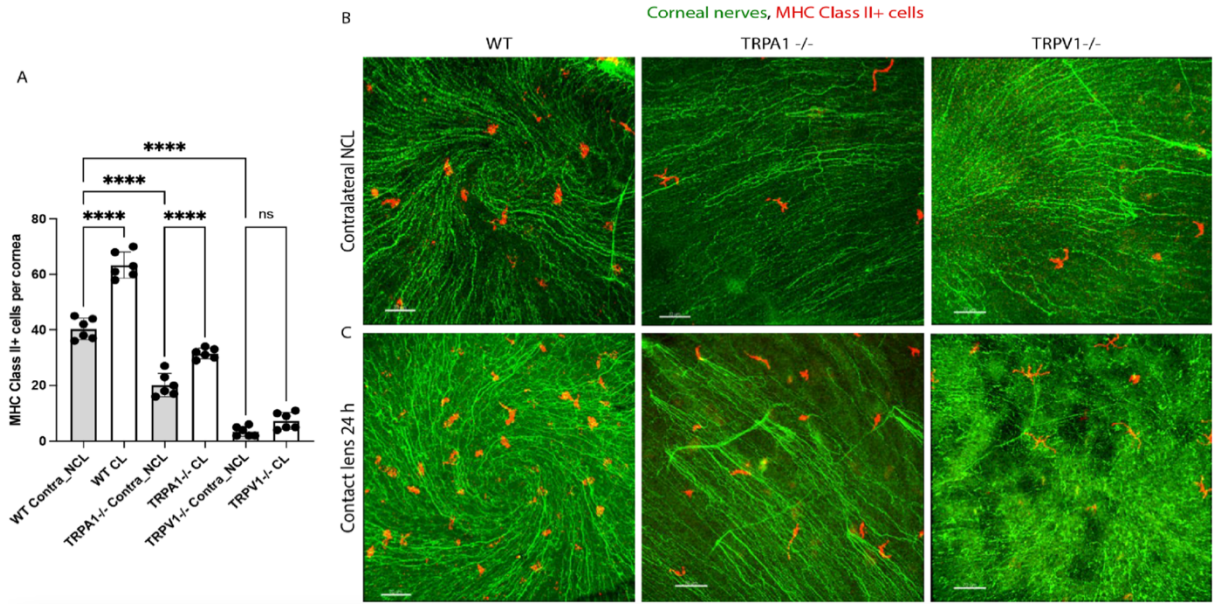


Figure 5. 6: *Quantification (A) and representative immunofluorescence imaging (B, C) of MHC class II+ cells (red) in the corneas of wild-type or gene-knockout mice in TRPA1 (−/−) or TRPV1 (−/−) with or without contact lens wear for 24 hours. Corneal nerves were labeled with β-tubulin III (green). Scale bar = 70 μm. MHC class II+ cell responses to lens wear (white bars) were present in WT and TRPA1 (−/−) mice, but not TRPV1 (−/−) mice. Contralateral eyes of TRPA1 (−/−) and TRPV1 (−/−) mice (grey bars) showed a significant reduction in baseline numbers of MHC class II+ cells. **** P < 0.0001, ns = not significant (One-way ANOVA with Tukey's multiple comparisons test)*

essential for eliciting contact lens-induced corneal parainflammatory responses but also for maintaining baseline levels of certain resident immune cell populations.

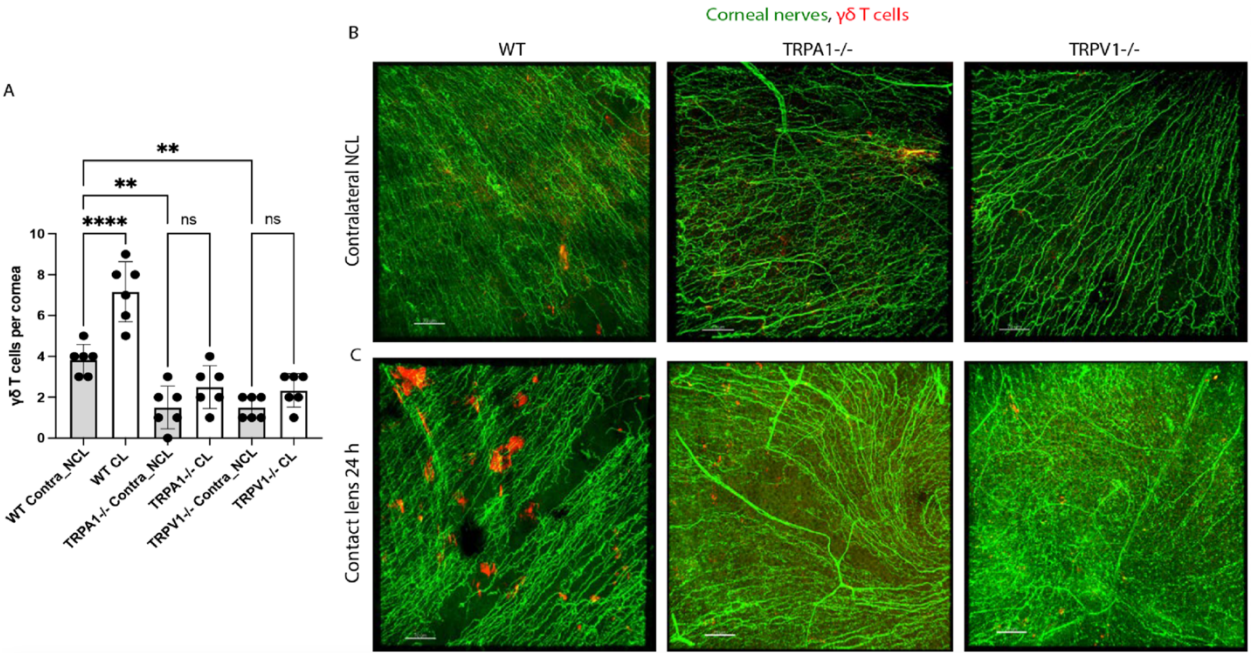


***Figure 5.** 7: Quantification **(A)** and representative immunofluorescence imaging **(B, C)** of γδ T cells (red) in the corneas of wild-type or gene-knockout mice in TRPA1 (−/−) or TRPV1 (−/−) with or without contact lens wear for 24 hours. Corneal nerves were labeled with β-tubulin III* (green). *Scale bar = 70 μm. The γδ T cell responses to lens wear* (white bars) *were absent in both TRPA1 (−/−) and TRPV1 (−/−) mice. Contralateral eyes of TRPA1 (−/−) and TRPV1 (−/−) mice* (grey bars) *each also showed a significant reduction in baseline numbers of γδ T cells.* ** P < *0.01,* **** P < *0.0001, ns = not significant [One-way ANOVA with Tukey's multiple comparisons test]*

5.5. Discussion

Contact lens wear can trigger a parainflammatory response in the cornea, characterized by subtle yet persistent inflammation. In this study, we explored the significance of parainflammation in defending the cornea against commensal bacteria (*Macrococcus epidermidis*) colonization and the potential involvement of TRPA1/V1 channels in mediating this response. Our findings revealed that murine corneas subjected to prolonged lens wear (up to 6 days) exhibited enhanced resistance to commensal bacterial adhesion, despite the bacteria's ability to penetrate deeper into the epithelial layer. This heightened defense coincided with an increase in the corneal Lyz2+ cell population following lens wear. Additionally, we observed that TRPA1 and TRPV1 channels were important for modulating both baseline levels of resident immune cells and lens-induced parainflammatory responses. TRPV1 was found to maintain the population of resident MHC-II+ immune cells, as their numbers decreased in its absence even after lens wear. Furthermore, both TRPA1 and TRPV1 were implicated in regulating the gamma delta T-cell response during lens wear, with gene knockout corneas also exhibiting reduced cell numbers compared to WT.

We have previously demonstrated the involvement of various immune cell types in the parainflammatory response induced by lens wear. Our findings here show enhanced resistance to commensal bacteria colonization post-lens wear, suggesting immune cells within the corneal epithelium contribute to heightened host defenses (Figure 5.1). Given that contact lenses act as reservoirs for commensal bacteria and impede tear flow, potentially harboring bacteria or their ligands under lenses, it is plausible that the observed parainflammatory response is part of the cornea's strategy to prevent colonization. Indeed, following 6 days of lens wear, Ly6G+ neutrophils are recruited to the murine cornea, suggesting a protective response in the absence of pathology, likely initiated by commensal bacteria/ligands associated with conjunctiva (Datta *et al.*, 2022). A possible mechanism of defense includes parainflammatory cells engaging in direct phagocytosis or releasing antimicrobial compounds, with Ly6G+ cells potentially having mechanisms like extracellular traps (Biswas *et al.*, 2023). Supporting this, a direct role for commensals modulating protective neutrophil responses is also observed at the conjunctiva, albeit against pathogens during active infection (St. Leger *et al.*, 2017).

While commensal bacteria are typically harmless and even provide benefits to mucosal sites during health, disruption of mucosal barrier function (in this case with continuous lens wear) may lead to commensals developing pathogenic tendencies. This may relate to our findings that *M. epidermidis* resided deeper in the corneal epithelium of lens-wearing corneas within 4 h (Figure 5.2). While the adherent bacteria were still very superficial, the findings may indicate the start of the traversal process, a notion that coincides with our previous observation that *P. aeruginosa* inoculated onto lenses can start to traverse the corneal epithelium (Metruccio *et al.*, 2019). Additionally, we have previously reported a role for antimicrobial peptides in enhancing the corneal barrier function against bacterial traversal (Alarcon *et al.*, 2011). Interestingly, lens exposure can inhibit the activity of certain antimicrobial peptides *in vitro* (Maltseva *et al.*, 2007), potentially serving as a mechanism that facilitates *M. epidermidis* ability to adhere deeper in corneas subjected to lens wear compared to those without lenses *in vivo*.

In this study, we explored the involvement of Lyz2+ cells, previously shown to be involved in lens-induced parainflammation. As expected, we observed an increase in corneal Lyz2+ cell infiltration following 6 days of continuous lens wear that coincided with decreased bacterial binding (Figure 5.4). Dendritic cells, some sub-types being Lyz2+ cells, have been implicated in antimicrobial defense against *P. aeruginosa* adhesion during superficial injury (Metruccio *et al.*, 2017). These cells not only possess direct antimicrobial effects but also produce cytokines that recruit other immune cells to the site of infection. For example, dendritic cells can secrete IL-12, promoting the differentiation of T cells. Additionally, γδ T cells are recruited during continuous lens wear and are required for Ly6G+ cell parainflammatory responses (Datta, *et al.*, 2023), and certain subsets are known to exhibit cytotoxic capabilities during infection (Marischen *et al.*, 2009; de Koning *et al.*, 2010).

Moreover, we observed that infiltrating Lyz2+ cells exhibited a more spherical morphology, were smaller in the center but larger in the periphery, and migrated closer to the epithelium following continuous lens wear and bacterial inoculation (Figure 5.5). This observation aligns with our previous findings demonstrating that Lyz2+ cells become more motile and showed increased circularity (referred to as sphericity in this study) after

lens wear (Metruccio *et al.*, 2019; Datta, Lee, *et al.*, 2023). Interestingly, in Chapter 4, we observed Lyz2+ cells were also smaller, and moving closer to the epithelium following *M. epidermidis* inoculation. This observation may be attributed to *M. epidermidis* being a commensal bacterium, similar to those associated with lens wear or the conjunctiva that we previously showed can drive a lens-induced parainflammatory response (Datta *et al.*, 2022). Thus, it remains unclear whether inoculation with *M. epidermidis* or existing commensals associated with lens wear induced these observed morphological changes. Nonetheless, the responses observed with lens wear in this study and without lens wear and injury in Chapter 4 are comparable, suggesting that bacteria and lens/lens-associated bacterial ligands can trigger similar parainflammatory responses. Despite these findings, the functional implications of recruitment of, and morphological changes in Lyz2+ cells during lens wear and bacterial challenge require further elucidation.

Contact lenses exert multiple effects on the ocular surface, impacting tear flow and corneal sensitivity which is partly reliant on TRP receptors. We and others have identified TRPA1 and TRPV1 can participate in corneal defense against colonization during health (Wan *et al.*, 2021), and infection (Lin *et al.*, 2021), suggesting their defense occurs in a context-dependent manner. During health, potential mechanisms for their antimicrobial role identified in this dissertation include parainflammation (Chapters 2 and 3) and tear fluid/ocular surface-associated (antimicrobial) proteins (Chapter 4), the latter correlating with *M. epidermidis* defense. Consistent with the parainflammatory mechanism, this current study shows a modulatory effect for TRPA1 and TRPV1 on specific immune cell subsets involved in the parainflammatory response, such as MHCII+ and γδ T cells, also confirmed using RTX (Figures 5.6 and 5.7, included from Datta *et al.,* 2023).

Furthermore, in the study of Datta *et al.*, 2023, baseline levels of corneal MHC-II+ cells were also reduced in the absence of TRPV1 suggesting a role for the receptor in maintaining their numbers at homeostasis. MHC-II+ cells can sample antigens from the microbiome and present them to T cells, shaping the immune response to commensal microbes and enhancing resistance to pathogens at mucosal sites (Roland, Mohammed and Kubinak, 2020; Eshleman *et al.*, 2023). Given that MHC-II+ cells include subsets of resident corneal immune cells, this finding may be linked to TRPV1's contribution to the cornea's amicrobic state observed in our current and previously published findings (Chapter 2) (Wan *et al*, 2021).

Our previous work implicated IL-1R and MyD88 in lens-induced parainflammation and the maintenance of the cornea's amicrobic state (Wan *et al.*, 2018; Metruccio *et al.*, 2019). IL-1R expression on sensory nerves suggests potential crosstalk between IL-1R and TRPA1/TRPV1 in driving parainflammatory responses during lens wear or maintaining baseline immune cell levels (Mailhot *et al.*, 2020). Furthermore, TRPV1 sensory nerves can be sensitized by pro-inflammatory cytokines such as TNF-α, potentially modulating local and adaptive immune responses, including MHC-II+ cells (Devesa *et al.*, 2011; Sooampon, Phoolcharoen and Pavasant, 2013; Feng *et al.*, 2017). The alteration of corneal TNF-α expression by contact lens wear, which can be attenuated by RTX, further suggests a potential mechanism (Datta *et al.,* 2023). Possibly related, TNF-α and IL-17A can also sensitize TRPV1+ nociceptors (Lin *et al.*, 2017; Luo *et al.*, 2021) and are also overexpressed in the tear fluid of contact lens wearers (Chao *et al.*, 2017). Additionally, we found γδ T cells were also attenuated at baseline but required both

TRPA1 and TRPV1. These are well known to produce IL-17, which has been associated with the induction of antimicrobial peptides in epithelial cells of mucosal tissues (Liang *et al.*, 2006; Archer Nathan K. *et al.*, 2016). Together these findings further emphasize the multifactorial nature of lens-induced parainflammation, and suggest a mechanism by which TRPV1 participates in preventing corneal microbiome formation during health.

5.6. Limitations

While the murine model used in this study aimed to mimic human conditions, sample sizes were limited and the phenotypes observed may not fully replicate the complexity of the human ocular immune response to long-term lens wear and microbial colonization. Additionally, our study focuses on Lyz2+ immune cells, which do not fully represent the entire immune response involved in corneal parainflammation such as γδ T, Ly6G+, and Langerhans cells. Further, although the study identifies a correlation between contact lens-related parainflammation and the cornea's defense against bacterial colonization, further mechanistic studies are needed to establish the underlying pathways/mechanisms involved. Our study also primarily focused on bacterial adhesion, immune cell numbers, and morphological changes. Other factors influencing corneal susceptibility to bacterial colonization, such as cytokine levels, or host factors such as antimicrobial peptides would be important topics for further investigation.

5.7. Conclusion

In conclusion, this study suggests contact lens-induced parainflammation in our murine model can protect superficially-injured corneas against commensal bacteria adhesion. Moreover, this parainflammatory response can be mediated by TRPA1/V1 receptors highlighting an important relationship between corneal sensory nerves, lens-induced parainflammation, and bacterial defense. However, the precise role of lens-induced parainflammation in safeguarding against bacterial colonization, as well as the significance of deeper adherent bacteria in this process, requires further investigation, with potential implications in better understanding and managing contact lens-associated bacterial infections.

5.7. Acknowledgement

This work was supported by the National Institutes of Health; EY011211(SF) and EY030350 (SF)

Chapter 6

Conclusions

Despite the cornea's constant exposure to environmental factors and potentially harmful microbes, it effectively avoids pathogenic bacterial colonization and infection. A complete understanding of this unique intrinsic defense is crucial for preventing sight-threatening infections such as contact lens-related bacterial keratitis. In previous research, our lab reported many contributing factors to this defense (Fleiszig *et al.*, 2020). Recently, we discovered that corneal sensory nerves and their TRPA1/V1 receptors can contribute to the tissue's intrinsic defense against the pathogen *Pseudomonas aeruginosa,* a Gram-negative causative agent of keratitis, and environmental colonization (Wan *et al.*, 2021). This dissertation tested the hypothesis that TRPA1/V1 receptors can confer specificity related to bacterial status as a pathogen versus commensal, or Gram type. This section summarizes the significant dissertation findings and proposes a new theoretical model for the basis of future directions.

In Chapter 2, I showed that TRPV1 played a role in countering ocular pathogen *Staphylococcus aureus* (Gram-positive) adhesion which differed from the TRPA1-mediated defense for *P. aeruginosa* (Gram-negative). This finding suggested that TRPA1/V1-dependent defense did not necessarily correlate with bacterial status as a pathogen versus commensal. I also compared the immune responses elicited by the two pathogens and explored how those responses related to TRPA1 and TRPV1, which revealed different parainflammatory responses involving CD11c+ and CD45+ cells. TRPA1/V1-mediated defense also correlated with specific requirements for sensory nerve firing, which was only implicated in the defense against *P. aeruginosa*. These findings extend our understanding of TRP-dependent corneal defense showing it varies by ocular pathogen and mediates specific parainflammatory responses.

CD11c+ cells were previously found to play a crucial role in countering *P. aeruginosa* adhesion following superficial injury (Metruccio *et al.*, 2017). Moreover, this defense was associated with the differential expression of immune mediators such as cytokines and chemokines. For *P. aeruginosa,* I investigated the impact of corneal sensory nerve firing on a set of cytokines and chemokines that can modulate immune cell responses (Chapter 3). The results indicate a potential role for corneal sensory nerves in modulating corneal cytokine/chemokine response to *P. aeruginosa* that correlated with altered expression of immune mediators such as chemokines CXCL5 and CCL7. Preliminary results show other impacted factors including IL-6 and IL-1β.

Our previous finding that TRPV1 can prevent environmental colonization (most likely Gram-positive commensal bacteria) suggests an antimicrobial role under homeostasis that may also relate to the cornea's lack of a microbiome (Wan *et al.*, 2018, 2021). Despite observing a distinction between TRPV1-mediated and TRPA1-mediated defense that might be based on bacterial Gram-type in Chapter 2, the results from Chapter 4 suggest more complexity. I showed that corneal defense against a Gram-positive eyelid commensal (*Macrococcus epidermidis*) involved both TRPA1 and TRPV1, but also did not require sensory nerve firing. Meanwhile, neither receptor were involved in corneal defense against a conjunctival Gram-positive commensal *Corynebacterium mastitidis*. These results indicate that there are complexities that dictate the role of these receptors and therefore the defenses they mediate in preventing adhesion of different types of bacteria - needed because they differ in many ways aside from their Gram-type.

Nerve firing not being required for both *S. aureus and M. epidermidis* suggests that the responsible TRPV1-mediated factors are already present at homeostasis to fend off commonly encountered bacteria, or that TRPV1 acts locally to prevent adhesion upon activation by either. In Chapter 4, I showed that TRPV1 modulated tear fluid/ocular surface proteins (some recognized for their antimicrobial properties) at baseline and following *M. epidermidis* challenge, revealed by quantitative mass spectrometry analysis (TMT). These included the anterior gradient protein 2 (AGR2) an important mucin-producing factor (Park *et al.*, 2009) and the Polymeric Immunoglobulin Receptor (pIgR) for SIgA (Masinick *et al.*, 1997; Willcox Dr and Lan, 1999; Mantis, Rol and Corthésy, 2011). Chapter 4 results further revealed factors that are TRPV1-dependent at homeostasis (in the absence of deliberate bacterial supernatant challenge) including Lipocalin 11 and S100 A11 calcium-binding protein. Together, the results of Chapter 4 suggest that TRPA1 and TRPV1 can modulate corneal defense against an eyelid commensal bacterium and tear fluid-associated proteins at the ocular surface.

Using a murine contact lens model, our lab previously showed the participation of various immune cell types (Ly6G+, CD11c+, γδ T, and Lyz2+) in mediating lens-induced parainflammation, as observed in humans (Metruccio *et al.*, 2019; Datta *et al*, 2023; Datta *et al.*, 2024). However, the mechanisms underlying parainflammation and its significance remain poorly understood. Subsequent work that I contributed to indicated lens-induced parainflammation can be modulated by TRPA1/V1 (Chapter 5) (Datta, Lee, *et al.*, 2023). Chapter 5 also showed this contact lens-induced parainflammation can prime corneal defense to prevent commensal bacterial adhesion, suggesting a protective role for the response.

Collectively, the findings in this dissertation suggest bacteria-specific ligands can selectively activate TRPA1 and/or TRPV1 on sensory nerves at the corneal epithelia to trigger distinct antimicrobial responses. The results also indicate a pattern recognition mechanism at the corneal surface, driven by sensory nerves and TRP receptors to discern different types of challenges. This is especially critical during contact lens wear and/or pathogenic bacteria challenges where an appropriate response is required to reduce the risk of infections while maintaining clarity.

6.1. Theoretical Model

The specificity of the activities of the two types of nociceptors compared to one another may relate to the importance of which one is being triggered (frontend) or relate to a different antimicrobial response regulated by each nociceptor (backend). While I did not explicitly investigate the former, the results in this dissertation (summarized in Table 6.1) support the latter possibility. Specifically, TRPA1-mediated defense correlated with sensory nerve firing and parainflammatory responses, while TRPV1-mediated defense correlated with locally secreted factors independent of sensory nerve firing or robust parainflammatory responses. This was further supported by the observation that both receptors play a crucial role in corneal defense against *M. epidermidis* (a Gram-positive commensal), yet defense mechanisms seem to be related to TRPV1-mediated responses.

Given these findings, a proposed theoretical model of how TRPA1/V1 may specifically carry out their protective role is as follows: **1)** during deliberate challenge, TRPA1 activation and/or sensory nerve firing modulates immune mediators/ parainflammatory responses that can clear pathogens in injured and uninjured corneas; **2)** Gram-positive bacteria/bacterial ligands trigger TRPV1 to modulate antimicrobial activity at the tear fluid/corneal epithelium interface under homeostasis; and **3)** TRPA1/V1 modulates lens-induced-parainflammation as a corneal defense mechanism against bacterial colonization and potential infection (Figure 6.1).

6.2. Innovation

6.2.1. Conceptual

1) The idea that corneal sensory nerves and TRPA1/V1 receptors actively prevent pathogenic and commensal colonization as part of normal homeostasis at mucosal sites introduces several novel concepts. It suggests a pattern recognition mechanism within the corneal defense, which may extend beyond the ocular surface and relate to microbiome composition across different tissue types; **2)** This dissertation focuses only on intrinsic corneal resistance to bacterial colonization during health. This differs from other research in the fields of vision science, neurobiology, and immunology, where the role of sensory nerves/TRP receptors and immune cells are investigated during outright infection after defense mechanisms have been breached; **3)** The role of parainflammation during lens wear has not been previously studied in the context of corneal defense or susceptibility to infection.

6.2.2. Technical

1) This dissertation combines several methods that span the fields of microbiology, cell biology, immunology, neuroscience, and vision science. These included methods to directly inhibit TRP receptor function to show an impact on corneal defense and a novel mouse contact lens model to explore the significance of lens-induced parainflammation; **2)** To address the inherent challenge of low tear fluid volume in mice, we developed a novel methodology for collecting tear fluid/eyewash samples. This innovative approach also involved the application of Quantitative Tandem Mass Tagging Mass Spectrometry, enabling the identification and quantification of multiple relevant tear fluid/ocular surface proteins modulated by TRPV1.

6.3. Suggested Future Directions

6.3.1. Determine immune cell contributions to corneal defense against pathogens

While this dissertation showed that pathogenic bacteria can trigger different TRP and sensory nerve responses in the cornea, including parainflammation, the underlying mechanisms remain unclear. Exploring the phenotypes of the impacted or responsible immune cell subtypes will improve our understanding of the mechanisms influencing corneal defense against each pathogen. Additionally, it would be worth determining which aspects of these responses are dependent on TRPA1 versus TRPV1 and sensory nerve firing. Once the specific contributions of TRPA1, TRPV1, and nerve firing are determined, investigations can focus on determining how immune cells and parainflammatory response participate in preventing bacterial adhesion. It would also be worth exploring whether the TRPA1/V1-mediated defenses are connected to other aspects of ocular immunity known to prevent pathogenic infection in the cornea, as suggested by previous research (St. Leger *et al.*, 2017).

6.3.2. Determine the antimicrobial activity of tear fluid/ocular surface factors modulated by TRPV1

Although mass spectrometry experiment was not conducted for TRPA1 in Chapter 4, the finding that TRPA1-mediated defense for Gram-negative *P. aeruginosa* correlated with immune cell recruitment (Chapter 2), while TRPV1-mediated defense correlated with locally secreted factors for a Gram-positive strain (Chapter 4), suggests the existence of distinct defense mechanisms for TRPA1 versus TRPV1 that may relate to Gram type. To explore this hypothesis and characterize their distinct mechanisms, future studies could investigate whether the TRPV1-dependent corneal defense against Gram-positive *S. aureus* is also associated with the secreted factors identified in Chapter 4.

While these secreted factors may contribute to the TRPV1-mediated corneal defense, the role of directly released factors from sensory nerves, such as neuropeptides, or modulation of known antimicrobial peptides at the epithelia cannot be ruled out. Performing additional proteomic studies aimed at identifying specific peptides would be necessary to begin to establish the TRPV1 (and/or A1) mediated corneal defenses. Once these are established, siRNA knockdown and/or complementation studies would be important to determine the antimicrobial roles of these and already identified secreted factors in Chapter 4. Additionally, investigating the sequence of events and further characterizing the cell types or systems, such as those involved in lacrimation, within this sequence would be important for a full characterization.

6.3.3. Explore the link between lens-induced parainflammation and TRPA1/V1-dependent corneal defense against pathogens

Given that contact lens wear can desensitize corneal sensory nerves (Murphy, Patel and Marshall, 2001), it is plausible that the sensory nerve/TRP receptor protective response against adhesion may be impacted during lens wear. Thus, increasing the risk of pathogenic bacteria colonization and infection of the otherwise amicrobic and infection-resistant cornea. Three current and previous findings support this hypothesis; **1)** Lens-induced-parainflammation is dependent on TRPA1/V1, which, in turn, correlated with defense against commensal bacteria adhesion (Chapter 5); **2)** Deliberately

inoculated pathogens can also induce TRPA1/V1-dependent corneal parainflammation (Chapters 2 and 3); and **3)** TRPA1/V1 can participate in preventing pathogenic and commensal bacteria adhesion (Chapters 2 and 4).

Supporting this hypothesis, we also recently reported the parainflammatory response that occurs ~24 h after lens wear can be dampened with extended lens wear (Datta *et al.*, 2024), suggesting an adaptation caused by the desensitization of sensory nerves/TRP receptors that drive this response. Further, both parainflammation and defense against pathogen *P. aeruginosa* depend on sensory nerve firing, can be inhibited following TRPA1/V1 ablation, and are absent in TRPV1/A1 genetic knockout mice. CD11c+ cells and associated cytokines, which are mediated by sensory nerves, and implicated in lens-induced parainflammation, are also involved in the corneal defense against *P. aeruginosa* adhesion. Given these findings, it would be worth investigating the link between TRPA1/V1-parainflammation and TRPA1/V1-defense mechanisms against *P. aeruginosa* and other keratitis-causing pathogens such as *S. aureus*. Exploring this link might reveal a role for parainflammation in either promoting or preventing *P. aeruginosa* infections during lens wear, offering novel insights into the pathogenesis of, and potential therapeutic strategies for bacterial keratitis.

Table 6. 1: *Summary of findings. White boxes represent published or unpublished findings related to TRPA1/V1-mediated corneal defense. Grey bars represent remaining gaps*

Experimental Conditions	**Healthy Cornea:** Defense vs. *Pseudomonas aeruginosa* adhesion	**Blotted Cornea:** Defense vs. *Pseudomonas aeruginosa* adhesion	**Healthy Cornea:** Defense vs. *Staphylococcus aureus* adhesion	**Healthy Cornea:** Defense vs. *Macrococcus epidermidis* adhesion	**Blotted Cornea:** Defense vs. *Macrococcus epidermidis* adhesion
Gene KO mice	Lost in TRPA1 (-/-) mice: TRPA1 required.	Lost in TRPA1 (-/-) mice: TRPA1 required.	Lost in TRPV1 (-/-) mice: TRPV1 required.	Lost in TRPA1 (-/-) and TRPV1 (-/-) mice: BOTH required.	
Bupivacaine treatment	Inhibits defense: Sensory nerve firing required.	Inhibits defense: Sensory nerve firing required.	No effect on defense: Sensory nerve firing NOT REQUIRED	No effect on defense: Sensory nerve firing NOT REQUIRED	
Testing *Ex vivo*	Defense lost: Requires *In vio*	Defense lost: Requires *In vivo*	Defense lost: Requires *In vivo*	Defense lost: Requires *In vivo*	
RTX treatment	Defense lost: TRPV1/TRPA1 required	Defense lost: TRPV1/TRPA1 required	Defense lost: TRPV1/TRPA1 required.	Defense lost: TRPV1/TRPA1 required.	
Specific TRP antagonist			TRPV1-Specific JNJ-17203212 blocks defense: TRPV1 required.	TRPV1-Specific JNJ-17203212 blocks defense: TRPV1 required.	
CD45+ cell quantitative response (Morphology)	YES. Correlates. Gene double KO mice show TRPV1/TRPA1 required. RTX also. (Cells more spherical, smaller than controls. After RTX, cells larger)		YES. Correlates. RTX shows TRPV1/TRPA1 required. (Cells more spherical after bacterial challenge. After RTX cells less spherical and larger)		
CD11c+ cell quantitative response (Morphology)	YES. Correlates. RTX shows TRPV1/TRPA1 required. Bupivacaine shows sensory nerve firing required. (Less spherical, closer to epithelium, larger vs. controls)	YES. Correlates. RTX shows TRPV1/TRPA1 required. Bupivacaine shows sensory nerve firing required. Bupivacaine shows nerve modulated cytokines/chemokines are involved	NO Response. (Fewer spherical cells, further from epithelium vs. controls)	NO Response.	
Lyz2+ cell quantitative response (Morphology)	No response. (No morphology change, but closer to epithelium)		No response. (Cells were more spherical,larger vs. controls)	No response. (Cells were more spherical, and closer to epithelium)	No response. (Cells were more spherical, and closer to epithelium)
Contact lens wear					Enhanced following prolonged lens wear. Correlates with TRPA1/V1-mediated parainflammatory responses (MHCII+ and gamma delta T cells)

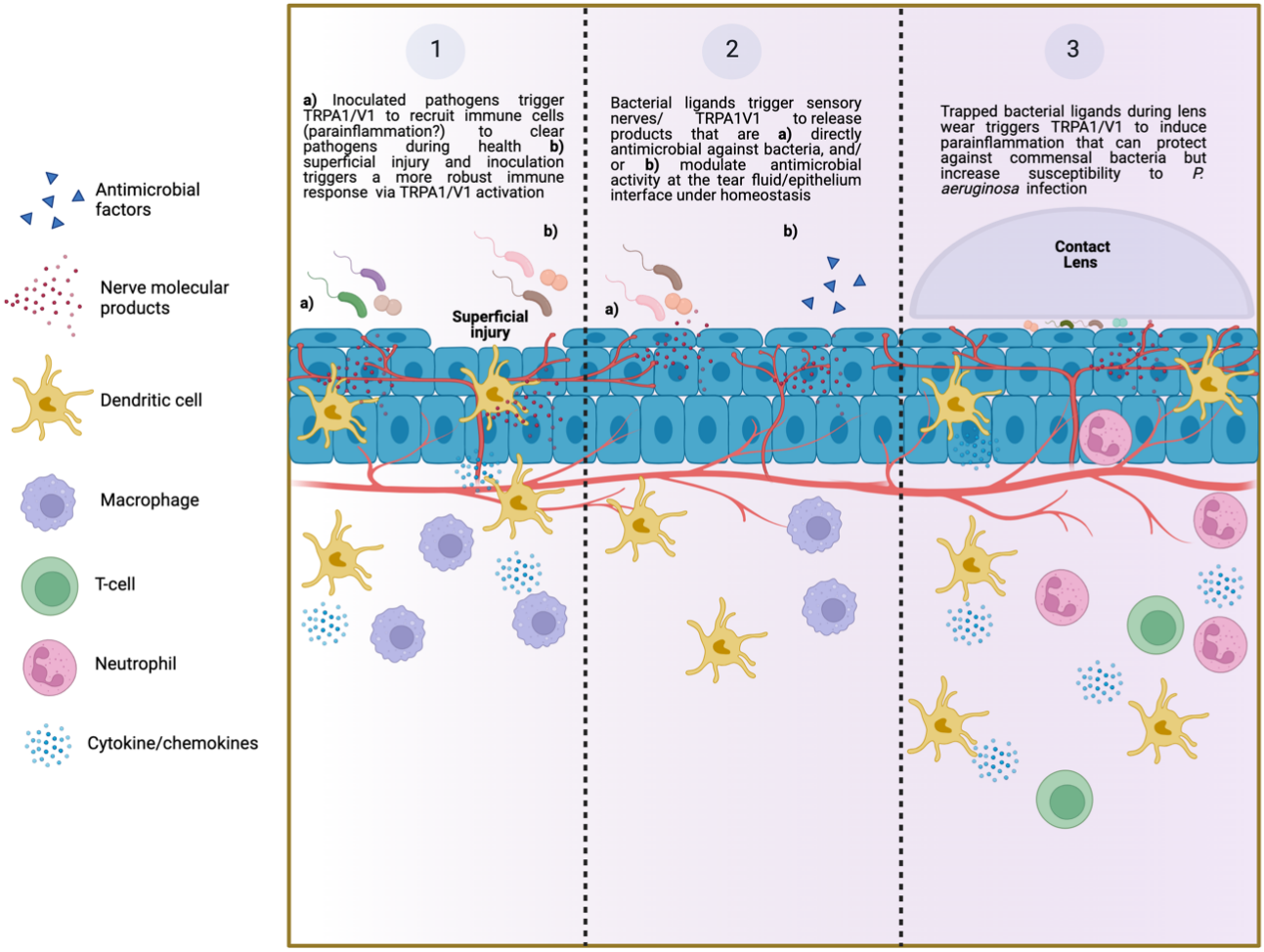


Figure 6. 1: *Schematic of theoretical model based on dissertation findings*

Chapter 7

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