Pseudomonas Aeruginosa Inducing Nociception Increases Susceptibility to Bacterial Keratitis

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Pseudomonas aeruginosa Inducing Nociception Increases Susceptibility to Bacterial Keratitis

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

Harvard University
May 2019
Abstract

Understanding nociceptive signaling during *Pseudomonas* keratitis and its impact on the immune response can prove to be useful for this very painful infection. While the cornea is one of the mostly densely innervated organs in the body, the role that nociception has during bacterial keratitis has yet to be elucidated. In this study, I utilize a novel experimental mouse model, using systemic treatment of Resiniferatoxin (RTX), a potent capsaicin analog that ablates TRPV1 positive neurons to assess the role of sensory neurons during *Pseudomonas* keratitis. While this model has been used to study lung, skin and gut pathologies, it has yet be confirmed whether this would prove to be useful in the context of the eye. Treated mice were used for an *in vivo* bacterial keratitis experiment to assess the impact that sensory neurons have on bacterial clearance and inflammation. Using *in vitro* experiments such as calcium imaging of primary trigeminal ganglia neurons, I assessed whether the pathogen can directly activate neurons depending on virulence factors. My results demonstrate that nociceptors can be directly activated by *Pseudomonas* and mediate immune response to infection. This could be due to CGRP, where we found that the presence of the neuropeptide impeded neutrophil dependent bacterial killing and reduced bacterial clearance in mice.
Dedication

Each of us has cause to think with deep gratitude of those who have lighted the flame within us.

To my mother. My rock and my light.
Acknowledgments

My deepest gratitude to Dr. Mihaela Gadjeva, Dr. James Morris, Dr. Jerry Pier and Dr. Isaac Chiu for their kindness, support and invaluable assistance to this thesis.
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Chapter I.

Introduction

In 2010, nearly one million clinical visits occur in the United States for infectious keratitis, of which 76.5% were due to microbial keratitis (Beach et al., 2010). Treating contact lens related keratitis costs $175 million in healthcare expenditures and utilizes over 250,000 hours of clinical time per year (Beach et al., 2010). The incidence of bacterial keratitis leading blindness is higher in marginalized communities in the United States and worldwide (Al-Mujaini et al., 2009, Ung et al., 2018), as it was deemed that corneal ulceration is a silent epidemic in the developing world (Srinivasan et al., 1997). This may be due to significant financial burden on the patient for medical visits, diagnostic testing and costly and ineffective treatment (Shaheen et al., 2014). *Pseudomonas aeruginosa,* a principal Gram-negative pathogen that causes microbial keratitis and is oftentimes characterized by intense pain and a rapid onset of infection that could lead to blindness (Al-Mujaini et al., 2009; Austin et al., 2017) and its ubiquitous pathogenicity and the growing prevalence of multi-resistant drug organisms foreshadows a potential ocular emergency worldwide (Ung et al., 2018). Thus, it is imperative to develop a more comprehensive therapeutic plan to reduce treatment cost and complications.

Definition of Terms

“Afferent pathway”: signals that neurons carry to the central nervous system.

“Avascular”: the absence or minimal presence of blood vessels
“Bacterial Keratitis”: the infection of the cornea by the proliferation of bacteria.

“Bowman’s Layer”: the a-cellular and non-replicating layer right located between the epithelium and stroma.

“Calcitonin Gene-Related Peptide (CGRP)”: neuropeptide released by central and peripheral neurons.

“Corneal perforation”: a compromised corneal integrity and tissue destruction from the epithelium to the endothelium.

“Corneal ulcer”: an open sore on the cornea

“Corticosteroids”: man-made drugs that resemble the cortisol, a hormone produced by the adrenal cortex

“Colony Forming units per milliliter (CFU/ml)”: unit to measure the number of viable bacteria or fungal cells in a sample.

“Conjunctiva”: composed of loose connective tissue in the lamina propria, beneath the epithelium

“Cytokines”: a class of small proteins that help regulate inflammation and modulate cellular activities.

“Endotoxins”: LPS released from bacteria that can be harmful to the host.

“Etiology”: the cause of a disease.

“Exocytosis”: a cellular process in which the contents of a vacuole are released extracellularly through the fusion of the vacuole membrane and the cell membrane.

“Fibroblast”: the principal cell in connective tissue.

“Flagella”: a lash like appendage for eukaryotic cells and bacteria

“Gram-negative”: a category of bacteria that are characterized for their thin peptidoglycan cell wall.

“in vitro”: in the glass, refers to studies performed with isolated cells or organisms from their normal biological environment.

“in vivo”: studies that occur within a living organism.
“necrotic”: premature death of cells in living tissue
“neutrophils”: a first responder granulocyte that is involved in the innate immune system
“neurotrophins”: a family of proteins that regulate the development, maintenance and function of neurons.
“nociceptors”: a sensory neuron that responds to damaging stimuli
“Ophthalmic branch”: One of three branches of the trigeminal nerve located in the facial region.
“photophobia”: abnormal intolerance to light
“pili”: plural for pilus, hair like appendages for bacteria
“Polymerase Chain Reaction (PCR)”: A laboratory technique used to sequence DNA.
“Pericentral”: arranged around the center
“Polymorphonuclear infiltration (PMN)”: white blood cells that comprise of neutrophils, eosinophils and basophils.
“sensory neurons”: afferent neurons that convert stimuli into internal electrical signaling
“type III secretion system (T3SS)”: a needle like structure found in many Gram-negative bacteria used to detect and infect eukaryotic organisms.

Bacterial Keratitis

Bacterial Keratitis, characterized by heightened inflammation and rapid tissue destruction, is caused by the proliferation of bacteria in the cornea (Mannis et al., 2017) and during acute stages of infection, by the mismanagement of the innate immune response (Lin et al., 2018). Common symptoms and signs include intense pain, redness, blurred vision, discharge, corneal infiltrates; ulceration, photophobia and anterior chamber inflammation (Mah et al, 2018). Blindness, secondary glaucoma, cataracts can occur due to the structural integrity of the corneal epithelium and stroma being compromised when left untreated or mistreated (Mannis et al., 2017; Shaheen et al.,
Predisposing factors to bacterial keratitis include the misuse of contact lenses as well as ocular surface diseases implicated by immunocompromised pathologies including diabetes mellitus (Dart et al., 1988, Mah et al. 2018).

*P. aeruginosa* keratitis is the most commonly identified Gram negative bacterial keratitis in epidemiological studies (Dart et al., 1988, Al-Mujaini et al., 2009; Lin et al., 2018). It is characterized by a rapid pathogenesis (within 24 hours), dense filtration in the stroma and massive tissue destruction (Mannis et al., 2017) by diffusing rapidly and spreading necrotic lesions (Srinivasan et al., 2008). Keratitis induced ulcers can be caused by the host’s inflammatory response, bacterial toxins and toxicity from antibiotic treatment. *P. aeruginosa* induced ulcers are oftentimes rapidly progressing (Dart et al., 2016) and have been found to respond poorly to common antibiotic treatment and exhibited worse clinical outcomes than other bacterial ulcers (Badawi et al., 2017).

**Animal Models of Bacterial Keratitis**

In order to study the mechanistic aspects of bacterial keratitis, experimental animal models have been used, by topical bacterial application after abrading the epithelial surface of the cornea (Dart et al., 2016). The most common animals that have been used for studying *Pseudomonas* keratitis are rabbits and mice (Marquart, 2011). Advantages to using mice is the accessibility to numerous genetically modified mice as well as available tools to measure mouse specific factors (Marquart, 2011).

**Bacterial vs Viral Keratitis**

One of the more common viral keratitis studied is Herpes simplex virus keratitis (HSK). In contrast to bacterial keratitis, HSK is marked by decreased corneal sensation in
patients (Srinivasan et al., 2008; Yang et al, 2018). In epidemiological studies, HSK is the leading cause of neurotrophic keratitis (Sachetti & Lambiase, 2014). Patients with HSK were found to have significantly decreased nerve densities in the infected and non-infected cornea, which in turn correlated with reduced corneal sensitivity (Hamrah et al., 2010). These findings have been supported in mouse models (Yun et al., 2014). While neurotrophic keratitis is more commonly associated with viral keratitis rather than bacterial keratitis, in a longitudinal study on infectious keratitis patients, there was a significant decrease in neuronal density in the subbasal plexus of the central cornea during bacteria keratitis and despite treatment, and the density of neuronal fibers were never fully restored (Müller et al., 2015).

Current Treatments for Bacterial Keratitis

The primary mode of treatment for initial diagnosis of bacterial keratitis involves antibiotic therapy. However, patients must be closely monitored during treatment, as prolonged use of topical antibiotics can cause toxicity, worsened inflammation and thus tissue destruction (Mah et al., 2018). Bacterial keratitis oftentimes implicates cellular infiltrates, neutrophils that have been recruited to kill the pathogen, but its uncontrolled presence can cause severe damage to the cornea (Dart et al., 2016). Furthermore, despite antibiotic treatment, P. aeruginosa keratitis can exhibit increased inflammation during the first 24 to 48 hours of infection (Mah et al., 2018). Topical corticosteroids have been suggested as a solution to control inflammation for adjunctive treatment to bacterial keratitis, but studies of its effectiveness are mixed (Yang et al., 2018) and its optimal dosage can vary depending on the type of pathogen, the severity of the infection and the patient’s medical history (Mah et al. 2018). Furthermore, corticosteroids are not
recommended for the neurotrophic keratitis as it has been found to impede nerve regeneration (Shaheen et al., 2014).

There are few therapeutic targets available to effectively control inflammation to promote tissue repair (Dart et al., 2016). In conjunction, keratitis has been shown to cause nerve degeneration in the cornea with no therapeutic targets in sight (Shaheen et al., 2014). Thus, there is a limited set of tools to address keratitis from the aspect of nerve and epithelial tissue destruction caused by an abundance of inflammatory cell infiltrates, both of which are crucial to preserve vision. Furthermore, growing antibiotic resistance to many of the commonly used antibiotics for bacterial keratitis including fluoroquinolones and aminoglycosides (El-Tantawy et al., 2017), may position bacterial keratitis to become a greater risk (Mannis et al., 2017). Finding specific targets that help modulate the innate immune response, without interfering its capacity to fend off infection can provide a more comprehensive treatment.

The architecture of the cornea

The cornea forms the outer most surface of the eye and consists of the epithelium, Bowman’s layer, stroma, Descemet’s membrane and endothelium. The primary physiological function of the cornea is to allow light to enter the eye and thus its transparency and structural integrity is essential for vision (Mannis et al., 2017). One of the predominant cells in the cornea is the keratocyte, which promote the structural integrity of the epithelial and stroma, and when activated by injury or infection, keratocytes transform into myofibroblasts which produce collagen-degrading enzymes and cytokines to promote stromal tissue repair (Mannis et al., 2017).

Corneal Innervation and Pain
The cornea is avascular but is known to be one of the most highly innervated organs in the human body (Shaheen et al., 2014), mostly derived from the sensory system as well as the peripheral system (Mannis et al., 2017). Eye pain results from nociception, the stimulation of noxious stimuli of the axons of sensory neurons in the cornea (Belmonte et al., 2015).

Sensory nerves originate from the ophthalmic division of the trigeminal ganglion (cranial nerve V), branching into nerve fibers forming dense bundles that terminate into nerve endings in the corneal epithelium (Shaheen et al., 2014). Sensory neurons found in the cornea can be categorized by morphology: small diameter and slow conducting unmyelinated C-fibers and medium diameter thinly myelinated fast conducting Aδ fibers (Belmonte et al., 2004) and by stimuli: while some neurons are stimulus-specific (mechanoreceptors), the majority of the sensory neurons in the cornea are often times polymodal in function, allowing the detection and response to external stimuli, including thermal, mechanical and chemical (Yang et al, 2018). Once stimulated, sensory neurons release neuropeptides including substance P, Calcitonin Gene-Related Peptide (CGRP) and Neurokinin A, neurotrophins and growth factors that have been found to regulate epithelial growth and regeneration (Mannis et al., 2017; Yang et al, 2018).

Voltage gated sodium, potassium and calcium channels are essential for the spike initiation and propagation of neurotransmission (Hunt & Koltzenburg, 2005). The expression of specific channels has been studied and characterized in nociceptive pain, including Transient Receptor Potential Cation Channel, subfamily V, member 1 (TRPV1). TRPV1 is a calcium permeant channel gated by thermal heat and capsaicin and has been mostly found in polymodal Aδ fibers whose stimuli includes heat, pH, ATP,
prostaglandins, 5HT, and endogenous mediators such as inflammatory cytokines (Belmonte et al., 2015; Alamri et al., 2018; González-González et al., 2017). TRPV1 positive neurons has been found to be co-expressed with CGRP and the calcium influx of neuronal cells correlated with CGRP exocytosis (Meng et al., 2009). Thus, studies have used CGRP as a marker for TRPV1 channel specific nociception. Capsaicin application evokes CGRP release in trigeminal ganglia (TG) derived sensory neurons (Murata & Masuko, 2016). Furthermore, the release of CGRP increased the responsiveness of TG derived sensory neurons (Meng et al., 2009).

An absence of sensory innervation can result in neurotrophic keratitis which is generally caused by the impairment of the trigeminal ganglia and is characterized by reduced corneal sensation, epithelial breakdown, and reduce tear film production (Sacchetti & Lambiase, 2014).

Corneal Immunity

Unlike most organs in the body, the cornea is immune privileged, meaning that it attempts to limit local immune and inflammatory response in order to preserve the structural integrity of the cornea and thus vision. Thus, at baseline, the ocular microenvironment generally attributes toward a more immunosuppressive milieu (Zhou et al., 2010). Despite this, studies have demonstrated that leukocytes, including macrophages and dendritic cells are present in the cornea (Liu et al., 2017). A significant number of resident macrophages have been identified in the pericentral epithelium as well as the stroma of the cornea (Liu et al., 2017). Recent studies have shown that there is a close association between nerve trunks and resident macrophages the stroma of the cornea (Seyed-Razavi, Chinnery and McMenamin, 2014). The presence of inflammatory
cells including polymorphonuclear cells (PMNs) has been correlated with wound healing in non-infectious models (Marazzo et al., 2011; Li, Burns, & Smith, 2006). The presence of neutrophils that migrate from the limbal tissues into the stroma is necessary to promote epithelial repair during non infectious wound healing (Marazzo et al., 2011). The cornea also depends upon the tear film by the lacrimal gland as well as the conjunctiva for immune support, as numerous immune cells for the innate response have been found (macrophages, neutrophils, mast cells, lymphocytes, dendritic cells and plasma cells (Knop et al., 2005). This dynamic milieu with immune cells reiterate the importance to understanding the specific mechanisms in the context of bacterial keratitis.

Pathogenesis of Pseudomonas Keratitis

During Pseudomonas keratitis, corneal surface injury is necessary for microbial invasion (Dart et al., 2016). Once these organisms can adhere to the epithelial surface of the cornea, it can invade into the stroma of the cornea (Dart et al., 1988). The toll like receptors (TLR) of the host will respond to microbial components or pathogen-associated molecular patterns (PAMPs), which result in activating cellular signally pathways leading to a rapid innate immune response (Dart et al., 2016).

Macrophages as well as keratocytes respond to the bacteria by releasing a panel of pro-inflammatory cytokines and chemokines including: IL-1β, IL-4, IL-6, IL17A, TNF-α, MCP-1, KC, MIP-2 and NE to recruit and promote PMN infiltration (Marquart et al., 2011). Bone marrow derived PMNs are recruited into the eye and is essential for host defense, which involves phagocytosis, lysosomal degranulation and bacterial killing within the acidic lysosomal compartment of the cell, which involve oxidative attack, producing toxins such as oxygen metabolites, triggering respiratory bursts, hydrogen
peroxide (Dart et al., 2016). Studies have been able to demonstrate that *P. aeruginosa* keratitis triggers significant expression of IL-1β, IL-6, MIP-2 and KC, particularly invasive strains such as 6294 who have the capacity to invade epithelial cells (Xue et al., 2002). Excessive expression of these mediates correlate with the uncontrolled inflammatory response to the pathogen and implicates tissue damage (Bian et al., 2012). IL-1β, MIP-2 and KC are known to be implicated in the migration and the activation of neutrophils (Xue et al., 2002). IL-6 can stimulate neutrophil adhesion and enhance neutrophil activation.

Virulence Factors

*Pseudomonas aeruginosa* possesses various virulence factors including type III secretion system (T3SS), which allows the bacteria to directly inject toxic proteins into the host cytosol to hijack cellular signaling pathways (Soscia et al., 2007). The motility factors that are involved include single polar flagella (fliC gene encodes for the major flagellin subunit), which are essential for cell adhesion, swimming and biofilm formation and the type IV pili (TFP) (pilA gene is its major subunit), which are polarly localized appendages that allow the bacteria to move, act as adhesins to host cells and contribute as a phage receptor during the early steps of biofilm formation (Bucior, Pielage and Engel, 2012). Once *P. aeruginosa* is in direct contact with a cell, exsA, the transcriptional activator initiates the T3SS regulon (Soscia et al., 2007). While the relationship between *P. aeruginosa* virulence factors and nociceptors are unknown, recent studies in virulence factors of *Staphylococcus aureus*, particularly those that promote hemolytic activity have been found to directly activate dorsal root ganglia (DRG) derived sensory neurons, which help the pathogen to evade the host immune system (Chiu et al., 2013).
Neuro-immune axis

Sensory neurons have been extensively studied in wound healing, diabetes and viral keratitis (Shaheen et al., 2014; Yang et al, 2018). Most of the studies of nociceptor and immune cell interaction in the context of bacterial infection have been in other pathology models, such as in the skin, lung and gut (Baral et al., 2018; Blake et al., 2018; Chiu et al., 2013; Pinho-Ribeiro et al., 2018), which involve DRG nociceptors rather than TG. There has been some understanding to the impact that the inflammatory response has on the functional activity sensory neurons, that it sensitizes and increases the activity of nociceptive signal transduction (Hunt & Koltzenburg, 2005) and that neurons express functional receptors for cytokines (Li et al., 2006). Studies have suggested that a certain degree of inflammation can promote corneal nerve regeneration, but excessive inflammation can lead to neurotrophic keratopathy (Shaheen et al., 2014).

In the context of the migraine, CGRP is the most abundant pain mediator in TG nociceptors and its presence is correlated with neurogenic inflammation (Shaheen et al., 2014). Resiniferatoxin, a TRPV1 ultra potent agonist was been assessed as analgesia for neuropathic pain by ablating nociceptive neurons (Shaheen et al., 2014) as well as utilizing a potent CGRP receptor antagonist, BIBN4096 (Durham & Vause, 2010). Nociceptors have been found to interact with immune cells during bacterial infection in other pathologies. In vivo and in vitro studies have demonstrated that the presence of CGRP promotes the polarization towards M2 macrophages, increased bacterial load, the suppression of inflammatory cytokines as well as neutrophil recruitment and functionality (Lim et al., 2017; Pinho-Ribeiro et al., 2018). The degeneration of nociceptors has been correlated with increased immune response, including heightened presence of dendritic
Research Aims, Goals and Hypothesis

The primary research goal of this thesis is to assess how sensory neurons impact the outcome of bacterial keratitis in mouse models and in vitro experiments. Based on previous literature, I hypothesize that sensory neurons regulate the magnitude and molecular characteristics of the inflammatory response during Pseudomonas keratitis. This occurs by direct interaction between Pseudomonas aeruginosa and nociceptors in the cornea, which correlate with increased levels of CGRP, which in turn alter the functionality of recruited PMNs in the cornea.

Specific Aim 1: Evaluate whether Resiniferatoxin (RTX) treatment significantly ablates sensory neurons in the cornea.

Methods: To affirm whether sensory neurons in the cornea are significantly ablated after RTX treatment, immunohistochemistry staining of corneal whole mounts will assess the presence of neuronal networks and the capsaicin eye wipe test will address neuronal functionality.

Specific Aim 2: Assess the functional significance of nociceptors during the progression of bacterial keratitis.

Methods: in vivo bacterial keratitis, ELISA panel to measure cytokine and chemokine levels in the corneas and pathology scoring after infection.

Specific Aim 3: Evaluate if P. aeruginosa can directly induce sensory neurons firing and if this is dependent upon virulence factors, type IV pili, flagella and type III secretion system.
Methods: Calcium imaging of primary trigeminal neurons with Fura-2AM by using mutant strains that lack type IV pili, flagella and type II secretion.

Specific Aim 4: Evaluate whether the outcome of bacterial keratitis may be dependent upon the release of CGRP.

Methods: Measuring the release of CGRP in infected corneas in RTX cohorts, opsonophagocytic assay in the presence of the CGRP protein and *in vivo* bacterial keratitis with mice treated with a CGRP receptor antagonist, BIBN4096.

Implications of Research: The current gap in specific therapeutic targets to modulate excessive tissue damage caused by inflammation during infection calls for a better understanding of the mechanistic role that sensory neurons play during bacterial keratitis. While RTX has been used previously to assess the absence of sensory neurons in other pathological models, studies have only demonstrated that systemic injection of RTX ablates dorsal root ganglia (DRG) derived sensory neurons. If I am able to demonstrate that RTX significantly ablates sensory neurons in the cornea, this mode of pharmacological ablation poses to be a useful model to assessing neuropathy.
Chapter II.

Materials and Methods

Animals

C57BL/6 mice were purchased from Taconic Farms (Germantown, NY). For infection, behavioral experiments and *in vitro experiments* adult (7-10 weeks old) female mice were used. Age matched littermate controls were used for all experiments.

Animal colonies were maintained in a specific pathogen free animal facilities at Brigham and Women’s Hospital. All animal experiments were performed following approval by the committee on microbiological safety and Institutional Animal Care and Use Committee (IACUC).

Resiniferatoxin Treatment

To chemically ablate TRPV1+ neurons, C57BL/6 female mice were treated with Resiniferatoxin (RTX) (Sigma-Aldrich, St. Louis, MO, USA). Four-week-old mice (with an average weight of 17 grams) were anesthetized by inhalation of isoflurane and injected subcutaneously with the following dosages in three consecutive days: 30ug/kg, 70ug/kg, and 100 ug/kg (Baral *et al.*, 2018). Control littermates were injected with vehicle solution (0.5%EtOH, 1% EtOH, 1.5 % EtOH).

BIBN4096 treatment
To evaluate the outcome of bacterial keratitis is CGRP dependent, we injected CGRP receptor antagonist, BIBN4096 (Tocris, Bristol, UK) intraperitoneally (30 mg/kg) (Pinho-Ribeiro et al., 2018) into 7 week old female mice (an average weight of 19 grams), 1 hour after infection. BIBN4096 were solubilized per the manufacturer’s instructions. Controls were injected with the vehicle (20% DMSO 1.5% Tween-80 in PBS).

**Immunohistochemistry**

After washing with PBS, harvested corneas were stained with rabbit anti-CGRP (Sigma, C8198, St. Louis, MO, USA) and rabbit anti-beta III tubulin (Tuj1) (Abcam, ab18207, Cambridge, UK) overnight in 4°C. After washing, the corneas were incubated with secondary antibodies Alexa 594 donkey anti-rabbit IgG (Abcam, Cambridge, UK) and DyLight-488 donkey anti-rabbit IgG (Abcam, Cambridge, UK) in the dark at room temperature for 2 hours. Corneas were subsequently washed without exposure to light and using a scalpel blade, flattened under a light microscope (Claybon & Bishop, 2011). Flattened cornea were kept in Prolong anti-fade without DAPI (ThermoFisher, Waltham, MA USA). Images were taken with an inverted Nikon Ti-E microscope using a 40x objective. Quantification of the neuronal density was done with collapsed z-stacks of the corneas were quantified for neuronal presence and density by using ImageJ. A calibrated line was applied perpendicularly via Image J and the number of intersections of nerve fibers were counted (Dvorscak & Marfurt, 2008). Beta-tubulin III was used as a general neuronal marker (Baral et al., 2018).
Capsaicin Eye Wipe Test

The Capsaicin Eye wipe test was performed three weeks after RTX treatment. Animals were acclimated to the behavioral testing tools three days before commencing the behavioral test and were performed within the same room as their cage to reduce stress. While anaesthetized mice were gently restrained, 3 mM of a Capsaicin (Sigma Aldrich, St. Louis, MO, USA) was applied to the left cornea of the mouse and its behavior was recorded for 1 minute immediately after application (Neubert et al., 2008). Blinded observers counted the number of wipes (via the ipsilateral forepaw). Normal facial grooming behavior was not included.

In vivo Bacterial Keratitis Model

For RTX cohorts, mice were rested for 2-3 weeks before commencing the in vivo bacterial keratitis model, at the age of 6-7 weeks. Nav1.8 mice and BIBN4096 mice were infected at 7 weeks of age with littermate controls. Mice were anesthetized with filtered xylazine (1.3 mg/ml) (Henry Schein, Melville, NY, USA) and ketamine (6.7 mg/ml) (Henry Schein, Melville, NY, USA) in sterile saline. Under a stereomicroscope, 3 abrasions were made on the central cornea with a 26G needle and pipetted the inoculum onto the cornea (Wilcox). Only the right eye was infected. Inoculums were prepared by plating the invasive strain *P. aeruginosa*, 6294 on a TSA 5% Sheep Blood plates at 37°C overnight, in which the lawn was re-suspended in sterile saline. By measuring an OD of 0.45 at 650 nm, I serially diluted to the desired inoculum (5x10^5 CFU/ml). Before harvesting the corneas, pathology scores, eyes were monitored by a blinded investigator for corneal opacity by visual observation and graded as follows: 0 = clear cornea, 1 =
slight corneal haze, 2 = moderate corneal opacity, 3 = severe corneal opacity but iris visible, 4 = severe corneal opacity with iris obscured.

There were three separate RTX cohorts and female mice were sacrificed 8 hours, 24 hours and 48 hours post infection. After CO2 inhalation, corneas were harvested and homogenized in filtered Phosphate buffered saline (PBS) containing 0.05% TritonX-100. Bacterial burdens were determined by serially diluting the corneal lysates in DMEM-F12 (ThermoFisher, Waltham, MA USA) 5% Fetal Bovine Serum (FBS) (ThermoFisher, Waltham, MA USA) and plating onto MacConkey plates for overnight incubation at 37°C.

To obtain corneal lysates for protein quantification, homogenized corneas were spun down at the 13.2 rpm at 4°C for 10 minutes to remove debris, in which the 1X protein inhibitor (Sigma-Aldrich, St. Louis, MO, USA) was added. Mice studies were randomized as appropriate. During infection, investigators involved in performing infection as well as assessing pathology scores were blinded and cages were randomized.

ELISA

Protein quantification of the corneal lysates was performed with the Bradford assay (BIORAD, Hercules, CA, USA) using a BSA standard (Fisher Scientifc, Hampton, NH, USA), mouse ELISAs were performed (R&D systems, Minneapolis, MN, USA) on the corneal lysates by following the manual’s instructions.

CGRP release assay from corneas

Corneas were excised with a blade and were rapidly transferred to a 24 well plate containing 300 ul of DMEM-F12 (Dulbecco’s Modified Eagle Medium/Ham’s F-12 (ThermoFisher, Waltham, MA USA). Three corneas per group were combined in one
well. Explants were incubated 32°C with gentle shaking (150 rpm) for 30 minutes (Pinho-Ribeiro et al., 2018). After incubation, the bath supernatant was collected and assayed to determine CGRP concentration with the CGRP EIA kit (Cayman Chemical, Ann Arbor, MI, USA) according to manufacturer’s instructions.

Opsonophagocytic Assay

Following euthanasia, femurs and tibias from adult C57BL/6 mice were dissected. Bone marrow cells were flushed out using Recommended Media (filtered 0.5% BSA 1 mM EDTA in phosphate buffered saline without calcium and magnesium (PBS -/-) pH 7.2) in a syringe and 26G needle. Cells subsequently went through a 100 µm strainer, centrifuged 600xg for 10 minutes at 4°C, decanted and added 2 mL of red blood cell lysis buffer (R&D systems, Minneapolis, MN, USA), shaking gently until incorporated and incubated for 5 minutes at room temperature. Recommended media was added and the cells were centrifuged for 10 min at 300xg at 4°C. After the supernatant was removed, the cells were re-suspended in recommended media at a concentration of 1x10^7 cells/mL Neutrophils were isolated using the immune magnetic negative selection kit, MojoSort (Biolegend, San Diego, CA, USA) according to the manufacturer’s instructions. For the opsonophagocytic killing assays, mouse neutrophils were used immediately after isolation. At the time of harvesting for bone marrow cells, cardial blood was collected with a syringe and a 26G needle. After letting the blood settle for an hour, the tubes were spun down at 4.6 rpm at 15°C for 24 minutes to collect serum P. aeruginosa (6294) inoculum was added (MOI 0.01) to mouse neutrophils (1x10^6 cells per well) with 10 ug of anti-Psl, 10% mouse serum in Hanks Balanced Salt Solution
containing calcium and magnesium (HBSS+/+) (Fisher Scientific, Hampton, NH, USA). Rat CGRP (Tocris, Bristol, UK) was added to cultures immediately before 6294 at different concentrations 100nM, 500nM and 1µM. Conditions and controls corresponding can be seen in Table. All conditions were transferred to 0.65mL sterile Eppendorf and incubated for 90 minutes at 37°C on a rotator (setting 6). The amount of extracellular and intracellular bacteria was determined by serial dilution plating on MacConkey plates (Fisher Scientific, Hampton, NH, USA), and bacterial colonies were counted after overnight incubation at 37°C. To determine the effectiveness of neutrophil killing, we took the average CFU counted for the condition and divide it by the average CFU counted for control conditions, which takes into the account of growth in the presence of mouse serum and antibody. For each assay, there were triplicates of each condition, including controls (Table 1). Controls contained all components except for PMNs to calculate neutrophil killing while considering the growth of bacteria during the 90 min. incubation.

Calcium Imaging of Trigeminal Ganglia Primary Neurons

Adult female and male 7-10-week-old C57 BL/6 mice and TRPV1 genetically ablated mice (by crossing TRPV1-cre+ with DTA+ mice) were euthanized by CO2 inhalation and were perfused with cold HBSS-/. The trigeminal ganglia (TG) dissected from the base of the skill and were enzymatically dissociated by incubating at 37°C HEPES-buffered saline (Sigma-Aldrich) containing collagenase A (Sigma-Aldrich) (1mg/kg), and Displase II (Thermo-Fisher) (2.4 U/mL) for 20 min at 37°C twice (Pinho-Ribeiro et al., 2018). Cells were transferred to a tube of DMEM/10% FBS (Thermo Fisher, Waltham,
MA, USA) containing DNase I (Thermo Fisher, Waltham, MA, USA) (150U/mL) and cells were dissociated with syringe needles in decreasing tip diameters to create single cell suspensions. Single cell suspensions were carefully pipetted over 10% bovine serum albumin (BSA) gradient (Fisher Scientific, Hampton, NH, USA) (Pinho-Ribeiro et al., 2018). The top layers of the cellular debris were removed and the pellet was washed, pelleted and resuspended in neuro-basal-A medium (NBM) (Thermo Fisher, Waltham, MA, USA). Cells were plated onto laminin coated cell culture dishes (Westnet, Canton, MA, USA) (3,000 cells per dish) in NBM with 50 ng/mL nerve growth (Thermo Fisher, Waltham, MA, USA). TG neurons were used for calcium imaging within 24 hours after plating. Cultured TG neurons were washed and loaded with 5 uM Fura-2 AM (LifeTechnologies, Carlsbad, CA, USA) in NBM for 30 minutes at 37°C, washed three times and imaged with Krebs-Ringer solution (Boston BioProducts, Ashland, MA, USA) (KR: 120mM NaCl, 5.5 mM HEPES, 1mM D-glucose, pH 7.2 +/- 0.15) at room temperature, using an Eclipse Ti-S/L100 inverted microscope with a Zyla sCMOAS camera. Cells were immulinated by an ultraviolet light source (Lambda XL lamp, Sutter Instrument), 340 nm and 380 nm excitation alternated by LEPMAC5000 filter wheel (Spectra services), and the fluorescence emission captured by Zyla sCMOS camera (Andor). 340/380 ratiometric images were processed, background corrected and analyzed with NIS-elements Advance Research software (Nikon). To measure calcium flux in response to bacterial application, bacterial strains, including 6294 (clinical isolate), PAK, pilin (PilA) KO, translocated flagellum (FliC) and type III secretion system KO (exsA mutant)) overnight in TSA 5% Sheep Blood plates (Fisher Scientific, Hampton, NH, USA) at 37°C overnight, re-suspending the lawn in Krebs-Ringer and measuring for an
OD 0.45 at 650 nm for 6294 and an OD 1.2 at 650 for the PAK bacterial strains, which was an inoculum of $9 \times 10^9$ CFU/ml for each mutant strain and plated the inoculum on a blood agar plate to confirm. Measurements were standardized by time of applications of Krebs-Ringer, the inoculum, Mustard oil, Capsaicin and KCl. NIS-elements software was used to image and process 340/380 ratiometric images from neurons and excel was used for analysis. The ratios obtained were normalized by the baseline measurement (2 minutes before application of the inoculum) An increase in 340/380 ratio of 25% or more from baseline levels was considered a positive response.

Statistical Analysis

All quantitative values plotted in figures are expressed as the mean ± standard error of the mean (s.e.m.) with individual mice representing individual symbols, conditions (for OPK) or plates (for calcium imaging). For analysis of bacterial burden, neuronal intersections for neuronal density quantification and cytokine concentrations between groups, two tailed unpaired t-test was used. When comparing pathology scores, Mann-Whitney U tests was used. When comparing with more than 2 groups (phenol red thread test, OPK assay, and calcium imaging), one-way ANOVA with Kruskal-Wallis’ post-test was used. P-values were considered significant if $p \leq 0.05$. Outliers detected by the ROUT method was not included in the analysis. Graphpad Prism software (California) was used to analyze and plot data.
Chapter III.

Results

Resiniferatoxin as a mouse model for studying sensory neurons

Systemic RTX treatment significantly ablates neuronal density in the cornea. As demonstrated in the corneal whole mounts where, there was significantly less immunoreactivity to beta-tubulin III, as well as with CGRP (Figure 2A), indicating significantly less neuronal presence and density in RTX treated mice. The collapsed z-stacks of the corneas of RTX treatment mice had significantly less number of nerves intersected than the z-stacks of the vehicle treated mice for CGRP staining (p<0.0005) as well as for beta-tubulin III (p=0.0079) (Figure 2B). Assessing their functionality with the capsaicin eye wipe test demonstrated that treated mice had significantly less sensitivity to the TRPV1 analog, capsaicin, as there was significantly less eye wipes counted in the RTX treated mice as compared to vehicle groups (p<0.0001) after the application of 100mM of capsaicin (Figure 3). Corneas were harvested 2 weeks after treatment for IHC staining. Behavioral tests occurred 3 weeks after treatment.

RTX mice during Bacterial keratitis

Our preliminary data suggested that neuropathy is developed during experimental keratitis (data not shown). This prompted us to evaluate the impact of nociceptors on disease pathology. After the depletion of sensory nociceptors with RTX treatment, we observed milder pathology. Infected corneas presented with 1-log reduction in bacterial burden and milder opacity (Figure 4 and Figure 5). Despite these changes, disease developed in the RTX-treated cohort and was similar in phenotype at later time points such as 48h post infection, where bacterial clearance and corneal pathology were no
longer significantly different (Figure 4). Consistent with bacterial levels in the infected corneas, the profiling of infection-associated cytokines showed lower levels of IL-1β (p=0.0401) and MIP-2 (p=0.0111) but no differences in NE at 24h post infection (Figure 6). In contrast, no significant changes in the cytokine levels were detected at later time points (Figure 7). Cumulatively, these data illustrate delayed and milder disease in the absence of nociception.

Direct interaction between *Pseudomonas* and TG derived sensory neurons

Through calcium imaging, we observed that the invasive strain 6294 has the capacity to directly activate TG primary sensory neurons (Figure 8). When exposing neurons to filtered inoculums of 6294, an absence of live bugs, but contains factors and materials shedded from the pathogen, there was less but substantial activation, particularly at a higher dose (5x10^8 CFU/ml) (Figure 8C). In contrast, at a lower dose, but in the presence of live bacterial challenge, significant neuronal activation was observed, suggestive of the presence of virulence factors carried by live bacteria. This direct neuronal- bacterial activation may be type IV pili dependent, as TG primary sensory neurons responded significantly less to *P. aeruginosa* PAK pilA KO (p=0.02) was significantly less, as compared to PAK WT (Figure 9C). Sensory neurons responded to *Pseudomonas* within 15 minutes of incubation, as can be seen with representative images of the field of view (Figure 8A and 9A) and trends of the neuronal calcium traces analysis (Figure 8B and 9B).

To assess whether activation was proton based, as sensory neurons have been found to be activated by the presence of protons (Mamet *et al*, 2002), each inoculum was measured for pH. All inoculums were at a normal range of pH, an average pH of 7, hence
no significant acidification was observed in the *in vitro* experiments. Calcium imaging experiments were performed twice and independent experiments were combined for statistical analysis.

Neuropeptide CGRP impedes defense against *Pseudomonas*

Based on recent studies, to assess whether the presence of CGRP impacts the outcome of corneal infection, we first evaluated CGRP levels of RTX treated mice before and after infection. Total CGRP levels were significantly different in the RTX-treated controls when compared to the vehicle treated mice before infection, which correlated with decreased neuronal presence detected in the corneas (Figure 2B) and after infection (Figure 10).

To evaluate the impact of CGRP, cohorts of mice were either treated with BIBN4096 to block CGRP-induced signaling or vehicle control. Post infection treatment of CGRP receptor antagonist BIBN4096 resulted in significantly lower bacterial burden in the infected corneas (p=0.0482) during 17 hours of infection, with a half log reduction in CFU counts (Figure 11). This suggests that CGRP hinders the host’s bactericidal capacities during infection.

To determine if CGRP elicited direct effect on neutrophils, OPK assays were carried out in the presence of increasing concentrations of CGRP. CGRP significantly hindered neutrophil’s bactericidal activities at 500nM and 1uM of CGRP (p=0.0293 and p=0.0342, respectively), as demonstrated by moderate but significant drop in the average percent killing as compared to the absence of CGRP (Figure 12).
Chapter IV
Discussion

Significance of Results

The goal of this study was to evaluate the role of sensory neurons on the outcome and susceptibility to *Pseudomonas* keratitis. My results demonstrate that nociceptors can be directly activated by *Pseudomonas* and mediate immune response to infection. This could be due to CGRP, where we found that the presence of the neuropeptide impeded neutrophil dependent bacterial killing and reduced bacterial clearance in mice.

Nociception can be directly activated by *Pseudomonas aeruginosa* with Type IV pili. Previous studies in nociceptive pain signaling have only involved the indirect consequences of bacterial keratitis: epithelial injury, nerve damage and inflammation (Belmont et al., 2008). Studies have found that calcium influx is correlated with the stimulation and subsequent exocytosis of neuropeptides in neurons (Hunt & Koltzenburg, 2005). Through calcium imaging, I have demonstrated that *Pseudomonas aeruginosa* has the capacity to directly activate TG derived sensory neurons through type IV pili, essential for sensing and initiating infection by surface contact to the host (Persat et al., 2015). This suggests that *Pseudomonas* induced nociception may rely upon direct contact with sensory neurons. This contrasts with a recent study that demonstrates the direct activation of dorsal root ganglia (DRG) derived sensory neurons by *Streptococcus pyogenes* (Pinho-Ribeiro et al., 2018). The study demonstrated that *Streptococcus* nociception was indirect, through the release of virulence factor, streptolysin (Pinho-Ribeiro et al., 2018).
Nociception can impede host defense and bacterial killing

The results have shown that the mouse models to study the absence of the sensory neurons, including RTX treated mice had delayed susceptibility to *Pseudomonas* infection. RTX treated mice had significantly less pathology scores and bacterial burden at 24h post infection, but by 48 hours of infection, this protective quality was lost, as the bacterial burden of RTX mice were at similar levels as vehicle treated groups. We saw the same tendency with mice whose sodium channel Nav1.8 was genetically deleted at 24h post infection (data not shown). A subset of Nav1.8 neurons are also positive for TRPV1, illustrating that the two different mechanisms of nociceptor ablation yield comparable functional outcomes.

The significant differences in neutrophil recruiting cytokines in the corneas and lower bacterial burden, suggests improved neutrophil functionality at early time points of infection in RTX treated mice. These data are in contrast to the recently reported *Pseudomonas* induced pneumonia model, where the ablation of sensory neurons did not improve survival (Baral et al., 2018), suggestive of ocular-specific phenotype. Nonetheless, the results do resonate with bacterial infections in the *Staphylococcus aureus* lung infection and *Streptococcus pyogenes* skin infection where RTX treated mice had shown improved disease outcome and bacterial clearance (Baral et al., 2018; Pinho-Ribeiro et al., 2018). Yet in those studies, the effect of nociceptors was on the recruitment of PMNs and functionality, where in our studies PMNs functionality is affected locally.

Neuropeptide CGRP mediates *Pseudomonas* keratitis
The calcium imaging result demonstrate that sensory neurons have the capacity to be directly activated by *P. aeruginosa* which implicates neuropeptide release, including CGRP. I demonstrated that there was less CGRP present in RTX treated mice without infection (Figure 2). During 24h of infection, the concentration of CGRP in the corneal lysate was significantly less in RTX female groups as compared to controls (Figure 10). Similarly, the CGRP released from the corneas, as measured by the corneal bath, showed a similar trend, despite the lack of significance. This suggests that an absence of TRPV1+ neurons correlates with decreased CGRP release during keratitis.

In *Streptococcus pyogenes* skin infection (necrotizing fasciitis), mice treated with CGRP receptor antagonist, BIBN4096 impeded suppression of host defense (Pinho-Ribeiro *et al.*, 2018). We found the same tendency in keratitis when treating with BIBN4096 one hour after infection that there was improved bacterial clearance. Yet, lack of significant pathology differences, suggests similar levels of cellular infiltration but differential functionality. Consistently, CGRP priming of mouse neutrophils *in vitro* hindered bactericidal activities as seen in other studies (Baral *et al.*, 2018; Pinho-Ribeiro *et al.*, 2018). This suggests that sensory neurons that release CGRP hinder the host’s ability to kill bacteria. Studies on *Pseudomonas* keratitis in relation to substance P, a neuropeptide that is simultaneously released with CGRP during nociception (Schlereth *et al.*, 2016) have demonstrated a similar outcome of disease in inflammatory cytokines, bacterial burden and pathology score in BALB/C and B6 mice (Hazlett *et al.*, 2007; Mcclellan *et al.*, 2008). A recent study demonstrated that both CGRP and SP alter neutrophil response to *Moraxella catarrhalis* bacterial infection *in vitro* by inhibiting the degranulation of neutrophils (Augustyniak *et al.*, 2018).
Study Limitations

While using mouse models is highly advantageous in terms of accessibility in numbers as well as tools for research, the use of inbred mice as well as and species differences such as the divergence in the lacrimal gland architecture and function between humans and mice, prove troublesome for translational medicine (Marquart, 2011). Thus, while there are more possibilities to assess the mechanistic roles in bacterial keratitis, the gaps between mouse models and patients may pose difficulties.

Furthermore, there is a lack of specificity in the ablation of nociceptors in mouse models that are currently available. The mode in which we ablate TRPV1+ sensory neurons during Resiniferatoxin treatment is by opening the calcium channels in a sustained manner until they undergo neuronal cell death. This treatment is highly immunogenic and thus requires the mice to rest before commencing experiments. It is unclear whether this episode of systemic overactivation of TRPV1 neurons may impact the overall physiological state of the mouse. However, in combination with the findings that corneal sensitivity as well as neuronal density are significantly decreased in RTX cohorts at baseline, RTX treatment could pose to be a useful mouse model for neurotrophic keratitis.

Future Research Directions

To further test my hypothesis, I would propose to measure in vitro CGRP from TG derived primary neurons that were exposed to 6294, to affirm that bacterial interaction does indeed induce significant CGRP release, as previous studies have
established (Pinho-Ribero et al., 2018) as well as to understand how CGRP induces neutrophil related bactericidal reduction by analyzing degranulation.

In lung and skin infection, there was a significant difference in neutrophil recruitment in the absence of sensory neurons. We did not make these observations, but in order to better understand the kinetics of this neuro-immune interaction, I would like to do flow cytometry or qPCR on digested corneas to detect the immune cells involved. Furthermore, it was observed that RTX treatment increased induction and resolution of cytokines, so that at an early point of infection there was higher levels of inflammatory cytokines in lung infections, but at 12h of infection, there was significantly lower IL-1β (Baral et al., 2018). It would be useful to measure cytokine levels at an early time point, such as at 8hr post infection to determine whether we observe a similar trend.

In order to strengthen RTX treatment as a viable mouse model for neuropathy, it would be ideal further assert the ablation of TRPV1+ neurons by running qPCR on the TGs from RTX treated mice to affirm that TRPV1+ sensory neurons are no longer present. Because there were some difficulty staining with TRPV1 antibody, I would also like to obtain cre-floxed TRPV1 mice that would be crossed with a reporter mice to try to do live confocal imaging after RTX treatment after various time points to further support my findings. I would also look for mouse models where sensory neuron markers such as Nav1.8 or TRPV1 can be deleted specifically in the trigeminal ganglia. To assess whether RTX treatment could be used a pharmacological mouse model for neurotrophic keratitis, I would need to further assess corneal sensitivity, possibly through the Cochet-Bonnet behavioral test.

Conclusion
My hypothesis that sensory neurons play a crucial role in maintaining the cornea as a ‘immune privileged’ by hindering immune response during bacterial keratitis was supported by the disease outcome of RTX treated mice as well as demonstrating the direct interaction between bacteria and neuron. My results demonstrate that sensory neurons play a crucial role during bacterial keratitis and this proves to be initially disadvantageous, as it primes neutrophils from being able to effectively fend off *Pseudomonas aeruginosa* via CGRP. Improved bacterial clearance after CGRP antagonist treatment post infection suggests that modulating neuronal response can be another therapeutic target to treat bacterial keratitis. Thus, elucidating further the nociceptive pathway during bacterial keratitis can help provide therapeutic targets to treat this very painful infection.
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Figure 1. Resiniferatoxin Experimental Plan

*C57BL/6 4 week old female mice were given three consecutive doses (30ug/kg, 70ug/kg and 100 ug/kg) and were subsequently rested for 2-3 weeks before beginning experiments.*
Figure 2. RTX treatment ablates corneal nociceptors

A. Corneal whole mounts were stained for CGRP and Beta-III tubulin. Z-stacks were taken with an inverted Nikon confocal using a 40x objective. Images are representative out of 10 analyzed mice per group. z-stacks were taken for the central and peripheral areas per cornea.
B. Quantification of the neuronal density was done with collapsed z-stacks of the corneas using ImageJ. A calibrated line was applied perpendicularly via Image J and the number of intersections of nerve fibers were counted (Dvorscak & Marfurt, 2008). Vehicle treated mice (Cntrl) mice had significantly more intersections counted (p<0.001) as compared to RTX mice. Values plotted in figures are expressed as the mean ± s.e.m. with each z-stack taken representing a single plot. Unpaired t-test was performed for statistical analysis.
Figure 3. Resiniferatoxin significantly reduces corneal sensitivity

The Capsaicin Eye wipe test was performed three weeks after RTX treatment. 3 mM Capsaicin was applied to the left cornea and mouse behavior was recorded for 1 minute immediately after application (Neubert et al., 2008). Vehicle treated mice (Cntrl) group exhibited more wipes after capsaicin application, consistent with decreased sensitivity in the RTX group. Values plotted in figures are expressed as the mean ± s.e.m. with symbols representing individual mice. Unpaired t-test.
A. RTX and vehicle-treated cohorts were infected with $5 \times 10^5$ CFU/ml P. aeruginosa 6294 and infected corneas were harvested at 8 hours, 24 hours and 48 hours. Data are representative of three independent experiments with 5-7 mice per group. Symbols represent individual animals. The vehicle group (cntrl) had significantly higher bacterial burden than RTX treated mice in the early hours of infections. Unpaired t-test.

Pathology scores were assessed by a blinded investigator, and was graded based on rubric: 0 = clear cornea, 1 = slight corneal haze, 2 = moderate corneal opacity, 3 = severe corneal opacity but iris visible and 4 = severe corneal opacity with iris obscured. At 24h of infection, RTX treated mice had lower pathology score than vehicle treated mice. This significant difference was lost at 48h post-infection. Mann-U Whitney test.
Figure 6. Cytokine profiling of the infected corneas showed significant differences in IL-1b, MIP-2 and CGRP at 24 h post-infection.

Cytokine concentrations were measured by ELISA and were normalized to protein concentration. Vehicle treated mice (Cntrl) had significantly higher levels of neutrophil recruiting cytokines IL-1b, MIP-2 and CGRP. Values plotted in figures are expressed as the mean ± s.e.m with symbols representing individual mice. Unpaired t-test.
Figure 7. No significant differences in the cytokine profiles of the infected corneas at 48h post-infection

Cytokine concentrations were measured by ELISA and were normalized to protein concentration. Values plotted in figures are expressed as the mean ± s.e.m with symbols representing individual mice. Unpaired t-test.
Figure 8. *Pseudomonas aeruginosa* (6294) can directly activate TG sensory neurons

A. Representative Fura-2 ratiometric fields were taken from a 10x or 20x objective. Before commencing measurements, fields were chosen to optimize the number of non-activated neurons in view.

B. Representative trends of calcium traces were obtained by selecting neurons using the NIS-software and the ROIs were measured and the 340/360 ratio measurements were exported to Microsoft Excel, where calcium trends were created. The arrows represent the timepoint of application of Pseudomonas (P), Capsaicin (C), and KCl (K).
C. Capsaicin and KCl were used as positive controls for neuronal response. Responding neuron were characterized as neurons that displayed a ratio was 25% over the baseline, which is the measurements taken before any application. Inoculums 1x10^8 CFU/ml and 1x10^7 live (L) and filtered (F) are show. Values plotted in figures are expressed as the mean ± s.e.m.
Figure 9. *Pseudomonas* activation is dependent upon Type IV pili

A. Representative Fura-2 ratiometric fields were taken from a 20x objective. Before commencing measurements, fields were chosen to optimize the number of non-activated neurons in view.
B. Representative trends of calcium traces were obtained by selecting neurons using the NIS-software and the ROIs were measured and the 340/360 ratio measurements were exported to Microsoft Excel, where calcium trends were created. The arrows represent the timepoint of application of Pseudomonas (PAK or ΔpilA), Capsaicin(C), and KCl (K).
C. The graph is an accumulation of 2 independent experiments. Krebs-Ringer buffer was used as a negative control. Capsaicin, mustard oil, and KCl were used as positive controls for neuronal response. Responding neuron were characterized as neurons that displayed a ratio was 25% over the baseline, which is the measurements taken before any application. Mann-U Whitney was used to determine significance between groups. There was significantly less response to the pilA KO mutant as compared to PAK WT (p<0.05). Values plotted in figures are expressed as the mean ± s.e.m.
Figure 10. Decreased presence and release of neuropeptide CGRP during 24 hours of infection

A. To measure CGRP concentration of corneal lysates, CGRP EIA kit (Cayman) was used. Values plotted in figures are expressed as the mean ± standard error of the mean (s.e.m.). Symbols represent individual mice.

B. To measure the amount CGRP released from the cornea, infected corneas were bathed in DMEM-F12. The supernatants were analyzed for CGRP using EIA kit. Values plotted in figures are expressed as the mean ± standard error of the mean (s.e.m.). Symbols represent pooled data for 3 corneas. Unpaired t-test was performed for statistical analysis.
Figure 11. CGRP receptor antagonist BIBN4096 decreases bacterial burden in the cornea treated mice 17h post-infection

*C57BL/6 female mice were infected with 6294 5x10^5 CFU/ml for 17 hour. One hour after infection, BIBN4096 (30mg/kg) or vehicles were injected intraperitoneally. Corneas were harvested bacterial burden. Values plotted in figures are expressed as the mean ±s.e.m. with symbols representing individual mice Vehicle treated mice (Cntrl) had significantly higher bacterial presence as compared to BIBN4096- treated mice (Unpaired t-test, p=0.04).*
Figure 12. The presence of CGRP inhibited Neutrophil opsonophagocytic killing.

Each symbol represents an individual sample. The graph is an accumulation of three independently performed experiments. At 500 nM and 1μM of rat CGRP, there was significantly reduced killing ($p<0.05$ for both) as compared to the absence of CGRP. One way ANOVA with Holm Sidak’s post-test was performed to determine significance.
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