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Characterizing the role of the Hv1 voltage-gated proton channel in Pseudomonas aeruginosa corneal infection

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Characterizing the role of the Hv1 voltage-gated proton channel in
*Pseudomonas aeruginosa* corneal infection

DISSERTATION

submitted in partial satisfaction of the requirements
for the degree of

Doctorate of Philosophy

in Biomedical Sciences

by

Priscila A. Rodriguez

Dissertation Committee:
Professor Eric Pearlman, Chair
Professor Melissa Lodoen
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2023

# DEDICATION

To my sons Alex and Adan

To my parents Priscila and Adan

To my furry babies Sage, Bailey, and Teddy

To all my family and friends

"Success is no accident. It is hard work, perseverance, learning,

studying, sacrifice and most of all, love of what you are doing or learning to do."

– Pele

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# ACKNOWLEDGEMENTS

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# ABSTRACT OF THE DISSERTATION

Characterizing the role of the Hv1 voltage-gated proton channel in
*Pseudomonas aeruginosa* corneal infection

by

Priscila A. Rodriguez

Doctorate of Philosophy in Biomedical Sciences

University of California, Irvine, 2023

Professor Eric Pearlman, Chair

Microbial infections of the cornea (keratitis) cause an estimated 1 million cases of visual impairment and blindness worldwide each year. Treatment includes antimicrobial agents followed by corticosteroids to reduce inflammation and inhibit fibrosis. However, corticosteroids have undesirable side effects including increased intraocular pressure and risk for glaucoma. The Hv1 voltage-gated proton channel functions in the regulation of pH and NOX-mediated ROS production and has been implicated in cancer, ischemic stroke, CNS demyelination, traumatic brain injury and spinal cord injury and presents a target for therapeutic intervention.  However, the role of this proton channel in ocular disease has not been examined. The immune response to invading microorganisms is characterized by recruitment of neutrophils to the site of infection which produce reactive oxygen species (ROS). Nevertheless, if produced in excess or prolonged periods of time, ROS can cause tissue damage. Using a recently discovered potent inhibitor of the Hv1 proton channel called Hv1 Inhibitor Flexible (HIF), we show that in PMA-, curdlan-, zymosan- and *ΔpscD*-stimulated murine bone marrow and human neutrophils, HIF inhibits ROS production in a dose-dependent manner. Our data also

show that *Hvcn1-/-* mice, which lack the Hv1 voltage-gated proton channel, develop more severe corneal disease. However, we found that there was no difference in neutrophils and monocytes infiltration into the corneas, indicating that the inability of *Hvcn1-/-* mice to eliminate the bacteria is not due to a defect in cell migration and infiltration. Our results suggest that HIF targeting of the Hv1 voltage-gated proton channel together with antibiotics has a strong potential for treatment of microbial keratitis.

# CHAPTER 1:


# Introduction

**Neutrophil biology and its role in disease**

The innate immune system is composed of anatomical and physical barriers, effector cells, antimicrobial peptides, soluble mediators such as cytokines and complement proteins, and cell receptors[1]. Cells of the innate immune system include cells from myeloid and lymphoid lineage. Myeloid cells differentiate from common progenitors derived from hematopoietic stem cells in the bone marrow and include mononuclear and granulocytic phagocytes[2–4]. Upon microbial invasion, phagocytes are quickly recruited to the tissues by chemotactic cytokines generated by resident cells where they are activated to phagocytose and release pro-inflammatory cytokines and chemokines that recruit and modulate other cells[5]. Phagocytes are comprised mainly of macrophages and neutrophils[2]. However, neutrophils will be the focus of this chapter.

Neutrophils (also called polymorphonuclear [PMN] cells) are the most abundant leukocyte in human blood and along with basophils and eosinophils are characterized by dense granules on their cytoplasm[6,7]. They are generated in the bone marrow from granulocyte-monocyte progenitor cells (GMPs), a myeloid committed progenitor cell and circulate in the periphery under homeostatic conditions[7,8]. Mature neutrophils are terminally differentiated and are short-lived compared to other innate immune cells while circulating in the blood (<24 hr)[9]. However, when activated, they can live up to 5.4 days in tissues[10]. Through direct contact and the production of cytokines, neutrophils can shape the immune landscape by activating macrophages, dendritic cells, and lymphocytes (natural killing cells, B and T cells)[6]. The main role of neutrophils is to rapidly travel to the site of inflammation or infection, recognize invading microbes, phagocytose and kill these pathogens through cytotoxic mechanisms that include Neutrophils Extracellular Traps (NETs), degranulation, reactive oxygen species (ROS)

and nutritional immunity[7,11]. Neutrophils also release serine and matrix

metalloproteinases that degrade the tissue and prevent dissemination of microbes[12].

*Neutrophil recruitment*

Neutrophils are the first to the site of infection and enter the tissues through a

process known as extravasation in response to signals generated at the site of

inflammation[13]. Extravasation proceeds in the following stages: tethering, rolling,

adhesion, crawling, and transmigration on vascular endothelial cells[14]. There are two

mechanisms by which neutrophils transmigrate: paracellularly (between endothelial

cells) and transcellularly (through an endothelial cell)[15]. Neutrophils mostly transmigrate

paracellularly.

When neutrophils come into contact with pathogens, they release inflammatory

mediators such as inflammatory cytokines that include IL-1β and TNF-α, which

stimulate endothelial cells[14,16,17]. Endothelial cells can also be activated by bacteria

through pattern-recognition receptors (PRRs)[14,18].  Activated endothelial cells

upregulate P- and E-selectins which bind to their neutrophil p-selectin glycoprotein-1

(PSGL1), leading to the low affinity tethering. When neutrophils encounter a chemokine

gradient, integrins that include LFA-1 and Mac-1 (CR3) change their conformation and

bind to intracellular adhesion molecules I and 2 (ICAM-1 and -2) on endothelial

cells[19,20]. At this point, neutrophils crawl to the nearest cell-cell junction, preferably

positioned where the basement membrane expresses lower levels of matrix proteins, in

a process known as locomotion[18]. The neutrophils associate with upright microvilli-like

endothelial projections enriched with ICAM-1 that form a cuplike structure that

surrounds the site to form a dome[16]. The cytoskeleton of the neutrophils is reorganized

in a way that allows them to spread over endothelial cells and extend pseudopodia.

Neutrophils transmigrate between endothelial cells in a process that is mediated by

PECAM-1/PECAM-1 interactions [21]. After transmigration, the neutrophils follow

chemokine gradients that lead to the site of inflammation.

*Neutrophil effector functions*

Neutrophils are the first responders to the site of infection and have an arsenal of

effector functions to destroy microorganisms (**Fig. 1.1**).
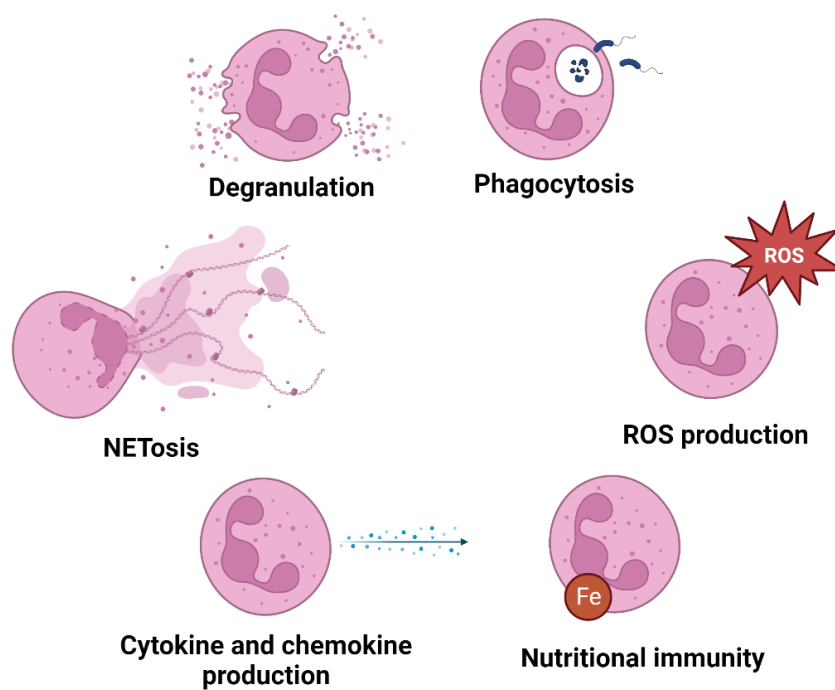


**Fig. 1.1. Neutrophil effector functions.** Neutrophil effector functions include phagocytosis, Neutrophil Extracellular Traps (NETs) and reactive oxygen species (ROS) production, degranulation, the production of cytokines and chemokines, and nutritional immunity. Created with Biorender.com.

***Phagocytosis***

The main function of neutrophils is to phagocytose microorganisms, which are

subsequently destroyed in phagolysosomes. Neutrophils can phagocytose both

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opsonized and non-opsonized bacteria [22]. Neutrophils express Fc and complement receptors that bind to antibody- or complement opsonized microbes including the β2 integrins MAC1/CR3 (CD11b/CD18)[22,23]. Phagocytosis of non-opsonized microbes is accomplished through pattern-recognition receptors (PRRs) in mammals and include C-type lectin receptors[24]. These receptors recognize pathogen-associated molecular patterns (PAMPs) from microbes or danger-associated molecular patterns (DAMPs) from damaged cells. Once neutrophils have engaged the microbe or the damaged cell through these specialized receptors, the pseudopodia extend around and engulf the particle, pinching off the plasma membrane to form phagosomes. [22]. The nicotinamide adenine dinucleotide phosphate (NADPH) oxidase 2 (NOX2) enzyme is assembled at the membrane of the phagosome. After sealing of the phagosome, azurophilic (primary) and specific (secondary) granules fuse with the phagosome, releasing their antimicrobial contents at the membrane of the phagosome[25]. Early alkalization of neutrophil phagosomes leads to the oxidative burst by NOX in phagosomes which produces reactive oxygen species (ROS)[26]. However, ROS will be discussed in another section.

### Neutrophil extracellular traps

Neutrophils also release Neutrophil Extracellular Traps (NETs) which comprise DNA histones and granular proteins such neutrophil elastase, cathepsin G, and myeloperoxidase (MPO) that entrap and kill microorganisms[27]. NETosis is an essential mechanism to entrap and kill microbes. However, when produced in excess, NETs can cause tissue damage. In suicidal NETosis, azurophilic granules are released into the cytoplasm which include the protein complex "azurosome". ROS causes dissociation of

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azurosomes which releases the serine proteases that include neutrophil elastase (NE), cathepsin G, azurocidin, and myeloperoxidase (MPO)[28]. Serine proteases, degrade cytoskeletal elements such as actin filaments, which cause plasma membrane disintegration. Subsequently, the serine proteases travel to the nucleus where they digest the nuclear lamina and histones contributing to chromatin decondensation[29]. In addition, peptidyl-arginase deaminase 4 (PAD4) is transferred from the cytoplasm to the nucleus to citrullinate histones, which further decondenses chromatin and promotes expulsion of the chromosomal DNA coated with antimicrobial peptides[30]. NETosis concludes in the disintegration of the nuclear membrane, disassembly of the cytoskeleton and nuclear lamina, and chromatin decondensation leading to the release of NETs and death of the neutrophil[31].

### *Degranulation*

There are four types of granules in neutrophils: azurophilic (primary), specific (secondary), gelatinase (tertiary), and secretory vesicles. Azurophilic granules contain NE, MPO, cathepsins, and defensins while specific granules contain lactoferrin and collagenase among others[32]. Tertiary granules contain matrix metalloprotease 9 (also known as gelatinase B) while secretory vesicles in human neutrophils contain human serum albumin, among others[13,33]. Exocytosis of the granules occurs in a series of steps[34,35]. In the first step, through the changes in the cytoskeleton and assembly of microtubules, granules are recruited from the cytoplasm to the target membrane. Vesicle tethering and docking leads to contact between the outer surface of the lipid bilayer surrounding the granule with the inner surface of the lipid bilayer of the target membrane. After granule priming, the granules are fusion-competent and a structure

known as the fusion pore develops between the granule and the target membrane. This fusion pore enlarges to allow granule fusion leading to degranulation[36]. ROS production by NOX2  inhibits degranulation that can lead to tissue damage by providing a negative feedback loop[37].  Degradation of infected tissues by these serine and matrix metalloproteinases serves to wall off the microbes and limit dissemination that can otherwise lead to sepsis.

### Cytokine production

Neutrophils also produce cytokines and chemokines which recruit more neutrophils and other immune cells to the site of infection[38]. Although neutrophils were not considered to be immune regulatory cells, recent studies clearly demonstrate that they produce multiple cytokines, including the pro-inflammatory cytokines IL-1α and IL-1β, chemokines CXCL1 and CXCL2 (and CXCL8/IL-8 by human neutrophils), and immunoregulatory cytokines that include IL-10, IL-12 and IL-23[39].

### Nutritional immunity

Metal cofactors are necessary for numerous fundamental processes such as DNA replication and transcription, relief from oxidative stress, and cellular respiration that are essential for both pathogens and host cells[40]. Neutrophils scavenge $Fe^{++}$ (transferrin and lactoferrin) and $Mg^{++}$ and $Mn^{++}$ (S100A8/A9, calprotectin), which competes with microbial siderophores to inhibit bacterial and fungal growth in a process termed 'nutritional immunity'[40,41].

### Reactive oxygen species

 In addition to these non-oxidative anti-microbial activities, neutrophils generate active oxidative anti-microbial reactive oxygen species (ROS), which are highly reactive

chemicals formed from molecular oxygen ($O_2$) and include superoxide ($O_2\bullet-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical ($\bullet OH$)[42]. ROS are potent microbicidal agents that can directly eliminate invading pathogens. This is evident in patients with Chronic Granulomatous Disease (CGD), who lack a functional NOX2 protein and are more susceptible to recurrent infections[43]. ROS also regulates inflammation by regulating immune cell proliferation, differentiation, and activation[44,45]. Like other effector functions of neutrophils, if ROS is produced in excess or for prolonged periods of time, it can lead to tissue damage.

ROS is produced by the NOX2 enzyme which has 6 hetero-subunits that associate in a stimulus-dependent manner[46,47]. gp91[phox] and p22[phox] are the integral membrane proteins that together make up the flavocytochrome b558 (cytb$_{558}$) subunit. cytb$_{558}$ resides at the membranes of phagosomes, secretory vesicles, specific granules, and the plasma membrane. Under unstimulated conditions, the regulatory subunits, p40[phox], p47[phox] and p67[phox], exist in the cytosol. Upon stimulation, p47[phox] undergoes phosphorylation, and the entire cytosolic complex translocates to the membrane and associates with cytb$_{558}$ to form the active NOX2[48]. At the same time, Rac2 binds guanosine triphosphate (GTP) and translocates to the membrane along with the cytosolic complex[47].

**The Hv1 voltage-gated proton channel**

The Hv1 voltage-gated proton channel is a transmembrane protein with four subunits that extrudes protons across the cell membrane and is encoded by the *Hvcn1* gene. Its role is to regulate cellular pH homeostasis and to extend and sustain ROS production by NOX enzymes[49,50].
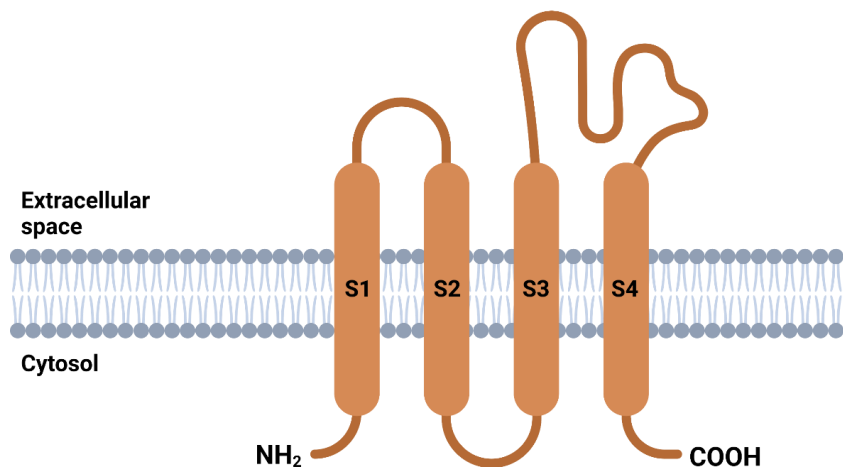
**Fig. 1.2. The human Hv1 voltage-gated proton channel.** The hHv1 proton channel is composed of four transmembrane domains segments (S1–S4), which form the voltage-sensing domain (VSD) and has an N and C terminus that lie inside the cell. Created with Biorender.com.

The Hv1 voltage-gated proton channel is a member of the superfamily of proteins containing voltage-sensing domains (VSDs) that includes voltage-dependent, sodium, potassium, and calcium channels and it is highly conserved among species[51]. The Hv1 voltage-gated proton channel is expressed in phagocytes, glial cells, cardiomyocytes, pancreatic islet β-cells, and sperm[52]. The protein has four transmembrane domains segments (S1–S4) that form the voltage-sensing domain (VSD), and has an N terminus and C terminus that lie inside the cell[50] (**Fig. 1.2**). Unlike other proton channels, Hv1 does not have a pore domain[49]. Instead, the transmembrane segments contain an intrinsic conduction pathway for $H^+$ ions and gates that open when the membrane is depolarized and the cytosol is acidified[50,52].

The Hv1 voltage-gated proton channel is detrimental in central nervous system (CNS) diseases and injuries, including ischemic stroke, traumatic brain injury, spinal

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cord injury, and demyelinating lesions[53]. However, in diabetes, the Hv1 voltage-gated proton channel promotes insulin secretion of pancreatic β-cells and glucose homeostasis[54]. There are relatively few studies that examine Hv1 in the context of microbial infection. Ramsay *et al.* generated *Hvcn1-/-* mice (which they kindly sent to us) and showed that following intraperitoneal infection with $1\times10^8$ or $1\times10^9$ *Staphylococcus aureus*, there were more bacteria recovered from *Hvcn1-/-* mice than WT mice after 6h, although there was no difference at 24h post infection (hpi)[55]. Similarly, Okochi et al found that *Hvcn1-/-* mice infected intranasally with *Candida albicans* had elevated pulmonary inflammation; however, there was no effect of Hv1 deficiency on fungal killing [56].

During the respiratory burst, NOX2 generates ROS superoxide ($O_2^-$) by accepting electrons ($e^-$) from cytoplasmic NADPH and donating them to molecular oxygen ($O_2$), leading to membrane depolarization and accumulated protons in the cytoplasm, which in turns leads to acidification of the cytosol[57] (**Fig. 1.3**). NOX2 activity, membrane depolarization, and intracellular acidification are key stimuli that activate the Hv1 voltage-gated proton channel. Membrane depolarization and cytosol acidification inhibit the activity of NOX2. However, this can be reversed by the Hv1 voltage-gated proton channel-mediated extrusion of protons from the cytoplasm to the outside of the cell, thereby maintaining physiological membrane potential and re-establishing normal pH[50,51,58] (**Fig. 1.3**).

$$2O_2 \xrightarrow{e^-} 2O_2^- \xrightarrow{H^+} 2H_2O_2$$
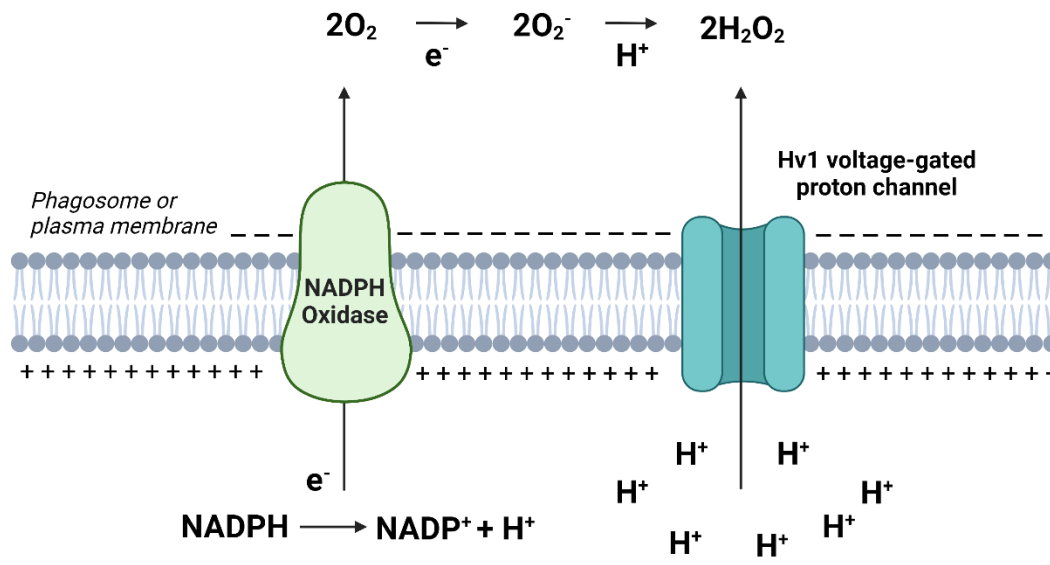
**Fig. 1.3. Role of the Hv1 voltage-gated proton channel in NOX2-mediated ROS production.** NOX transfers electrons from cytoplasmic NADPH to molecular oxygen, causing membrane depolarization and accumulation of protons in the cytoplasm. The Hv1 proton channel extrudes the accumulated protons to the outside of the cell repolarizing the membrane and re-establishing physiological pH. Created with Biorender.com.

## Drugs targeting the Hv1 voltage-gated proton channel

Hv1 activity has been reported to hinder recovery from ischemic stroke, traumatic brain injury, and spinal cord injury, and to increase the metastatic potential of different types of cancer, motivating the development of Hv1 antagonists as potential neuro-protective agents and anticancer drugs[51,59].

Thus far, two major strategies have proved successful in developing Hv1 antagonists, one focused on the use of peptide toxins binding the channel extracellular side, such as Hanatoxin (HaTx), Corza6 (C6) and AGAP$_{W38F}$, the other focused on small organic molecules targeting the channel intracellular side, such as 2GBI, ClGBI, and more recently, HIF (Hv1 Inhibitor Flexible) compounds[60–64]. Some of these ligands

have found applications as pharmacological tools to study the gating mechanism of
conducting VSDs and the role of Hv1-mediated proton currents in cellular physiology.
Drug screening approaches on native proton currents, have led to the identification of
additional potential antagonists. However, the mechanism of action of these compounds
is not well understood.

2GBI (2-guanidinobenzimidazole) and its derivatives can bind the Hv1 VSD only
in the open conformation and act as open-channel blockers[65]. HIF (3-(2-amino-5-
methyl-1H-imidazol-4-yl)-1-(3,5-difluorophenyl)propan-1-one) (**Fig. 1.4**) and its analogs
are structurally related to 2GBI and were developed to improve Hv1-ligand
interactions[64]. These molecules interact with the channel VSD in both open and closed
conformations[63]. They do not need channel activation in order to bind, and can diffuse
inside the cell to inhibit Hv1 localized in phagosomes in addition to the channels
localized on the plasma membrane. Experiments described in Chapter 2 examine the
role of HIF on ROS production by human and murine neutrophils.
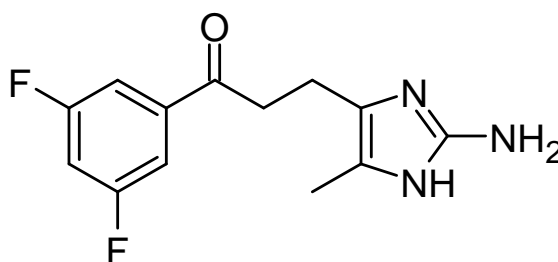


**Fig. 1.4. Hv1 inhibitor flexible (HIF).**  HIF is composed of a phenyl ring and a 2-
aminoimidazole ring separated by a flexible linker.

**Microbial infections of the cornea (keratitis)**

Microbial corneal infection (keratitis) is caused by pathogenic microbes such as
bacteria, fungi, and parasites and is the fifth leading cause of blindness worldwide[66,67].

Pathogenic microbes penetrate the corneal stroma following disruption of the epithelial cell barrier where they cause inflammation, loss of function, visual impairment and blindness[68]. The most common risk factors for development of infectious keratitis in the USA and other industrialized countries are poor contact lens care and hygiene, ineffective disinfecting solutions, and ocular surgery[66,69]. In contrast, ocular trauma is the major risk factor for microbial infections in developing countries[70]. Early antibiotic treatment is required to prevent vision impairment and eye loss as the disease progresses quickly[71]. Symptoms of microbial keratitis generally include redness, tearing, rapid onset of pain and blurred vision. However, clinical presentation depends on the causative agent[72].

Bacterial keratitis accounts for approximately 90% of microbial keratitis cases worldwide and *Pseudomonas aeruginosa* is the main causative agent[73,74]. Other bacteria that cause corneal infections are *Staphylococcus aureus*, *Streptococcus pneumonia* and *Serratia* species[66]. *Pseudomonas aeruginosa* is a Gram-negative bacterium and an opportunistic pathogen that causes multiple types of infections, including lung, skin, and keratitis[75]. The clinical presentation of *P. aeruginosa* keratitis is more severe than clinical presentations of keratitis caused by other bacteria[76]. Clinical manifestations of *P. aeruginosa* keratitis include decreased vision, photophobia, pain, redness, tearing, discharge, and stromal inflammation and ulceration[77]. A hallmark of *P. aeruginosa* keratitis is the ring-shaped abscess caused by accumulation of neutrophils which leads to corneal opacity surrounding the central lesion[78].

*Pseudomonas aeruginosa* release virulence factors that allow for adaptation to the harsh environment of the host enabling it to establish infection and disease[79]. One

of the main virulence factors for *P. aeruginosa* is their type III secretion system (T3SS), a specialized protein export system that forms a needle-like complex that injects exotoxins into the host's cell membrane that cause cell damage and disrupt immune responses (**Fig. 1.4**). The toxins secreted by the T3SS include ExoS, ExoT, ExoY, and ExoU. However, most strains of *P. aeruginosa* express either ExoS or ExoU, exclusively[80].  ExoS has been found to suppress ROS production in human neutrophils by ADP-ribosylation of Ras which prevents it from interacting with and activating phosphoinositol-3-kinase (PI3K), which is required for assembly of the NOX2 enzyme complex at the plasma or phagosome membrane[81].
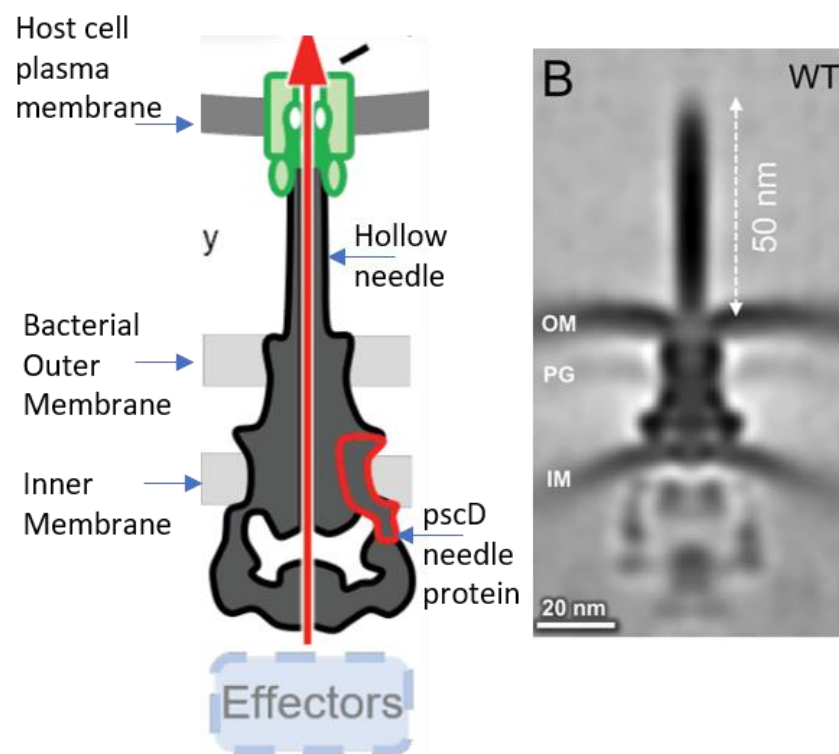


**Fig. 1.5. Type III Secretion System of *P. aeruginosa.*** The T3SS consists of a needle complex, translocating apparatus, and effector toxins (ExoS, ExoT, ExoY, and ExoU) that are injected directly from the bacterium to the cytosol of the host cell.

**An essential role for NOX2 and ROS in *P. aeruginosa* keratitis**

Neutrophils are the first line of defense against bacterial infections and the generation of ROS is part of the effector functions used to clear bacteria. The importance of ROS in *P. aeruginosa* killing in infected corneas was demonstrated by Vareechon and Pearlman[81]. Mice that lack the gp91$^{phox}$ subunit of the NOX2 enzyme exhibit impaired NOX2 dependent ROS production in neutrophils. Corneas of C57BL/6 and gp91$^{phox-/-}$ were infected with wild-type *P. aeruginosa* (PA01) or with a mutant strain that lacks the essential T3SS needle structure component PscD (*ΔpscD*) and corneal opacity due to neutrophil infiltration and colony forming units (CFU) were measured 24hpi. gp91$^{phox-/-}$ had exacerbated corneal opacity and more CFU/eye than the C57BL/6 mice. They also found that in *ΔpscD* mice, corneal opacity was less severe compared to PA01 in C57BL/6 mice[81]. These results indicate that ROS production is required to kill *P. aeruginosa* in infected corneas and that the T3SS is used by *P. aeruginosa* to repress ROS in production in neutrophils and promote survival (**Fig. 1.5**).
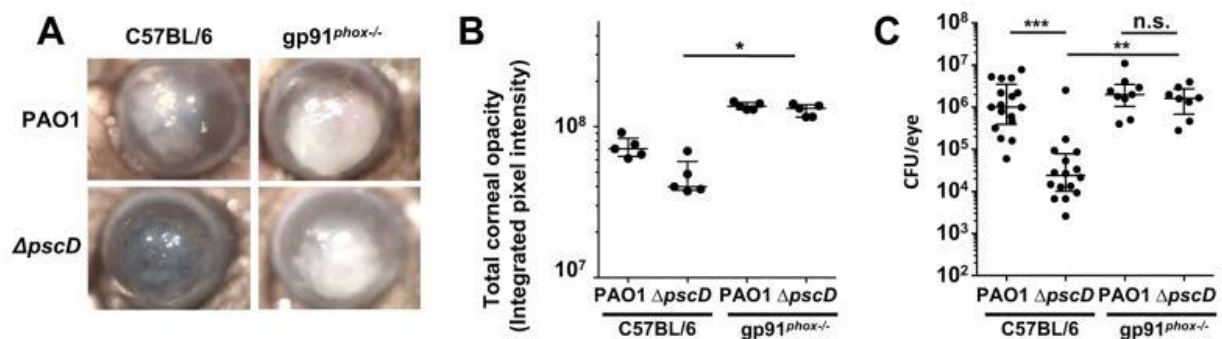
**Fig. 1.6. NOX2 mediates ROS production by neutrophils and facilitates clearance of *P. aeruginosa* during bacterial keratitis.** A) Representative images of corneal opacification 24hpi of C57BL/6 and gp91$^{phox -/-}$ (CGD) mice infected with 1x10$^5$ CFU PAO1 (WT) or with the *ΔpscD* (T3SS null) mutant strain. B) Quantification of corneal opacity by determining average pixel intensity of corneas described previously (Sun et al., 2012) (n=5 mice). C) Colony forming units (CFU) recovered from infected corneas 24 h post-infection (n=9 mice). [from Vareechon, Pearlman and Rietsch, Cell Host & Microbe 2017]

Experiments described in Chapter 2 examined the role of the Hv1 voltage-gated proton channel in NOX2-mediated ROS production in the context of *P. aeruginosa* corneal infection. In addition, we examined inhibition of ROS production in murine bone marrow and human neutrophils using HIF after infection with *P. aeruginosa* strain PA01 and *ΔpscD* or following stimulation with PMA.

# CHAPTER 2


**_Pseudomonas aeruginosa_ corneal infection is regulated by the Hv1 voltage-gated proton channel and a novel small molecule Hv1 inhibitor (HIF)**

**Abstract**

Assembly of the NADPH oxidase 2 (NOX2) associated proteins in neutrophils plays an essential role in controlling microbial infections by producing high levels of reactive oxygen species (ROS) at the plasma membrane and in phagolysosomes. However, relatively little is known about the Hv1 voltage-gated proton channel that is required for sustained NOX2 activity. We examined the role of Hv1 in a murine model of blinding *Pseudomonas aeruginosa* corneal infection in which neutrophils comprise >80% total infiltrating cells. The corneal epithelium of C57BL/6 and *Hvcn1-/-* mice (that encodes Hv1) was abraded and infected topically with $5\times10^4$ *P. aeruginosa* strain PAO1 expressing green fluorescence protein (GFP). After 24h, corneal opacification and GFP were examined, and viable bacteria were quantified by CFU. We found that infected *Hvcn1-/-* mice develop more severe corneal disease consistent with impaired bacterial killing detected by elevated GFP and CFU. In vitro, we used a novel Hv1 inhibitor (Hv1 Inhibitor Flexible [HIF]) to examine its effect on ROS production by human and murine neutrophils. We found that HIF inhibits ROS production in a dose-dependent manner following infection with *P. aeruginosa* or stimulation with fungal cell wall products curdlan and zymosan or with PMA. Collectively, these findings demonstrate that Hv1 has an important role in controlling infection with *P. aeruginosa* and that HIF inhibits ROS production by human and murine neutrophils.

**Introduction**

Neutrophils play an essential role in killing and inhibiting dissemination of pathogenic bacteria. Release of serine and matrix metalloproteinases from neutrophil granules digests the tissue resulting in localization of the bacteria; however, these

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enzymes can also cause damage to sensitive tissues[82]. Neutrophils also have multiple

oxidative and non-oxidative effector functions for killing bacteria, including reactive

oxygen species (ROS) that kill bacteria in the phagolysosome[83].

The NADPH oxidase protein complex 2 (NOX2) that produces ROS comprises

the gp91[phox] and p22[phox] proteins together with Rac small G protein that comprise the

flavocytochrome b558 (cytb$_{558}$) subunit on plasma membranes and on phagosome

membranes[46]. The regulatory p40[phox], p47[phox] and p67[phox] proteins in the cytoplasm

translocate to the membrane following phagocytosis phosphorylation of p47[phox] and

associates with cytb$_{558}$ to form the active NOX2[26,48]. Once assembled, NOX2 generates

ROS superoxide ($O_2^-$) by accepting electrons from cytoplasmic NADPH and donating

them to molecular oxygen, leading to membrane depolarization and accumulation of

protons in the cytoplasm, which in turn leads to acidification of the cytosol[84]. The

importance of NOX2 for bacterial killing has been well described in patients with

mutations in these subunits, most commonly gp91[phox] who are highly susceptible to

infection, and also in mice with deletions in these proteins[43].

Although the role for NOX2 in infectious diseases has been well documented,

there are relatively few reports on the role of the Hv1 voltage-gated proton channel that

is required for sustained NOX2 activity. The Hv1 proton channel is a membrane protein

that maintains cellular pH homeostasis by releasing protons across the plasma

membrane and outside the cell[50,52]. Hv1 functions in a similar manner in phagosomes.

While NOX2 activity is inhibited by membrane depolarization and cytosolic acidification,

Hv1 is activated under these conditions and releases $H^+$ protons into the

phagolysosome, thereby sustaining NOX2 mediated ROS production[50,51,58].

Recently, Dr. Francesco Tombola from the Physiology and Biophysics department at the University of California, Irvine, using a rational design approach that combined experimental and computational methods, designed and created a novel potent inhibitor for the Hv1 voltage-gated proton channel called Hv1 inhibitor flexible (HIF). HIF is composed of a phenyl ring and a 2-aminoimidazole ring separated by a flexible linker (**Fig. 1.4**). Unlike other Hv1 inhibitors that are only able to bind within the intracellular vestibule of the channel transmembrane domain in the active/open state, HIF is also able to bind to the channel in the inactive/closed state. Additionally, HIF displays inhibitory properties superior to those of previous compounds; even though HIFs have lower affinity for Hv1 proton channel compared to peptide inhibitors, they have higher efficacy. Furthermore, because of its membrane permeability, HIF can also bind the Hv1 channels located in intracellular compartments unlike other peptide inhibitors that can only bind to the Hv1 channel exposed to the cell surface[63,64].

In the current study, we used *Hvcn1-/-* mice to examine the role of Hv1 in a clinically relevant murine model of blinding *Pseudomonas aeruginosa* corneal infection (keratitis). Our findings that *Hvcn1-/-* mice an impaired ability to clear *P. aeruginosa* from infected corneas compared to C57BL/6 mice demonstrate the importance of Hv1 and sustained ROS production. Also, our findings that a novel Hv1 proton channel inhibitor (Hv1 Inhibitor Flexible [HIF]) blocked ROS production by *P. aeruginosa* infected human and murine neutrophils illustrates the potential for targeting Hv1 therapeutically in keratitis and other infections.

**Materials and Methods**

*Mice*

20

Male and female C57BL/6J mice aged 6-8 weeks were purchased from The Jackson

Laboratory (Bar Harbor, ME). Two *Hvcn1-/-* breeding pairs were graciously provided

from Wu and colleagues, Harvard Medical School. Mice were bred under IACUC

approved conditions and all animals were housed in the University of California, Irvine

vivarium. Age-matched, male and female mice were used for all experiments.

*Bacterial strains and culture conditions*

PAO1-GFP, a *Pseudomonas aeruginosa* strain that expresses Green Fluorescent

Protein, was obtained from Dr. A. Rietsch (Case Western Reserve University). Bacteria

were grown to midlog phase ($\sim 1 \times 10^8$ bacteria/ml) in high-salt Luria-Bertoni (LB) broth,

at 37°C with 5% $CO_2$, 200 rpm. Bacteria were then resuspended in 1X PBS to $5\times10^4$

bacteria/2 µl for all in vivo infections.

*Murine model of Pseudomonas keratitis*

Three corneal epithelial abrasion of 10 mm were performed using a sterile 30-gauge

needle followed by topical infection of $5\times10^4$ PAO1-GFP in 2 µl 1X PBS as described

previously[85]. CFU was quantified at 2 hpi to verify the inoculum for each experiment. At

24 hpi, mice were euthanized and corneal opacity and GFP fluorescence were imaged

and quantified. Imaging and quantification of light reflected or emitted from infected

mouse corneas. Mice were euthanized by $CO_2$ asphyxiation and positioned in a 3-point

stereotactic mouse restrainer for eye imaging. Corneal opacity (brightfield [BF]) and

bacterial burden (GFP) were visualized in the intact cornea using a high-resolution

stereo fluorescence MZFLIII microscope (Leica Microsystems). ImageJ was then used

to calculate corneal opacity and GFP intensity. All images were obtained using the

same Spot Advanced Software under the same magnification (×20), exposure (BF, 0.4

seconds; eGFP, 2 seconds), gain (BF, 1; RFP/eGFP, 4/16), and gamma

(BF/RFP/eGFP, 1.85) parameters.

*CFU Quantification*

At 2 (inoculum) or 24 hpi, whole eyes were collected and homogenized in 1 ml 1X PBS.

Serial log dilutions of the homogenate were plated on LB plates and incubated overnight

at 37°C with 5% $CO_2$. Colonies were counted manually, and CFUs were calculated as:

(number of colonies × dilution factor × 100 [10 µl of the 1 ml was used for plating and

dilution])

*Flow cytometry*

Dissected corneas were incubated in 500 µl of 3 mg/ml collagenase (C0130;

SigmaAldrich) in RPMI (Gibco), with 1% HEPES (Gibco), 1% penicillin-streptomycin

(Gibco), 0.5% BSA (Fisher Bioreagents), and 2 µl of 1M Calcium Chloride for 1 h at

37°C. Cells were incubated for 10 min with anti-mouse CD16/32 Ab (BioLegend) to

block Fc receptors. Cells were then incubated 20 min at 4°C with anti-mouse CD45-PE-

Cy5, Ly6G-BV510, Ly6C-PE-Cy7, CD11b-PETxRed, CCR2-BV421, and F4/80-FITC

(BioLegend) and fixable viability dye (BD Biosciences). Cells were rinsed with FACS

buffer and fixed with Cytofix/Cytoperm (BD Biosciences) for 15 min at 4°C. Fixed cells

were washed with 1X PBS and resuspended in FACS buffer.

*Reactive Oxygen Species Assay*

Neutrophils were incubated with luminol (Sigma) for 30 min before stimulation. Curdlan

(100 ug/ml), PMA (100 ug/ml, Sigma), zymosan (100 ug/ml), PA01 (MOI 30), or *ΔpscD*

(MOI 30) were added and immediately read on the Cytation5 for 90 min. The curves of

technical replicates were used to calculate the area under the curve.

The Hv1 Inhibitor Flexible or HIF was design and produced by Dr. Francesco Tombola Lab in the department of Physiology and Biophysics in the School of Medicine at the University of California, Irvine. HIF was diluted in RMPI media and was used to treat neutrophils in 5, 10, 20, and 50 μM concentrations.

**Results**

**The Hv1 voltage-gated proton channel is required to kill *Pseudomonas aeruginosa* in infected corneas**

We reported that NOX2 is required kill *P. aeruginosa* in infected corneas as gp91$^{phox}$/CybB$^{-/-}$ mice are unable to clear ExoS expressing PAO1 strain or a T3SS mutant[81]. As Hv1 sustains release of ROS generated by NOX2, we determined if there is the role of the Hv1 voltage-gated proton channel in *Pseudomonas aeruginosa* keratitis. C57BL/6 (WT) and *Hvcn1$^{-/-}$* corneas were abraded and infected with 5×10$^4$ PAO1-GFP, an ExoS *Pseudomonas aeruginosa* strain that expresses Green Fluorescent Protein as we described[11]. At 24hpi, corneas were imaged and whole eyes were homogenized to quantify colony forming units (CFU). Images of the corneas were used to calculate corneal opacity, GFP-intensity as a quantification of total bacteria, and CFU as a measure of bacterial killing.
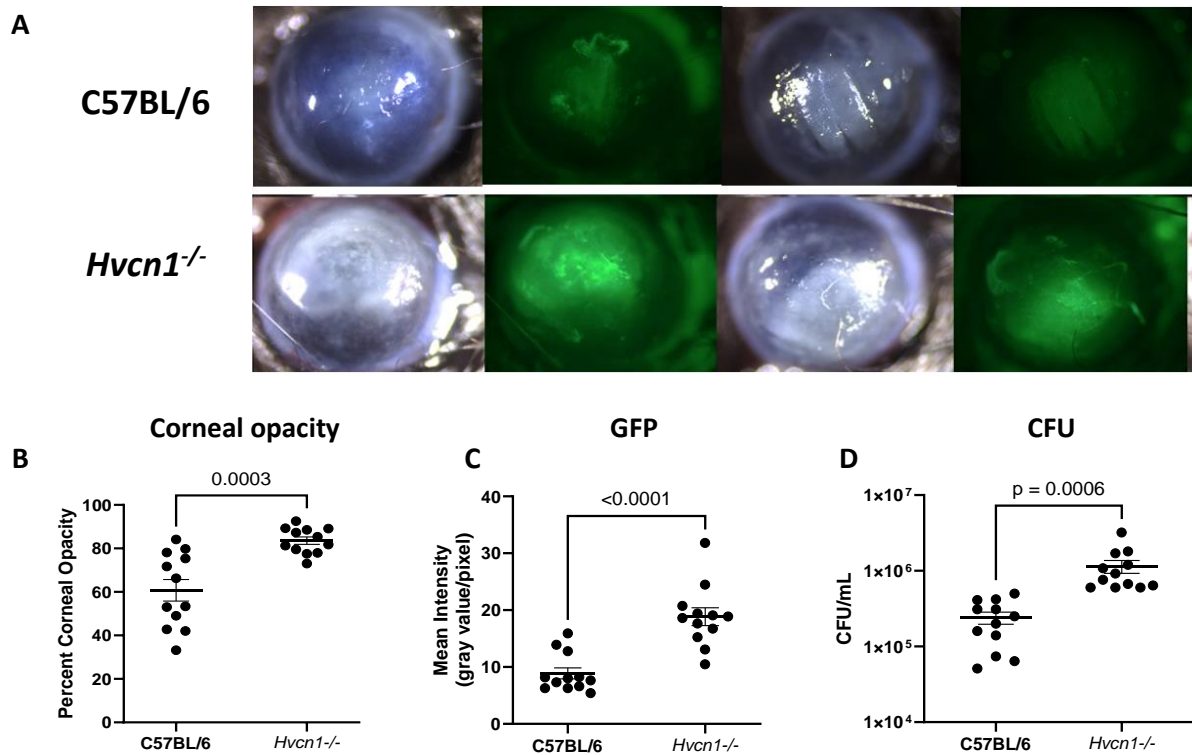
**Fig. 2.1. Role of Hv1 in *Pseudomonas aeruginosa* keratitis.** Corneas of C57BL/6 and *Hvcn1-/-* mice were abraded and infected with $5 \times 10^4$ bacteria/2 μl GFP-expressing PAO1, and corneas were examined after 24h. A) Representative images of corneal opacity (brightfield) and GFP (bacteria) in infected C57BL/6 and *Hvcn1-/-* mice. B) Quantification of corneal opacity by image analysis using ImageJ. C) Total GFP+ bacteria in infected corneas quantified by image analysis. D) Viable bacteria quantified by CFUs. Significance calculated using paired t-test, n=12, three experiments combined.

Although corneal opacification was observed in both strains of mice, infected *Hvcn1-/-* corneas had had significantly higher corneal opacity (**Figure 2.1A,B**). Total bacterial mass (GFP) and viable bacteria (CFU) were also significantly higher in infected *Hvcn1-/-* corneas compared with C57BL/6 (**Figure 2.1C,D**). Collectively, these results show that disease is exacerbated in *Hvcn1-/-* mice compared to control mice and indicate that the Hv1 proton channel is required to clear PA01 infection.

## Elevated cytokine production in infected *Hvcn1-/-* corneas

To determine the outcome of Hv1 deficiency on cytokine production, *Hvcn1^{-/-}* and C57BL/6 corneas were dissected 24hpi. Corneas were homogenized, and pro-inflammatory and chemotactic cytokines (chemokines) were quantified by ELISA. As shown in **Fig. 2.2**, the concentration of IL-1α, IL-1β and CXCL1 was significantly higher in infected *Hvcn1^{-/-}* corneas compared to C57BL/6 mice, and although CXCL2 was also elevated in *Hvcn1^{-/-}* corneas, the difference was not statistically significant. Collectively, these findings demonstrate that there is increased inflammation in infected *Hvcn1^{-/-}* corneas, which is associated with elevated corneal opacification.
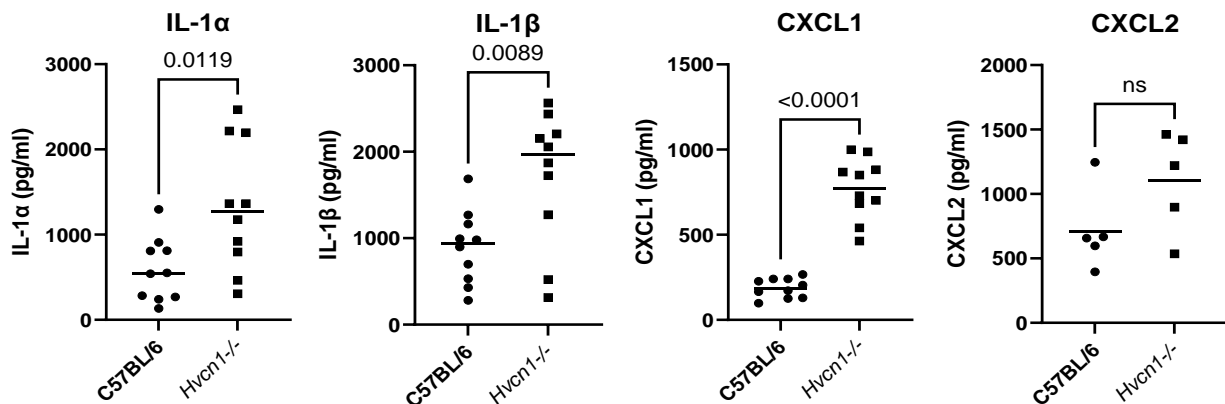


**Fig. 2.2. Cytokine production in PA01-infected corneas.** Corneas of C57BL/6 and *Hvcn1^{-/-}* mice were infected with $5 \times 10^4$ bacteria/2 µl PAO1. After 24h, corneas were homogenized and pro-inflammatory cytokines and chemokines were measured by ELISA. n=5-10, one or two experiments combined. Statistical analysis calculated using a paired t-test.

## Hv1 does not regulate neutrophil recruitment to infected corneas

Healthy corneas have relatively low numbers of resident dendritic cells and macrophages, but no resident neutrophils. However, following *P. aeruginosa* infection, >90% total cells in the cornea are CD45+ myeloid cells that primarily comprise

neutrophils and inflammatory monocytes in the first 72h[12,11]. As elevated cytokine

production is generally associated increased cellular infiltration, and Hv1 promotes

neutrophil migration by regulating calcium signaling, we examined the effect of Hv1

deficiency on neutrophil recruitment to infected corneas[7]. C57BL/6 and *Hvcn1*^{-/-} corneas

were collagenase digested 24hpi, and single cell suspensions were gated for live

singlets and incubated with antibodies to monocytes (CD45+/CD11b+/Ly6G-) and

neutrophils (CD45+/CD11b+/Ly6G+) were quantified by flow cytometry. Gating strategy

is shown in **Fig. 2.6**.

We found that neutrophils comprised ~85-90% of the total cellular infiltrate in

infected C57BL/6 corneas with monocytes comprising the remaining 10-15%. However,

there was no significant difference in the number or percentage of infiltrating neutrophils

or monocytes in *Hvcn1*^{-/-} and C57BL/6 mice (**Figure 2.3A,B**). We also visualized the

infected corneas by histology and IHC following formalin fixation and paraffin

embedding to generate 5 µm sections. While there was a pronounced cellular infiltrate

in the corneal stroma of C57BL/6 corneas, there was no apparent difference cell

numbers between C57BL/6 and *Hvcn1*^{-/-} corneas (**Figure 2.3C**).

In conclusion, the increased corneal opacification and cytokine production in

infected *Hvcn1*^{-/-} corneas does not appear to be due to increased neutrophil infiltration,

and is more likely due to more activated neutrophils.

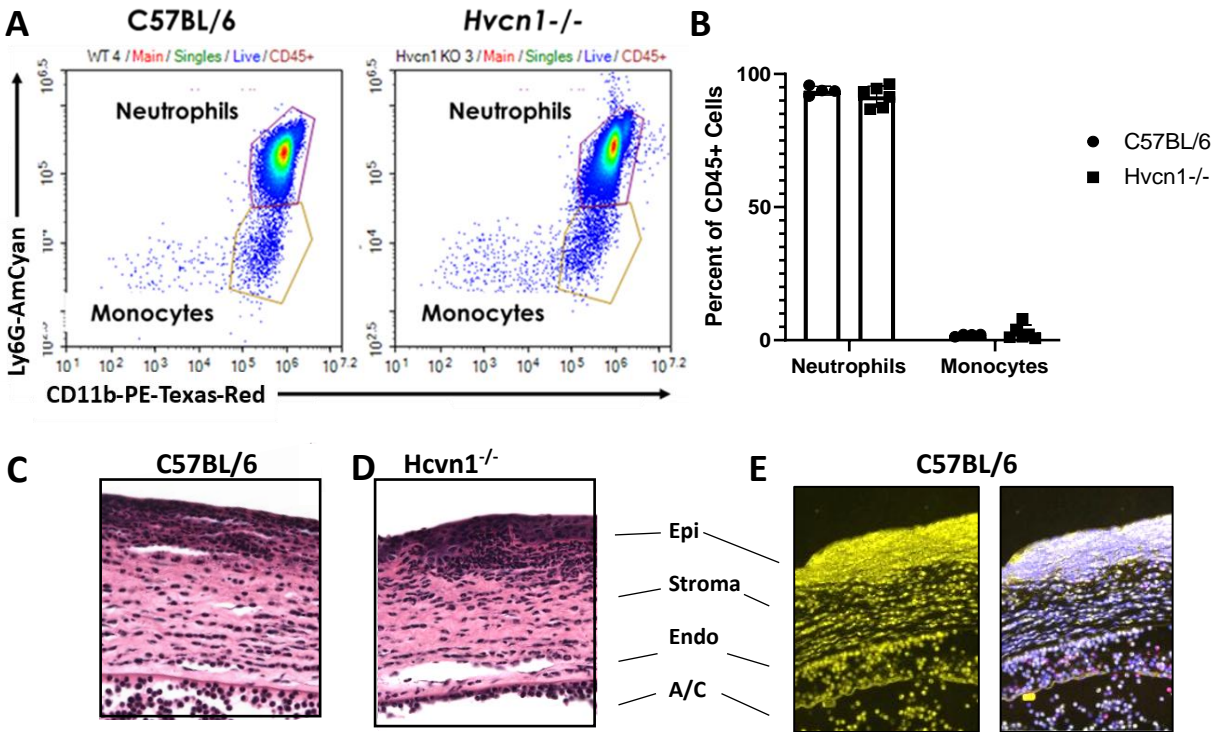**Fig. 2.3. Neutrophil and monocyte recruitment to PAO1-infected corneas.** Corneas of C57BL/6 and Hvcn1^{-/-} mice were infected with $5\times10^4$ bacteria/2 µl of PAO1. At 24h post infections, corneas were dissected and digested in collagenase, and the number of neutrophils and monocytes was assessed by flow cytometry. A) Representative flow cytometry plots of live, single, CD45+ cells. Neutrophils are CD11b+ Ly6G+ and monocytes are CD11b+Ly6G-. B) Neutrophils and monocytes were calculated as a percentage of total CD45+ cells. n=6, two experiments combined. C, D) H&E-stained sections of infected corneas from C57BL/6 (C) and Hcvn1^{-/-} mice (D). E) C57BL/6 immunostained with anti-Ly6G and DAPI that shows neutrophils in the epithelial layer. Original magnification is x400. Epi: epithelium; endo: endothelium; A/C: anterior chamber.

## The Hv1 Inhibitor Flexible (HIF) blocks ROS production by murine and human neutrophils and inhibits bacterial killing

Recently, the Tombola group at the University of California, Irvine, designed and generated a potent and highly selective Hv1 voltage-gated proton inhibitor called Hv1 Inhibitor Flexible (HIF) that directly interacts with the voltage sensing domains[62,86]. To test this novel inhibitor in neutrophil ROS production, bone marrow neutrophils from C57BL/6 mice were incubated with increasing concentrations of HIF and infected with

live PAO1 or with the Type III secretion mutant $\Delta pscD$. ROS was then quantified over 90 minutes using the Luminol reagent. Neutrophils were also incubated with the NOX2 inhibitor Diphenyleneiodonium (DPI).

We reported that *P. aeruginosa* ExoS inhibits NOX2 activity by ADP ribosylating RAS[81]; therefore, ROS is generated following infection with the T3SS injectosome mutant $\Delta pscD$, which does not form a needle structure and therefore cannot injected ExoS or other exoenzymes into host cells.

We found that DPI completely blocked ROS production as expected; however, HIF also inhibited ROS production in $\Delta pscD$ infected and PMA-stimulated neutrophils in a dose-dependent manner (**Fig. 2.4** and **Fig. 2.5**). HIF also inhibited ROS production by murine and peripheral blood human neutrophils stimulated with curdlan or zymosan (**Fig. 2.7** and **Fig. 2.8**).

**Murine neutrophils**



**Fig. 2.4. HIF inhibits extracellular ROS production in a dose-dependent manner in murine bone marrow neutrophils.** Murine bone marrow neutrophils were isolated and treated with 500 µM luminol and HIF then activated with A) PMA (100 µM), B) *ΔpscD* (MOI 30) or C) PA01 (MOI 30). Representative time course of ROS production with area under the curve calculated from technical replicates. n=3-6, one to two experiments combined, p<0.05 for PMA and *ΔpscD*.

**Human neutrophils**

**A** PMA

Legend: Untreated, 1 uM, 2 uM, 5 uM, 10 uM (HIF), DPI

**B** *ΔpscD*

Legend: Untreated, 5 uM, 10 uM, 20 uM, 50 uM (HIF), DPI

**C** PA01

Legend: Untreated, 5 uM, 10 uM, 20 uM, 50 uM (HIF), DPI

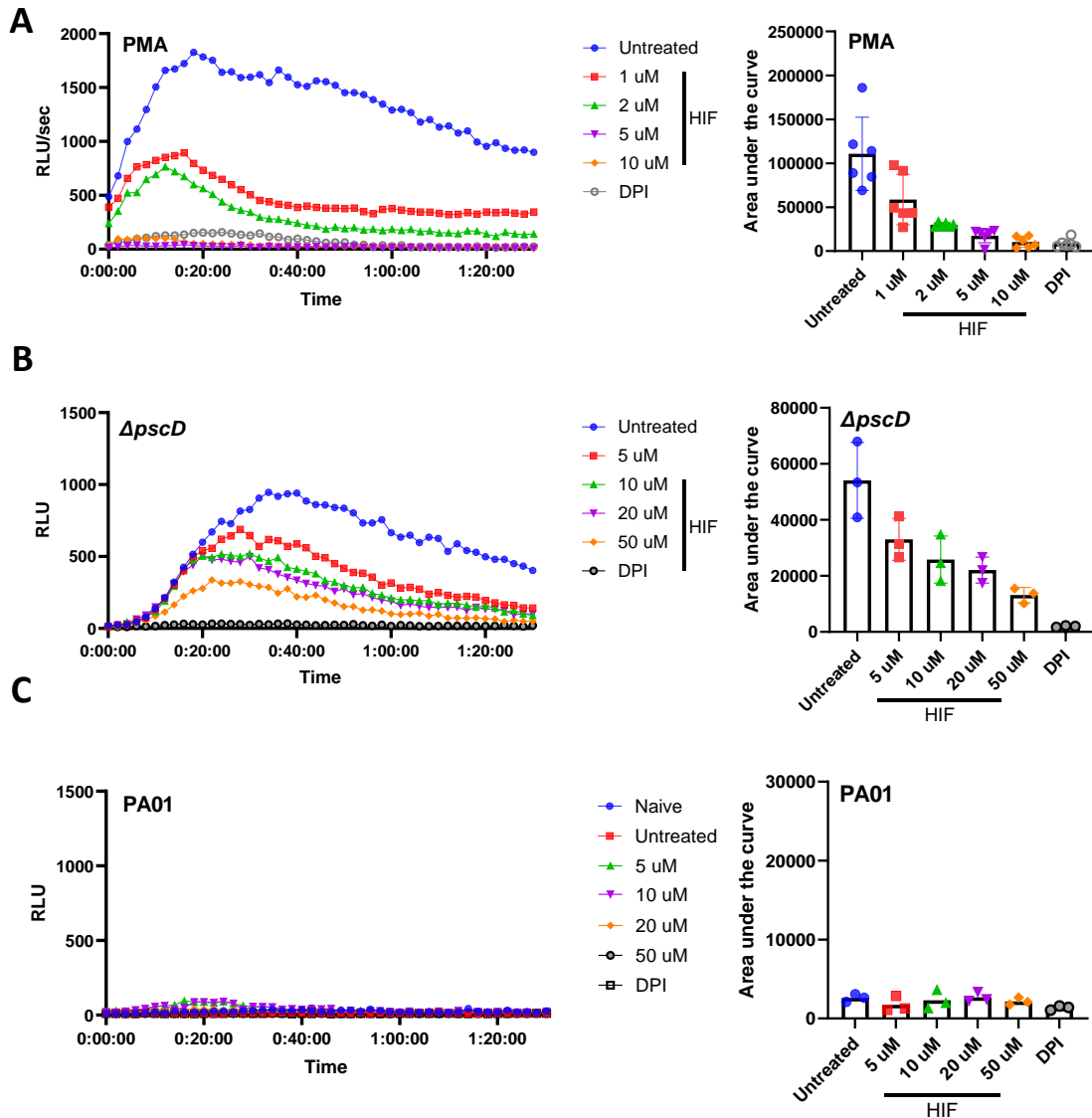**Fig. 2.5. HIF inhibits extracellular ROS production in a dose-dependent manner in human neutrophils.** Human neutrophils were isolated and treated with 500 μM luminol and HIF then activated with A) PMA (100 μM), B) *ΔpscD* (MOI 30) or C) PA01 (MOI 30). Representative time course of ROS production with area under the curve calculated from technical replicates. n=3-6, one to two experiments combined, $p < 0.05$ for PMA and *ΔpscD.*

## Supplemental figures
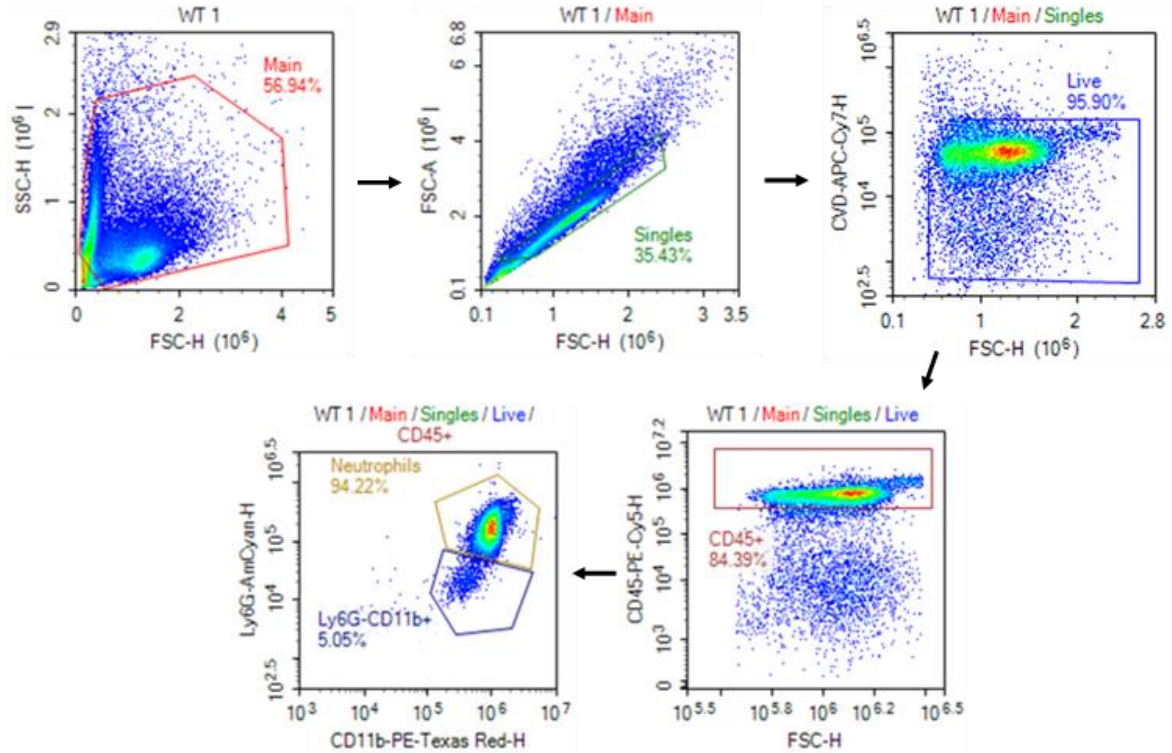


**Fig. 2.6. Gating strategy for detection of CD45+ myeloid cells in infected corneas.** After gating on total (main) cells, we gated for single cells and then viable cells using cell viability dye to identify dead cells. Live cells were gated for total CD45+ cells, from which neutrophil (CD11b+Ly6G+) and monocytes (Ly6G-CD11b+) were identified. Gating was performed using fluorescence minus one (FMO) controls (not shown).
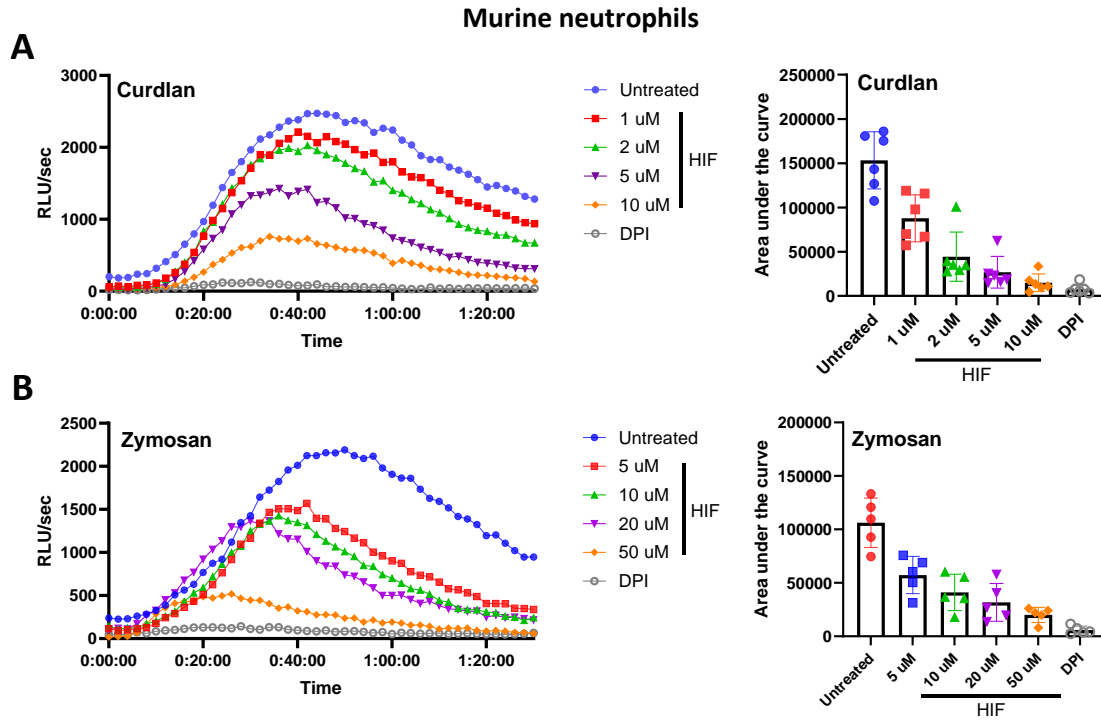
**Fig. 2.7. HIF inhibits extracellular ROS production in a dose-dependent manner in murine bone marrow neutrophils when stimulated with curdlan and zymosan.** Murine bone marrow neutrophils were isolated and treated with 500 µM luminol and HIF then activated with A) curdlan (insoluble b-glucan) (100 µg/ml), or B) zymosan (100 µg/ml). Representative time course of ROS production with area under the curve calculated from technical replicates. n=3-6, one to two experiments combined, p<0.05.
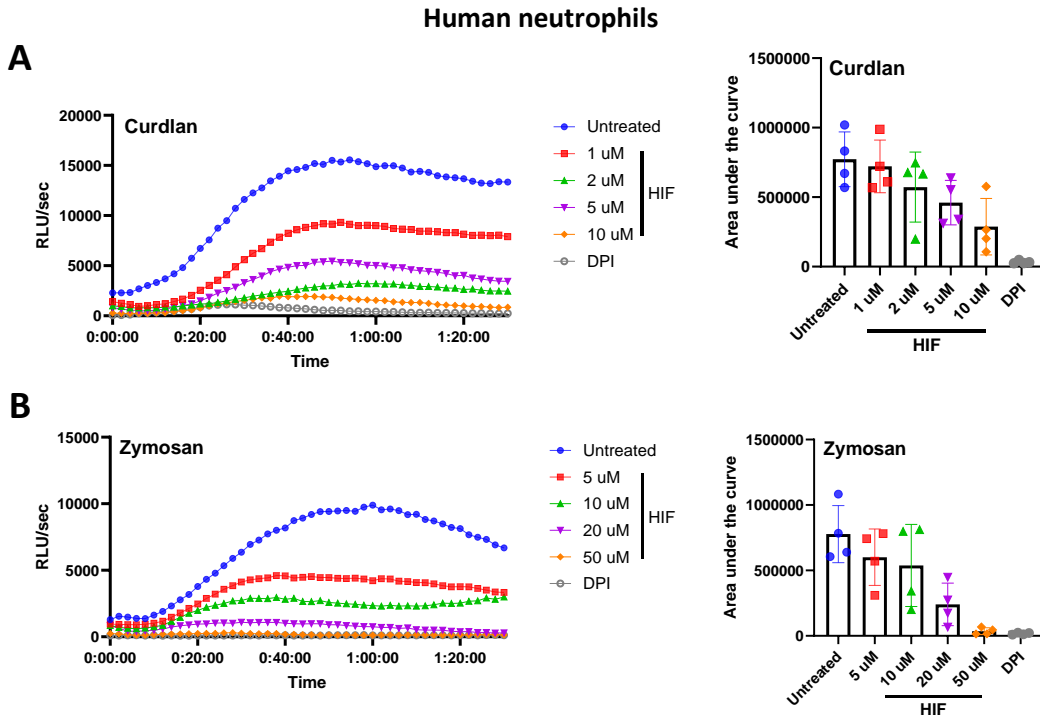
**Human neutrophils**

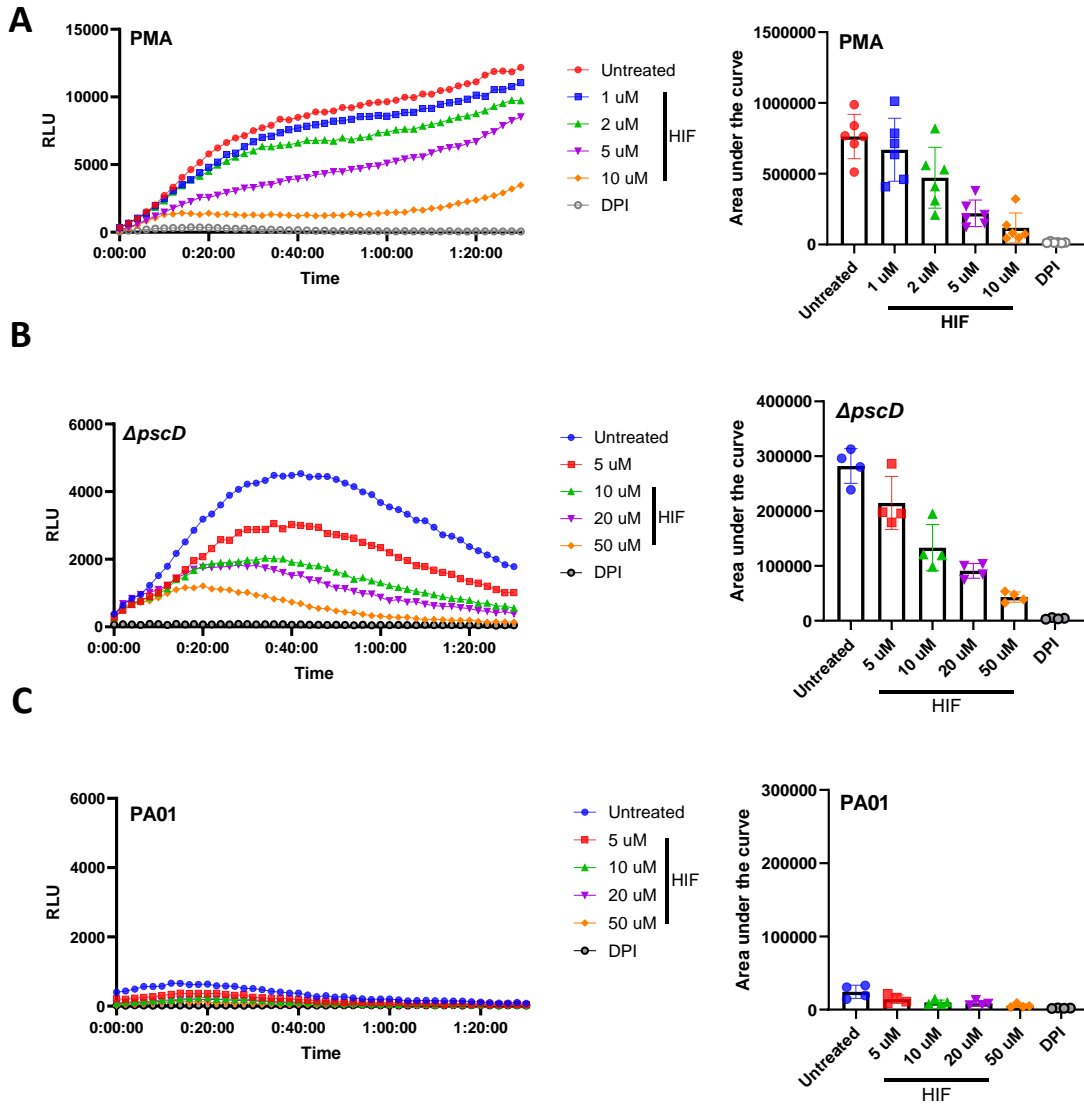**Fig. 2.8. HIF inhibits extracellular ROS production in a dose-dependent manner in human neutrophils when stimulated with curdlan and zymosan.** Human neutrophils were isolated and treated with 500 μM luminol and HIF then activated with with A) curdlan (insoluble b-glucan) (100 μg/ml), or B) zymosan (100 μg/ml). Representative time course of ROS production with area under the curve calculated from technical replicates. n=3-6, one to two experiments combined, p<0.05.

# CHAPTER 3:


# Conclusion and future directions

These findings identify an important role for Hv1 in bacterial keratitis, and demonstrate that HIF potently blocks ROS production and bacterial killing. Bacterial keratitis is a major cause of corneal blindness worldwide, and the current treatment following includes antibiotics followed by corticosteroids to reduce the inflammation and inhibit fibrosis. However, when corticosteroids are combined with an ineffective antimicrobial treatment, the bacteria or fungi return in higher concentrations due to the suppressed immune response caused by the corticosteroids. Additionally, there are undesirable side effects when treating with corticosteroids such as increased intraocular pressure and increased risk for glaucoma[87,88]. Thus, new and effective treatments to reduce residual inflammation in bacterial keratitis are needed. Some of this inflammation is caused by ROS production by neutrophils and inhibition of this ROS production by HIF and other Hv1 proton channel inhibitors present a potential therapeutic approach not only for bacterial keratitis but also for other inflammatory conditions.

Whether HIF can be used as a substitute for corticosteroids in the treatment of bacterial keratitis remains to be tested and presents a potential therapeutic option to prevent fibrosis in bacterial keratitis. To test this hypothesis, we would have to determine the best route of delivery (ideally by topical administration as eye drops) and the optimal HIF concentration to inhibit ROS production in *P. aeruginosa* corneal infection after treatment with antibiotics.

Topically applied compounds may enter the eye by two routes: trans-corneal or trans-scleral[89]. However, trans-corneal delivery provides a more direct route of absorption. The pore diameters of the cornea is 2.0 nm ± 0.2 which limits the

penetration of molecules to <500 Da in size[90,91]. While lipophilic drugs may penetrate through transcellular pathways, hydrophilic compounds cross the cornea by the paracellular route which is hindered by corneal epithelial tight junctions which prevent drug passage[89]. Theoretically, HIF, being a lipophilic compound should cross the epithelium of the cornea through the paracellular route. If we find that we cannot deliver HIF topically, we propose to use nanoparticles that could more readily cross the epithelial barrier of the cornea.

Another inhibitor for the Hv1 voltage-gated proton channels called Corza6 (C6) peptide was used by Zhao, Goldstein *et al.* to ameliorate ROS production in human white blood cells (WBC) *in vitro*, demonstrating that the Hv1 proton channel plays an important role in sustaining NOX2 activity in WBC[86]. These investigators also used an lipopolysaccharide (LPS)-induced acute lung injury (ALI) module and showed that the C6 peptide ameliorates bacterial infection in mice by suppressing neutrophil infiltration and production of ROS, and pro-inflammatory cytokines in LPS-challenged mice[62]. Zhao *et al.* have graciously provided C6 peptide and future directions will include testing in *P. aeruginosa* corneal infection.

Other therapeutic approaches use resolvins and protectins to enhance production of pro-inflammatory lipid mediators that accelerate resolution of inflammation[92]. In the cornea, Resolvin E1 was used by the Pearlman group to ameliorate neutrophil recruitment and cytokine production in response to tobramycin killed *Staphylococcus aureus*, *P. aeruginosa*, and LPS[93]. Targeting TLRs on corneal epithelial cells and neutrophils is another approach. Using the TRL4 antagonist Eritoran tetrasodium, the Pearlman group demonstrated that neutrophil recruitment and cytokine

production were reduced after treating with topical Eritoran in LPS- and *Pseudomonas aeruginosa*-induced corneal inflammation[94]. As neutrophil mediated tissue damage occurs as a result of release of neutrophil granules containing proteolytic serine and matrix metalloproteinases, Catz et al generated small molecule inhibitor called Nexinhibs, that target the small GTPase Rab27a that is critical for transport of granules to the plasma membrane for degranulation. These inhibitors were found to reduce neutrophil mediated inflammation by blocking degranulation of azurophilic granules[95]. Nexinhibs were kindly sent to the Pearlman lab and will be used in future studies to block inflammation in inflamed and infected corneas. While no single approach may be sufficient to regulate inflammation and prevent fibrosis, a combination of inhibitors in the pathway of inflammation presents potential new therapeutic options to lower inflammation in the cornea during bacterial keratitis. Also, although I have been focused on corneal disease, these approaches hold potential to regulate inflammation and tissue damage in other sites of infection.

In conclusion, we demonstrated that the Hv1 voltage-gated proton channel is needed to sustain NOX2-mediated ROS production. We also demonstrated that HIF inhibits ROS production in a dose dependent manner in murine and human neutrophils *in vitro* when stimulated with *ΔpscD*, PMA, curdlan, or zymosan. The Hv1 proton channel therefore presents a therapeutic target for the treatment of bacterial keratitis and HIF could potentially be used to inhibit fibrosis in *P. aeruginosa* corneal infection.

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