ANTIMICROBIAL PEPTIDE MEDIATED HOST DEFENSE IN BACTERIAL KERATITIS

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To

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CERTIFICATE

This is to certify that the thesis entitled "**Antimicrobial Peptide Mediated Host Defense in Bacterial Keratitis**" submitted by **Prerana Sharma** bearing reg. no. **16LAPH06** in partial fulfilment of the requirements for the award of Doctor of Philosophy in Microbiology and Immunology is a bonafide work carried out by her under our supervision and guidance. This thesis is free from plagiarism and has not been submitted previously in part or in full to this or any other University or Institution for the award of any degree or diploma. Parts of this thesis have been:

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1. Presented a poster entitled "Role of antimicrobial peptides in *Pseudomonas* keratitis" in IMMUNOCON, 2017, held at GITAM UNIVERSITY, Vishakhapatnam, India.
2. Presented a poster entitled "Host defense mediated by antimicrobial peptides in *Streptococcus pneumoniae* keratitis" in ARVO-IERG, 2018 held at L.V. Prasad Eye Institute, Hyderabad, India.
3. Presented a poster entitled "Role of type III secretion system inhibitor in *Pseudomonas* keratitis" in ARVO-IERG, 2019 held at Sankara Nethralaya, Chennai, India.

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This is to certify that this thesis entitled "**Antimicrobial Peptide Mediated Host Defense in Bacterial Keratitis**" submitted by **Prerana Sharma** bearing registration number 16LAPH06 for the degree of Doctor of Philosophy to the University of Hyderabad is a bonafide record of research work carried out by her at the L.V. Prasad Eye Institute, Hyderabad under my supervision. The contents of this thesis, in full or parts have not been submitted to any other University or Institution for the award of any degree or diploma. I hereby recommend her thesis for submission, for the award of the degree of Doctor of Philosophy from the University.

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DECLARATION

I **Prerana Sharma** hereby declare that this thesis entitled "**Antimicrobial Peptide Mediated Host Defense in Bacterial Keratitis**" submitted by me under the guidance and supervision of **Dr. Sanhita Roy** and **Dr. Suresh Yenugu** is an original and independent research work. I also declare that it has not been submitted previously in part or in full to this University or any other University or Institution for the award of any degree or diploma.

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DEDICATED

TO

THE EYE DONORS & THEIR FAMILY

PATIENTS & THEIR FAMILY

MY BELOVED PARENTS

MY DEAREST FAMILY

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ABBREVIATIONS

Abbreviations/units	Full form/Alternate name
Akt (PKB)	Protein kinase B
AMP	Antimicrobial peptides
AMR	Antimicrobial resistance
ATG	Autophagy related gene/protein
Bec	Beclin
BPE	Bovine Pituitary Extract
C57BL/6	C57 black 6
DAPI	4′,6-diamidino-2-phenylindole
DMEM-F12	Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRAMP	Data repository of antimicrobial peptides
EGF	Epidermal growth factor
ERK	Extracellular-signal regulated kinases
FBS	Fetal Bovine Serum
H_2DCFDA	2',7'-dichlorodihydrofluorescein diacetate
hBD	Human β- defensin
HCEC	Human Corneal Epithelial Cells
Hep	Hepcidin
IL	Interleukin
INP0341	Salicylidene acylhydrazide
JNK	c-Jun N-terminal kinases
KSFM	Keratinocyte Serum Free Media
LB	Luria Bertani
LCN	Lipocalin

LL-37	Leucine Leucine-37
MAPK	Mitogen-activated protein kinase
MG132	N-Benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal
ml	millilitre
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
NF-κβ	Nuclear factor kappa light chain enhancer of activated B cells
nm	nanometre
nM	nanomolar
Nrf2	The nuclear factor erythroid 2-related factor 2
PAMPs	Pathogen associated molecular patterns
PI	Propidium iodide
PI3K	Phosphoinositide 3-kinases
PRR	Pathogen recognition receptors
qPCR/QPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
RNase 7	Ribonuclease 7
ROS	Reactive oxygen species
RPMI 1640	Roswell Park Memorial Institute (RPMI) 1640
SAPK	Stress-activated protein kinases
siRNA	Small interfering RNA/ short interfering RNA/ silencing RNA
STAT-3	Signal transducer and activator of transcription 3
tBHQ	tert-Butylhydroquinone
THB	Todd Hewitt Broth
TLR	Toll-like receptor
U937	Human Monocyte cells
μm	micrometre
μM	micromolar

CHAPTER I

INTRODUCTION

1.1 Human Eye

Eye is the most important sense organ for sight that receives visual images and relays the visual signal to the brain. The human eye is shaped like an asymmetrical sphere and hence also termed as the eyeball. The eye is supported by extraocular muscles and bony socket (Kierstan Boyd, 2018) and is made up of many layers and compartments. Cornea is the anterior-most part of the eye which is in contact with the environment. It is over 0.5 mm thick and its diameter is 11-12 mm (**Figure 1.1**). Cornea is devoid of any blood vessels and maintains its hydration state with the help of the endothelium layer. The cornea is made up of cells and collagen fibers arranged orderly in the stroma. All these factors together makes the cornea tough and transparent (Maurice, 1957; McCaa, 1982). The transparent nature and dome shape of cornea aids in clear vision. Stromal structure of the cornea also governs the spherical shape of cornea. The cornea is one of the essential parts of the eye for focusing light onto the retina, the posterior layer of the eye, where the light is further relayed to the brain via optic nerve for image processing.

The cornea consists of five different layers:

1. Epithelium
2. Bowman's membrane
3. Stroma- constitutes 90% thickness of cornea
4. Descemet's membrane
5. Endothelium- Innermost part of cornea consisting of hexagonal monolayer cells.

The tear film layer is anterior to the corneal epithelium and essentially contains secretions from the goblet cells, lacrimal glands, meibomian glands and the glands of Moll and Zeis (McCaa, 1982).

Corneal epithelium is the anterior-most layer of the cornea that protects it from the invading pathogens and the outside environment, while maintaining its transparency for vision. It is made up of 5-6 layers of squamous non-keratinized epithelial cells and is 30-50μm thick. The tight junction between the epithelial cells prevents the penetration of microbes and fluids in the stroma. The anterior 2-3 layers of epithelium are made up of flat and polygonal cells with multiple microvilli and microplicae (ridges) coated with glycocalyx that interacts with and stabilizes the tear film. The next 2-3 layers of epithelium has wing or suprabasal cells with tight junction complexes between them. The posterior-most layer of the corneal epithelium is made up of perilimbal basal epithelial cells that differentiate and migrate

towards the anterior layers to replace the old cells (P. Asbell & Brocks, 2010; Eghrari et al., 2015; Forrester et al., 2016).

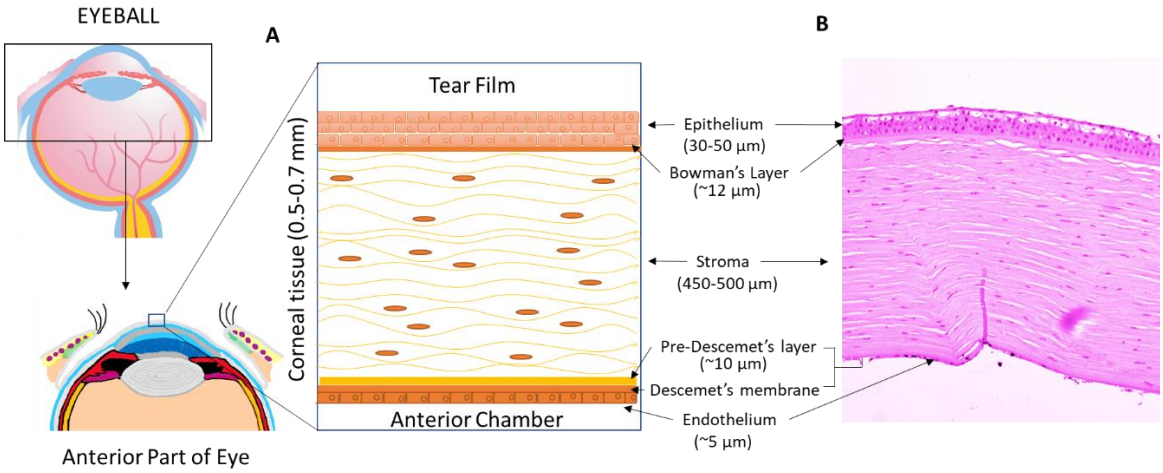


Figure 1.1 Representative images of cornea and bacterial keratitis **A**) Cartoon of corneal layers; B) Tissue section of cornea. Redrawn from (Urwin et al., 2020).

Posterior to the corneal epithelium is an acellular, non-regenerating layer, approximately 12µm in thickness, called Bowman's layer which is made up of randomly placed collagen fibrils within an extracellular matrix. It helps in maintaining the shape of cornea. The posterior surface of the bowman's layer merges with stromal collagen lamellae (Sridhar, 2018; Wilson, 2020). The corneal stroma comprises of dense, regularly arranged collagen fibrils. This layer helps provide mechanical strength to the cornea and maintain its transparency. The stroma helps in refraction of light onto the lens. The descemet's membrane comprises of resting endothelial cells and the corneal endothelium helps in maintaining hydration and clarity of cornea via various molecular transporters and channels (Sridhar, 2018) (**Figure 1.1**).

1.2 Corneal Infection (Keratitis)

Corneal infections, also known as keratitis, is an inflammatory disease of the cornea and may lead to the development of corneal ulcers and opacification due to neovascularization and scarring eventually resulting in loss of vision. Cornea normally remains intact and can repair minor abrasions. However, sometimes because of corneal abrasions, bacteria can breach the cornea and cause ocular trauma (Keay et al., 2006) or infections (**Figure 1.2**).In a study on cases published between 2010-2020 from India and western world, it was found that about 23.4 to 100% of the cases of infectious keratitis had bacterial etiology (Ting, Ho, Deshmukh, et al., 2021).

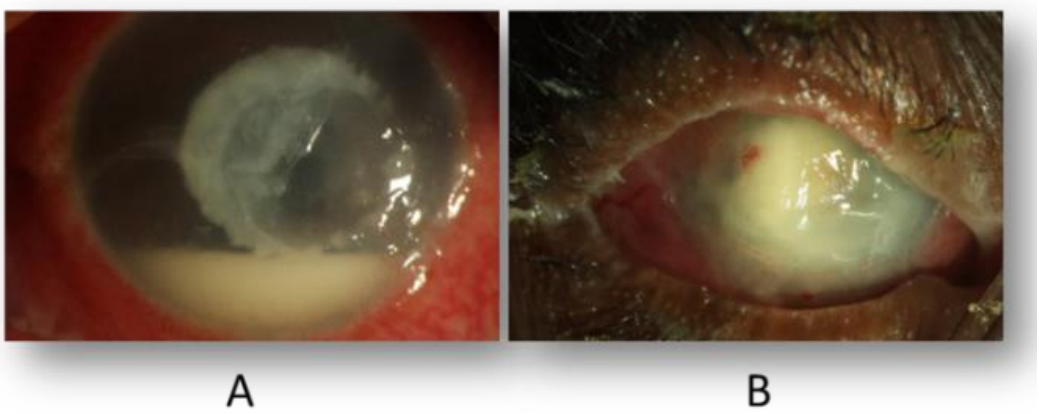


Figure 1.2 Representative images of bacterial keratitis. A) *P. aeruginosa* keratitis; B) *S. pneumoniae* keratitis

The most common predisposing factor for keratitis in developed countries is contact lens wear (Bourcier et al., 2003; Keay et al., 2006; Sagerfors et al.; Sauer et al., 2020), whereas in developing countries it is ocular trauma (Ting, Ho, Deshmukh, et al., 2021). Other risk factors include corneal graft failure (Okonkwo et al., 2018), and chemical or ultraviolet light exposure (*Estimated Burden of Keratitis — United States, 2010*, n.d.). Known risk factors for infective keratitis are given in **Table 1.1.**

Out of the many pathogens that are responsible for infectious keratitis, fungus and bacteria are the most common ones. Bacterial keratitis is one of the leading cause of corneal opacification and is the major cause of blindness burden both in India and worldwide (Ung et al., 2019).

Table 1.1 Risk factors for infectious keratitis (Acharya, Farooqui, Jain, et al., 2019)

LOCAL	SYSTEMIC
Trauma to intact epithelium	Immunodeficient states- HIV AIDS, Malignancy, Drug induced
Contact lens wear	Connective tissue disorder-Like rheumatic arthritis
Eyelid-Entropion, extropion, adnexal infection	Diabetes
Neurotrophic disease	Measles
Bullous keratopathy	Malnutrition
Ocular surface disease	Diarrhea
Topical medications- eg. steroids	

The most common pathogens causing bacterial keratitis are *Staphylococcus aureus*, *Pseudomonas aeruginosa*, coagulase-negative *Staphylococcus* and *Streptococcus* spp. The most prevalent bacteria in the cases of bacterial keratitis are given in **Table 1.2.**

Table 1.2 Bacterial keratitis causative organisms

Area/ Country	% Bacterial keratitis	Gram positive/ negative	Gram positive	Gram negative	Reported by
Tertiary care Centre, Eastern India	60%	Gram positive	*Streptococcus pneumoniae*	*Pseudomonas* spp.	(Das et al., 2019)
Aravind Eye Hospital, South India	7.5%	Gram positive	*Streptococcus pneumoniae*	-	(Chidambaram et al., 2018)
North India	1169 cases	Gram positive	coagulase negative *Staphylococcus*	*Pseudomonas* spp.	(Acharya, Farooqui, Singh, et al., 2019)
South Texas	95.1%	Gram positive	coagulase negative *Staphylococcus*	*Pseudomonas* spp.	(Puig et al., 2020)
Nottingham, UK	92.8% (4.5% polymicrobial)	Gram positive	*S. aureus*	*P. aeruginosa*	(Ting, Ho, Cairns, et al., 2021)
Sydney, Australia	65% (2.3% polymicrobial)	Gram positive	*Staphylococcus epidermidis*	-	(Khoo et al., 2020)
North India	54.2%	Gram positive	*Staphylococcus* spp.	*Pseudomonas* spp.	(Singh et al., 2020)
Nepal	43.4%, mixed 2.7%	95% Gram positive	*Streptococcus pneumoniae* 45.5%		(Bajracharya et al., 2020)
San Francisco	23.7%	65.3% Gram positive	Methicillin-sensitive *Staphylococcus aureus* 20.1%	34.7% Gram negative of which *P. aeruginosa* 10.9%	(Peng et al., 2018)

At present, the treatment of bacterial keratitis is done mainly by using conventional antibiotics (Mah & Baum, 2015), alone or in combination with few other adjunctive therapies like bacteriophage therapy (Ibrahim et al., 2020; O'Brien, 2003). Some of the antibiotics currently used for management of bacterial keratitis are cefazolin, vancomycin, bacitracin, fluoroquinolones, tobramycin, gentamicin, ceftazidime etc. (Lin et al., 2019).

1.3 Pathogenesis of causative agents of bacterial keratitis

1.3.1 *Pseudomonas aeruginosa*

P. aeruginosa is a gram-negative opportunistic pathogen and is responsible for many diseases (Lyczak et al., 2000). It is one of the main causes of nosocomial infections and infections in immunocompromised individuals. It is also one of the leading causes of contact lens wear-related infectious keratitis (Lap-Ki Ng et al., 2015). *P. aeruginosa* is known for causing more severe ulcers at presentation that are often difficult to cure, leading to a worse visual outcome.

P. aeruginosa harbors several virulence factors that is controlled by a complicated regulatory network (Balasubramanian et al., 2013). These virulence factors can also act together and play a crucial role in infection (D. G. Lee et al., 2006). The major virulence factors of *P. aeruginosa* includes lipopolysaccharide, alginate, flagella, hydrogen cyanide, pyocyanin, pyoverdine, rhamnolipid, type IV pili, various exotoxins and secretion systems (Fleiszig & Evans, 2002; Hilliam et al., 2020) (**Figure 1.3**). Till now six different classes of secretion systems (Type-I to VI) have been described that are present in *P. aeruginosa* (Bleves et al., 2010; Juhas, 2015). Amongst all, Type-3 secretion system (T3SS) is the most studied one and has been shown to play a critical role in the virulence of *P. aeruginosa* (Filloux, 2011). Additionally, pili of *P. aeruginosa* aids in the invasion of the corneal epithelial cells *in vitro* and helps in the colonization of the cornea *in vivo* (Zolfaghar et al., 2003). Secreted bacterial proteases like *P. aeruginosa* small protease (PASP) also play an important role in development of keratitis (Tang et al., 2018).

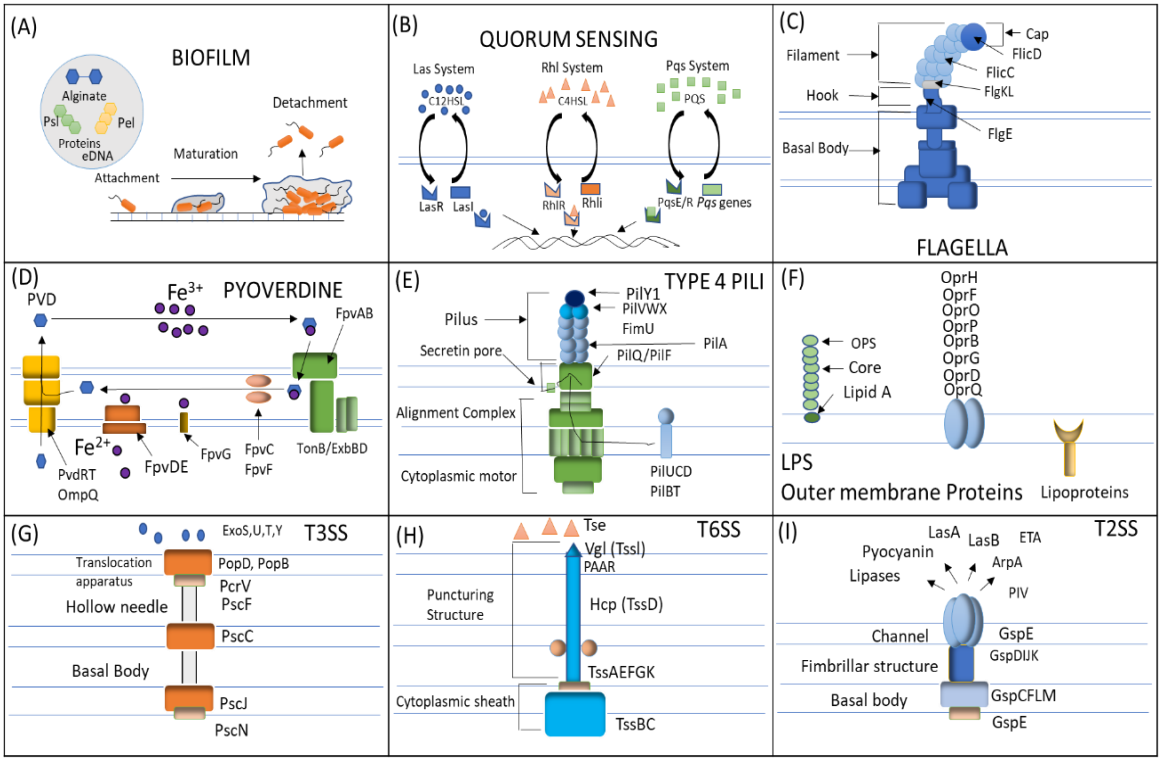


Figure 1.3 Different virulent factors and systems of *P. aeruginosa*. (A) Biofilm (B) Quorum sensing (C) Flagella (D) Pyoverdine iron uptake siderophore (E) Type 4 pili (F) Lipopolysaccharide and outer membrane proteins (G) T3SS (H) Type VI secretion system (J) Type II secretion system. Redrawn from (Jurado-Martín et al., 2021)

Pathogenesis of *P. aeruginosa* is facilitated by various virulent factors that help in adhesion, modulation or inhibition of host cell pathways and extracellular matrix. A brief flowchart about pathogenesis of *P. aeruginosa* is given in **Table 1.3**.

Table 1.3 Pathogenesis of *P. aeruginosa*

Adhesion	Invasion and cytotoxic effect	Stromal necrosis and ring infiltrate formation
• mediated by bacterial adhesins, glycocalyx	• Elastase A and B, modified elastase, alkaline protease, protease IV, PA small protease (PASP)- cleaves collagen and large exoprotease. MucD protease supress 1L1B, KC and MIP2 and neutrophil recruitment. • Metalloproteases-elastase B and alkaline protease • Protease IV cleaves host defense proteins like Igs, complement parts, AMPs and surfactants. • T3SS	• Bacterial exotoxins and proteases over a period of time causes stromal destruction. • LPS, an endotoxin on cell wall results in production of stromal rings which consists of polymorphonuclear leucocytes.

1.3.1.1 *P. aeruginosa* Type-3 Secretion System (T3SS)

The cell envelope of *P. aeruginosa* has an inner membrane and an outer membrane, separated by a hydrophilic space called periplasm. To facilitate the transport of large hydrophilic molecules via the hydrophobic parts of the membrane, the bacteria harbor several secretion systems. These secretion systems either follow one step mechanism (T1SS, T3SS, T6SS), where the molecules are transported directly to the cell surface, or two step secretion mechanism (T2SS, T5SS), where the molecules have a stopover at the periplasm (Filloux, 2011). T4SS subunits forms a channel in bacterial cell membrane that is often a pilus or pilus-like structure which facilitates horizontal gene transfer (Juhas, 2015).

T3SS or injectisome is a needle-like injection system that enable the translocation of bacterial exotoxins and enzymes into the host cells. T3SS is a virulence apparatus found in many pathogens and is specially conserved in gram-negative bacteria. It has evolved from flagella (Cornelis, 2006), a bacterial motility enabler. *P. aeruginosa* type III secretion system is made up of five major parts- first, the components that make up the secretion machine, second, the components that help in the translocation of secreted proteins into host cells, third, the components that helps in regulation of secretion process, fourth, the components that hold the secreted proteins to enable the secretion, known as chaperone proteins, and fifth, the effector proteins that are translocated into host cells (**Figure 1.4**). *P.*

aeruginosa type III secretion system secretes four effector proteins namely, ExoS, ExoU, ExoT and ExoY (Frank, 1997).

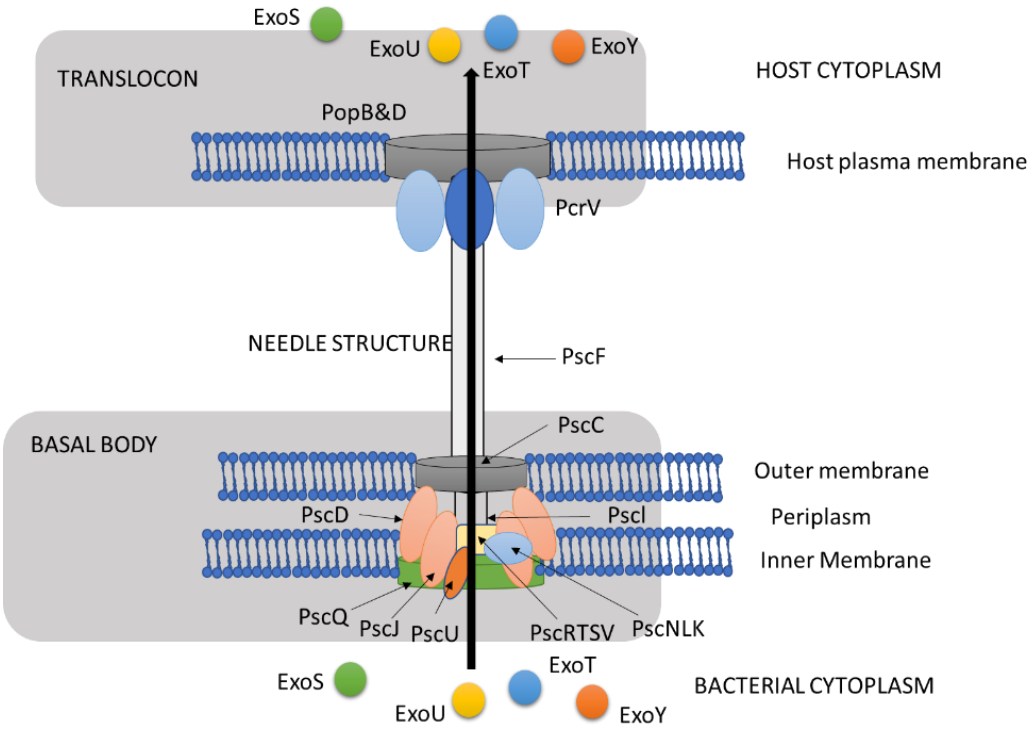


Figure 1.4 Representation of *Pseudomonas aeruginosa* T3SS. Redrawn from (Naito et al., 2017)

ExoS shows GTPase-activating and ADP ribosyl transferase activities that causes actin cytoskeleton disruption and cell death. ExoT, like ExoS also exhibit GTPase-activating and ADP ribosyl transferase activities, but, unlike ExoS it is directed against different substrates (Barbieri & Sun, 2004). ExoU shows phospholipase A_2 activity and leads to prompt lysis of several mammalian cells (Sato & Frank, 2004). ExoY, an adenylyl cyclase (Yahr et al., 1998) and has been shown to weaken early innate immune response, activate apoptosis, microtubule breakdown and endothelial cell proliferation (Balczon et al., 2013; Kloth et al., 2018; T. C. Stevens et al., 2014). ExoS and ExoT ADP ribosyltransferase activities promote neutrophil apoptosis and bacterial survival and has been shown to mediate *Pseudomonas aeruginosa* keratitis (Sun et al., 2012). ExoS also negatively regulates mTOR and autophagy in epithelial cells. ExoS-mediated ADP ribosylation of RAS leads to inhibition of mTOR. It also leads to an inhibition of autophagy by suppression of autophagic Vps34 kinase activity (Rao et al., 2021). Majority of invasive strains of *P. aeruginosa* were found to be ExoS and ExoT positive but ExoU negative (Karthikeyan et al., 2013). ExoU expressing strains are cytotoxic whereas ExoS expressing strains are

invasive in nature. Among all the *P. aeruginosa* strains the most common virulence genes were found to be *exoA* and *exoS*, followed by *exoU* and *lasB* (Heidari et al., 2018). Apart from effector protein-mediated pathogenicity, T3SS also shows independent pathogenicity. FliC, a protein secreted by T3SS of bacteria can trigger NLRC4-inflammation activation and consequently induce cell death by pyroptosis (Anantharajah et al., 2016).
ADP-ribosyltransferase (ADP-r) activity of *P. aeruginosa* T3SS exotoxin ExoS (Angus et al., 2010), allows it to survive within epithelial cells by forming bleb-niches in the plasma membrane (Fleiszig et al., 1996) that helps it evade acidic intracellular vacuoles and promotes its replication by suppression of vacuolar acidification (Heimer et al., 2013). Thus, T3SS mutants migrate to perinuclear vacuoles. *P. aeruginosa* ExoY adenylate cyclase activity can also help in the formation of bleb-niche in epithelial cells, similar to the ExoS ADP-r activity. However, the formation of bleb-niche mediated by ExoY does not help in intracellular replication *in vitro* but it enhances the bacterial virulence *in vivo* in susceptible mice (Hritonenko et al., 2011).

1.3.2 *Streptococcus pneumoniae*

S. pneumoniae is a gram-positive opportunistic pathogen that exists as a single coccus, a pair (diplococci) or forms lancet shaped chain. It's an aerotolerant anaerobic bacteria, catalase negative with the capsulated strains being pathogenic (Dion & Ashurst, 2021). Colonies on blood agar are α- hemolytic and often difficult to grow because of their ability to autolyze (Peter & Klein, 2008). *S. pneumoniae* is one of the major causes of corneal ulcers in the developing countries, comprising of about 13% to 44% of the bacterial cases (Deorukhkar et al., 2012; Kunimoto et al., 2000; Srinivasan et al., 1997; Suwal et al., 2016).

Since *S. pneumoniae* lacks enzymes like catalase and produces hydrogen peroxide as a by-product of its metabolism, they have certain oxidative stress proteins involved in enzyme detoxification, cation homeostasis and gene regulation (**Figure 1.5**) that protects the bacteria from reactive oxygen species (ROS) and nitric oxide stress (Yesilkaya et al., 2013). *S. pneumoniae* produces hydrogen peroxide due to the activity of the enzyme pyruvate oxidase, SpxB leading to DNA damage and induction of apoptosis in the host (Rai et al., 2015). SpxB is also involved in counteracting the oxidative stress.

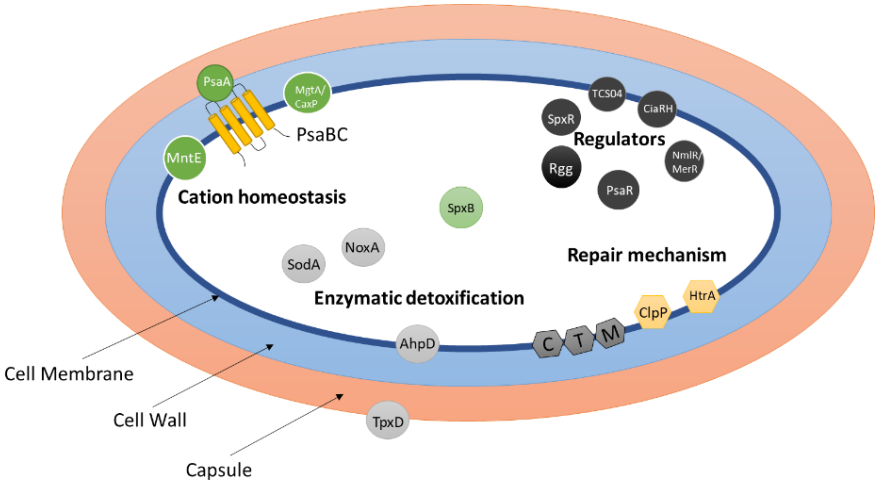
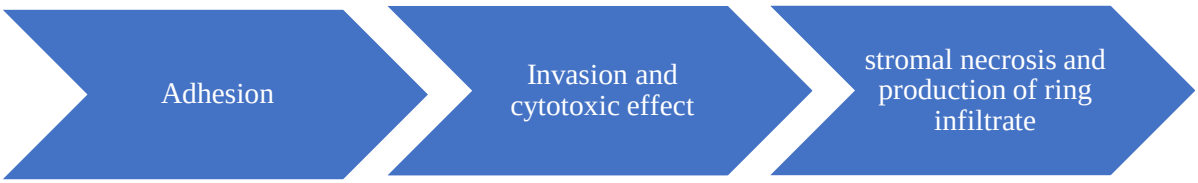


Figure 1.5 Proteins involved in oxidative stress defense in *S. pneumoniae*. TpxD* indicates that TpxD does not contain a known signal sequence or localization signals and it is predicted to be extracellular. Redrawn from (Yesilkaya et al., 2013).

S. pneumoniae harbor various virulent factors that help bacterial adherence and invasion to host cells, and evade host's immune system. For example, the cytoplasmic toxin, pneumolysin (Ply), helps in binding of bacteria to the host membranes and causes cell lysis by creating pores, induces inflammation and activation of complement (Brooks & Mias, 2018). Most of the damage caused by pneumococcal keratitis, *in vitro* and *in vivo*, is associated with Ply (Norcross et al., 2011). A brief flowchart about pathogenesis of *S. pneumoniae* is given in the **Table 1.4**.

Table 1.4 Pathogenesis of *S. pneumoniae* (Lakhundi et al., 2017)



Adhesion	Invasion and cytotoxic effect	stromal necrosis and production of ring infiltrate
• Surface proteins, plasmin and fibronectin binding protein A recognises host fibronectin. •Pneumococcal surface adhesin A, pneumococcal surface protein A, pneumolysin (ply), pneumococcal adherence and virurence factor A, choline-binding protein A, putative protease maturation protein A, IgAI protease and streptococcal lipoprotein rotamase A. Pneumococcal surface protein C •Pili and fibrils	•Pneumolysin binds to cholesterol, polymerizes and creates pores on host cell membrane. Also activates complement system and instigates inflammation •PspA and PspC, neuraminidase A •Metalloprotease, ZmpC, removes membrane-associated mucin from conjuctival and corneal epithelial cells.	•Bacterial exotoxins and proteases over a period of time causes stromal destruction.

1.4 Antimicrobial Resistance (AMR)

"Antimicrobial resistance is a phenomenon when bacteria, viruses, fungi and parasites change over time and no longer respond to antimicrobials including antibiotics, making infections harder to treat and increasing the risk of disease spread, severe illness and death (*Antimicrobial Resistance*, n.d.)." The microorganisms that show AMR are often referred to as "superbugs" (*Antimicrobial Resistance*, n.d.). According to the World Health Organization, AMR is one of the top ten health-threats faced by the world currently and the infections caused by antibiotic resistant bacteria has become a burden to healthcare system. The term "antibiotic" was coined in 1942. Antibiotics are produced by microorganisms or chemically synthesized and is known to be antagonistic to the growth of other microorganisms. The antibiotics can act as bactericidal or bacteriostatic medicines to cure a number of microbial infections. Penicillin, the first antibiotic discovered in 1928 by Alexander Fleming in the "pre-antibiotic" era, set the stage for the discovery of many others (Erdem et al., 2011; Tan & Tatsumura, 2015). The onset of an era of antibiotic discovery (Aminov, 2010) was marked with an enthusiasm in researchers all over the world to discover other novel naturally occurring antibiotics. Very soon the synthetic antibiotics

were made that were more efficient. Within a decade of the discovery of the first antibiotic, it was believed that humans have triumphed over bacterial diseases. But this sense of victory over the bacterial diseases did not last very long. The antibiotics began to fail, in curing some bacterial diseases, especially the ones acquired in hospitals, or nosocomial diseases. Today we are entering a "post-antibiotic" era where there is no stopping the antibiotic-resistant bacteria (Kwon & Powderly, 2021). It has been shown that antibiotic resistance commonly occurs in nature and dates back to times even before the antibiotics were used clinically (Dcosta et al., 2011).Therefore, WHO has identified AMR as "a serious threat to global public health." Additionally, the cost and feasibility of health care have risen due to AMR. WHO has put out new recommendations to fight the AMR, and one of the objectives is to "increase investment in new medicines, diagnostic tools, vaccines and other interventions." Global Antimicrobial Resistance and Use Surveillance System (GLASS), is the first initiative by WHO to integrate official national observation data of AMR in human, food chain, and environment (Organización Mundial de la Salud, 2015). A report by WHO -GLASS on emerging AMR during the year 2019, shows a list of confirmed events of AMR in *Pseudomonas aeruginosa, Staphylococcus aureus, Escherichia coli,* to name a few. In the recent GLASS report, high rates of resistance were recorded among antimicrobials commonly used to treat (World Health Organization (WHO), 2020).

In a report by WHO published in 2017, *P. aeruginosa* was identified as one of the top three priority-1 pathogens and *S. pneumoniae* was as one of the priority-3 pathogens needing urgent attention in research and development of antibiotics against them, due to emergence and re-emergence of AMR. Parallelly, the WHO, India branch along with the Department of Biotechnology (DBT) has also developed a list of drug resistant microbial pathogens of national relevance which will help the prioritization of research and development of new and effective antibiotics from Indian perspective (Sharma, 2021). According to this list *P. aeruginosa* comes under critical priority and *S. pneumoniae* under medium priority category. Infectious Diseases Society of America has identified some antibiotic-resistant bacteria (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) that has been given an acronym "the ESKAPE pathogens" – signifying their ability to "escape" the killing effect of antibiotics and exhibiting new models in pathogenesis, spread and resistance (Pendleton et al., 2013). The earliest reports of resistance towards

fluoroquinolones are found in bacterial keratitis causing strains (Goldstein et al., 1999; Schaefer et al., 2001). There have been reports of several multidrug-resistant (MDR) bacterial keratitis in recent years (Schubert et al., 2020). In one study *Pseudomonas* spp. was found to be the most common pathogen showing resistance to chloramphenicol (A. E. Lee et al., 2019). In other studies it was found that 100% percent of the ocular isolates of *P. aeruginosa* showed resistance to cefalotin/cephazolin and chloramphenicol (Mohammadpour et al., 2011; Watson et al., 2020). An rise in resistance to penicillin was also reported in both gram negative and gram positive ocular isolates (Ting, Ho, Cairns, et al., 2021). Keratitis caused by antibiotic-resistant bacteria shows worse clinical outcomes, prolonged disease and treatment regimen (Charani et al., 2021; Dimatatac et al., 2003).

1.4.1 Development of antibiotic resistance

Antibiotic resistance has been attributed to certain genes, that are known to cluster together at certain sites of the bacterial chromosome. Such clusters have been made possible by the transportation of AMR genes by transposons, integrons and plasmids. That means these genes are often foreign to the host bacteria and most possible sources can be mutation in housekeeping genes as an adaptation to new substrates, antibiotic production by microbes for self-protection, and natural resistance genes in soil (Mazel & Davies, 1999). Bacteria can develop resistance by either gene mutation (vertical evolution) or by incorporating genes involved in antibiotic resistance from other strains or species (horizontal gene transfer). A vast diversity of antibiotic resistance genes in the human microbiome was identified and it is highly possible that human pathogens could be developing antibiotic resistance by acquiring those genes by horizontal gene transfer (HGT) (Sommer et al., 2009). In one study it was concluded that there is a multitude of mechanisms and processes that leads to the persistence of chromosomal and plasmid-borne resistance factors and how we can target them to curb AMR. It was shown that a very low antibiotic concentration can enrich resistant bacteria and that its release into the environment could cause selection for resistance (Andersson & Hughes, 2011). The development of antibiotic resistance can thus be either because of natural selection, mutation or gene transfer in bacteria (**Figure 1.6**).

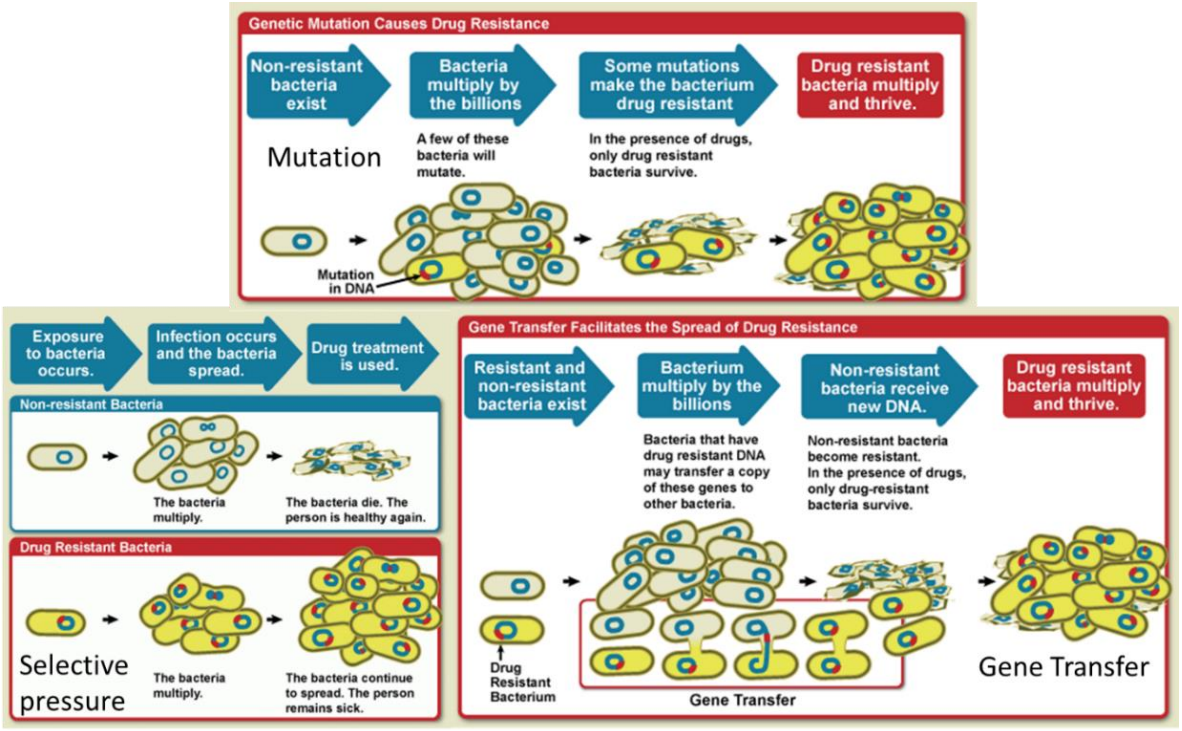


Figure 1.6 Development of antibiotic resistance in bacteria. Picture courtesy (*Causes of Antimicrobial (Drug) Resistance | NIH: National Institute of Allergy and Infectious Diseases*, n.d.)

1.4.2 Mechanism of antibiotic resistance

Bacteria employ four different mechanisms for exhibiting resistance to antibiotics (Blair et al., 2015; Mazel & Davies, 1999; P. K. Mukherjee, 2019):

1. The inactivation or modification of antibiotics. (E.g., Antifolates, Aminoglycosides, Glycopeptides, Amphenicols, β lactams, Rifamycins)
2. Alteration of antibiotic's target site & hence reducing its binding capacity. (E.g., β Lactams, Aminoglycosides, Glycopeptides, Fluoroquinolones, Rifamycins, Macrolides, Tetracyclines)
3. Alteration of metabolic pathways to inhibit the antibiotic effect. (E.g., Sulfonamides, Trimethoprim)
4. Reduction in intracellular accumulation of antibiotic by decreasing permeability (E.g., aminoglycosides, β Lactams) and/or increasing the active removal of antibiotics from the bacterial cell. (E.g., Aminoglycosides, β Lactam, Macrolides, Quinolones, Tetracyclines)

P. aeruginosa is known to show multidrug resistance to various antibiotics and this has been attributed to low permeability of its cell wall, expression of numerous resistance

mechanisms like biofilm formation, mutation in resistance regulatory genes and its ability to incorporate genes conferring resistance from other organisms via plasmids, transposons and bacteriophage (Lambert, 2002). Inherent presence of certain resistance genes like *catB* and *ampC* makes *P. aeruginosa* resistant to chloramphenicol and β-lactams, respectively (Livermore, 1995). *P. aeruginosa* also expresses efflux pumps like MexAB-OprM and MexXY-OprM that makes it resistant to certain other antibiotics (Poole, 2011). In one study it was found that Indian isolates of *P. aeruginosa* were more resistant to the antibiotics ciprofloxacin, levofloxacin, tobramycin, ceftazidime, piperacillin, imipenem, gentamycin and polymyxin than the Australian isolates (Poole, 2011). Additionally, Indian isolates also have additional resistance genes, in comparison to Australian isolates, that conferred resistance to fluoroquinolones, aminoglycosides.

1.4.3 Spread of antimicrobial resistance

Antibiotic resistance in bacteria is spread by misuse, overuse of antibiotics, unhygienic environment and poor infection control, misuse of antibiotics in agriculture, farms and dairies. Rampant usage and improper disposal of antibiotics in the environment facilitates the spread of antibiotic resistance (**Figure 1.7**). The misuse of antibiotics has been reported as a 'global crisis' (Bell, 2014). Recently, in a study conducted on AMR in companion birds, it was found that they are an important reservoir of antimicrobial resistance genes, and can transfer the resistance directly or indirectly to humans (Varriale et al., 2020).

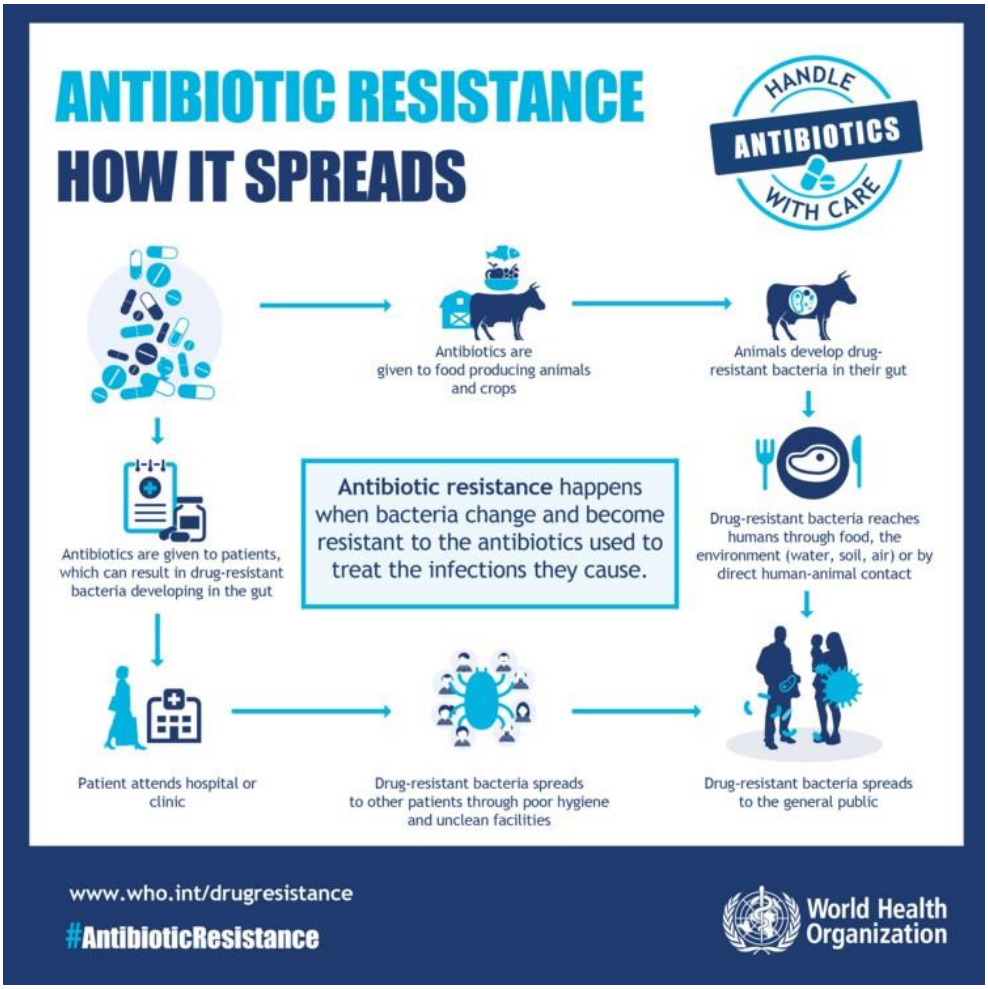


Figure 1.7 Spread of AMR. Image from World Health Organization (WHO)

1.5 Host Innate Immune Response to Pathogens

Innate immune responses against pathogens is induced when evolutionarily conserved pathogen associated molecular patterns (PAMPs), bind to germline-encoded pattern recognition receptors (PRRs) like TLRs, Triggering Receptor Expressed on Myeloid cells-1 (Hommes et al., 2014) and Nod-like receptors (Alhazmi, 2018) on host cell surfaces and triggers the intracellular signaling that leads to the activation of signaling pathways like MAPK, inflammasomes and transcription factors like nuclear factor- κβ (NF-κβ) and AP-1. These activated transcription factors consequently lead to the production of antimicrobial peptides (AMPs), inducible nitric oxide synthase, pro-inflammatory cytokines like IL-1, IL-2, TNF-α and chemokines. These cytokines may then induce various other signaling pathways leading to the expression of additional pro-inflammatory cytokines and activation of other innate and adaptive immune responses (**Figure 1.8**).

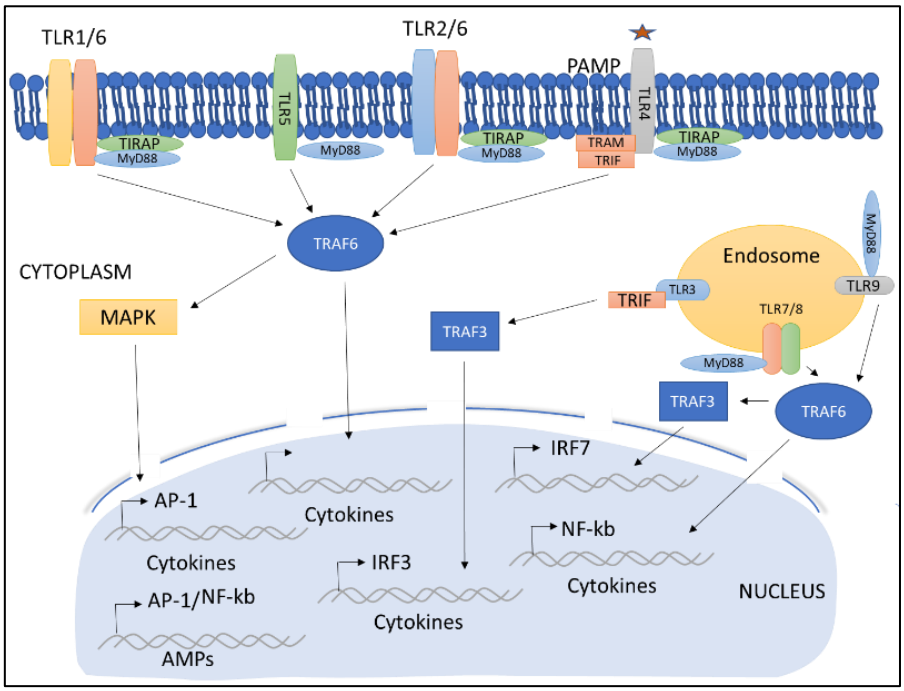


Figure 1.8 TLR signaling in humans. Image adapted from (J. Yang et al., 2020)

TLR2 and TLR4 are shown to be activated during *S. pneumoniae* keratitis. TLR2 putatively helps in bacterial clearance by recruiting neutrophils to the cornea and TLR4 is speculated to be essential to modulate the immune response for preventing cellular damage (Tullos et al., 2013). *S. pneumoniae* lipoproteins are major inducers of the macrophage TLR2 and NF-κβ–mediated inflammatory response via IL-1R–associated kinase (IRAK-4) (Tomlinson et al., 2014). The earliest upregulation of cytokines like IL-1α, IL-1β, TNF-α, KC and MIP-2 was shown to be dependent on transcription by NF-κβ RelA in myeloid cells during *S. pneumoniae* infection (Pittet et al., 2011). In another study it was found that the expression of TLR2, TLR4, TLR5 and TLR9 was upregulated in *P. aeruginosa* and *S. pneumoniae* infected patient corneas (Karthikeyan et al., 2013). It has been shown that TLR4 plays an important role in the activation of innate immune responses in response to *P. aeruginosa* infection (Skerrett et al., 2007). In addition to TLRs, Nod receptors also play a role in initiation and amplification of host innate immune response. Nod1, a cytosolic receptor that is activated by diaminopimelate-containing muropeptides from Gram-negative bacteria peptidoglycan, has been shown to detect the *P. aeruginosa* peptidoglycan causing NF-κβ activation and chemokine expression in epithelial cells (Travassos et al., 2005). MAP-kinase or MAPK pathway controls major cellular processes that includes growth, differentiation, apoptosis, stress response and immune defense. MAPK pathway is

present ubiquitously and is highly conserved in eukaryotes. There are three MAPK pathways identified in mammals:

1. ERK- activated by growth factors, hormones and pro-inflammatory cytokines
2. JNK- triggered by stress and pro-inflammatory cytokines
3. P38- triggered by stress and pro-inflammatory cytokines

P. aeruginosa has been shown to regulate gene expression by activating p38 MAPK signaling via TLR5 in human airway epithelial cells (HAECs) which further activates NF-κβ (Zhang et al., 2007). Glycosyl hydrolase 25 relating to invasion protein (GHIP), a virulent factor of *S. pneumoniae*, was shown to induce the expression of IL-6 via TLR2 mediated activation of JNK and p38 (Dong et al., 2014).

Another intracellular signaling pathway that plays an important role in host innate immune response during infection is phosphatidylinositol-3 kinases/Akt/mammalian target of the rapamycin pathway. This pathway plays a part in cell growth, differentiation, metabolism, proliferation, motility, and survival. It is also responsible for regulation of inflammation and is usually accompanied by the initiation of TLRs/NF-κβ, cytokine receptors and tyrosine kinase receptor signaling (B. Li et al., 2018; Saxton & Sabatini, 2017; Weichhart et al., 2015). It has been shown that the inhibition of this pathway increases the risk of infection significantly in tumor patients undergoing chemotherapy (Rafii et al., 2015). JAK-STAT pathway is an intracellular cell signaling pathway that also takes part in innate immune response of the host during infection. It is activated by cytokines that includes type-1 interferons like IFN-α/β, type-2 interferon like IFN-γ and IL-10 family of type II cytokines. The JAK or Janus family tyrosine is a family of kinases which are of four types: JAK1, JAK2, TYK2 and JAK3, out of which JAK1, JAK2 and TYK2 are expressed widely in a constitutive manner and JAK3 is inducible. In one study it was shown that *S. pneumoniae*, early on during the infection, induced the expression of Type I Interferons that consequently lead to the initiation of the JAK/STAT signaling pathway that regulated the expression of Interferon Stimulated Genes products having anti-tumor, anti-viral, immunomodulatory, and pro-apoptotic functions (Joyce et al., 2009). Autophagy is yet another innate immune response of the host, that triggers upon bacterial infection. One such example is the autophagy induced in mast cells during *P. aeruginosa* infection in lungs, where it helps in effective clearing of the bacteria both *in vitro* and *in vivo*. Hence, autophagy inducers like mTOR inhibitors (rapamycin), modulators of calcium dependent signaling and IP3 inhibitors can be used as a therapeutic for alleviating infection

(Junkins et al., 2013). Autophagy is also known to be activated during *S. pneumoniae* infection in human alveolar epithelial cells (P. Li et al., 2015), osteoblasts (J. Kim et al., 2017), microglia (Guan Wang et al., 2020) etc. One of the virulent factors of *S. pneumoniae*, PLY was found to induce autophagy by ROS mediated activation of AMPK and consequent inhibition of mTOR (J. Kim et al., 2017). ROS mediated downregulation of PI3K/AKT/mTOR pathway was also found to activate autophagy in *S. pneumoniae* infected cells *in vitro* (P. Li et al., 2015). All these findings show that there is an interplay of various signaling pathways and processes within the host during infection. ROS production is one of the early defenses of the host against the pathogen and plays an important role in inflammation. ROS is usually generated by NADPH oxidase complex (phagocyte oxidase (phox) complex), which is stimulated via various intracellular signaling pathways like PI3K upon interaction of PAMPs with PRRs including TLRs (Nguyen et al., 2017). Several pro-apoptotic signals also induce autophagy. Various virulent factors of *P. aeruginosa* like pyocyanin, exotoxin A, protease, T3SS etc., are known to induce apoptosis in the host via mitochondrial pathway, caspase 3, Bak pathway, cAMP, reactive oxygen intermediates etc. (Du et al., 2010; Kaufman et al., 2000; Usher et al., 2002).

P. aeruginosa is also shown to activate NF-κβ pathway in respiratory epithelial cells (DiMango et al., 1998) and it was found to be induced by ExoU (C. D. M. de Lima et al., 2012). Pyocyanin, a virulent factor of *P. aeruginosa* was found activate MAPK and NF-κβ pathways in differentiated human promonocytic cell line (U937) (Chai et al., 2014).

1.5.1 Antimicrobial Peptides (AMPs)

1.5.1.1 Background

The first AMP, lysozyme, was discovered by Alexander Fleming from the human nasal mucus. At that time, these peptides were not classified as AMPs and their major role in innate immunity was not identified. It was not until the 1970s, when Hans Boman group identified a non-specific humoral immune response in *Drosophila* and went on to figure out, for the first time, the structure an AMP, cecropin, from silk moth, which was published in Nature in 1981 (Boman et al., 2020; Steiner et al., 1981). He is recognized as a 'pioneer in peptide-mediated innate immune defense' (Pütsep & Faye, 2009). The Antimicrobial Peptide Database (APD) has a repository of 3283 antimicrobial peptides from six kingdoms (371 bacteriocins/peptide antibiotics from bacteria, 5 from archaea, 8 from protists, 22 from

fungi, 361 from plants, and 2431 from animals, including some synthetic peptides (https://aps.unmc.edu/AP/).

AMPs are produced by all the kingdoms of life (Ageitos et al., 2017) (**Figure 1.9**). plays a significant function in the host defense system, dating back to times when the immune system was not evolutionarily developed in the organisms. It's still one of the potent armors in protecting the organisms against any infiltration.

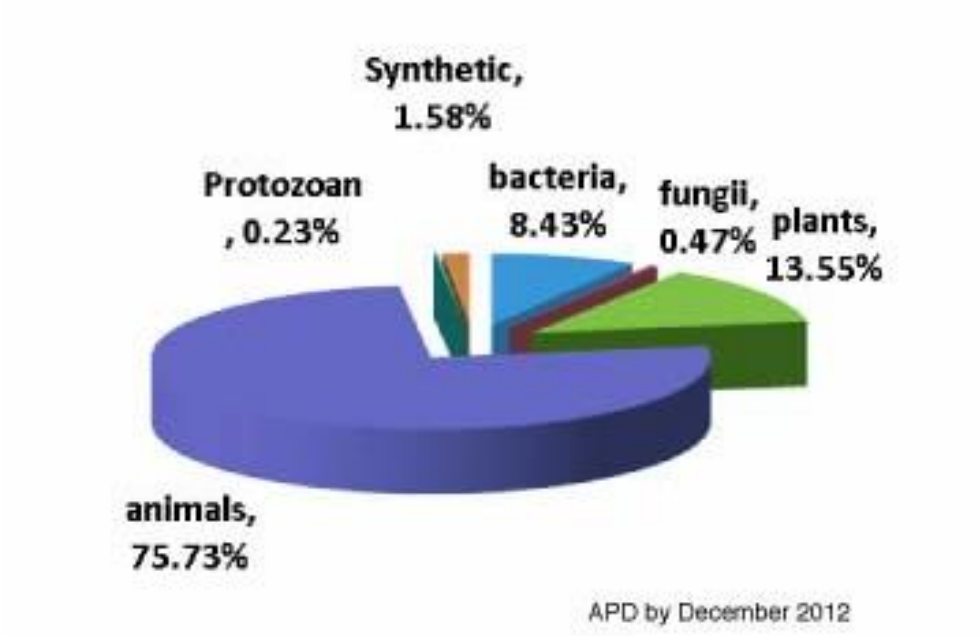


Figure 1.9 Sources of AMPs. Picture courtesy https://aps.unmc.edu/facts

AMPs are widely studied as an alternative to or complementary to the antibiotics that are already been used for treating bacterial infections. The advantage of AMPs over antibiotics are that AMPs have a broad spectrum of action, have immunomodulatory and wound healing actions on the host, and most importantly bacteria rarely develops resistance against it (Spohn et al., 2019). AMPs are expressed by neutrophils and epithelial cells (Gallo & Hooper, 2012; S. Mukherjee & Hooper, 2015; Prasad et al., 2019; Turner et al., 1998). Human corneal epithelium constitutively expresses human beta-defensins like *hBD-1*, *hBD-2*, *hBD-3* and *hBD-4* (O. J. Lehmann et al., 2000; McNamara et al., 1999). *hBD-9* was also found to be constitutively expressed on ocular surface epithelia with a high level of expression in conjunctival epithelium (Mohammed et al., 2010; Otri et al., 2012). Other constitutively expressed AMPs by human corneal epithelium are *S100A8*, *S100A9*, *S100A12*, *LL-37*/cathelicidin, *Rnase-7*, liver expressed antimicrobial peptide-1 and -2, and *S100A7*/psoriasin (Garreis et al., 2011; Gordon, Huang, et al., 2005; McIntosh et al., 2005;

Mohammed et al., 2011). During infection, various components of the bacteria like flagella, lipopolysaccharides and nucleic acids, upregulates the expression of the AMPs (Garreis et al., 2011; Kumar et al., 2006; G. Li et al., 2009; Q. Li et al., 2008), mediated by TLRs (Dua et al., 2014; Kumar et al., 2006). Activation of TLRs expressed on ocular surface has been shown to be responsible for the induction of *LL-37* and *hBD-2* (Redfern et al., 2011). Some intracellular signaling pathways are found to be involved in TLR mediated expression of AMPs. For example, *hBD-2* expression can be switched on by proinflammatory cytokines like IL1β and TNF-α, via MAPK and NF-κβ pathways. Since IL-1 increases on ocular surface post-injury, *hBD-2* expression is increased in regenerating corneal epithelium and is implicated in wound healing. IL-1β stimulates *hBD-2* expression via two pathways- one pathway involves the "direct" activation of NF-κβ through IκB kinase β whereas the other pathway is mediated by upstream signaling of p38 MAP kinase and JNK (McDermott et al., 2003).

1.5.1.2 Structure and properties of AMPs

AMPs are short peptides, contains cationic amino acids in the majority, and can hence adopt amphipathic conformation due to which it shows affinity to the negatively charged bacterial membranes. A higher negative transmembrane potential inside the bacteria increases electrostatic attraction even more. Unlike bacteria, the outer layers of eukaryotic membranes comprise zwitterionic (neutral) lipids. That's why AMPs act selectively on bacteria (Manrique-Moreno et al., 2020).

Even though AMPs are mostly cationic, various different groups have also been identified and they are classified as follows (Brogden, 2005):

Different classes of AMPs	Anionic peptides- E.g., Human dermicidin
	Linear cationic α-helical peptides- E.g., human LL37
	Cationic peptides enriched with specific amino acids- E.g., Human histatins
	Anionic and cationic peptides that has cysteine and forms disulphide bonds-E.g., Human β defensins (*hBD-1*)
	Anionic and cationic peptide fragments of largetr proteins- E.g., antimicrobial domains from human haemoglobin.

Structural conformation of different AMPs is represented in **Figure 1.10**.

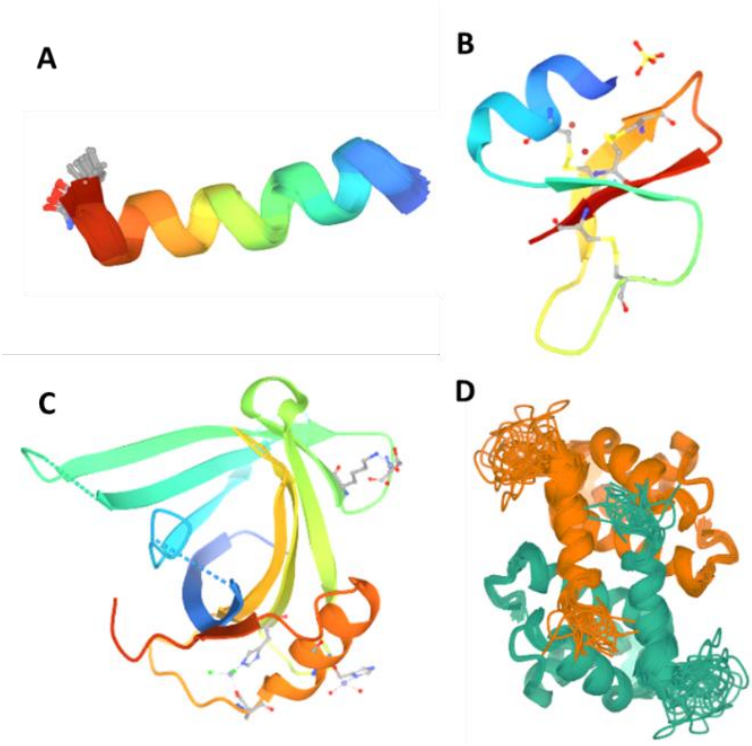


Figure 1.10 Structural conformations of different AMPs. (A) LL-37 (Guangshun Wang et al., 2012); (B) Human beta defensin-1 (Hoover et al., 2001); (C) Lipocalin-1 (Breustedt et al., 2005); (D) S100A12 ((Hung et al., 2013).

There are certain attributes of AMPs that influences its activity and specificity. These are listed as follows (Brogden, 2005):

1. **Size**: Short. 10-60 amino acids long.
2. **Sequence**: Mostly basic amino acids. Also contains hydrophobic and repetitive amino acids. The ratio of hydrophobic: charged amino acids ranges from 1:1 to 2:1.

3. **Charge**: Zinc complexed anionic peptides or the highly cationic peptides are more active compared to the neutral or lower-charged peptides.
4. **Shape and structure**: Amphipathic α-helical peptides are more active compared to the peptides with indistinct secondary structures.
5. **Hydrophobicity**: This property helps water soluble AMPs to intercalate within the lipid bilayer.
6. **Amphipathicity**: AMPs have hydrophilic amino acids on one side and hydrophobic amino acids on the other side of a helix.

1.5.1.3 Mechanism of action of AMPs

AMPs show various mechanisms of action depending on its structure, the proportion between AMP and lipid on the bacterial cell membrane, and the intrinsic properties of the bacterial cell membrane lipid. AMPs can also target the cell wall, impede the folding of protein or enzyme action, or target other cellular components or processes like modification of cytoplasmic membrane septum formation, binding of nucleic acid, inhibition of cell wall synthesis, inhibition of nucleic acid and protein synthesis (Brogden, 2005). Hence, AMPs interact with the outer membrane of the cell wall of gram-negative bacteria, and relatively porous cell wall of gram-positive bacteria before further acting on the plasma membrane and the internal targets of bacteria.

Cationic AMPs interact with different molecules on the gram-positive cell wall with different affinities before they reach the cytoplasmic membranes. These interactions possibly will diminish their effective concentration on the plasma membrane surface. Gram positive peptidoglycan cell wall layer, as pointed out earlier, is comparatively porous and facilitates penetration of AMPs. Hence it is reported (Malanovic & Lohner, 2016) that the peptidoglycan layer might act as ‘sponge’ enabling the AMPs to penetrate the cell wall of Gram positive bacteria, facilitating the interaction of AMPs with the bacteria plasma membrane. However, another component of gram positive cell wall, lipoteichoic acid, which is anionic in nature may strongly attract positively charged AMPs leading to either capture of AMPs or help AMPs reach the plasma membrane as a “ladder”(Malanovic & Lohner, 2016).

With the aid of electrostatic and hydrophobic interactions, the cationic AMPs have been found to be able to interact strongly with negatively charged gram negative bacteria cell

wall outer membrane surface lipopolysaccharides (LPS) that leads to self-promoted uptake and resultant passage of the AMPs to the bacterial plasma membrane. AMPs also interact with and enter into the membranes of liposomes in a similar manner. However, these interactions of AMPs with the cell wall outer membrane and liposome membrane did not damage them (Anunthawan et al., 2015).

The models proposed for interaction of cationic AMP with membranes are- Barrel Stave model, carpet model and Toroidal-pore model (Brogden, 2005; Sani & Separovic, 2016). The mechanism is dependent on the peptide and the lipid membrane composition and may vary depending on the concentration of peptide, and environmental conditions like pH and temperature. Different models like SMART model (Bechinger, 2015) have explained diverse possibilities of how the AMPs interacts with the dynamic lipids of the bacterial membrane, often forming transient structures and interactions, that may ultimately lead to the breakdown of the intact membrane.

Some studies suggest that intracellular biomass flocculation might be an important AMP killing mechanism supported by the fact that most AMPs require micromolar concentrations for their activity, selectively targets bacteria over mammalian cells, and there is a lower prevalence of bacteria showing resistance against them as opposed to the commonly used antibiotics (Chongsiriwatana et al., 2017). Some AMPs like defensins have been found to inhibit exotoxin production without affecting bacterial growth (Merriman et al., 2014). AMPs are also involved in immunomodulation (Koeninger et al., 2020). LL-37, the only human cathelicidin, has been found to modulate immune system by inhibiting the activation of lipid sensing toll-like receptors (TLRs) like LPS induced TLR4 and LTA induced TLR 1/2/6, and by activating the nucleic acid sensing TLRs , inflammasomes and autophagy (Scheenstra et al., 2020). LL-37 has been shown to prevent epithelial breach by *P. aeruginosa* by increasing lung epithelial cells stiffness and decreasing it's permeability (Byfield et al., 2011). Some antimicrobial peptides, like the one derived from lactoferricin B, can enter bacteria like *Escherichia coli* without disrupting the cell membrane, hence are known as cell-penetrating peptides (CPP). Membrane potential of the bacterial cell membrane helps in the passage of AMPs without damaging the membrane. Higher the membrane potential, easier it is for the CPP- AMPs to pass through (F. Hossain et al., 2021).

AMPs in combination with various antibiotics has been found to be effective against a broad spectrum of pathogens (Cote et al., 2020). Several AMPs show direct killing action against microbes and has also been found to be an essential part of the innate immune

system, hence making them potential substitute for commonly used antimicrobial agents. To further fight and possibly win the war against antimicrobial resistance, AMPs can also be used as adjuvants in combination with current antibiotics as these combination therapies reduce the likeliness of development of resistance or transmission of cross-resistance (Lewies et al., 2019). A concise representation of AMPs mechanism of action is given in **Figure 1.11**.

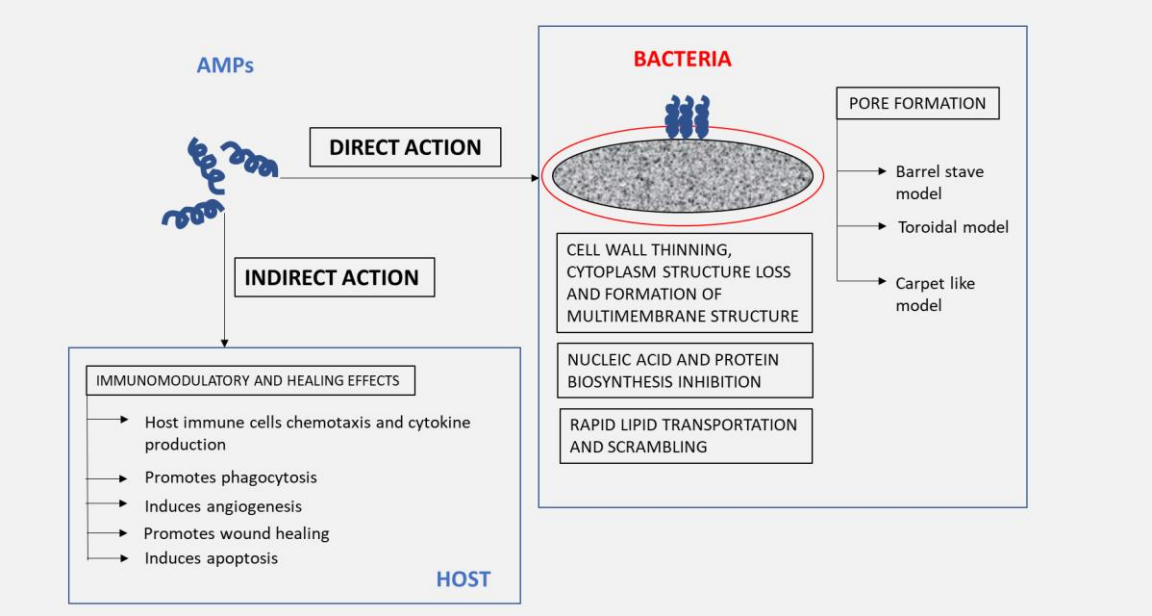


Figure 1.11 Mechanism of action of antimicrobial peptides.

1.5.2 Response of Pathogens to Host Innate Immunity

During infection, pathogens target the host intracellular signaling pathways like MAPK to modulate immune response (Roy & Mocarski, 2007). *P. aeruginosa* has been shown to inhibit ROS production in human neutrophils through the action of ExoS and ExoT of T3SS. ADP-ribosylation of Ras by ExoS prevents the interaction of Ras with phosphoinositol-3-kinase (PI3K). In the absence of PI3K activation by Ras, NADPH-oxidase in the neutrophil is not stimulated, hence ROS generation is suppressed. ADP-ribosylation activity of ExoT also disrupts PI3K signaling without any involvement of Ras (Vareechon et al., 2017). ExoY of *P. aeruginosa* has been shown to bind to actin filaments of the host and modulate the actin cytoskeleton both directly, by F-actin aggregation, and indirectly, by actin-activated nucleotidyl cyclase activity (Mancl et al., 2020) . Modulation, rearrangement and disruption of host actin filament is a means by which some pathogens

either evade the host innate immune response like phagocytosis or invade the host. The actin filament network of the host is also used by some pathogens like *Listeria monocytogenes* for its movement and dissemination (J. M. Stevens et al., 2006). *P. aeruginosa* evades phagocytosis by functional loss of flagellum-mediated swimming motility (Amiel et al., 2010). The capsule of *S. pneumoniae* helps in evading phagocytosis and complement pathway activity (Hyams et al., 2010). The pneumococcal capsule was also shown to partially dampen MyD88-mediated antibacterial defense by impairing the recognition of *S. pneumoniae* derived TLR ligands (Vos et al., 2015).

The expression of AMPs and other innate immune responses has also been shown to be regulated by various virulence factors of the pathogens like T3SS (Haneda et al., 2012). As discussed earlier, T3SS is one of the virulence apparatuses in gram-negative bacteria and has been extensively studied in *P. aeruginosa*. Studies have also confirmed that T3SS is also capable of dampening IL-17 mediated innate immune response against pathogen by exploiting NLRC4-coupled inflammasome (Faure et al., 2014). It has also been reported that bacterial proteins can disable the action of AMPs like defensins, and manipulate its expression via T3SS-mediated inhibition of the NF-κβ pathway (Menendez & Brett Finlay, 2007). Thus, targeting T3SS of *P. aeruginosa* with drugs or inhibitors can be an alternative or supplementary to the therapeutics currently used to treat keratitis (Sheremet et al., 2020).

1.6 Aim of The Study

Currently, combating antibiotic resistance is one of the major goals in the medical and scientific world. AMPs are considered to be the most potent amongst the many other alternatives that can be use in place of, or in conjunction with antibiotics. Even though many studies have been done on usage of AMPs as an alternative or supplementary therapeutics in treating bacterial diseases, and many AMPs have proven to be promising in preclinical trials, but the definitive role of AMPs is yet to be recognized (Gordon, Romanowski, et al., 2005). Moreover, there were no studies on AMPs expressed in patients' samples during *P. aeruginosa* and *S. pneumoniae* mediated corneal infections. Hence the aim of my thesis work is to decipher the role of antimicrobial peptides in bacterial keratitis.

1.7 Objectives

The objectives of my thesis are listed below:

1. To determine the expression of antimicrobial peptides (AMPs) during bacterial keratitis in patients.
2. To determine the role of AMPs in keratitis caused by gram-negative bacteria, *P. aeruginosa*.
3. To determine the role of AMPs in keratitis caused by gram-positive bacteria, *S. pneumoniae*.

The study has been divided into different chapters, with each objective covered in a separate one, for clarity. It begins with **Chapter I,** that covers the **Introduction** and **Chapter II** with detailed description of the **Materials and Methods**. **Chapter III** covers **Objective 1** where the expression of AMPs in patients' samples is discussed, to get an idea about the host AMP response during infection**. Chapter IV** covers **Objective 2** where we focus on the AMP related response of the host during gram negative bacterial infection, with *P. aeruginosa* as a representative organism. Here we also delve deeper into deciphering the role that *P. aeruginosa* T3SS plays in its pathogenesis. We also studied the host response both *in vivo* and *in vitro*, when T3SS is pharmacologically inhibited. **Chapter V** is dedicated to **Objective 3** where we focus on the AMP related response of the host during gram positive bacterial infection, with *S. pneumoniae* as a representative organism. We further focus on LL-37, the onlyAMP belonging to the cathelicidin group in humans, and study its expression, the signaling pathway involved and its antibacterial property. We also look into the Nrf2 mediated antioxidant response of the host during *S. pneumoniae* infection and the effect of Nrf2 inducers in the course of infection**.**

CHAPTER II

MATERIALS AND METHODS

2.1 Corneal scrapings and tissue sections

We collected corneal scrapings patients presented with keratitis after taking their informed consent in accordance to the practice established by Institutional Review Board of Hyderabad Eye Research Foundation, LVPEI, Hyderabad, following the tenets of Declaration of Helsinki. The identification of the causative organism was later confirmed from Jhaveri Microbiology Centre, LVPEI. Corneal tissue sections of *S. pneumoniae* keratitis patients that underwent therapeutic keratoplasty were obtained from the Pathology Department, L V Prasad Eye Institute, Hyderabad. For control, cadaveric corneas that were deemed unfit for transplantation were obtained from in-house Ramayamma International Eye Bank.

2.2 Identification of bacterial strains

The patient ulcer, collected in sterile condition, were used for isolation and identification of causative organism as done routinely at Jhaveri Microbiology Centre, LVPEI, following Institute's guidelines, as described earlier (Roy et al., 2015). In brief, the ulcer sample was smeared on slides to perform Gram's staining and potassium hydroxide with calcofluor white staining. The corneal scrapings were also inoculated in different specific media (chocolate agar, 5% sheep blood agar, potato dextrose agar, Sabouraud dextrose agar, thioglycolate broth, non-nutrient agar with *Escherichia coli* and brain heart infusion broth), and incubated in aerobic conditions at 37°C, except the inoculum in chocolate agar, that was grown in 5% CO_2 at 37°C (Das et al., 2019). The isolated colony was subcultured and purified. For identification purpose biochemical tests and sequencing was performed. The identity of homogenous pure culture was confirmed with VITEK® 2 compact system (bioMerieux Inc, Durham, NC, USA).

2.3 Bacterial culture

The bacteria used in the study are as follows (**Table 2.1**)

Table 2.1 Details of bacterial strains/isolates used in the study

Bacterial strain/isolate	Abbreviated as	Remarks
P. aeruginosa PAO1	PAWt	Has an intact Type-3 secretion system (T3SS)
P. aeruginosa ΔpscC	PA*ΔpscC*	T3SS is inactive
S pneumoniae American Type Culture Collection (ATCC) 49619	Sp ATCC	Pneumolysin positive
Clinical isolate of *S. pneumoniae*	Sp CS	Pneumolysin positive. Obtained from Jhaveri Microbiology Centre, L.V. Prasad Eye Institute

All isolates and strains of *P. aeruginosa* were grown in Luria Bertani (LB) media (MP Biomedicals, Mumbai, India) for experiments. The overnight culture (200µl in 5 ml LB) was used to give a subculture (200µl in 5ml) and incubated in 37ºC to get an optical density (O.D.) of 0.2 that corresponds to 10^8 CFU/ml (mid exponential phase). The bacterial culture was pelleted down at 10,000 rpm for ten minutes and re-diluted in 5 ml of 1X PBS or LB. This culture was further diluted in 1X PBS or LB to obtain the required dilution. Multiplicity of infection (MOI) for *in vitro* experiment was 1:10 (cell: bacteria).

All isolates and strains of *S. pneumoniae* were maintained on blood agar plates. For experiments, *S. pneumoniae* was cultured overnight in Todd Hewitt broth (THB) (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37ºC and 5% CO_2. The bacterial broth culture was grown till OD 0.28 that corresponds to 10^8 CFU/ml (mid exponential phase). The culture was pelleted down and 5ml of 1X PBS or THB was used to resuspend the pellet. This culture was further diluted to obtain required dilution. MOI for *in vitro* experiment was 1:10 (cell: bacteria).

2.4 Culture of HCEC and U937

The cell lines used for the study were immortalized human corneal epithelial cells (HCECs) 10.014 pRSV-T (Roy et al., 2015), human monocytes U937 and primary human corneal epithelial cells (primary HCECs) derived from cadaveric uninfected corneas.

HCECs were maintained in keratinocyte serum free media (KSFM) supplemented with bovine pituitary extract (BPE) and recombinant human epidermal growth factors (EGF) (cKSFM; Invitrogen, Carlsbad, CA, USA) or DMEM-F12 (Hyclone, USA/Lonza, USA) supplemented with 10ng/ml EGF (ThermoFisher Scientific, Waltham, USA), 2µg/ml Insulin (ThermoFisher Scientific, Waltham, USA) and 10% FBS (Biowest, Riverside, USA). U937 cell culture was maintained in RPMI 1640 (Hyclone, USA) with 10% FBS at 37^{o}C centigrade and 5% CO_2. For experiments, required number of cells were subcultured in cell culture grade plates. All the experiments were however performed in the presence of cKSFM.

Uninfected donor corneas collected from Ramayamma International Eye bank, that were unsuitable for transplantation were used to isolate primary HCECs, as described earlier (Roy et al., 2011). The isolation of primary HCECs is elucidated in **Section 2.3.1** and **Figure 2.1**.

2.4.1 Isolation of primary HCEC

1. Human cadaveric corneas were washed with 1X PBS thoroughly and the scleral part was removed with the help of scissors.
2. The corneas were placed in a 35 mm petri dish containing 1X HBSS with dispase II (10mg/ml) and gentamycin (5mg/ml) and incubated at 4^{o}C for 4 hours.
3. After incubation, the dispase containing buffer was discarded and 2ml of 1X trypsin was added to cornea and incubated at 37^{o}C for about 1 minute.
4. The corneal epithelium was scraped gently and collected in a 15 ml falcon tube containing 300µl of FBS, to stop the trypsinization.
5. The trypsinized epithelial cells were pelleted down at 1000 rpm for 3 minutes and pellet was re-diluted in 1ml of fresh cKSFM containing 1% of Penicillin-Streptomycin solution (Hyclone, USA).
6. The isolated primary HCECs resuspension was plated for further growth in cKSFM containing Penicillin-Streptomycin solution (1%).
7. Primary-HCECs were grown in cKSFM devoid of any antibiotics one day prior to the experiment.

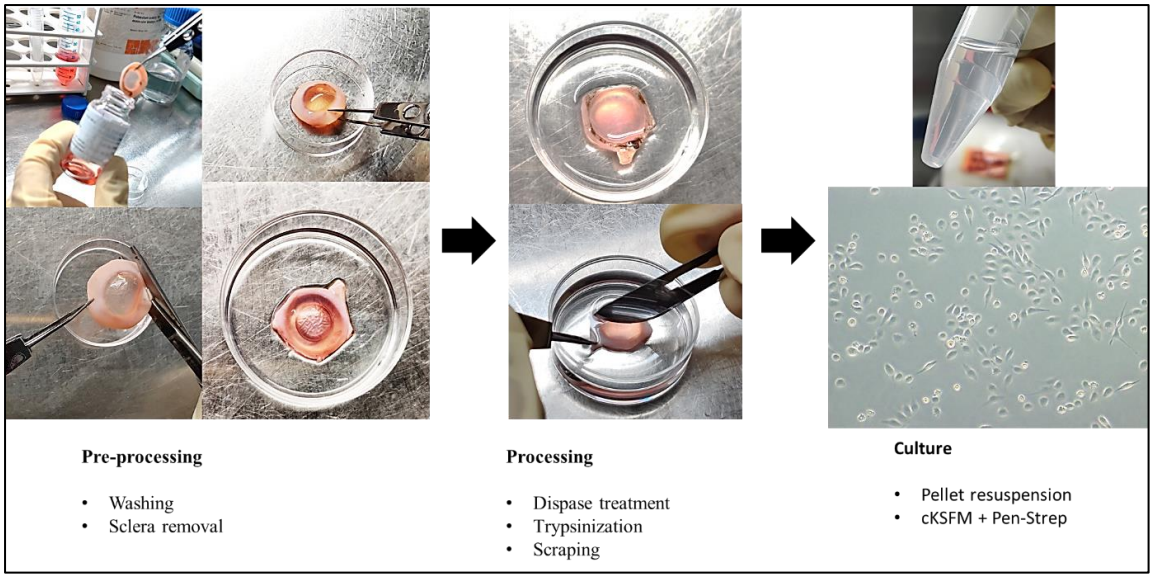


Figure 2.1 Brief representation of isolation and culture of primary HCEC.

2.5 RNA isolation, cDNA synthesis and quantitative PCR analysis

The overnight grown culture of 10^5 cells/well in a 12 wells plate (Thermo Fisher Scientific, Denmark) were infected with bacteria or treated with an inhibitor or tBHQ, and incubated at 37°C and 5% CO_2 for 3h or as specified, after which the cells were washed with 1X PBS and were incubated in gentamycin containing media (50µg/ml) for 1h at 37°C and 5% CO_2. The cells were then washed with 1X PBS and 1ml of TRIzol reagent (Invitrogen, Carlsbad, CA) was added and collected in 1.5ml microcentrifuge tubes (MCTs) and stored in -80°C for RNA isolation next day. The corneal scrapings from patients' samples were also processed similarly.

The frozen lysate from -80°C was thawed on ice and 200µl of chloroform was added to it, mixed well, incubated on ice for 2-5 minutes (1h incubation for tissue obtained from *in vivo* animal study) and was centrifuged at 12000 rpm in 4°C for 15 minutes. The uppermost aqueous layer was collected in another 1.5 ml MCT after centrifugation, to which 500 ul of isopropanol was added to precipitate RNA. This mix was incubated on ice for 10-15 minutes (overnight incubation in 4^0C for tissue obtained from *in vivo* animal study) and then centrifuged at 12000 rpm in 4°C for 10 minutes. The supernatant was carefully removed and the pellet was washed with 1ml of 100% ethanol. Then it was centrifuged at 7500-8000 rpm in 4°C for 5 minutes. Then the supernatant was removed, RNA pellet was kept for drying and then resuspended in 10-20µl of ultrapure nuclease free distilled water

and its purity (A_{260}/A_{280}) and concentration (µg/ml) were checked with Nanodrop (NanoVue, Holliston MA). The RNA sample was also run on 1.2% agarose gel to check for any contamination. Pure RNA was stored in -20°C for further use. TRIzol method was also used to isolate RNA from corneal scrapings obtained from patients.

Single strand of cDNA was synthesized from isolated RNA by reverse transcription by using kits (Reverse Transcriptase kit, Eurogentec, Belgium) or Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) using the protocol provided by the manufacturer. Concentration of synthesized cDNA (µg/ml) was measured using Nanodrop and around 220ng of cDNA sample was used as a template for quantitative PCR using SYBR Green PCR Master mix (Kapa Biosystems, Wilmington, MA/ Thermo Scientific, MA, USA) on ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Grand Island, NY). Primer sequences used in qPCR are given in **Annexure 1.**

2.6 *In vitro* susceptibility test of *S. pneumoniae* with LL-37

The overnight grown culture of Sp ATCC and Sp CS (O.D. 0.28) were centrifuged at 10000 rpm for 10 minutes and was redissolved in THB. This stock (containing 10^8 CFU/ml) was serially diluted in THB to get a dilution of 10^4 CFU/ml and was plated in a 96 wells plate with an increasing concentration of LL-37 (Invivogen, San Diego, CA, USA) at 37°C for 4h. After the incubation the culture from each well was serially diluted again, and plated on blood agar. CFUs were counted after 24h post plating and incubation at 37°C, to quantify the viable number of bacteria.

2.7 Assay for reactive oxygen species (ROS) generation

Overnight grown cultures of 10^4 HCECs or U937 in 96 wells plate were infected with PAWt or PA*ΔpscC* for 2h. After incubation, cells were washed with 1X PBS. Thereafter, the cells were stained with 2'2'-dichlorodihydrofluorescein diacetate dye (H_2DCFDA; Invitrogen, Carlsbad, CA) containing media (5µM) for 30 minutes. The cells were washed twice with 1X PBS and observed using fluorescent microscope (Olympus IX73, Zeiss, Germany) with FITC filters under 10X objective. For quantitative measure of ROS, cells were infected in a similar way and incubated with H_2DCFDA containing media for 30 minutes. H_2DCFDA, a dichlorofluorescin diacetate, is a chemically reduced form of fluorescein which is cell-permeant and used as an indicator for ROS in cells. Intracellular

esterases cleave and oxidize the acetate group of the nonfluorescent H_2DCFDA and convert it to the a highly fluorescent 2',7'-dichlorofluorescein (DCF), which can be easily detected by fluorescent microscope or measured spectrophotometrically (Excitation at 485nm and Emission at 525nm, cutoff none). The cells were washed with 1X PBS, media added and fluorescent intensity was measured in cells by using SpectraMax M3 (Softmax Pro 6.3).

In another experiment, 10^4 HCECs were seeded in 96 wells plates and infected with PAWt with or without INP0341 (100uM, 2% final DMSO concentration) or PA*ΔpscC* for 2h. Thereafter the supernatant was removed and H_2DCFDA containing media was added to cells and the cells were incubated for another 30 minutes. Then, the supernatant was removed and 1X PBS was added for observation using fluorescent microscope (Olympus IX73, Zeiss, Germany) under 10X objective. For quantitative measure of ROS, HCECs were infected similarly with PAWt with or without INP0341 (100µM, 2% final DMSO concentration) or PA*ΔpscC*, and incubated with H_2CFDA containing media for 30 minutes and quantitative fluorescence intensity was measured by SpectraMaxM3.

In another experiment, 10^4 HCECs were cultured in 96 wells plates and infected with Sp ATCC for 2h. Thereafter the supernatant was removed and H_2DCFDA containing media was added to cells and incubated for another 30 minutes. Then, the supernatant was removed and 1X PBS was added for observation under fluorescent microscope (Olympus IX73, Zeiss, Germany) under 10X objective. For quantitative measure of ROS, cells were infected similarly for 1h or 2h with Sp ATCC and incubated with H_2CFDA containing media for 30 minutes and fluorescence intensity of H_2CFDA dye was measured quantitatively by SpectraMaxM3.

We also measured ROS generation by flow cytometry (Beckman Coulter, IN, USA). In brief, 10^6 HCECs were treated with tert-butylhydroquinone (tBHQ) 1h prior to infection. Cells were then infected with Sp ATCC and were incubated for 2 hours. After infection cells were washed with 1 X PBS twice and 500µl of FACS buffer (1% FBS in 1X PBS) was added per well. The cells were gently scraped out without lysing and collected in MCTs and pelleted down at 1500 rpm for 5 minutes. To the control unstained cells only 300µl of the FACS buffer was added to resuspend the cell pellet. To the rest of the treated cell pellets 100µl of H_2DCFDA containing media (5µM) was added and the cells were incubated for 30 minutes in ice and dark. Treated cells were then washed twice with FACS buffer and

then re-diluted with 300µl of FACS buffer and flow cytometry analysis was performed using Beckman Coulter, IN, USA.

2.8 Immunostaining

5 X 10^4 cells were transferred on coverslips placed in a 12 wells plate, grown overnight and was infected with PAWt, PA*ΔpscC*, or *S. pneumoniae* and incubated for specified time period at 37°C, 5% CO_2. The cells were then washed with 1X PBS and 150µl of 4% Paraformaldehyde (PFA) was added for 15 minutes to fix the cells. The cells were then washed with 1X PBS and treated with 0.2% Triton X-100 for 1 minute for permeabilization. The cells were washed again with 1X PBS twice and primary antibodies for NFkβ-p65 (1:200, Novus Biologicals, Littleton, CO), LL-37 (1:100; BioLegend, San Diego, CA, USA), Nrf2 (1:200, Novus Biologicals, Littleton, CO), and LC3B (1:200 dilution; CST, MA) was added to the cells for 45 minutes. After washing the cells with 1X PBS twice, Alexafluor 488 labeled secondary antibody (1:500; Molecular probes, Eugene, OR) was added and incubated for 45 minutes. The cells were then washed with 1X PBS twice, and milliQ water once, counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Abcam, Cambridge) and images observed and captured on a fluorescent microscope (Olympus IX73, Zeiss, Germany).

2.9 Immunohistochemistry

5µm thick tissue sections of the paraffin-embedded corneas obtained from patients diagnosed with *S. pneumoniae* keratitis and underwent therapeutic penetrating keratoplasty was taken from Pathology department, LVPEI. Cadaveric, infection free corneas, that were deemed unsuitable for transplantation were taken as control. The sections were deparaffinised and immunostained with LL-37 antibody (1:100, BioLegend, SanDiego, CA, USA), anti-3 nitrotyrosine (1:50, Novus Biologicals, Littleton, CO) or anti-catalase (1:50, Novus Biologicals, Littleton, CO) for 45 minutes and then after washing with 1X PBS twice, with Alexafluor 488 labelled secondary antibody (1:250; Molecular probes, Eugene, OR) was added and incubation was done for another 45 minutes. The sections were then washed with 1X PBS twice, counterstained with DAPI (Abcam, Cambridge) or Propidium Iodide (Invitrogen, Carlsbad, CA) and images observed on a fluorescent

microscope (Olympus IX73, Zeiss, Germany) using 10X objective and imaged using Olympus DP71 camera.

2.10 Western blotting

10^6 cells (HCECs or U937) were grown in 6 wells plate, overnight and infected with bacteria with a MOI of 1:10 and incubated at 37°C and 5% CO_2 for different time points. Thereafter, the cells were washed with 1X PBS twice, and 110µl of 1X lysis buffer (CST, Danvers, MA) was added per well, incubated on ice for 10 minutes and the lysate was scraped out in 1.5 ml microcentrifuge tubes, out of which 5µl from each lysate was kept aside for total protein estimation by Bicinchoninic acid (BCA) method (Thermo scientific, CA) following the manufacturer's guidelines.

Equal concentration of protein samples was run on SDS-PAGE, transferred onto nitrocellulose membrane by semi-wet transfer apparatus (Amersham Biosciences, CA, USA) and probed with one of the following primary antibodies pIkB (1:2000 dilution; CST, MA; 1:2000 dilution, Novus Biologicals, Littleton, CO), pERK (1:2000 dilution; CST, MA; 1:2000 dilution, Novus Biologicals, Littleton, CO), total ERK (1:2000 dilution; CST, MA), pp38 (1:2000 dilution; CST, MA; 1:2000 dilution, Novus Biologicals, Littleton, CO), total p38 (1:2000 dilution; CST, MA), pJNK (1:2000 dilution, Novus Biologicals, Littleton, CO), total JNK (1:2000 dilution; CST, MA; 1:2000 dilution, Novus Biologicals, Littleton, CO), total STAT3 (1:2000 dilution; CST, MA), pSTAT3 (1:2000 dilution; CST, MA), pPI3K (1:2000 dilution; eLabscience, Houston, T), total PI3K (1:2000 dilution, Novus Biologicals, Littleton, CO), pAKT (1:2000 dilution, Novus Biologicals, Littleton, CO), LC3B (1:2000 dilution; CST, MA), LL-37 (1:2000 dilution; CST, MA; 1:2000 dilution, Novus Biologicals, Littleton, CO) and β-actin (1:2000 dilution; CST, MA).

The blots were then counterstained with IRDye-680 secondary antibody (1:6000 dilution: LI-COR Biotechnology, Lincoln, NE, USA) and was developed using Odyssey CLx Imaging System (LI-COR Biotechnology, NE) at 700nm. The band densities were checked using ImageJ (Schneider et al., 2012).

2.11 T3SS inhibitor (INP0341) formulation

Salicylidene acylhydrazide (INP0341) was kindly gifted by our collaborator Dr. Mikael Elofsson, Umeå University, Sweden. 25mM of stock solution was prepared in dimethylsulfoxide (DMSO) and stored in -20^{o}C in dark for further use. 5mM of intermediate solution was prepared in 1:1 Media: DMSO. The working concentrations were freshly prepared in media before the experiments.

2.12 Cytotoxicity test by lactate dehydrogenase (LDH) release

Overnight culture of 10^4 HCECs in 96 wells plate was infected with PAWt with or without INP0341 (100µM, 2% final DMSO concentration) or PAΔ*pscC* and was incubated for 6h. The cells were washed and imaging was done under phase contrast microscope. Parallelly, LDH assay was done using CytoTox nonradioactive cytotoxicity assay kit (Promega, Madison, USA) with the cell supernatant. For positive control, cells were treated with 0.3% Triton X-100 and/or 1X lysis buffer supplied in the CytoTox 96 cytotoxicity assay colorimetric kit (Promega, Madison, WI USA). The supernatant was taken from each well after incubation and the release of LDH was quantitatively measured at 490 nm using CytoTox 96 cytotoxicity assay colorimetric kit following manufacturer's guidelines. The LDH activity was analyzed by colorimetric method that is based on the principle of tetrazolium salt reduction to formazan violet crystals by LDH mediated NADPH formation (Ponsoda et al., 1991).

In a separate experiment, overnight grown 10^4 HCECs were incubated with different concentrations of INP0341- 50µM (1% DMSO), 100µM (2% DMSO), 250µM (5% DMSO) and 500µM (10% DMSO), in triplicates and incubated for 6h at 37^{o}C / 5% CO_2. The supernatant was taken from each well after incubation and the release of LDH was quantitatively measured at 490 nm as described above.

2.13 Cytotoxicity test by Propidium iodide staining (PI)

Overnight grown HCECs or U937 (10^4 cells/well) in 96 wells plate were infected with PAWt or PA*ΔpscC* for 2h, washed and stained with Propidium Iodide (PI) (500nM in 1X PBS) (Invitrogen, Carlsbad, CA) for 20 minutes and incubated at 37^{o}C and 5% CO_2. PI is a fluorescent nuclear and chromosome counterstain that intercalates between the bases of

DNA. It is not permeable in live cells and hence widely used to detect dead cells. The stain was removed, 1X PBS added and observed under fluorescent microscope.

In a separate experiment overnight grown culture of 10^4 HCECs in 96 wells plate were infected with Sp ATCC or Sp CS for 4h, washed and stained with Propidium Iodide (PI) as described above. The stain was removed, 1X PBS added and observed under fluorescent microscope.

In another experiment overnight grown culture of 10^4 HCECs in 96 wells plate were exposed to Sp ATCC with or without tBHQ for 6h, washed and further incubated with media containing antibiotics for 16h. Thereafter the cells were washed with 1X PBS and stained with Propidium Iodide (PI) as described above. The stain was removed, 1X PBS added and observed under fluorescent microscope.

2.14 Cell viability test by MTT

Overnight grown culture of 10^4 HCECs in 96 wells plate were infected with Sp ATCC or Sp CS for 4h at 37ºC and 5% CO2. Thereafter the cells were washed with 1X PBS and incubated in gentamicin containing media (50µg/ml) for 1h at 37ºC and 5% CO_2. The cells were then washed with 1X PBS and incubated with 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) (Molecular probes, Eugene, OR) containing incomplete media (0.5mg/ml) for 1h. MTT is used to detect cell viability as metabolically active cells convert the water-soluble MTT to an insoluble formazan that forms purple precipitate. The formazan is then solubilized in DMSO and its concentration checked by measuring O.D. at 595nm.

2.15 Actin filament staining

5 X 10^4 HCECs grown on coverslips were infected with PAWt for 6h with or without INP0341 or PAΔ*pscC* only. The cells were washed with 1X PBS twice after infection. Cells were fixed using 4% PFA and immunostained with Alexa Fluor 488 phalloidin (ThermoFisher Scientific, Waltham, USA) for 15 minutes, nucleus counterstained with DAPI (Vector laboratories, Burlingame, USA) before observing under fluorescent microscope (Olympus IX73, Zeiss, Germany) using 100X oil immersion objective.

2.16 Murine model of corneal infection

Experiments were performed on C57BL/6 mice at Vivo Bio Tech Ltd, Hyderabad, after approval from the Institutional Animal Ethics Committee (VB/IAEC/09/2018/ Mouse/C57BL/6). The animal chosen for experiment were all 6-8 weeks old and were bred in pathogen-free microisolator and handled in agreement to the ARVO Statement for the Use of Animals in Ophthalmic and Vision research. Ketamine (8.7 mg/ml) and xylazine (0.5 mg/ml) with a dosage of 0.01 ml/g body weight was injected intraperitoneally to anesthetize the mice. Three parallel scratches were made on cornea using a 26-gauge needle. In the first group of mice, 2.5µl of media containing approximately 10^5 PAWt was topically applied to one eye, and 1X PBS to the other eye. In the second group 5µl of 500µM INP0341 (10% DMSO) was added following the exposure to PAWt, and only 500µM INP0341 was added to the other eye. In the second group second dose of INP0341 was added 6h post-infection. 24 hours later, the mice were euthanized and the eye was examined under the stereomicroscope and given clinical scores as observed. The criteria for assigning the clinical score are given in **Objective 2, Chapter IV** (**Table 4.1**).

To check the bacterial load, CFUs of bacteria was calculated by plating the homogenized lysates (Homogenizer, Genetix Biotech, Hyderabad, India) along with the serial dilutions.

In a separate experiment, mice were infected with PAWt with INP0341 treatment at 0h and 6h post infection (group I) or at 3h and 6h post infection (group II). Mice were euthanized 24h post infection and corneal opacification was observed under stereomicroscope and clinical scores were assigned according to **Table 4.1**. CFU was counted by plating whole eye homogenates 24h post infection.

2.17 Histology and immunohistochemistry of murine eye sections

The mice eyes were collected in 10% formalin after infection and/or treatment. The corneal sectioning was done by Vivo Bio Tech Ltd, Hyderabad and Pathology department, LVPEI. The sections were sent to the Pathology Department, LVPEI for deparaffinization and staining with hematoxylin and eosin (H&E) to check for cellular infiltrations in the cornea.

2.18 Cytokine quantification by ELISA

HCECs were infected with Sp ATCC with or without tBHQ for 6 h and supernatants were collected. Cytokines like IL-1β and IL-6 in the supernatant were quantitatively measured using Quantibody® Human Inflammation Array, a kit available commercially (RayBiotech, GA, USA) following the protocol provided by the manufacturer. In detail, glass slide was equilibrated at room temperature (RT) for about 20-30 minutes and air dried for another 1-2h. Meanwhile the standards were prepared using sample diluent provided with the kit. The array surface was blocked with 100µl of sample diluent at RT for 30 minutes. Then the sample diluent was decanted and 100µl of standard or sample was added to each well and left undisturbed at RT for 2h. The samples were decanted and the wells washed with 1X wash buffers I and II provided with the kit. Biotinylated Detection Antibody Cocktail was added to each well and stored at RT for 2h. After washing the wells as mentioned before, Streptavidin-Conjugated Fluor was added to each well and left at RT and dark for 1h. The wells were washed, slides were dried and scanned with a gene microarray laser scanner. Densitometry analysis were done with the help of a software.

2.19 Bacterial load measurement by colony forming unit (CFU) and Optical density (O.D.)

HCEC (10^4 cells/well) were infected with Sp ATCC or Sp CS, in the presence or absence of tBHQ, for 6h, washed and lysed using 0.3% triton X-100. The lysate was diluted serially and plated onto blood agar. CFUs were counted manually and plotted graphically. The supernatant from a similar experiment where HCEC were exposed to Sp ATCC with or without tBHQ were also diluted serially and plated onto blood agar plates for determining the CFUs. In another experiment Sp ATCC was incubated with or without tBHQ for 24h and OD measured at 600nm using SpectraMax M3.

2.20 RNA interference of *Nrf2* and *LL-37* in HCEC

HCEC (10^4 cells/well) were transiently transfected with *Nrf2* siRNA, or *LL-37* siRNA or control siRNA using lipofectamine 3000 TM transfection agent (Invitrogen, Carlsbad, CA) for RNA interference. 10µM of stock siRNAs were reconstituted following the manufacturer's protocol (Santa Cruz, Dallas, TX) and cells were transfected using Lipofectamine kit with working concentration of 150nM. In some experiments, cells were

transfected with *Nrf2* siRNA or control siRNA, exposed to Sp ATCC with or without tBHQ as mentioned in text and LC3B expression was checked by immunostaining. In separate experiments, cells were transfected with *LL-37* siRNA, and then infected with *S. pneumoniae* with or without tBHQ. The cells treated with 0.3% triton X-100 and cell lysates were diluted and plated onto blood agar to determine the CFUs. In another experiment, HCEC were similarly transfected with control siRNA or *LL-37* siRNA and infected with Sp ATCC for 4 h in presence or absence of tBHQ and cell cytotoxicity was checked by lactate dehydrogenase assay, as described earlier.

2.21 Wound healing activity of LL-37 in HCECs

Scratch was made on the monolayer of overnight grown culture of HCECs in a serum free media, with a 10µl tip head. Thereafter the cells were incubated with media only or different concentrations of LL-37 (100ng/ml, 250ng/ml or 500ng/ml). The wound closure was observed and images were taken under phase contrast microscope using 5X objective, at different time intervals (0h, 8h, 12h, 24h, 30h) and the percentage wound closure was calculated using ImageJ. In another experiment we made a scratch wound in a monolayer of HCECs as described above and incubated with LL-37 for 24h. Thereafter we fixed the cells and immunostained the cells for Ki-67, a proliferation marker, and observed under fluorescent microscope (Olympus IX73, Zeiss, Germany), to check if proliferation contributes to LL-37 mediated wound healing.

2.22 Statistical analysis

Means are represented as bar graphs and error bars denotes SEM. Statistical analysis was done using either one-way ANOVA, two-way ANOVA, paired one-tailed t-test or unpaired t-test (GraphPad Software, La Jolla, CA, USA). Significant difference was considered when P-values were less than 0.05 or as otherwise mentioned.

***The list of materials used in the study is given in Annexure 2.**

CHAPTER III

OBJECTIVE 1: TO DETERMINE THE EXPRESSION OF ANTIMICROBIAL PEPTIDES (AMPs) DURING BACTERIAL KERATITIS IN PATIENTS

3.1 Introduction

Corneal opacity is the fifth major cause of blindness and visual impairment worldwide (Flaxman et al., 2017) with an estimated 4.2 million people suffering from it (*Blindness and Vision Impairment*, n.d.). One of the major causes of corneal opacity is keratitis, which is a condition presented with severe inflammation of cornea caused either by infectious or non-infectious sources. Infectious keratitis is acute or chronic, transient infection of the cornea that causes inflammation, corneal ulceration and opacity which may lead to blindness. The common pathogens responsible for causing infectious keratitis are bacteria and fungi. The most common bacteria isolated from cases of bacterial keratitis are gram negative *Pseudomonas aeruginosa* and gram positive *Staphylococcus aureus*, followed by gram positive *Streptococcus pneumoniae* (Ting, Ho, Deshmukh, et al., 2021). The prevalence of *P. aeruginosa* keratitis ranges from 18.2% to 68.2 %, with the maximum prevalence in contact-lens related microbial keratitis. In India, the average percentage of isolation and prevalence of *P. aeruginosa* from ocular infections is 76.3% and 22.7% respectively (Subedi et al., 2018; Vazirani et al., 2015).

The current regimen of treating bacterial keratitis majorly includes the usage of antibiotics. However, emerging and re-emerging antibiotic resistance in bacteria worsens the clinical outcome of keratitis (Fernandes et al., 2016). In 2017, World Health Organization (WHO) published a report on global antibiotic resistance, placing antibiotic resistant pathogens in three different priority groups (critical, high or medium). In this report, *P. aeruginosa* and *S. pneumoniae* were placed in the critical and medium priority group respectively and therefore needs immediate attention (*WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed*, n.d.). In 2019, CDC in its "Antibiotic Resistance Threats in the United States (AR) report," placed multidrug-resistant *P. aeruginosa* and drug-resistant *S. pneumoniae* in 'serious threats' category (*Biggest Threats and Data | Antibiotic/Antimicrobial Resistance | CDC*, n.d.).

There are various alternative therapeutics that are being studied to combat the antibiotic resistance. Some of these alternatives include small molecular inhibitors of bacterial lectins, quorum sensing and virulent factors (Hauck et al., 2013), metal chelators (Qiu et al., 2011), vaccination (Asadi Karam et al., 2019; Sigurdsson et al., 2017), nanoparticles (Bayroodi & Jalal, 2016), electrochemical scaffolds (Raval et al., 2019; Sultana et al., 2015), phage therapy (Khalifa et al., 2015) and anti-inflammatory drugs (Dutta et al., 2007; Oliveira et

al., 2019). One such alternative that shows huge potential is the usage of antimicrobial peptides (AMPs) (Galdiero et al., 2015; Magrone et al., 2018; Nuti et al., 2017). AMPs are naturally expressed by prokaryotes and eukaryotes (Yazici et al., 2018). They can also be designed and synthesized chemically (P. G. Lima et al., 2021). AMPs are also one of the crucial players of innate immunity. They are short, cationic with broad spectrum activity against several pathogens (Q. Y. Zhang et al., 2021). The direct mode of action of AMPs includes creating pores on the bacterial cell membrane, causing disruption, cell lysis and death (Luo & Song, 2021). AMPs can also act intracellularly by binding to and inhibiting the function of host's nucleic acids or proteins (Le et al., 2017). They also have immunomodulatory and wound healing functions (Hilchie et al., 2013; Niyonsaba et al., 2007). AMPs can initiate both pro-inflammatory and anti-inflammatory responses in the host depending on the host's nature, environmental interactions and the peptide's concentration (Brown et al., 2011; Paranjape et al., 2013). In a study by Chen *et. al.*, LL-37, the only human AMP belonging to the group cathelicidin, was found to modulate immune response of cytokine or dsDNA induced keratinocyte.

The different layers of cornea are known to constitutively express various antimicrobial peptides and other host defense mediators like cytokines, pattern recognition receptors, surfactant proteins, surface mucins etc. Additionally, an intact corneal surface and basement membrane also act as physical barrier against the invading pathogen. The resident immune cells like macrophages, dendritic cells and keratocytes in corneal epithelium and stroma also help in defense against the invading pathogens (Evans & Fleiszig, 2013). The constitutively expressed AMPs by the corneal surface include beta defensins (*hBD-1,2,3*), *HNP 1-3*, *LL-37*, *RNase-7*, psoriasin, histatin-5 and dermicidin (Haynes et al., 1999; Ling C. Huang et al., 2009; McDermott, 2009; McIntosh et al., 2005). During infection, the expression of various AMPs are induced that act against the pathogens together with various other innate immune responses (Gordon, Huang, et al., 2005; McIntosh et al., 2005; Mohammed et al., 2017) (**Figure 3.1**). β-Defensins are primarily expressed in humans by epithelial cells at mucosal surfaces and play an important role as modulators of infection, inflammation and wound healing (Semple & Dorin, 2012). Human corneal epithelium was earlier shown to express *hBD-1* and *hBD-3* constitutively, and *hBD-2* when stimulated by pro-inflammatory cytokines (McDermott et al., 2003). Inducible *hBD-3* expression is also reported in corneal and conjunctival samples from infected patients (McIntosh et al., 2005). Defensin is upregulated in corneal stroma of eyes with infectious keratitis (Gottsch et al.,

1998). *hBD-1*, *hBD-2* and *hBD-3* is induced by microbial-stimuli in human epidermal keratinocytes (Sørensen et al., 2005). *hBD-2* expression is also induced in human alveolar epithelial cells during *Mycobacterium tuberculosis* infection (Rivas-Santiago et al., 2005). *hBD-2* gene and protein expression is also induced in renal epithelium with chronic infection (J. Lehmann et al., 2002).

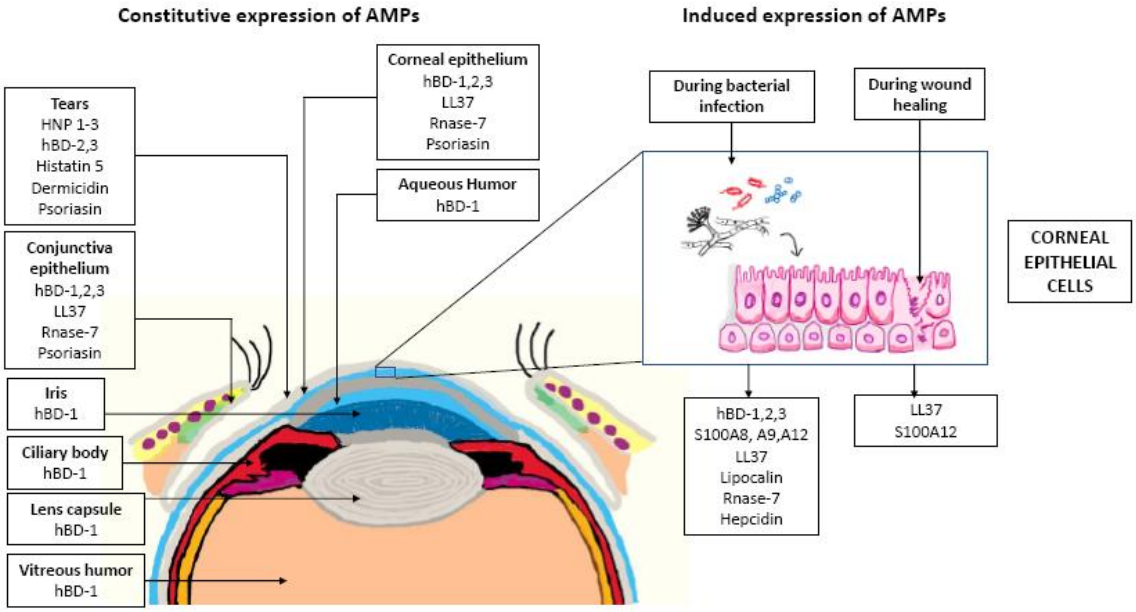


Figure 3.1 Representative image of constitutively expressed and induced expression of AMPs in the eye.

In addition, humans express LL-37, a single antimicrobial peptide belonging to the cathelicidin group which is produced as an inactive pro-form of 18kDa holoprotein (hCAP-18) in salivary glands, neutrophils, lung, mast cells, squamous epithelia, seminal fluid, keratinocytes and urogenital and gastrointestinal tracts (Dürr et al., 2006). Upon stimulation by infection this pro-form is converted to an active form. The S100 proteins are calcium-binding proteins that regulates proliferation, apoptosis, inflammation, cell migration, differentiation, energy metabolism, and Ca (2+) homeostasis (Donato, 1999). Lipocalin-2, also known as neutrophil gelatinase-associated lipocalin was first discovered as a major component of the neutrophil secondary granules. Later, it was found that lipocalin-2 can also be synthesized *de novo* by epithelial cells and macrophages during inflammation (Jung et al., 2012; Nielsen et al., 1996). RNAse-7, a ribonuclease, is expressed in various epithelial tissues including skin, respiratory and genitourinary tract, and has been shown to play an crucial role in epithelial innate immune response (Harder & Schröder, 2002; Spencer et al., 2013). Interleukin (IL)-1β, a pro-inflammatory cytokine, stimulated *RNAse7*

expression was reported in the superficial layers of ocular surface cells (Mohammed et al., 2011). Hepcidin, originally discovered in hepatocytes, is a key regulator of iron metabolism and mediator of innate immunity (Ganz, 2003).

Although, many studies have shown the induced expression of AMPs during infection, a complete picture of the expression of endogenous AMPs during bacterial keratitis, specifically caused by *P. aeruginosa* and *S. pneumoniae*, is lacking. This chapter focuses on **objective 1**, which is to determine the expression of AMPs during bacterial keratitis in patients, to address this lacuna.

3.2 Results

3.2.1 Clinical characteristics of patients with *P. aeruginosa* keratitis

We collected corneal scrapings from patients with their informed consent. The identification of the causative organism was later confirmed from Jhaveri Microbiology Centre, LVPEI, and the characteristics of patients with *P. aeruginosa* keratitis were listed out (**Table 3.1**). The mean age was 36 years with a standard deviation of 19. About 54% of the patients were female and 62% of the patients had the presence of hypopyon. The majority of the patients were housewife, agriculture or manual labor and 53% of the patients had ulcers less than 5 mm in size.

Table 3.1 Clinical characteristics of patients with *P. aeruginosa* keratitis	
Characteristics	
Age	2 to 81
Mean (SD)	36.692 (19.011)
Sex	
Male (%)	46
Female (%)	54
Hypopyon	
Yes (%)	62
No (%)	38
Occupation	
Housewife (%)	31
Agriculture/Manual Labor (%)	31
Desk Jobs (%)	23
Unspecified (%)	15
Size of Ulcer	
<5 mm (%)	53.84
5–15 mm (%)	46.15
>15 mm (%)	0

3.2.2 Antimicrobial peptides are expressed differentially in patients with *P. aeruginosa* keratitis

The expression of AMPs like β-defensins (BD-1,2,3 and 4), S100 peptides (S100A7,8,9 and 12), LL37, lipocalin-2, RNAse-7 and hepcidin in patient's corneal scraping samples was checked by quantitative PCR. Uninfected cadaveric corneas not suitable for transplantation surgery were used as control.

In this study, we found a significant upregulation of *hBD-1* (>21-fold), *hBD-2* (>5-fold) and *hBD-3* (>10-fold). *hBD-4* was not expressed in any of the samples obtained from patients. The expression of *S100A7*, *S100A8*, *S100A9* and *S100A12* was significantly up regulated with *S100A12* showing the maximum value (>90-fold). *RNAse7* showed decreased expression and there was a significant upregulation of *LL-37* (>600-fold), lipocalin 2 (>60-fold) and hepcidin (>20-fold). (**Figure 3.2**) Primer sequences are given in **Annexure 1**.

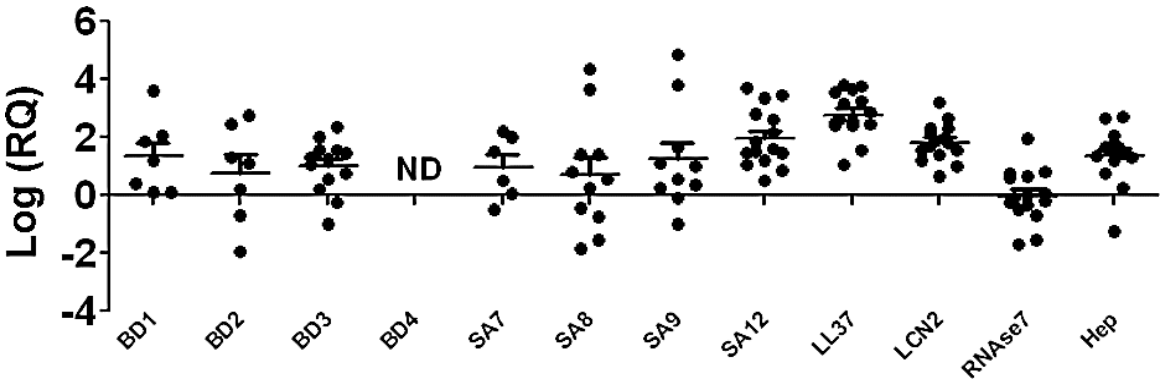


Figure 3.2 Expression of AMPs in *P. aeruginosa* keratitis patient samples. The expression was checked by quantitative PCR and the values plotted as log relative gene expression (RQ) as compared to uninfected cadaveric corneas. Each data point represents individual patient. GraphPad Prism software was used to calculate ANOVA and P<0.001 was considered significant. ND: Not detected.

This is the first report on AMP expression in *P. aeruginosa* keratitis patients' corneal scrapings.

3.2.3 Clinical characteristics of patients with *S. pneumoniae* keratitis

We collected corneal scrapings from twelve patients with their informed consent. The identification of the causative organism was later confirmed from Jhaveri Microbiology Centre, LVPEI, and the characteristics of patients with *S. pneumoniae* keratitis were presented in **Table 3.2**. The mean age was 52 years with a standard deviation of about 20. About 25% of the patients were female. The hypopyon was present in 25% of the patients. The majority of the patients were involved in agriculture or manual labor. Majority of the patients had ulcers either less than 5 mm or greater than 15 mm in size.

Table 3.2 Clinical characteristics of patients with *Streptococcus pneumoniae* keratitis	
Characteristics	
Age	25 to 90
Mean (SD)	52.25 (20.176)
Sex	
Male (%)	75
Female (%)	25
Hypopyon	
Yes (%)	25
No (%)	75
Occupation	
Agriculture/Manual Labor (%)	75
Desk Jobs (%)	25
Size of Ulcer	
<5 mm (%)	37.5
5–15 mm (%)	25
>15 mm (%)	37.5

3.2.4 *S. pneumoniae* keratitis patients' samples show differential expression of antimicrobial peptides

We took corneal scrapings from *S. pneumoniae* keratitis patients and analyzed the expression of same panel of AMPs by quantitative PCR. Uninfected cadaveric corneas not suitable for transplantation were used as control. We found a significant upregulation of AMPs *hBD-2*, *hBD-3*, *hBD-4*, *S100A7*, *S100A8*, *S100A9*, *S100A12*, *LL-37*, hepcidin, lipocalin-2 and *RNAse-7*. However, *hBD-1* expression was downregulated (**Figure 3.3**) in contrast to its upregulation found in *Pseudomonas* keratitis patients. This is the first report of AMP expression in patients with *S. pneumoniae* keratitis.

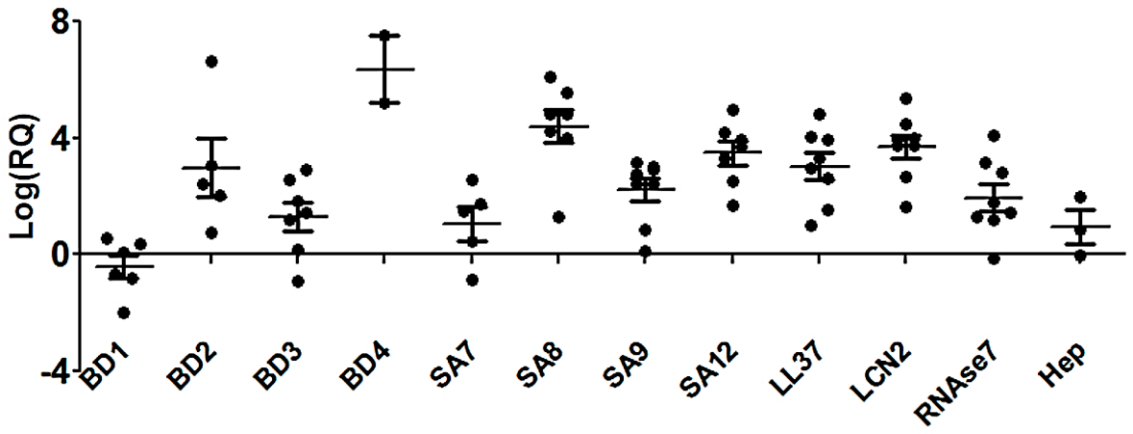


Figure 3.3 Differential expression of AMPs in *S. pneumoniae* keratitis patient's samples. Corneal scrapings of SP keratitis patients were used to determine AMPs expression by quantitative PCR. The log of relative gene expression (log (RQ)) is plotted in the graph. P<0.0001 is considered as significant. Each data point represents each patient.

3.3 Discussion

The emergence and re-emergence of antibiotic resistance in bacteria have compelled researchers to look for new alternatives. Antimicrobial peptides have shown adequate promise as an alternative or adjunct therapeutic that studying its role in infection has been a burning topic since past few years. To understand the significance of AMPs during infection, it is necessary to study its expression profile in patients. There have been several reports of endogenous expression of AMPs in patients of atopic dermatitis (Ong et al., 2002), ulcerative colitis (Fahlgren et al., 2003), infective cellulitis (Stryjewski et al., 2007), joint infections (Gollwitzer et al., 2013) and several other diseases. Differential expression of AMPs was also reported in patients with chronic skin wound and gastric mucosa infection (Bauer et al., 2013; Dressel et al., 2010). However, there were no report on the endogenous expression of AMPs in keratitis patients during *P. aeruginosa* or *S. pneumoniae* infection. In this objective we checked a panel of AMPs in patients' samples obtained from cases of *P. aeruginosa* or *S. pneumoniae* keratitis for the first time. We checked the expression of beta-defensins, S100A proteins, cathelicidin, hepcidin, lipocalin and *RNase-7*. We found significant upregulation of *hBD-2, 3, S100A7, S100A8, S100A9, S100A12,* lipocalin-2 and hepcidin in both *P. aeruginosa* and *S. pneumoniae* patients' samples. *hBD-1* was upregulated in *P. aeruginosa* patients' samples whereas it was downregulated in *S. pneumoniae* patients' samples. We also found reduced expression of *RNase7* in *P. aeruginosa* keratitis patients' samples whereas it was upregulated in *S.*

pneumoniae patients' samples. We found the expression of *hBD-4* in *S. pneumoniae* patients' samples, whereas, it was not detected in *P. aeruginosa* keratitis patients' samples. In another study done by Roy *et. al.*, the expression of AMPs like *S100A8, S100A9* and *hBD-1* was found to be significantly upregulated in corneal ulcers obtained from patients infected with *Corynebacterium pseudodiphtheriticum* (Roy et al., 2015). *hBD-2* was also found to be upregulated in response to *Helicobacter pyroli* infection in patients (Bauer et al., 2013). Otri *et. al.* first reported the differential expression of *hBD-3* and *hBD-9* in the human ocular surface in response to bacterial infection (Otri et al., 2012) showing that not all AMPs respond similarly to infection. In another study, *LL-37* was found to be differentially expressed in tissue samples of periodontitis patients (Türkoğlu et al., 2011).

To conclude, we reported the expression of AMPs in gram negative *P. aeruginosa* and gram positive *S. pneumoniae* patients' samples for the first time. The expression profile of AMPs was markedly distinct suggesting that the induced expression of AMPs in bacterial keratitis is also unique for each type of infection. For future studies, the knowledge of AMPs expression profile in keratitis patients will help understand the AMP-mediated innate immune response of the host and the impact of AMPs expression in the patients' clinical outcome.

* **This chapter has been published in parts in the journal 'Pathogens and Disease' (Sharma et.al., 2018) and 'Pathogens' (Sharma et.al., 2019). The citations are given below and the publications are attached.**

Sharma, P., Guha, S., Garg, P., & Roy, S. (2018). Differential expression of antimicrobial peptides in corneal infection and regulation of antimicrobial peptides and reactive oxygen species by type III secretion system of *Pseudomonas aeruginosa*. Pathogens and disease, 76(1), 10.1093/femspd/fty001. https://doi.org/10.1093/femspd/fty001

Sharma, P., Sharma, N., Mishra, P., Joseph, J., Mishra, D. K., Garg, P., & Roy, S. (2019). Differential Expression of Antimicrobial Peptides in *Streptococcus pneumoniae* Keratitis and STAT3-Dependent Expression of LL-37 by *Streptococcus pneumoniae* in Human Corneal Epithelial Cells. Pathogens (Basel, Switzerland), 8(1), 31. https://doi.org/10.3390/pathogens8010031

CHAPTER IV

OBJECTIVE 2: ROLE OF ANTIMICROBIAL PEPTIDES IN KERATITIS CAUSED BY GRAM NEGATIVE BACTERIA *Pseudomonas aeruginosa*

4.1 Introduction

Pseudomonas aeruginosa is a gram negative pathogen, opportunistic in nature and one of the leading causes of bacterial keratitis (Bharathi et al., 2003; Bourcier et al., 2003; Fong et al., 2007; Kaliamurthy et al., 2013; Mun et al., 2019; Ting, Ho, Deshmukh, et al., 2021). Almost 8-21% of all bacterial keratitis is caused by *P. aeruginosa.* Corneal infections caused by *Pseudomonas* spp. is often hard to treat and are presented with serious phenotypes. It harbors various virulence factors that make it one of the most dreaded pathogens. Some known virulent factors include outer membrane proteins and lipopolysaccharides, siderophore like pyoverdine, various secretion systems, quorum sensing and biofilm formation, flagella and type 4 pili (Jurado-Martín et al., 2021; Newman et al., 2017; Vasil, 1986). Consequently, *P. aeruginosa* corneal ulcers have a more severe presentation and are difficult to treat (Lin et al., 2019). Moreover, keratitis caused by a drug resistant strains show poor prognosis (Vazirani et al., 2015).

There are six types of secretion systems (T1SS-T6SS) in *P. aeruginosa* that have been recognized till now (Filloux, 2011; Juhas, 2015) out of which type-3 secretion system (T3SS) (**Figure 4.1**) is the most studied one and have been shown to play an important role in its pathogenesis (Galle et al., 2012). The T3SS is made up of five major parts- the components that make up the secretion machine (PscF, PscN, PscJ, PscC, PscW, PscP), components that help to translocate the secreted proteins into host cells (PopB, PopD, and PcrV), components that helps in regulation of secretion process (ExsA-transcription factor, ExsC, ExsD, and ExsE), components that hold the secreted proteins to enable the secretion (chaperone proteins) (SpcS, SpcU, PcrH, PscE and PscG), and the proteins that are translocated into host cells, known as effector proteins (ExoS, ExoU, ExoT and ExoY) (Galle et al., 2012; Hauser, 2009). The secretion system comprises of 20 different proteins (Psc, Pcr or Pop components). PscC is a secretin like protein that forms oligomers with lipoprotein PscW to form a channel in the outer membrane (Galle et al., 2012).

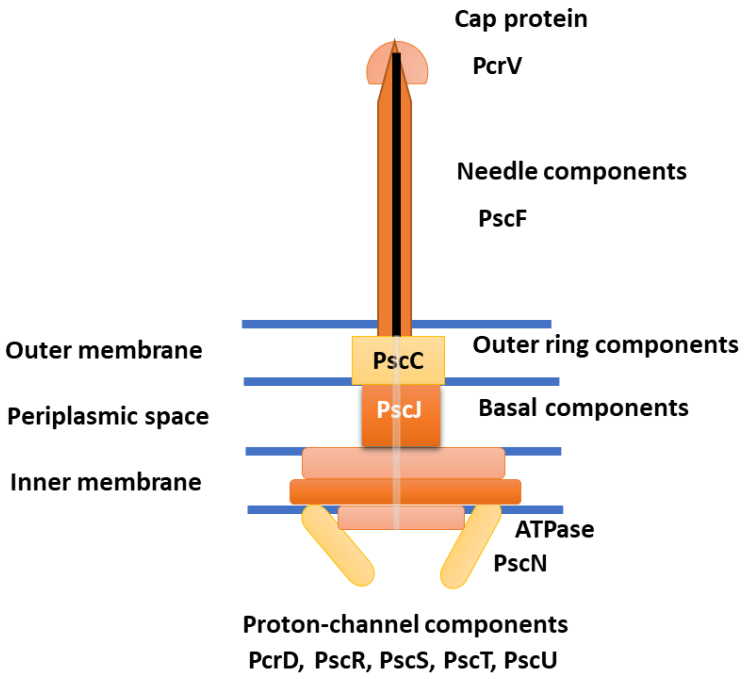


Figure 4.1 Major components of *P. aeruginosa* T3SS: cap component (PcrV), needle component (PscF). an outer membrane component (PscC) and basal components (PscJ, ATPase PscN etc.). Redrawn from Sawa, 2014 (Sawa, 2014).

P. aeruginosa T3SS secretes four effector proteins namely, ExoS, ExoU, ExoT and ExoY (Hauser, 2009). Strains that express ExoU causes rapid cell lysis whereas, ExoS secreting strains are invasive and causes membrane bleb formation in epithelial cells which serves as a site for bacterial motility and replication. Thus, invasive *P. aeruginosa* can enter epithelial cells where they form plasma membrane bleb-niches that acts as intracellular compartments via ADP-ribosyltransferase (ADPr) activity of ExoS (Heimer et al., 2013). In one study it was shown that invasive strains are associated with poorer visual acuity at presentation and clinical outcomes when compared to the cytotoxic strains. Furthermore, invasive strain associated keratitis occurred more frequently in elderly males and was significantly associated with previous ocular trauma or surgery. Further, cytotoxic strains are found to be associated significantly with contact lens wear, when compared to the invasive strains (Borkar et al., 2013; Shen et al., 2015). ExoS and ExoT play a very important role in the pathogenesis of *P. aeruginosa* keratitis by promoting apoptotic cell death of neutrophils, thereby subverting an important host response. These exotoxins also help in the survival of the pathogen inside the host neutrophils (Sun et al., 2012). T3SS apparatus was also shown to dampen the host defense, independent of the exotoxins, via NLRC4-coupled inflammasomes (Faure et al., 2014).

Since, T3SS contributes significantly to the pathogenicity of *P. aeruginosa*, several inhibitors of T3SS are being studied that ranges from antibodies (Hotinger & May, 2020) to small molecules (Keyser et al., 2008). Blocking of virulence factors can serve as an alternative to antibiotics in treating infections caused by antibiotic resistance bacteria. Recently, it was shown that a T3SS inhibitor, fluorothiazinon, was able to inhibit T3SS of *Chlamydia* spp., *P. aeruginosa*, and Salmonella *in vitro* and provided therapeutic and prophylactic protection from *Salmonella* oral infection (Zigangirova et al., 2021). In another study it was shown that, subsets of tanshinones, a natural herbal compound used in traditional Chinese medicine, inhibited the biogenesis of *P. aeruginosa* T3SS and consequently reduced the cytotoxicity and pathogenicity of the bacteria *in vitro* and reduced the severity of infection *in vivo* (Feng et al., 2019). Some other inhibitors of T3SS, like N-hydroxybenzimidazole that inhibits ExsA-DNA binding, was also shown to have anti-virulent properties against *P. aeruginosa* (Grier et al., 2010). One of the inhibitors of T3SS is INP0341, a salicylidene acylhydrazide. Earlier studies with this small molecule inhibitor of T3SS has shown that it inhibits the expression and secretion of ExoS (**Figure 4.2**), inhibits bacterial motility and biofilm formation. It was also shown to reduce *P. aeruginosa* infected mice mortality *in vivo* (Uusitalo et al., 2017).

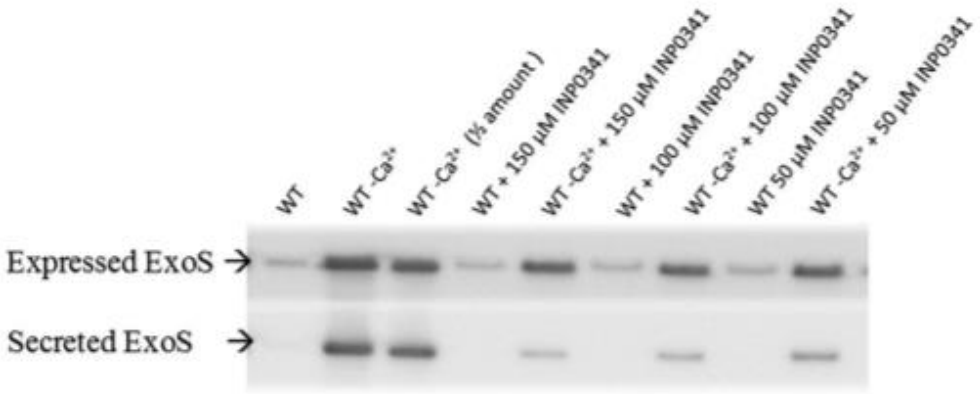


Figure 4.2 INP0341 inhibits the expression and secretion of ExoS. Expression of ExoS was induced in wild type *P. aeruginosa* by depleting Ca^{2+} from the growth media. Bacteria were incubated with 50µM, 100µM or 150µm of INP0341 in calcium depleted or normal media and the expression of ExoS was determined from whole sample and secretion of ExoS was determined from the supernatant. Image courtesy (Uusitalo et al., 2017).

Toll-like receptor (TLR) signaling is one of the most important innate immune responses during infection. TLRs are a type of pattern recognition receptors (PRRs) that identify the various components of invading pathogens, also known as pathogen associated molecular patterns (PAMPs). These receptors not only act as the initiator of innate immune responses but also acts as a bridge between innate and adaptive responses. *P. aeruginosa*

and its PAMPs acts as a ligand to TLR1/2, TLR2/6, TLR4, TLR5 or TLR9 (McIsaac et al., 2012). The activation of TLRs further lead to the initiation of downstream signaling pathways via NF-κβ and MAP-kinase, that consequently lead to the expression of effectors of innate immunity like cytokines, chemokines and AMPs (J. Zhang et al., 2005). TLRs activation in corneal resident macrophages by *P. aeruginosa* during keratitis leads to the expression of proinflammatory cytokines like IL-1α and IL-1β. This cytokine influx leads to onsite neutrophil recruitment and production of reactive oxygen and nitrogen species (ROS, RNS), matrix metalloproteinases and neutrophil extracellular traps. This sequence of events is mostly responsible for the inflammatory damages and opacification of corneal stroma during keratitis (Pearlman et al., 2013). However, the lack of these innate immune responses leads to dissemination of bacteria in systemic circulation and other vital organs (Thanabalasuriar et al., 2019). Therefore, a regulated innate immune response is required for the alleviation of the disease. Many pathogens including *P. aeruginosa* have mechanisms to evade host immune responses or invasion. *P. aeruginosa* is known to cause cytoskeletal rearrangement *in vitro* in human macrophages to facilitate its invasion and cause cell death (Mittal et al., 2016). Exoenzymes secreted by T3SS of *P. aeruginosa* play a major role in this. Rho GAP activity of Exoenzyme S (ExoS) is known to stimulate actin cytoskeletal rearrangement (Krall et al., 2002). ExoS and ExoT has been implicated in host cell rounding up and actin cytoskeletal disruption. In other studies it was shown that ExoT and ExoY causes the alteration or disruption of the host cell actin cytoskeleton and inhibits early bacterial internalization (Cowell et al., 2005; Garrity-Ryan et al., 2000).

Since we observed differential expression of various AMPs like beta defensins, cathelicidin, S100A proteins, hepcidin etc., in patients with *P. aeruginosa* keratitis (**Objective 1, Chapter III**), we further checked the expression of AMPs in our *in vitro* infection model for which we used human corneal epithelial cells (HCECs) and pro-monocytic cell line U937. We also checked the effect of T3SS on the activation TLR related signaling pathways, NF-κβ and MAP-kinase. These signaling pathways were particularly chosen as they have been shown to play an important role in the expression of AMPs. For infection we used a wild type *P. aeruginosa* (PAWt), having an intact T3SS and a mutant *P. aeruginosa* (PAΔ*pscC*), lacking a functional T3SS. We further did *in vivo* studies with our well-established mouse model of keratitis using C57BL/6 mice. Hence our goal in this section is to decipher the role of T3SS on the host innate immune response *in vitro* and *in vivo* with the focus being on AMPs.

4.2 Results

4.2.1 T3SS dependent suppression of antimicrobial peptide expression *in vitro*

To check the expression of AMPs, *in vitro* and determine if T3SS of *P. aeruginosa* plays a role, HCECs were either infected with wild type *P. aeruginosa* (PAWt) having an intact T3SS apparatus or mutant *P. aeruginosa* lacking a part of T3SS, PscC (PAΔ*pscC*). The multiplicity of infection (MOI) was 10 and the cells were incubated for 4h (3h infection followed by 1h gentamycin) or 24h (6h infection followed by 1h gentamycin followed by overnight media). A significant increase in the expression of AMPs like *hBD-1* (>3-fold), *hBD-2* (>8-fold), *hBD-3* (>8-fold), hepcidin (>5- fold), *RNAse7* (>15-fold) and *LL-37* (>60-fold) was observed in HCECs in response to infection with PAΔ*pscC* when compared to HCECs infected with PAWt at early time (4h) (**Figure 4.3 A and 4.3 B**). Similar to the patients' sample, *hBD-4* was not detected in infected HCECs. There was also an increased expression of *S100A7* (>2-fold), *S100A8* (>50-fold), *S100A9* (>25-fold) and *S100A12* (>40-fold) in HCECs infected with PAΔ*pscC* compared to cells infected with PAWt at an early time (4h) (**Figure 4.3 C**). We did not see any significant difference in AMP expression in HCECs in response to PAWt or PAΔ*pscC* at 24 h. Primers sequences are presented in **Annexure 1**.

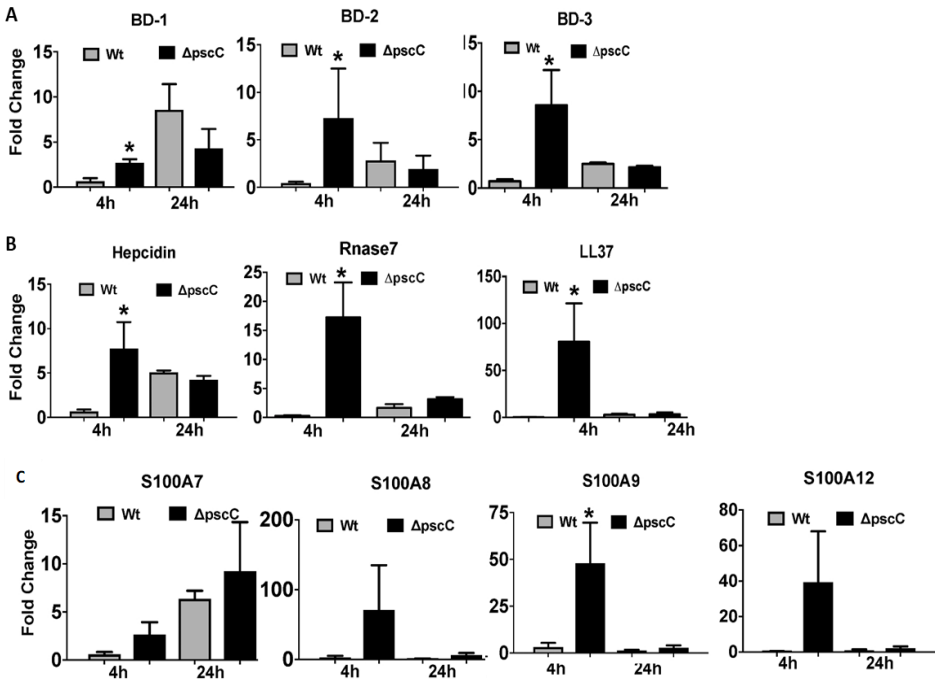


Figure 4.3 *In vitro* expression of antimicrobial peptides (AMPs) in HCECs. HCECs were infected with PAWt or PAΔ*pscC* for 4h or 24h, and AMP expression was checked by quantitative PCR, with GAPDH as an housekeeping gene. The data shown was done in duplicates and analyzed using GraphPad Prism software. (*P < 0.05, **P < 0.005)

To check if the supression of AMPs expression is limited to epithelial cells or not, we did a similar study on U937 cell line as well, because monocytes and macrophages are also one of the effectors of innate immunity and act a a "bridge" between innate and adaptive immune responses (Plüddemann et al., 2011). We found supressed expression of *hBD-2*, *hBD-3*, *S100A8*, *S100A9* and *S100A12* (**Figure 4.4A and 4.4C**) in U937 infected with PAWt when compared with the cells infected with mutant PAΔ*pscC*. There was a significant reduction in the expression of *LL-37*, *Rnase7* and hepcidin in cells infected with PAWt bacteria when compared to cells infected by mutant PAΔ*pscC* (**Figure 4.4B**).

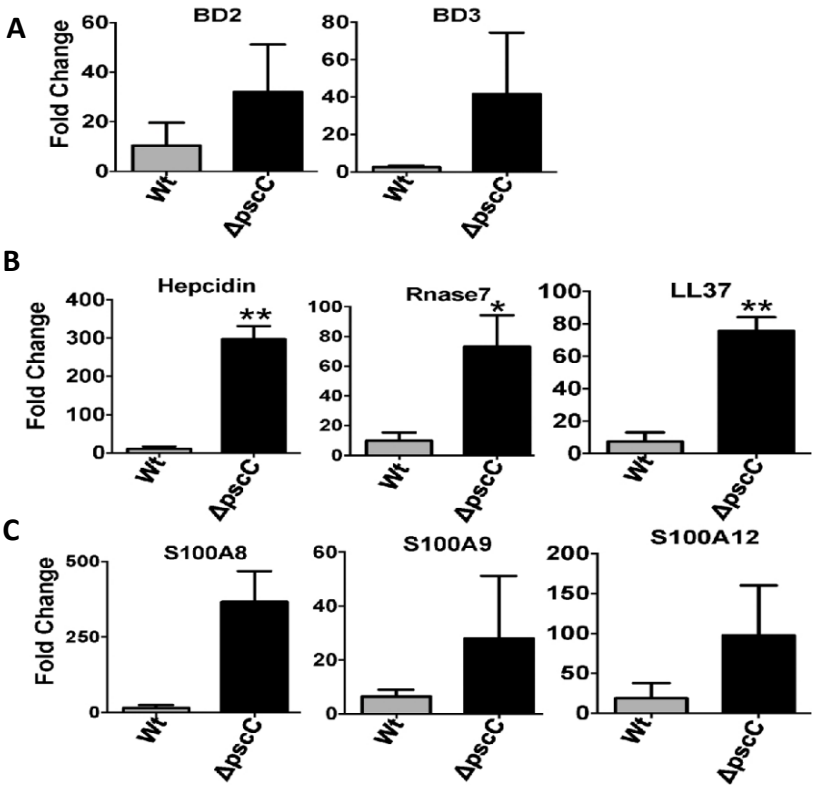


Figure 4.4 *In vitro* expression of AMPs in U937 in response to *P. aeruginosa.* U937 infected with PAWt or PAΔ*pscC* for 3h and gene expression checked by quantitative PCR. GAPDH was used as a housekeeping gene. Experiment was done twice in duplicates. (* $P < 0.05$, ** $P < 0.005$)

Similar to the findings in HCECs, *hBD-4* was also not detected in U937. *hBD-1* and *S100A7* expression was also absent in U937. These observations were in cells infected with bacteria for 3h. However, the AMPs expression did not show any significant difference in U937 infected with PAWt or PAΔ*pscC* for a longer time period (6h) (**Figure 4.5**). The sequences of primers used in the study is represented in **Annexure 1**.

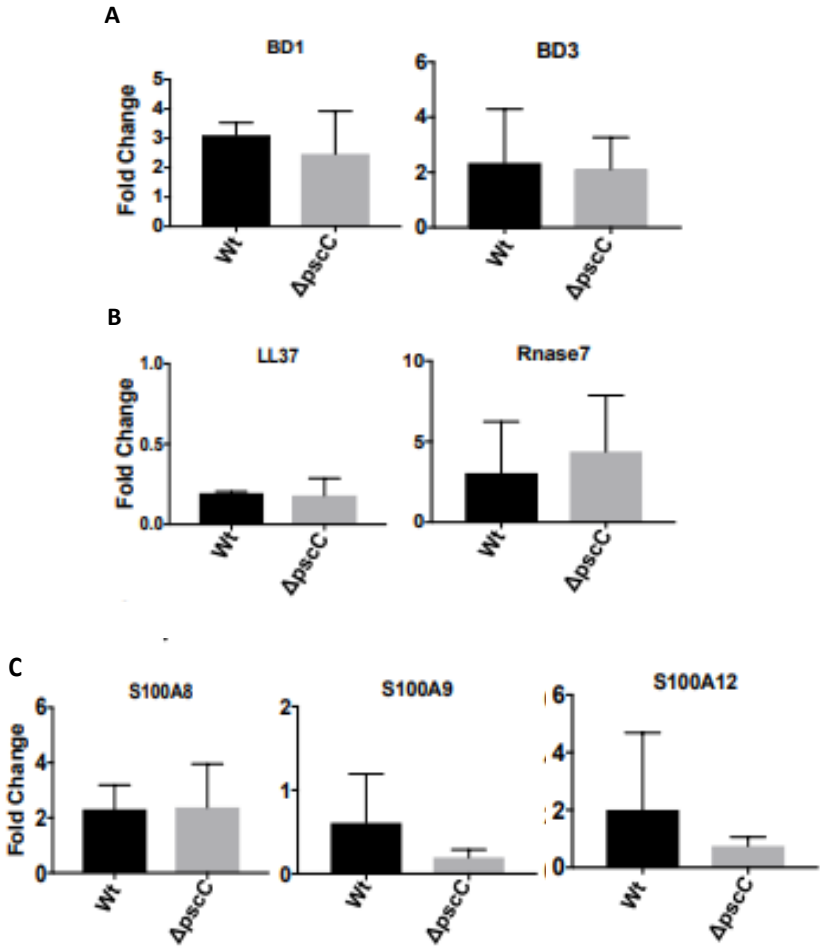


Figure 4.5 *In vitro* expression of AMPs in U937 in response to *P. aeruginosa* for 6h. Expression of AMPs in U937 infected with PAWt or PA*ΔpscC* done by quantitative PCR. The cells were infected with bacteria for 6h. Housekeeping gene being GAPDH.

4.2.2 Suppression of NF-κβ signaling is T3SS mediated in HCECs and U937

Since we observed that *P. aeruginosa* subdues AMP expression in HCECs and U937 in T3SS dependent manner, we further checked the effect of T3SS on NF-κβ signaling pathways. NF-κβ is one of the important host responses during infection and many pathogens are known to manipulate this signaling pathway (Krachler et al., 2011). NF-κβ also plays a role in AMP expression (Cunliffe & Mahida, 2004; G. Li et al., 2009; Prasad et al., 2019). During normal conditions NF-κβ is kept in the cytoplasm by an inhibitory protein, IκB, which is phosphorylated and degraded upon stimulus, thereby releasing NF-κβ. An enhanced phosphorylation of Iκβ, indicative of activation of NF-κβ, began at 30 minutes and continued till 2h in HCECs and U937 infected with PA*ΔpscC*. However, there was a delayed phosphorylation of Iκβ in the cells infected with PAWt. (**Figure 4.6A and B**).

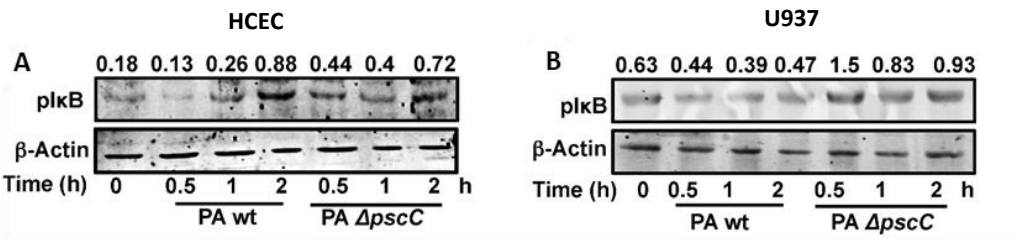


Figure 4.6 Effect of T3SS on activation of NF-κβ pathway. Western blot analysis of pIκβα in HCECs (A) and U937 (B) infected with PAWt or PAΔ*pscC*. The quantitative result was calculated as a ratio of pIκβ and β-actin band density.

Immunofluorescence microscopy was done to check the localization or nuclear translocation of p65 in infected cells, and it was observed that p65 translocated to the nucleus as early as 30 minutes in cells infected with PAΔ*pscC*. Whereas, the nuclear translocation of p65 occurred relatively later in cells infected with PAWt as compared to the cells infected with PAΔ*pscC*. (**Figure 4.7A and B**).

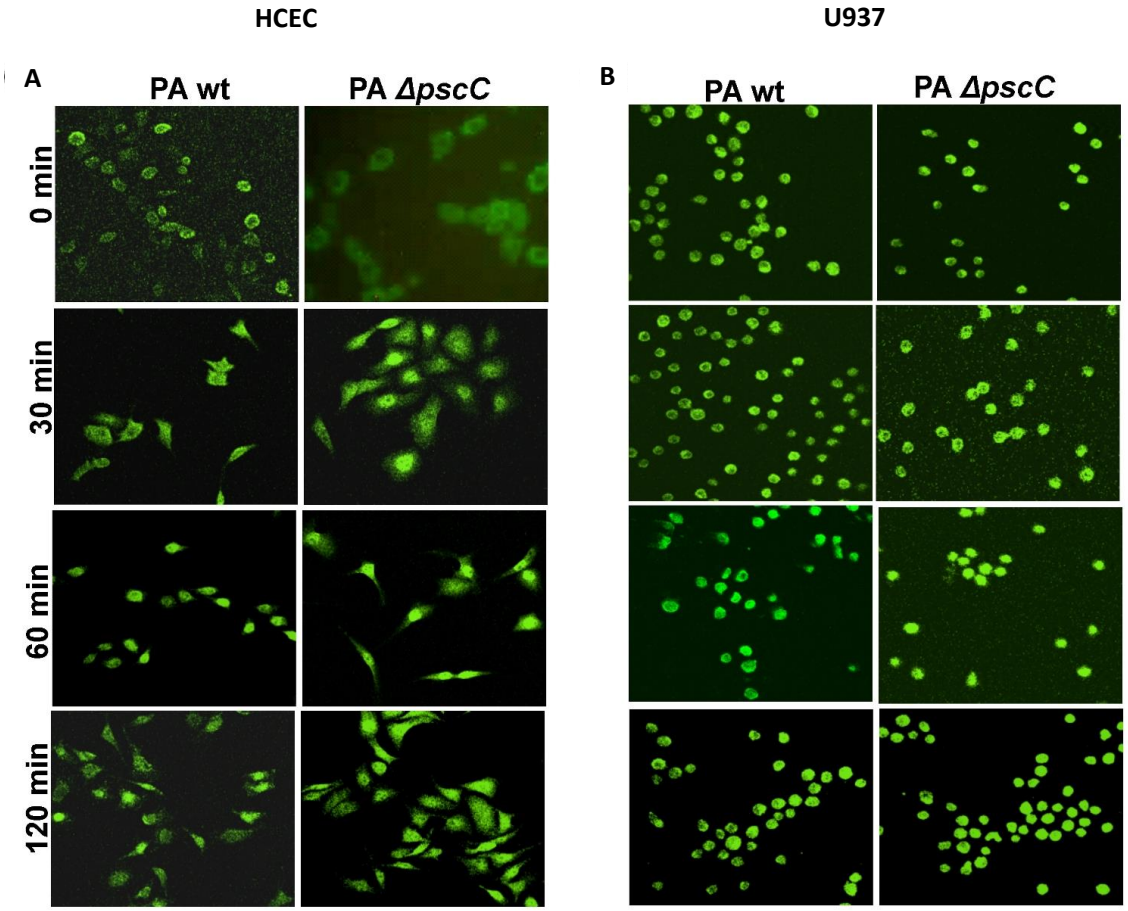


Figure 4.7 Effect of T3SS on p65 nuclear translocation. p65 translocation was determined by immunostaining and observing under florescence microscope in HCECs(A) and U937(B) infected for 30minutes,1h and 2h with either PAWt or PAΔ*pscC*.

Quantitative analysis was done by counting the number of cells with p65 staining in nucleus and we observed a significant increase in p65 translocation in cells infected with PAΔ*pscC* than in cells infected with PAWt (**Figure 4.8A and B**).

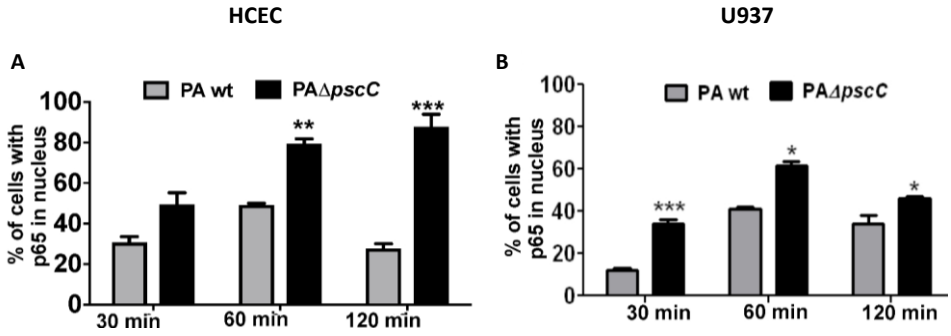


Figure 4.8 Effect of T3SS on NF-κβ signaling pathway. Quantitative data for p65 nuclear translocation in HCECs (A) and U937 (B) was determined by manually counting the number of cells (at least 100 cells were counted) showing nuclear translocation. (* $p < 0.05$, **$p < 0.005$, *** $p < 0.0005$).

These results indicate that T3SS dependent suppression of NF-κβ signaling pathway might play a role in consequent AMP suppression.

4.2.3 T3SS dependent inhibition of p38 and ERK activation in HCECs and U937

Like NF-κβ signaling pathway, MAPK signaling is also one of the important signaling pathway activated during infection in the host and is also known to mediate AMPs expression (Hippenstiel et al., 2000; Y. J. Kim et al., 2013; Schauber et al., 2003; Shibata et al., 2005). Therefore, we checked the activation of three MAPK families: extracellular signal- regulated kinase (ERK), Jun kinase (JNK/SAPK) and p38 MAPK. ERK is a serine/threonine protein kinase that acts as a signal transduction protein. ERK1 and ERK2 are the two important components of the MAPK/ERK pathway, with molecular weights of 44 and 42kDa, respectively (Guo et al., 2020). MAPK/ERK activation plays an important role in proliferation, differentiation and development in mammals (Wei & Liu, 2002). c-Jun N-terminal kinases (JNKs) or stress-activated kinases (SAPKs) activation also plays a role in proliferation, embryonic development and apoptosis (Behrens et al., 1999; Dérijard et al., 1994). p38 mitogen-activated protein kinases gets activated during stress and are involved in cell differentiation, apoptosis and autophagy (Zarubin & Han, 2005). We observed by western blotting that phosphorylation of p38 occurred as early as 30 minutes and continued up to 2h in HCECs infected with PAΔ*pscC* when compared to the cells infected with PAWt (**Figure 4.9A**). Similarly, we also saw an early activation of p38 in U937 which continually increased upto 2h in cells infected with PAΔ*pscC* when compared

to the cells infected with PAWt (**Figure 4.9D**). Infection of HCECs by PAΔ*pscC* also resulted in early activation of ERK as compared to cells infected with PAWt. (**Figure 4.9B**). Similarly, infection of U937 by PAΔ*pscC* also resulted in early activation of ERK that continues upto 2h, as compared to cells infected with PAWt (**Figure 4.9E**). There was no difference in activation of JNK in HCECs (**Figure 4.9C**) and U937 (**Figure 4.9F**) infected with either PAΔ*pscC* or PAWt. This indicates that *P. aeruginosa* is able to suppress the activation of p38-MAPK and ERK-MAPK signaling in a T3SS dependent manner.

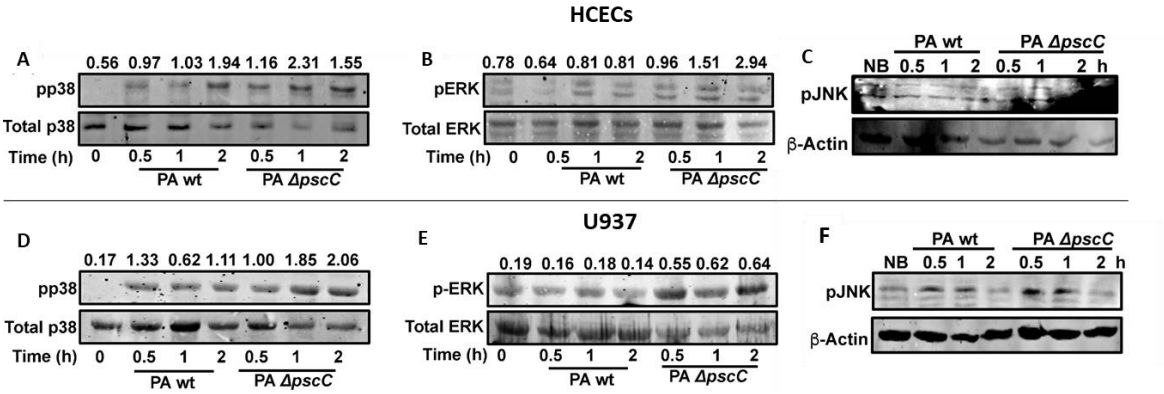


Figure 4.9 Effect of PAWt T3SS in MAPK signaling. Western blot analysis of phosphorylation of p38 (A), ERK (B) or JNK (C) was done in HCECs infected with either PAWt or PAΔ*pscC*. Similar analysis was done in U937 to check the phosphorylation of p38 (D), ERK (E) or JNK (F) upon infection. The represented number was calculated as the ratio of band density of phosphorylated to total protein.

4.2.4 T3SS dependent suppression of reactive oxygen species generation in HCECs and U937

The partial reduction of oxygen leads to the formation of reactive oxygen species (ROS) such as superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical ($HO^\bullet$). ROS is generated intracellularly during the process of mitochondrial oxidative phosphorylation (Bayir, 2005; Murphy, 2009). Oxidative stress occurs when the balance between the endogenous ROS production and expression of antioxidants is disrupted (Krumova & Cosa, 2016). During bacterial infections, the host neutrophils, macrophages and epithelial cells generates ROS that directly kills or inhibits the pathogen (Keyer et al., 1995) and also regulate key signaling pathways like MAPK (Ray et al., 2012). Since we saw that the innate immune responses like AMP expression, NF-κβ and MAPK pathway were subdued during *P. aeruginosa* infection in a T3SS- dependent manner, we further checked the effect of T3SS on ROS generation in HCECs or U937. The cells were infected for 2h with PAWt or PAΔ*pscC* and stained with 2',7'-dichlorodihydrofluorescein diacetate

(H2DCFDA), a membrane permeable fluorescent dye, and was checked under microscope and spectrophotometrically. Significant reduction in ROS generation was found in HCECs in response to PAWt infection compared to the cells infected with PAΔ*pscC* (**Figure 4.10A**). The quantitative spectrophotometric readings also showed a significant increase in ROS in HCECs in response to PAΔ*pscC* infection when compared to cells infected with PAWt (**Figure 4.10B**. Similarly, we found a significant decrease in ROS generation in U937 in response to PAWt infection compared to the cells infected with PAΔ*pscC* (**Figure 4.10C**). The quantitative spectrophotometric readings also showed a significant increase in ROS in HCECs infected with PAΔ*pscC* when compared to cells infected with PAWt (**Figure 4.10D).** Cells infected with *Escherichia coli* DH5α served as the positive control. This indicates that there is a T3SS mediated suppression of ROS generation in HCECs.

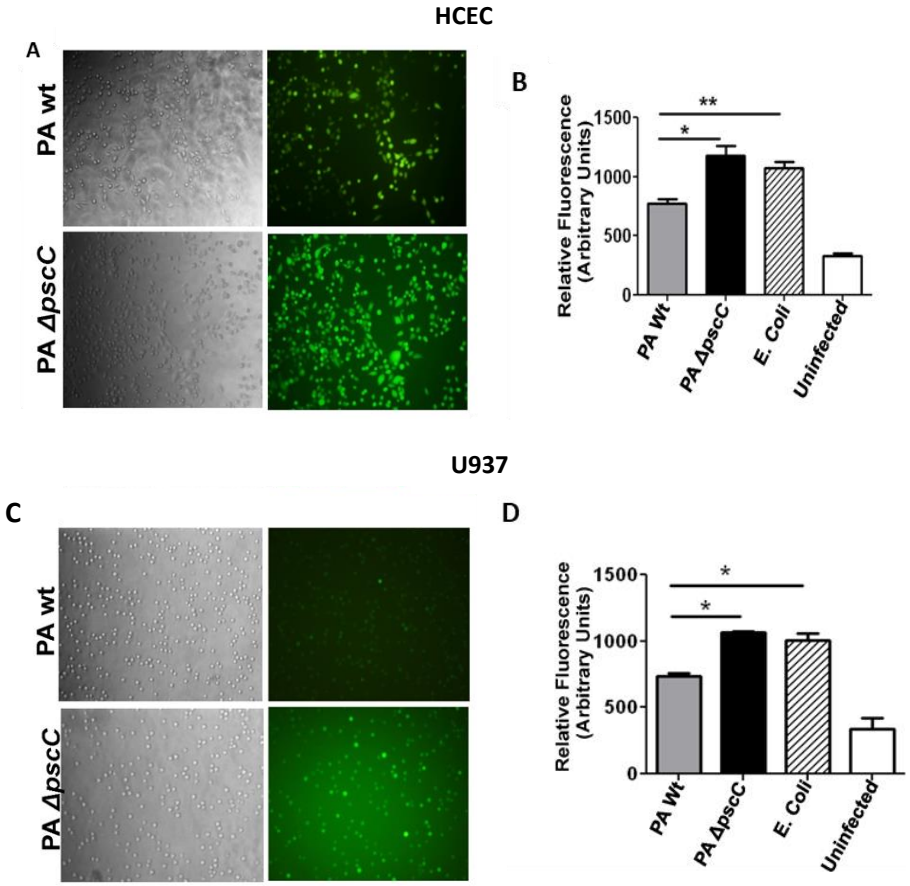


Figure 4.10 ROS generation in response to *P. aeruginosa*. HCECs and U937 was infected with PAWt or PAΔ*pscC* for 2h, incubated with H_2CFDA dye and ROS production was checked under microscope using 10X objective (A and C); similar experiment was done and quantitative measurement of fluorescence was taken using plate reader (B and D). *E. coli* was used as a positive control. The experiment was repeated thrice (* $P < 0.05$, ** $P < 0.005$).

To confirm that the difference in ROS was not due to loss of cells from cell death, we checked the viability of cells by propidium iodide (PI) staining. There was no difference in

the viability of cells infected either with PAWt or PAΔ*pscC* at the mentioned time point (**Figure 4.11**).

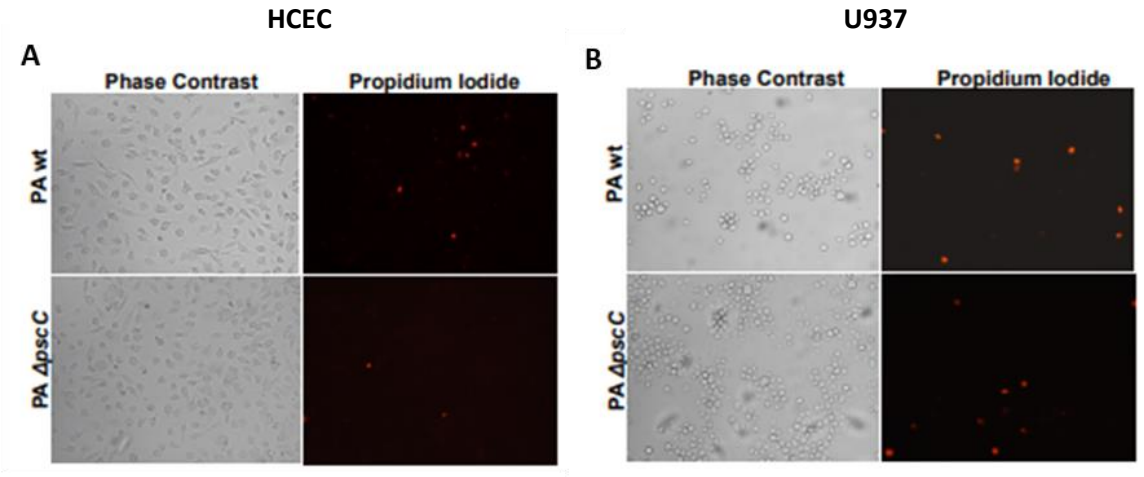


Figure 4.11 Cell viability test by propidium iodide (PI) staining. HCECs (A) or U937 (B) cells were infected with PAWt or PAΔ*pscC* for 2h, washed and stained with PI for 20 minutes, removed and observed under microscope using 10X objective.

Since T3SS plays an important role in the pathogenesis of *P. aeruginosa*, it has emerged as an attractive target for antimicrobial therapeutics. Our data also convincingly showed the important role exerted by T3SS in pathogenesis of *P. aeruginosa* infection of corneal cells. Many small molecule inhibitors acting against T3SS, have been discovered, synthesized and studied upon (Anantharajah et al., 2016). The idea of inhibiting the virulence rather than the survival of the bacteria is attractive as this approach may immensely reduce the selection pressure on pathogens to develop antibiotic resistance (Hotinger et al., 2021). Some T3SS inhibitors that have been studied till now that includes guadinomines, thiazolidinone, N-Hydroxybenzimidazole etc (Duncan et al., 2012). One such well-studied class of T3SS inhibitors are salicylidene acylhydrazides. It has been previously shown that INP0341 (**Figure 4.12**), a salicylidene acylhydrazide, inhibits transcriptional activation of T3SS of *P. aeruginosa* (Uusitalo et al., 2017).

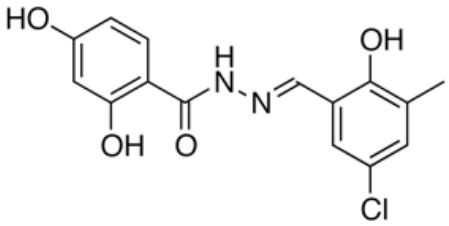


Figure 4.12 Chemical structure of INP0341.

4.2.5 T3SS inhibitor, INP0341, prevents cytotoxic effects of *P. aeruginosa* on HCECs

Since we saw that T3SS of *P. aeruginosa* plays an important role in its pathogenesis *in vitro*, by evading host immune responses via suppression of the expression of various antimicrobial peptides (AMPs), inhibition of NF-κβ signaling and MAPK pathway mediators like ERK and p38 along with inhibition of ROS generation, we next checked if T3SS inhibitor can inhibit the pathogenicity of *P. aeruginosa* both *in vitro* and *in vivo*. Our collaborator had selected few small molecule inhibitors through high throughput screening (Nordfelth et al., 2005) and showed that INP0341 inhibits the expression and secretion of ExoS, bacterial motility and biofilm formation *in vitro* (Uusitalo et al., 2017). Hence, we chose INP0341 to see if it could help inhibit *P. aeruginosa* mediated cytotoxicity in corneal cells both *in vitro* and *in vivo*.

We first checked the cytotoxic effect of INP0341 on HCECs. Cells were incubated with different concentrations of INP0341 and the percentage of enzyme lactate

dehydrogenase (LDH) release was measured spectrophotometrically. The percentage was calculated relative to the positive control (HCECs treated with 0.3% triton X), where the positive control value was considered as 100 percent. There was no significant LDH release in HCECs even in the presence of higher concentrations of INP0341 (**Figure 4.13A**) indicating that INP0341 is not cytotoxic to HCECs. Also, no significant inhibition in the growth of PAWt was noted in the presence of INP0341 6h and 24h post incubation *in vitro* (**Figure 4.13B**). This indicates that there is a very low propensity in *P. aeruginosa* of developing resistance against INP0341 as it does not affect its growth directly.

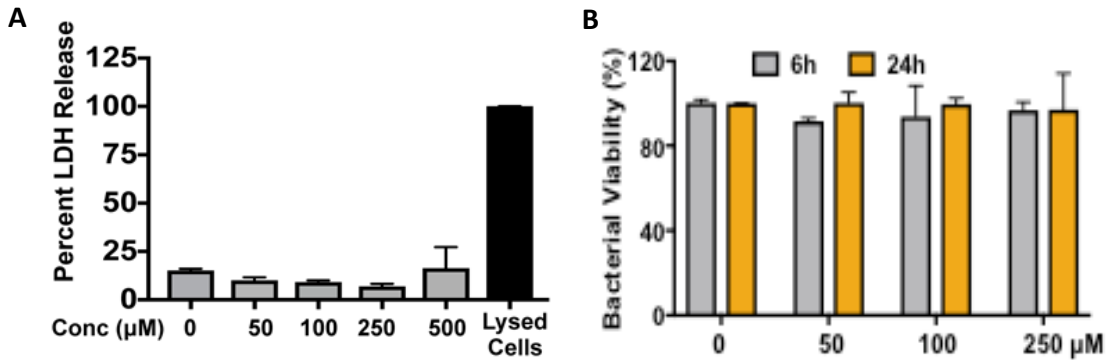


Figure 4.13 Cytotoxic effect of INP0341 on HCECs and its effect on bacterial viability. (A) Cytotoxicity test in HCECs treated with different concentrations of INP0341 for 6h by LDH assay. (B) PAWt was treated with different concentration of INP0341 for 6 or 24 h and bacterial viability was checked by the optical density measurement at 600 nm. The absorbance of bacteria without the inhibitor was considered as 100 percent viable. The experiments were repeated at least 3 times.

Next, the ability of INP0341 on inhibiting the cytotoxic effect of PAWt in HCECs was checked. HCECs were exposed to PAWt with or without INP0341 (100µM) or PAΔ*pscC* for 6h. The infection with PAΔ*pscC* was used as a control to compare the effect of INP0341. Thereafter the cell phenotype was checked under microscope. HCECs infected with PAWt underwent a change in their morphology with about 70% of the cells rounding up (**Figure 4.14Ai**) and this morphology change significantly decreased with inhibitor treatment (**Figure 4.14Aii**). However, the uninfected and untreated HCECs (**Figure 4.14Aiv**) or HCECs infected with PAΔ*pscC* only (**Figure 4.14Aiii**) did not cause any morphological change or rounding up of the cells. Release of LDH from HCECs as a direct indicator of cytotoxicity, was also determined with triton-X treated cells as positive control and it was found that HCECs infected with PAWt released 50% of maximum LDH. This percentage lowered significantly in the presence of INP0341. HCECs infected with PAΔ*pscC* did not show any significant increase in LDH release and was similar to that of

cells exposed to PAWt alongwith INP0341. HCECs incubated with INP0341 alone showed less than 15% of maximum LDH release. The uninfected-untreated cells (No Bac) also showed a lower LDH release (**Figure 4.14B**).

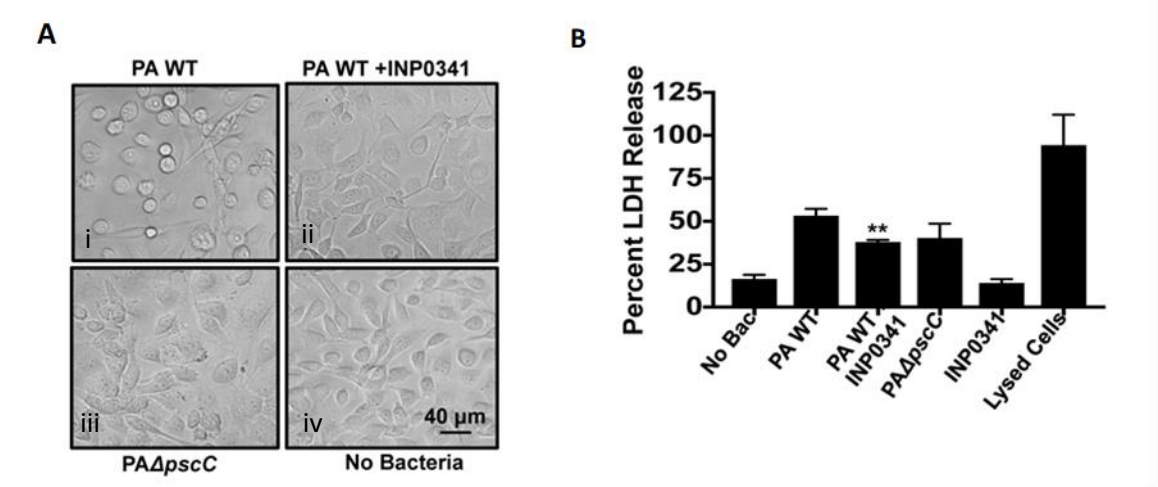


Figure 4.14 Effect of INP0341 on *P. aeruginosa* induced cell cytotoxicity. (A) Cell morphology of HCECs infected with PAWt with or without INP0341 (100µM) or PAΔ*pscC* for 6h. Checked by bright field microscopy under 20X objective. (B) HCECs infected with PAWt with or without INP0341(100µM) or PAΔ*pscC*. Cell cytotoxicity was checked by LDH assay. The experiments were repeated at least 3 times. (**p< 0.005).

The rearrangement and disruption of actin cytoskeleton serves as a survival mechanism adapted by various pathogens in order to invade, survive intracellularly, or exit the hosts (Colonne et al., 2016). Additionally, some pathogens disrupt actin polymerization to evade phagocytosis by the hosts (Uribe-Quero & Rosales, 2017). As described earlier, *P. aeruginosa* is also capable of T3SS mediated redistribution and disruption of host cell cytoskeletal system (Cowell et al., 2005; Garrity-Ryan et al., 2000). To check if T3SS inhibitors can block this effect efficiently, HCECs were exposed to PAWt with or without INP0341 for 6h. The infected cells showed a marked rearrangement of actin cytoskeleton resulting in peripheral assembly of actin and rounding up of HCECs (**Figure 4.15ii**) which was inhibited with INP0341 treatment (**Figure 4.15iii**). HCECs infected with PAΔ*pscC* alone also did not show much cytoskeletal rearrangement (**Figure 4.15iv**).

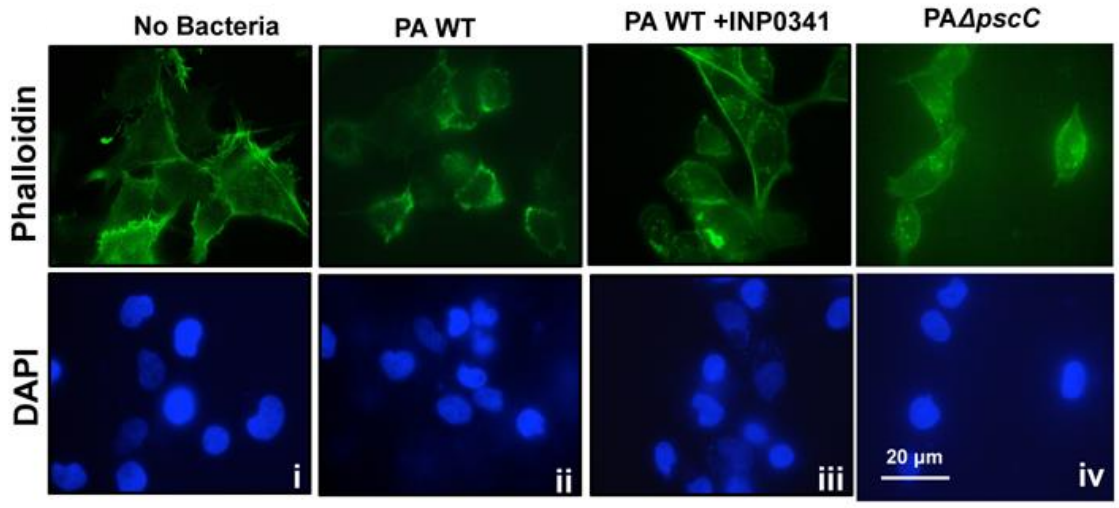


Figure 4.15 F-actin staining. HCECs were infected with PAWt in the presence or absence of INP0341, or PAΔ*pscC* for 6h. Phalloidin-Alexa fluor 488 stained HCECs was viewed by fluorescent microscopy under 100X oil immersion objectives.

4.2.6 Upregulation of antimicrobial peptide expression in HCECs infected with *P. aeruginosa* in presence of T3SS inhibitor INP0341

We observed that the expression of AMPs like *hBD-1, hBD-2, hBD-3,* hepcidin, *Rnase7, S100A9* and *LL-37* was suppressed in HCECs infected with PAWt and increased significantly upon infection with PAΔ*pscC*, a T3SS mutant. This observation made it imperative to study the effect of INP0341 inhibition of T3SS on the expression pattern of AMPs. For this purpose, we infected cells with PAWt and two others clinical isolates of *Pseudomonas* with or without INP0341 (100µM). Like PAWt these clinical isolates also show expression of ExoS, T and Y but no ExoU. Hence, we checked AMP expression in HCECs in response to PAWt infection in presence of INP0341 and found significant upregulation of *hBD-2, hBD-3, LL-37, RNase7, S100A9* and *S100A12* (**Figure 4.16A**). Similarly, AMP expression was also upregulated in HCECs infected with clinical isolates alongwith INP0341 when compared to HCECs infected but not treated with INP0341 (Figure **4.16B, C**). The sequences of the primers are given in **Annexure 1**.

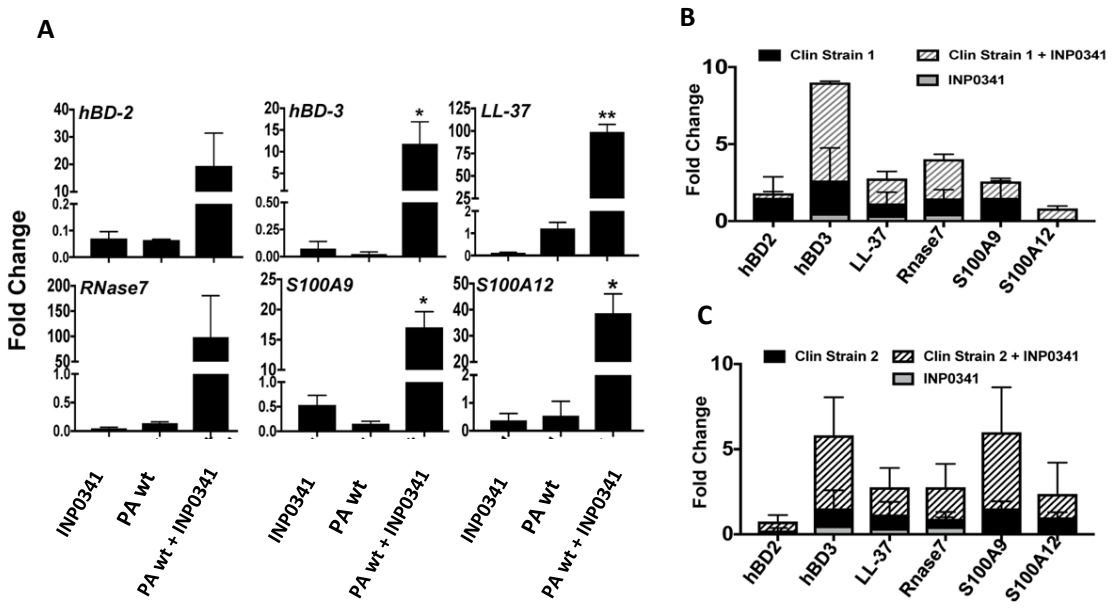


Figure 4.16 AMPs expression in HCECs in presence or absence of INP0341. HCECs were exposed to PAWt (a), Clinical isolates (b, c) with or with or without INP0341 for 4h and quantitative PCR was done to find the expression of AMPs. $2^{-\Delta\Delta ct}$ method was used to calculate fold change with GAPDH as housekeeping gene. Experiments were repeated thrice in duplicates. (* $p < 0.05$, ** $p < 0.005$).

4.2.7 Significant increase in ROS generation by HCECs in response to infection by *P. aeruginosa* in presence of INP0341

During infection, ROS generation is induced in the host that helps in pathogen clearance directly or indirectly by activation of signaling pathways related to inflammation and host immune response (Sareila et al., 2011). However some pathogens are able to evade host's ROS response by secreting or inducing antioxidant defenses like including superoxide dismutase, catalase, thioredoxins, etc., induction of bacterial virulence or increasing its tolerance towards the effects of ROS (H. Li et al., 2021). Since we saw a suppression of ROS generation by PAWt in a T3SS dependent manner, we further checked the effect of INP0341 on ROS production in infected HCECs. We infected HCECs with PAWt in presence or absence of INP0341 for 2h. Thereafter, generation of ROS was checked by incubating the infected cells with H_2CFDA dye, a cell-permeant dye. It exists in a reduced form of fluorescein which is cleaved by the intracellular esterases and oxidation and is converted to fluorescent 2',7'-dichlorofluorescein, which can be used as a direct indicator of ROS. We observed a suppression of ROS generation in HCECs in response to PAWt (**Figure 4.17Aii**) when compared to the HCECs infected with PAΔ*pscC* (**Figure 4.17Aiv**), as seen earlier. However, we found that there was a significant increase in the level of ROS

in PAWt infected HCECs in the presence of INP0341 (**Figure 4.17Aiii**) when compared to the level of ROS generation in HCECs infected with PAWt alone. We did not see any increase in ROS generation in the uninfected control (**Figure 4.17Ai**) or only INP0341 treated cells (**Figure 4.17Av**). The quantitative measurement of ROS by spectrophotometer also showed similar result (**Figure 4.17B**).

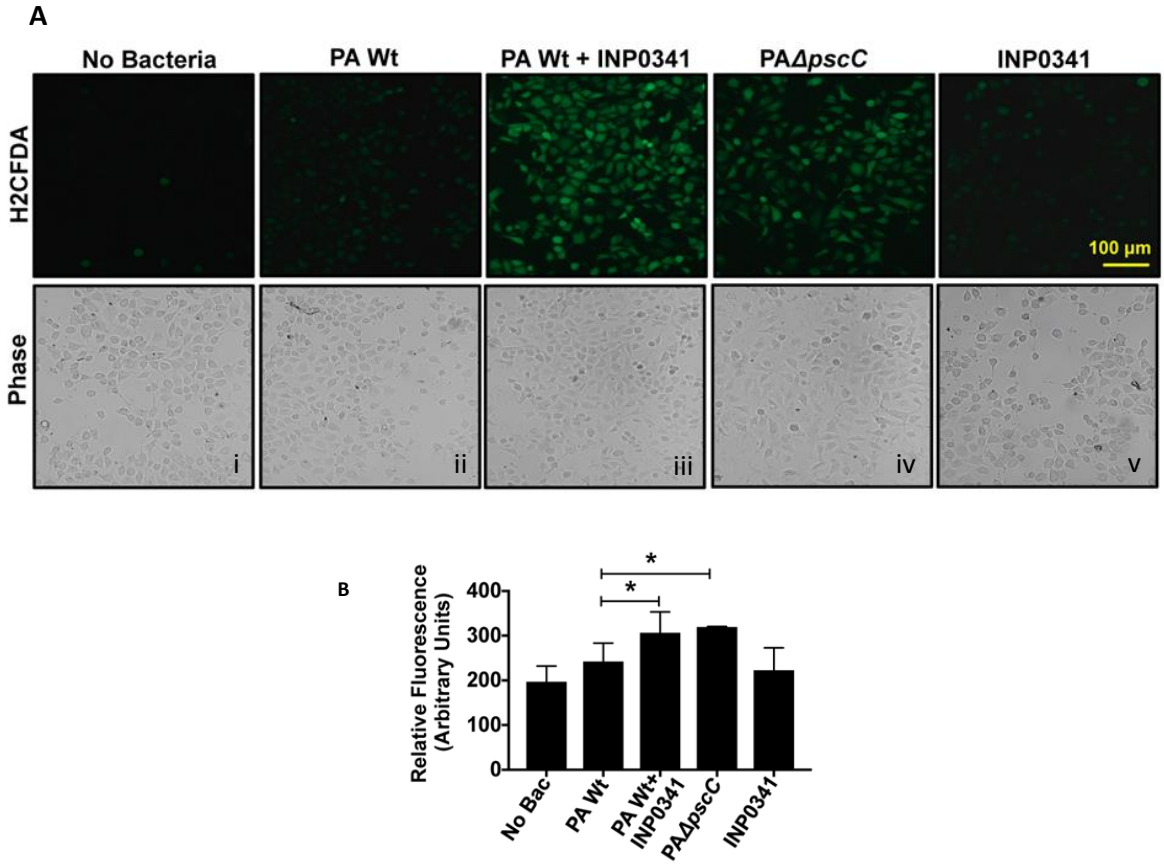


Figure 4.17 ROS generation in HCECs in presence or absence of INP0341. ROS generation was detected by infected HCECs with PAWt with or without INP0341 or PAΔ*pscC* for 2h and then incubated with H2CFDA dye for 15 minutes. Imaging was done under fluorescent microscope using 10X objective (A) or quantitative reading taken using a plate reader (B). Experiments were repeated thrice in duplicates. (* $p < 0.05$).

4.2.8 Protective effect of INP0341 in murine model of *P. aeruginosa* keratitis

To determine the protective capability of INP0341 *in vivo*, further studies were performed with C57BL/6 mice using our established infection model (Roy et al., 2014). We used 500µM of the inhibitor for the *in vivo* study as it was found to be effective earlier and the concentration of the inhibitor could not be increased further due its solubility properties (Uusitalo et al., 2017). The mouse corneas were scratched with 26 gauze needle and infected with PAWt with or without INP0341 (500µM in 10% DMSO), topically added at 0h and 6h. The corneas of the other group were also scratched and infected with PA*ΔpscC*

to serve as a positive control. Mice were euthanized 24h post infection. The corneas were imaged under stereomicroscope and checked for opacity. The clinical score for corneal opacity was assigned as described earlier (Pan et al., 2003) (**Table 4.1**).

Table 4.1 Clinical scores for corneal opacity

CLINICAL SCORES	
1	No opacity
2	Slight opacity, details of iris clearly visible
3	Some details of iris no longer visible
4	Pronounced opacity, pupil still recognisable
5	Total opacity

For checking the bacterial load in the eye by CFU method, the eyes were homogenized and plated on Luria Bertani (LB) agar plates. We saw no opacification in uninfected control eyes (**Figure 4.18Aiv**) and maximum opacification in the eyes infected with PAWt only (**Figure 4.18Ai**). We saw a considerable reduction in opacity in the eyes infected with PAWt with INP0341 treatment (**Figure 4.18Aii**). The opacity in the eyes infected with PA*ΔpscC* only was also less when compared to those infected PAWt (**Figure 4.18Aiii**). Consequently, the average clinical score of the eyes infected with PAWt only was the highest and those uninfected or infected with PA*ΔpscC* was the lowest. When compared to the eyes infected with PAWt only there was significant decrease in the average clinical score of the eyes infected with PAWt in the presence of INP0341 (**Figure 4.18B**).

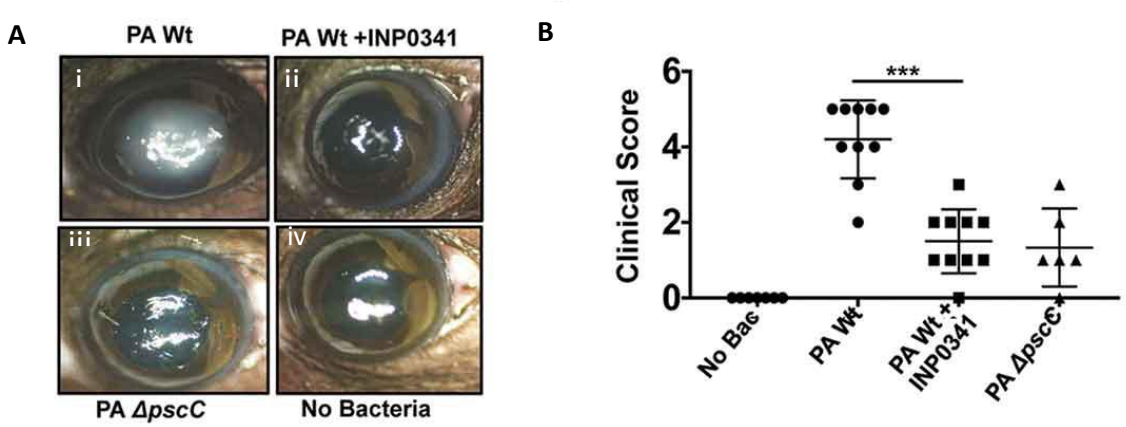


Figure 4.18 INP0341 alleviates *P. aeruginosa* infection in murine model of keratitis. C57BL/6 mice were infected with PAWt with INP0341 treatment at 0h and 6h after infection. After 24h mice were euthanized and opacification of cornea was imaged using dissection microscope (A). The clinical score was assigned (B) according to the Table 4.1 (*p < 0.05; ** p < 0.005; p<0.0005)

We also observed a significant reduction in CFU per eye that were infected with PAWt along with INP0341 when compared to eyes infected with PAWt only. The CFU per eye

that were infected with PA*ΔpscC* only was also less than the eyes infected with PAWt (**Figure 4.19**).

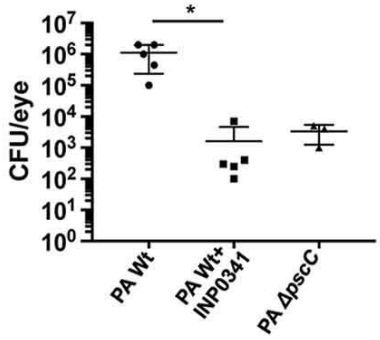


Figure 4.19 INP0341 alleviates *P. aeruginosa* infection in murine model of keratitis. C57BL/6 mice were infected with PAWt with INP0341 treatment at 0h and 6h after infection. The whole eye homogenized lysate was used for CFU counting (n=5 mice); each data point represents individual cornea (*$p < 0.05$).

Corneal sections of mice eyes stained with hematoxylin and eosin showed decreased infiltration of immune cells in PAWt infected eyes treated with INP0341 (**Figure 4.20ii**) when compared to PAWt only infected eyes (**Figure 4.20i**). We observed less cellular infiltration in eyes infected with PAΔ*pscC* infected eyes as well (**Figure 4.20iii**). The control sections showed no cellular infiltrations (**Figure 4.20iv**).

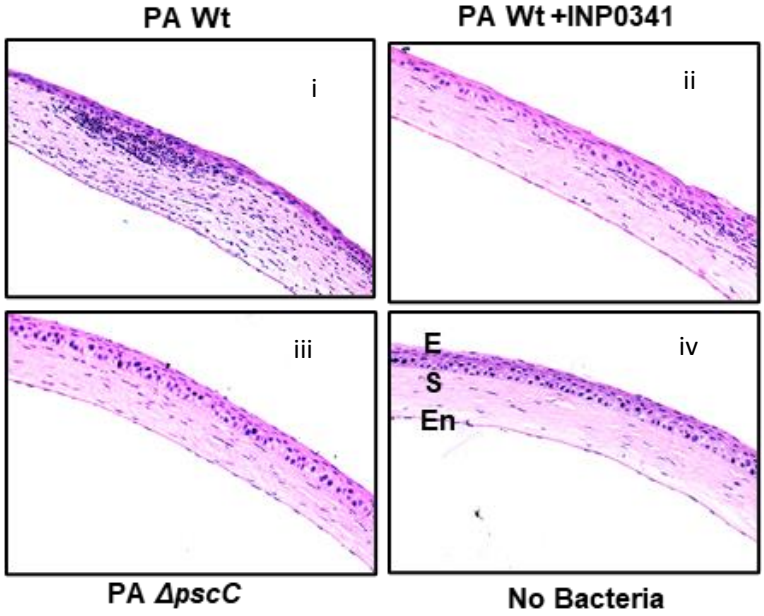


Figure 4.20 INP0341 alleviates *P. aeruginosa* infection in murine model of keratitis. C57BL/6 mice were infected with PAWt with INP0341 treatment at 0h and 6h after infection. Haematoxylin and eosin staining of corneal sections for checking immune cells infiltration under microscope using 10X Objective. E, Epithelium; S, Stroma; En, endothelium.

To determine the optimum time for addition of INP0341 during the infection, further experiments were performed where mice corneas were scratched and infected with PAWt

along with INP0341 (500µM in 10% DMSO) at 0h and 6h (group I) or at 3h and 6h (group II) after infection. After 24h mice were sacrificed and corneal images were taken. Control uninfected eyes showed no opacity (**Figure 4.21i**). When compared to PAWt only infected group (**Figure 4.21ii**), corneas of PAWt infected group with INP0341 treatment, i.e., group I (**Figure 4.21iii**) and group II (**Figure 4.21iv**) mice displayed significantly less opacification. The opacity of the corneas in group I (**Figure 4.21iii**) mice was comparatively less than that of the group II mice (**Figure 4.21iv**).

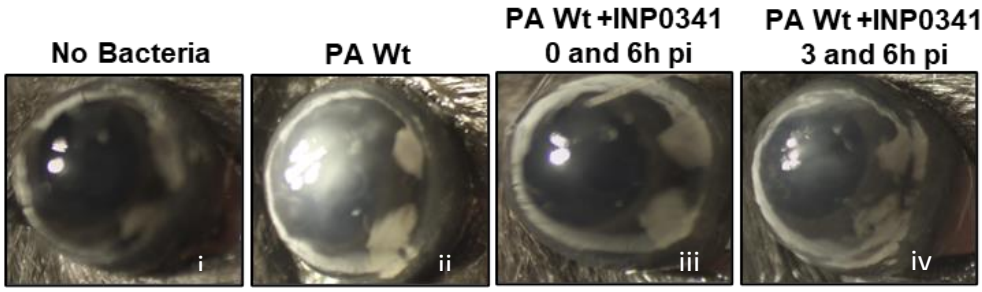


Figure 4.21 Effect of INP0341 on C57BL/6 mouse model of *P. aeruginosa* keratitis- corneal opacity. Mice were scratched and infected with PAWt in presence of INP0341 for 0h and 6h post infection in group I and 3h and 6h post infection in group II. Cornea was imaged after euthanizing the mice after 24h.

There was a significant reduction in the opacity of corneas in group I mice, where the INP0341 inhibitor was added at 0h and 6h post infection, when compared to the PAWt infected group without any INP0341 treatment, as shown in the clinical score graph (**Figure 4.22**). The clinical score of the eyes of group II mice that were infected with PAWt with the INP0341 inhibitor added 3h and 6h post infection was also lower than those infected with PAWt only, but not significantly (**Figure 4.22**).

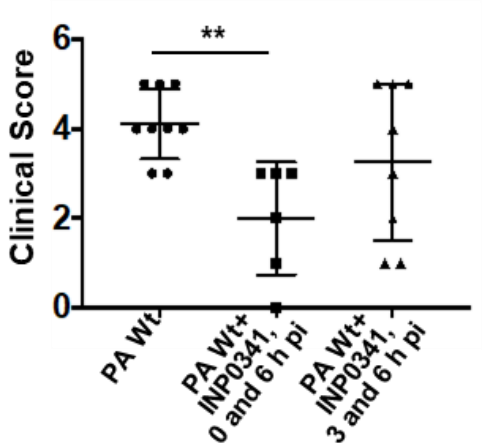


Figure 4.22 Effect of INP0341 on C57BL/6 mouse model of *P. aeruginosa* keratitis-clinical score. Mice were scratched and infected with PAWt in presence of INP0341 for 0h and 6h post infection in group I and 3h and 6h post infection in group II.). Clinical scores were assigned for severity of infection. (** $p<0.005$).

CFU/eye of group I mice was also less when compared to that of the only PAWt infected group (Figure **4.23**).

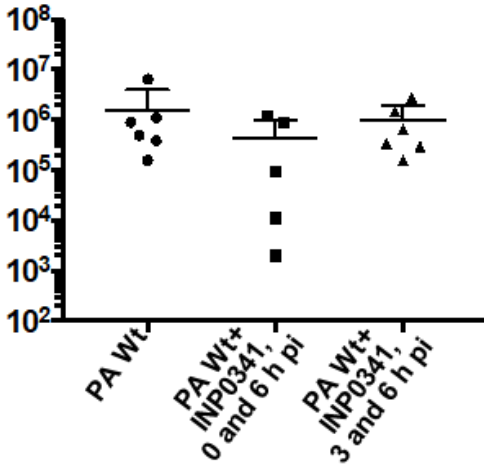


Figure 4.23 Effect of INP0341 on C57BL/6 mouse model of *P. aeruginosa* keratitis- bacterial load. Mice were scratched and infected with PAWt in presence of INP0341 for 0h and 6h post infection in group I and 3h and 6h post infection in group II. Eye homogenates were plated and CFU counted (n=6 mice) with each data point representing individual cornea.

The hematoxylin and eosin staining of corneal sections from group I (**Figure 4.24ii**) and group II (**Figure 4.24iii**) mice also showed reduced cellular infiltrations when compared to the sections from PAWt infected group mice eyes (**Figure 4.24i**), with group I mice eyes sections (**Figure 4.24ii**) showing the less infiltration when compared to group II mice eyes (**Figure 4.24iii**).

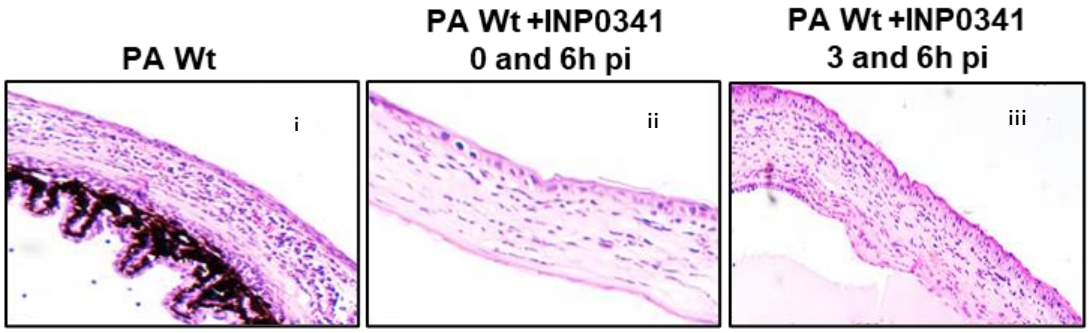


Figure 4.24 Effect of INP0341 on C57BL/6 mouse model of *P. aeruginosa* keratitis- cellular infiltration. Mice were scratched and infected with PAWt in presence of INP0341 for 0h and 6h post infection in group I and 3h and 6h post infection in group II. Hematoxylin and eosin staining of corneal sections was done and imaged using 10X objective to check infiltrates (D).

Since we found that AMP expression was upregulated in HCECs in response to PAWt in presence of INP0341 when compared to the cells infected with PAWt, we further checked the expression of AMPs *in vivo*. The corneas of mice were scratched and infected with PAWt with or without INP0341 and AMP expression was determined 24h post infection

by qPCR. Like *in vitro* experiment, we saw decrease in the expression of *mBD-1*, *mBD-2*, *mS100A9*, lipocalin (*mLCN2*) and *CRAMP* (mouse homologue of *LL-37*) in corneas infected with PAWt which significantly increased in corneas in response to PAWt infection in the presence of INP0341 (**Figure 4.25**). Primer sequences are given in **Annexure 1.**

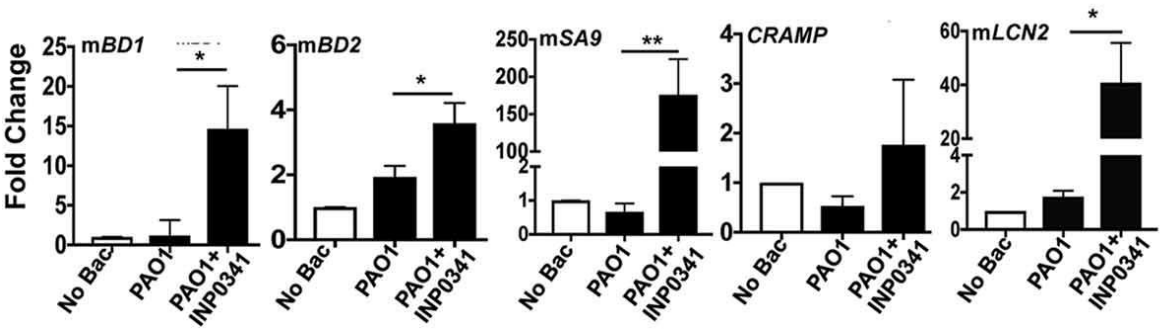


Figure 4.25 Effect of INP0341 on C57BL/6 mouse model of *P. aeruginosa* keratitis- AMP expression. C57BL/6 mice were infected with PAWt with INP0341 treatment at 0h and 6h after infection. RNA isolation was done from whole eye (n=3) and AMP expression determined using quantitative PCR by 2-ΔΔCt method with mouse GAPDH as housekeeping gene. (*p < 0.05; ** p < 0.005)

4.3 Discussion

P. aeruginosa is a notorious gram negative pathogen because of the presence of many virulent factors including T3SS (Jurado-Martín et al., 2021). The most common infections caused by *P. aeruginosa* includes those of soft tissue and urinary tract, bacteremia, diabetic foot, respiratory pneumoniae, otitis externa, otitis media folliculitis and keratitis (Gellatly & Hancock, 2013). It is an opportunistic pathogen and one of the major causes of nosocomial infections (Sikora & Zahra, 2021). In this study we tried deciphering the role of AMPs in *P. aeruginosa* infection, with a focus on keratitis.

Since we saw that there was a differential expression of AMP in *P. aeruginosa* keratitis patient samples (Objective1, Chapter III), we further checked the expression of AMP in *P. aeruginosa* infection *in vitro*. We found that *P. aeruginosa* was able to suppress AMP expression, during the early time point, in both HCECs and U937 in a T3SS dependent manner. Earlier studies have shown that innate immune response against pathogens in epithelial cells activate MAPK and NF- κβ pathways, that leads to AMP expression along with pro-inflammatory cytokines like IL-6, IL-8 and TNF-α (Hop et al., 2017; Kumar et al., 2004). However, several pathogens are known to evade these host responses (Niu et al., 2018). Several PAMPs are known to manipulate host kinase signaling like inhibition of NF-κβ and MAPK leading to suppression of ROS production, growth arrest, induction of apoptosis or inhibition of proinflammatory responses (Alto & Orth,

2012; Krachler et al., 2011). Since we saw suppression of AMP expression in response to PAWt infection, we further checked the expression or activation of these signaling pathways as well. We found *P. aeruginosa* caused the suppression of cell signaling, like delayed activation of NF-κβ, p38 and ERK, in a T3SS dependent manner. However, there was no significant change in the activation of JNK, indicating that JNK pathway probably does not play any role in expression of AMP. In another similar study, *P. aeruginosa* virulence factor, PumA, was found to mediate the inhibition of NF- κβ and Tumor necrosis factor receptor 1 (TNFR1) signaling pathways (Imbert et al., 2017). *P. aeruginosa* is also able to evade host immune responses by secreting several proteases, toxins and lipases that directly leads to inactivation or inhibition of host immune cells and molecules (Kharazmi, 1991). T3SS has also been implicated in dampening host immune responses by killing phagocytes or interfering with phagocytosis (D. Dacheux et al., 1999; Denis Dacheux et al., 2000). ROS generation is also one of the early host defenses against the pathogens. However, many pathogens are known to show antioxidant response to protect itself from the harmful effects of ROS (Piacenza et al., 2019). In an earlier study it was shown that suppression of LPS-induced ROS generation *in vitro* consequently resulted in the suppression of NF- κβ and MAPK pathways and modulation of pro-inflammatory cytokines (Park et al., 2015). Since the NF- κβ and MAPK pathways were suppressed in response to *P. aeruginosa* infection, we further checked if there is any effect of infection on ROS generation or not. We found that the *P. aeruginosa* suppress ROS generation in T3SS dependent manner, indicating that the pathogen is able to suppress the interconnected host responses. In another supporting study, ExoS was implicated in actively suppressing ROS production in neutrophils by ADP-ribosylation of Ras, thereby inhibiting its interaction with phosphoinositol-3-kinase (PI3K) (Vareechon et al., 2017). There are several studies where pathogens have been shown to evade ROS mediated killing, either by avoiding, inhibiting or neutralizing ROS generation. (Eze, 1991; McCaffrey & Allen, 2006). For example, *Staphylococcus aureus* harbors antioxidant enzymes like catalase and peroxidase that neutralizes H_2O_2 (G. Y. Liu et al., 2005). . Group A *Streptococcus* has a multitude of ROS resistance mechanisms like surface/ secreted proteins, intracellular and secreted enzymes, metal sequesters and the transcription factors that is involved in antioxidant gene regulation (Henningham et al., 2015).

As T3SS was found to play an important role in the pathogenesis of *P. aeruginosa* and the host response *in vitro*, we further checked the effect of T3SS inhibition on the host

response. INP0341, a salicylidene acylhydrazide, has been earlier reported to inhibit T3SS transcription (Anantharajah et al., 2016). In a previous study, small molecule inhibitors were used to inhibit *Salmonella enterica* T3SS-1 that consequently led to suppression of inflammatory responses *in vitro* (Hudson et al., 2007). In another study a small molecule inhibitor of bacterial T3SS was shown to have a protective effect *in vivo* from *Citrobacter rodentium* infection (Kimura et al., 2010). Studies have shown that INP0341 inhibited the expression and secretion of ExoS, inhibited bacterial motility and biofilm formation *in vitro* and reduced the severity of *P. aeruginosa* infection *in vivo* (Uusitalo et al., 2017). Since we observed that *P. aeruginosa* was able to suppress AMP expression, ROS generation and other innate immune host responses in T3SS dependent manner, we pharmacologically inhibited T3SS of *P. aeruginosa* with INP0341. There have been several studies in the recent past on the inhibitors of T3SS. The inhibition of virulent factors of the pathogen is a promising approach to counteract antibiotic resistance as the inhibitors of virulent factors does not affect the growth of the pathogen, thereby removing the selective pressure to develop resistance (Kolář et al., 2001). We observed that in the presence of INP0341, AMP expression increased, which was suppressed in response to PAWt infection. We also saw that there was an elevation in ROS generation in PAwt infected HCECs in the presence of INP0341. There was also a marked reduction in *P. aeruginosa* induced cell cytotoxicity in the presence of INP0341. We also observed that the inhibition of T3SS by INP0341 prevented *P. aeruginosa* induced actin cytoskeletal rearrangement. It was recently reported that ExoY can bind to actin filaments and can modulate actin cytoskeleton directly by bundling F-actin and indirectly by actin-activated nucleotidyl cyclase activity (Mancl et al., 2020). Earlier, ExoT was also shown to disrupt actin cytoskeleton and inhibit internalization of *P. aeruginosa* by epithelial cells and macrophages (Garrity-Ryan et al., 2000). Hence, inhibition of T3SS restricted the actin modulation. These results indicate that T3SS of *P. aeruginosa* plays a very important role in its pathogenesis and its suppression can help upregulate the host responses like AMP expression, phagocytosis and ROS generation.

In some studies it was shown that AMPs were effective against clinical ocular isolates in *in vitro* setup, but were ineffective in *in vivo* study (Mannis, 2002). Therefore, we did an *in vivo* study to check if the inhibitor has similar effect as *in vitro* or not. Similar to the *in vitro* observation we found that there was an upregulation of AMPs expression *in vivo* in the presence of INP0341. INP0341 was also able to reduce the severity of the infection *in vivo*

by reducing *P. aeruginosa* induced corneal opacity. We also saw concurrent reduction in bacterial load in the infected mice eyes and cellular infiltration in the infected mice corneas in the presence of INP0341.

To conclude, *P. aeruginosa* evades host response by suppressing AMP expression, ROS generation and various related signaling pathways in T3SS dependent manner. Pharmacological inhibition of T3SS by INP0341 results in reversal of this suppression and leads to upregulation of AMPs and increase in ROS generation, inhibition of actin cytoskeletal rearrangement and reduction in *P. aeruginosa* induced cell cytotoxicity *in vitro.* INP0341 was also found to alleviate the infection by reduction in corneal opacity, bacterial load and cellular infiltration and upregulation of AMPs expression *in vivo*.

* **This chapter has been published in parts in the journal *Pathogens and Disease* (Sharma et al., 2018) and *Virulence* (Sharma et al., 2020). The citations are given below and the publications are attached.**

Sharma, P., Guha, S., Garg, P., & Roy, S. (2018). Differential expression of antimicrobial peptides in corneal infection and regulation of antimicrobial peptides and reactive oxygen species by type III secretion system of *Pseudomonas aeruginosa*. Pathogens and Disease, 76(1), 10.1093/femspd/fty001. https://doi.org/10.1093/femspd/fty001

Sharma, P., Elofsson, M., & Roy, S. (2020). Attenuation of *Pseudomonas aeruginosa* infection by INP0341, a salicylidene acylhydrazide, in a murine model of keratitis. Virulence, 11(1), 795–804. https://doi.org/10.1080/21505594.2020.1776979

CHAPTER V

OBJECTIVE 3: ROLE OF ANTIMICROBIAL PEPTIDES IN KERATITIS CAUSED BY GRAM POSITIVE BACTERIA *Streptococcus pneumoniae*

5.1 Introduction

Streptococcus pneumoniae is a gram-positive, catalase-negative and facultative anaerobic bacteria that exists as single coccus, diplococci (lancet shaped) or in chains (*Streptococcus Pneumoniae: For Clinicians | CDC*, n.d.). The bacteria optimally grow in 5% carbon dioxide or anaerobic conditions. The bacterial colonies grown in blood agar are α-hemolytic with green or brown discoloration at the periphery caused by partial destruction of red blood cells (Peter & Klein, 2008). One of the unique characteristics of *S. pneumoniae* is that it produces H_2O_2 when grown in aerobic conditions that causes oxidative stress and cytotoxic effects in the host cells that results in DNA damages and apoptosis (Rai et al., 2015). H_2O_2 produced by *S. pneumoniae* is also shown to have bactericidal and bacteriostatic effect on other bacteria. However, the bacteria itself is resistant to oxidative effects of H_2O_2 due to its enzyme pyruvate oxidase (SpxB), that is also responsible for endogenous production of H_2O_2. *spxB* mutants were found to be killed in the presence of H_2O_2 due to rapid ATP depletion. SpxB contributes to an alternate H_2O_2-resistant energy source, hence protecting the bacteria (Pericone et al., 2003). α-hemolysis or green-halo around the *S. pneumoniae* colonies in blood agar is due to the oxidation of oxy-hemoglobin to met-hemoglobin, the nonbinding oxygen form, by the hydrogen peroxide synthesized by the bacteria (Z. Hossain, 2014; McDevitt et al., 2020).

One of the host responses towards an increased oxidative stress is the activation of NF-E2–related factor 2 (Nrf2) (K. M. Kim & Ki, 2017; J. M. Lee & Johnson, 2004). It belongs to the cap 'n' collar/basic leucine zipper (CNC-bZIP) protein family and a key transcription factor that mediates host's antioxidant responses by binding to antioxidant response element (ARE) and regulating the expression of various antioxidant genes (Itoh et al., 1997; Jaiswal, 2004; Tsan, 2006). Nrf2 is known to regulate the transcription of glutathione and thioredoxin antioxidant systems components, the phase I and phase II detoxification enzymes, NADPH regeneration, and heme metabolism. Nrf2 also helps in other cellular processes like autophagy and unfolded protein response (Ma, 2013). Intracellular Nrf2 activation is controlled by Kelch-like ECH-associated protein 1 (Keap1), a redox-sensitive E3 ubiquitin ligase substrate adaptor that binds to Nrf2. Keap1 acts as an adaptor for Cul3 E3 ubiquitin ligase, that helps in continuous ubiquitylation and degradation of Nrf2. During oxidative stress, reactive cysteine residues of Keap1 are oxidized, causing its inactivation that leads to Nrf2 stabilization and nuclear translocation

where it can bind to the ARE and activate the expression of target genes. Nrf2 activated genes that play a role in host antioxidant defense includes those involved in reactive oxygen species (ROS) catabolism (Superoxide dismutase 3, Glutathione peroxidase, Peroxiredoxin), metal binding protein (Metallothionein, Ferritin), stress response protein (Heme oxygenase), reduction (NAD(P)H:quinone oxidoreductase) and so on (Ma, 2013).

Autophagy is also one of the innate immune responses of the host against bacterial infections (**Figure 5.1**). ROS generation is known to have a cross-talk with autophagy via various signaling pathways like ROS–Nrf2–P62–autophagy, ROS–FOXO3–LC3/BNIP3–autophagy, ROS–HIF1–BNIP3/NIX–autophagy and ROS–TIGAR–autophagy (L. Li et al., 2015).

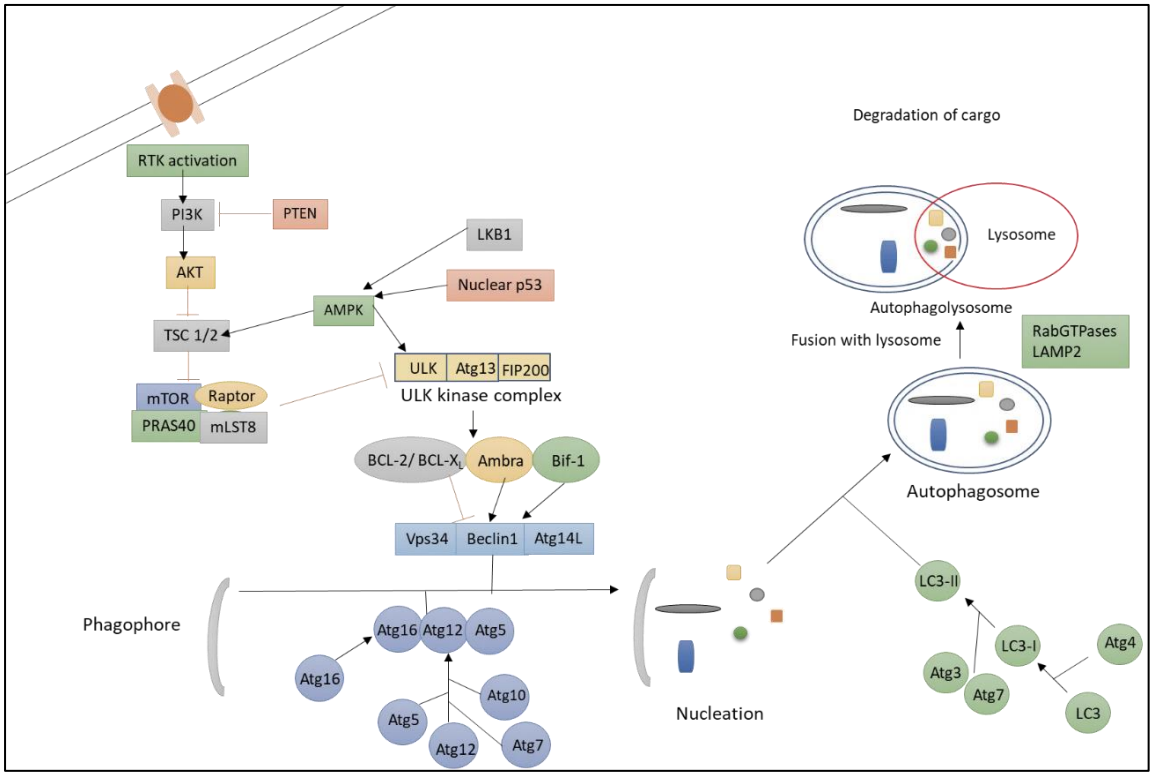


Figure 5.1 Autophagy signaling pathway. Various signals activate Autophagy in mammals like biding of receptor tyrosine kinase (RTK) to the ligand, activation of AMP-activated protein kinase (AMPK) by p53 etc. LC3-II initiates the formation and lengthening of the autophagosome and is a major marker of autophagy. Adapted from (Z. J. Yang et al., 2011).

Autophagy plays a role in clearing the irreversibly damaged biomolecules due to oxidative stress. Also, the phosphorylation of p62, an autophagy adaptor protein, increases its affinity for Keap1 thereby interacting and sequestering it, consequently releasing and activating Nrf2, consequently activating host antioxidant response (Ichimura et al., 2013; Komatsu et al., 2010; Lau et al., 2010). H_2O_2 has been shown to oxidize ATG4, an autophagy related

gene that causes enhanced lipidation of LC3/ATG8 leading to initiation of autophagy. ROS can also regulate autophagy via MAPK or AKT/ mTOR (mechanistic target of rapamycin) and AMPK signaling pathways. It has been shown that ROS generated by mitochondrial NADPH oxidase (NOX) complexes in response to infection play an important role in employment of the microtubule-associated protein light chain 3 (LC3) on phagosomes, thus activating antibacterial autophagy (J. Huang et al., 2009).

Host innate immune response against *S. pneumoniae* is activated upon binding of the bacterial PAMPs to either TLR1/2, TLR4 or cytosolic Nod2 (Malley et al., 2003; Opitz et al., 2004; Schmeck, Huber, et al., 2006; Schröder et al., 2003). There are some reports on the activation of MAPK and NF-κβ pathways in the hosts in response to *S. pneumoniae* infection (N'Guessan et al., 2006; Schmeck et al., 2004). It was shown that IL-8 expression was induced in epithelial cells infected with *S. pneumoniae* via the activation of JNK (Schmeck, Moog, et al., 2006). The bacteria is also shown to activate host innate immune response, like induction of IL-6 and TNF-α secretion via MAPK and PI3K/AKT signaling (L. Wang et al., 2020).

AMPs play an important role in innate immunity against bacteria. Cathelicidins are one of the many families of antimicrobial peptides that have been identified. They have a conserved N-terminal cathelin domain and a variable C-terminal antimicrobial domain which can be cleaved from the precursor protein by proteinases (Zaiou et al., 2003). LL-37 (**Figure 5.2**) is a part of C-terminus of the human cationic antimicrobial protein (hCAP-18), the only cathelicidin-derived antimicrobial peptide found in humans. It is a 37 amino acid long peptide, amphipathic in nature, with its helical form present throughout the body. It exhibits a broad spectrum of antimicrobial activity (Denardi et al., 2021). LL-37 has been shown to be effective in eliminating both extracellular and intracellular *Staphylococcus aureus* (Noore et al., 2013). LL-37 also shows antibacterial activity against other bacteria like *Helicobacter pylori* (Hase et al., 2003), *Acinetobacter baumannii* (Thomas-Virnig et al., 2009), *Burkholderia pseudomallei* (Kanthawong et al., 2009), *Brucella suis* (Dudal et al., 2006), *Mycobacterium tuberculosis* (Martineau et al., 2007), *P. aeruginosa, Escherichia coli, Salmonella typhimurium, Listeria monocytogenes, Staphylococcus epidermidis* (Turner et al., 1998) etc. It is expressed in epithelial cells and leukocytes and regulates inflammatory responses and wound healing (Hase et al., 2003; Simonetti et al., 2021). The expression of many AMPs in response to bacterial infection is also known to

be mediated via the MAPK and/or NF-κβ pathways (Krisanaprakornkit et al., 2002; Scharf et al., 2010).

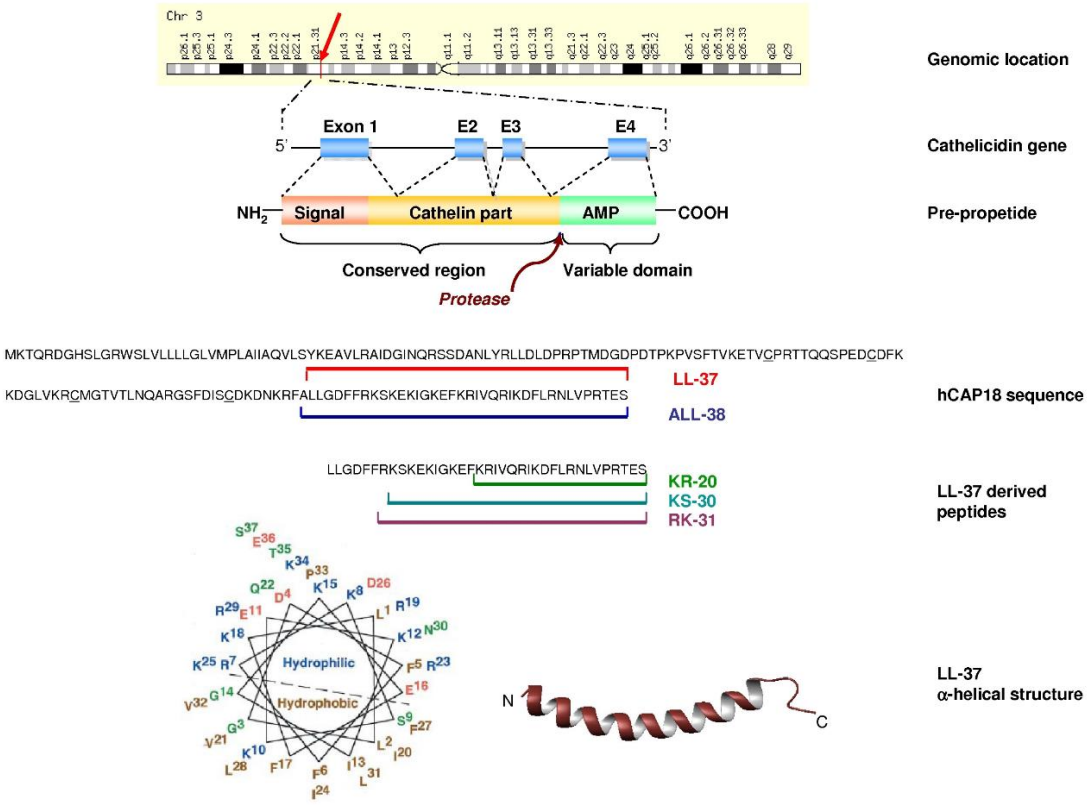


Figure 5.2 Genomic location and peptide structure of LL-37. Picture courtesy (Seil et al., 2010)

MEK1/2 and p38 MAPK signaling pathways has been shown to induce *LL-37* gene expression in human epithelial cells in response to *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) (Méndez-Samperio et al., 2008). In another study it was shown that *LL-37* expression is induced via activation of transcription factors AP-1 and NF-κβ during inflammatory bowel disease (Kusaka et al., 2018). It was also shown that skin commensal bacteria, Staphylococci, induces expression of the antimicrobial peptides, *hBD-3* and *RNase7* via NF-κB activation, and pathogenic staphylococci suppresses NF-κβ signaling while activating MAPK and PI3K/AKT signaling pathways (Wanke et al., 2011). Miraglia *et. al.* also identified sites in *LL-37* gene promoter region where Signal transducer and activator of transcription 3 (STAT3) binds and induces *LL-37* expression in response to etinostat (Miraglia et al., 2016).

Since we observed differential expression of various AMPs like beta defensins, S100A proteins, cathelicidin, hepcidin etc., in patients with *S. pneumoniae* keratitis (**Objective1**, **Chapter III**), we further studied the expression of AMPs in our *in vitro*

infection model for which we used primary and immortalized human corneal epithelial cells (HCECs) and human pro-monocytic cell line (U937). We also checked the activation of TLR related signaling pathways, NF-κβ and MAPK in response to *S. pneumoniae* infection since *S. pneumoniae* is known to activate TLR2. Further we focused on the role of LL-37 in *S. pneumoniae* infection and the importance of STAT3 signaling in its expression. We also looked into the wound healing effects of LL-37 in HCECs. We also checked the effect of *S. pneumoniae* induced oxidative stress and autophagy response in HCECs, and how the usage of tBHQ, a known Nrf2 inducer, can play a role in the outcome of the disease. In some experiments we used an ATCC strain (Sp ATCC 49619) and a pneumolysin-positive ocular clinical isolate (Sp CS) of *S. pneumoniae*, for infection, to compare the host responses against these two isolates. In most of the experiments we used Sp ATCC for infection. Hence, our goal in this chapter is to decipher the role of pneumococcal infection on the host innate immune response *in vitro* with the focus being on AMPs.

5.2 Results

5.2.1 *In vitro* expression of AMPs in *S. pneumoniae* infected immortalized HCECs

As we found differential expression of AMPs in *S. pneumoniae* keratitis patient corneal scrapings, we further studied the expression of AMPs in HCECs infected with a laboratory strain of *S. pneumoniae* (Sp ATCC) and an ocular clinical isolate of Sp (Sp CS). HCECs were infected with either of the strains for 3h and then incubated in gentamicin containing media for 1h, to kill any extracellular adherent bacteria. Sp CS is a pneumolysin positive, like ATCC strain and showed resistance towards amikacin. The expression of AMPs was determined by qPCR. There was a significant upregulation of beta-defensins in Sp ATCC infected HCECs, specially *hBD-2* (30-fold) and *hBD-4* (50-fold), when compared to control. There was an increase in expression of S100A group of AMPs too during Sp ATCC infection. An increase of 10-fold in the expression of *S100A7* and more than 6-fold increased expression of *S100A8* was observed. However, *S100A12* expression was not detected in Sp ATCC infected HCECs unlike in the case of patients' corneal scrapings. There was a significant upregulation of *LL-37* (6-fold) and hepcidin (5-fold) also showed increased expression in response to Sp ATCC infection. In comparison to the expression of AMPs in HCECs infected by Sp ATCC there was significantly reduced expression of AMPs in HCECs infected with Sp CS (**Figure 5.3**).

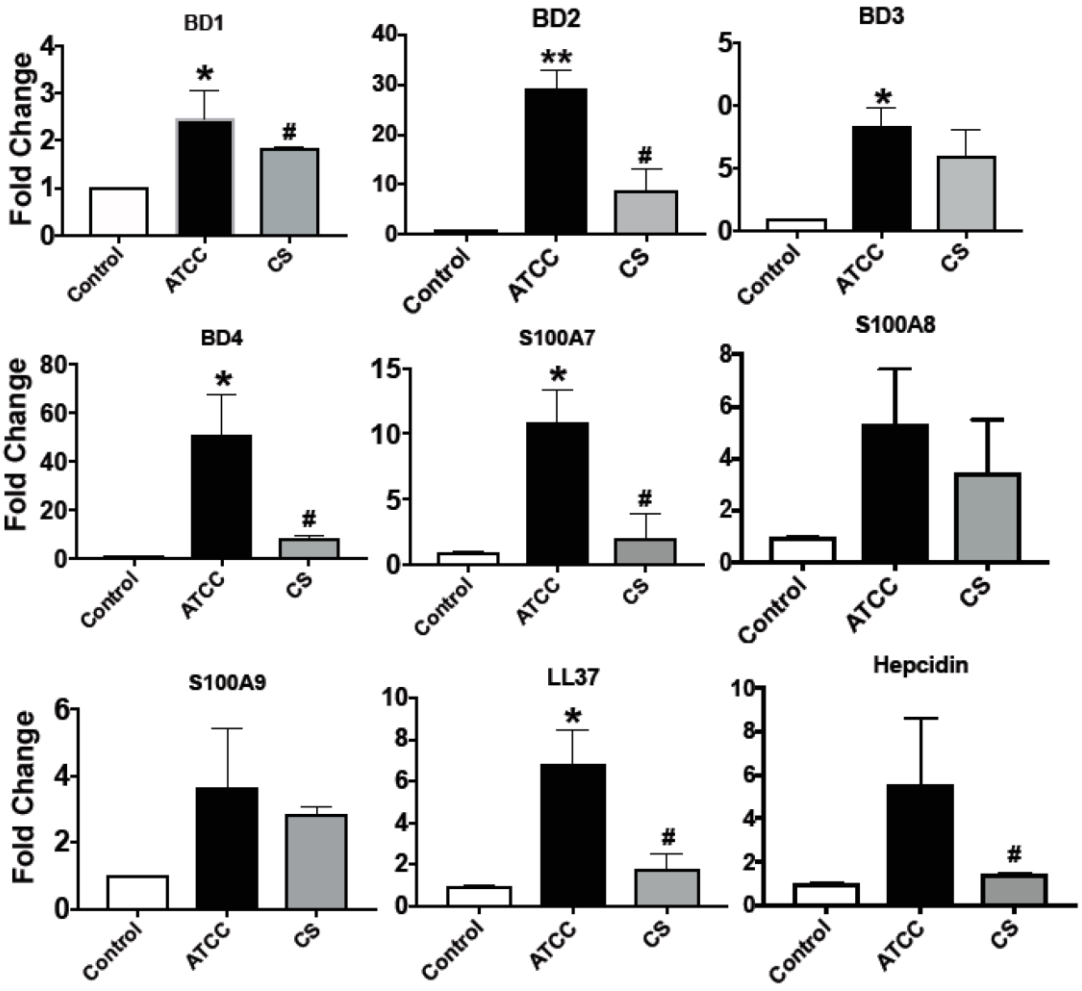


Figure 5.3 *In vitro* expression of AMPs. HCECs were infected with either Sp ATCC or Sp CS for 3h and AMPs expression was checked by quantitative PCR with GAPDH as housekeeping gene. The experiment was repeated twice and data taken in technical duplicates. (* $p < 0.05$, ** $p < 0.005$ compared to control; # $p < 0.05$ compared to ATCC)

To confirm that the lowered expression of AMPs in HCECs infected with Sp CS was not because of *S. pneumoniae* mediated cell cytotoxicity, we did a MTT assay (**Figure 5.4 A**) and PI staining (**Figure 5.4 B**). In separate experiments, HCECs were infected with Sp ATCC or Sp CS for 4h and cell viability checked by MTT assay and PI staining. We found no significant change in cell viability in HCECs infected by Sp ATCC or Sp CS indicating that the difference in the expression pattern of AMP in cells infected with Sp ATCC and Sp CS is not due to the any cell death or cytotoxicity.

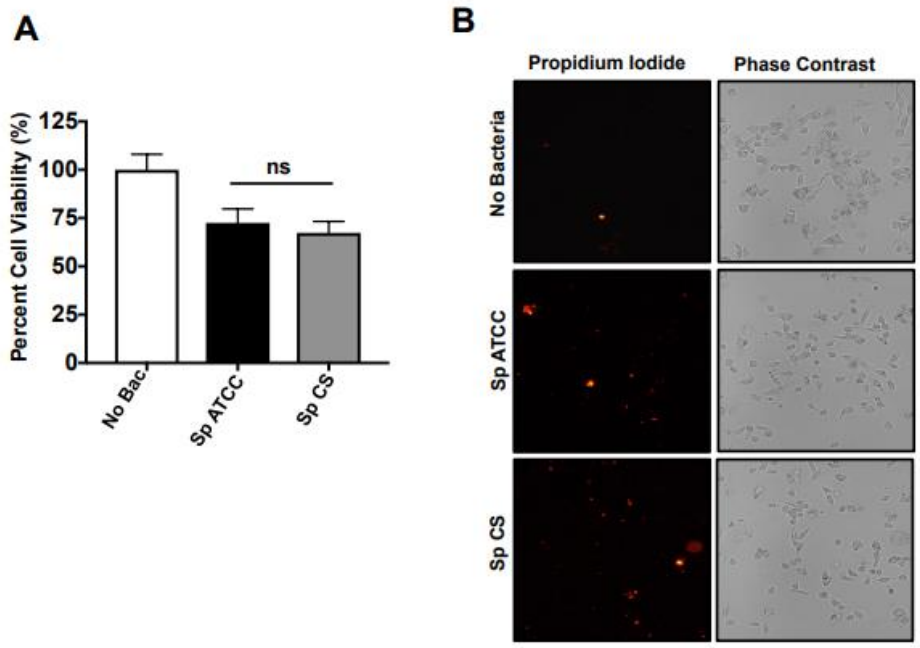


Figure 5.4 Cell viability tests of SP infected HCECs. HCECs were infected with Sp ATCC or Sp CS for 4h and cell viability was checked by MTT assay (A) and propidium iodide staining (B). Cells were observed under fluorescent microscope using 10X objective.

5.2.2 *In vitro* expression of AMPs in *S. pneumoniae* infected primary HCECs

The expression of AMPs during *S. pneumoniae* infection was also determined in primary corneal epithelial cells, as the primary cells are the direct representation of the state of native tissue from where it is isolated and acts as the closest mimic of the *in vivo* system. HCECs were infected with either Sp ATCC or Sp CS for 3h and then incubated in gentamicin containing media for 1h. The expression of AMPs was checked by qPCR and we found that the expression of *hBD-3*, *S100A8*, *S100A9* and *LL-37* were significantly upregulated in primary HCECs infected with Sp ATCC. No significant increase in the expression of *S100A12* and *RNase7* expression was observed in primary HCECs in response to Sp ATCC infection. We did not see any expression of hepcidin in primary HCECs infected with Sp ATCC. There was also a significant upregulation in the expression of *S100A9* in primary HCECs infected with Sp CS. We also saw a significant upregulation of *S100A9* and downregulation of *RNase7* in response to Sp CS (**Figure 5.5**). This indicates that the pattern of expression of AMPs in both immortalized and primary HCEC is similar in response to *S. pneumoniae* infection.

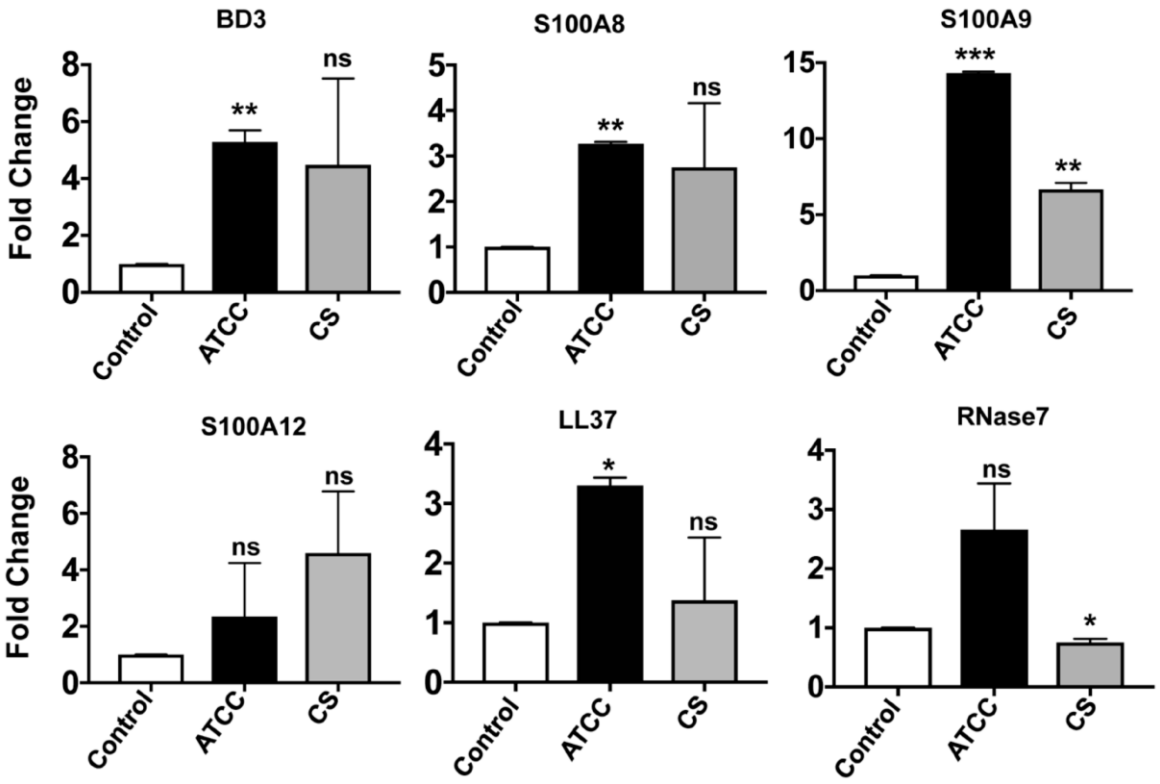


Figure 5.5 The AMPs expression in SP infected primary HCECs. Donor corneas were used to isolate epithelial cells and culture them. These primary HCECs were infected with SP at an MOI of 10 for 3h. Quantitative PCR was done to check the AMPs expression with GAPDH as the housekeeping gene. The experiment was done thrice and data obtained from technical duplicates. (* $p < 0.05$, ** $p < 0.005$, ns is not significant).

5.2.3 *In vitro* expression of AMPs in immortalized human monocytes in response to *S. pneumoniae* infection

In addition to the epithelial cells, macrophages and neutrophils also act as the first line of defense during ocular infections (Akpek & Gottsch, 2003). Hence, the expression of AMPs during *S. pneumoniae* infection was also checked in a human pro-monocytic cell line, U937. The cells were infected with Sp ATCC or Sp CS for 3h and then incubated in gentamicin containing media for 1h. The expression of AMPs was checked by qPCR. We found no significant difference in the expression of AMPs in U937 either in response to Sp ATCC or Sp CS when compared to uninfected cells (**Figure 5.6**). This indicates that macrophages might not play a role in AMP expression in response to *S. pneumoniae* infection.

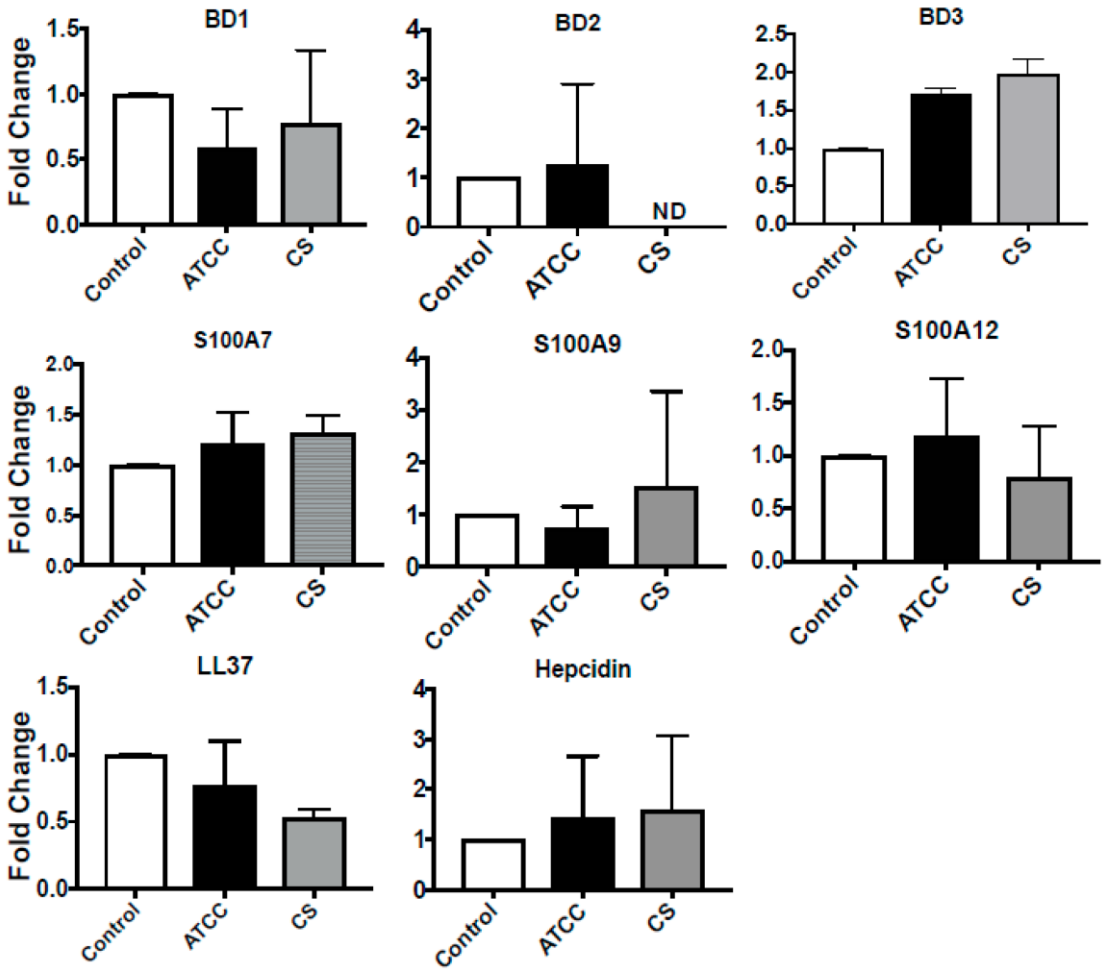


Figure 5.6 AMPs expression in U937 during SP infection. U937 were infected with Sp ATCC or Sp CS at an MOI of 10 for 3h and quantitative PCR was performed to check the expression of AMPs with GAPDH as the housekeeping gene.

5.2.4 *LL-37* expression is upregulated in HCECs in response to *S. pneumoniae* infection

LL-37 is the only human AMP belonging to the cathelicidin group. Since we saw an upregulation of *LL-37* in *S. pneumoniae* patients' samples and in Sp ATCC infected primary and immortalized HCECs, we further focused our study on the expression of LL-37 in HCECs during *S. pneumoniae* infection. Tissue sections from *S. pneumoniae* keratitis patients (n=4) were immunostained for LL-37 to check for the protein level expression and localization. Increased LL-37 immunostaining or expression was seen in the corneal sections of patients, removed during penetrating keratoplasty surgery when compared to the control cadaveric corneal sections. The LL-37 was seen to be localized at the epithelium and stromal layers. Uninfected cadaveric corneas deemed unfit for transplantation were used as controls where we did not see any specific staining for LL-37 (**Figure 5.7 A**). To

check if there is a time dependent upregulation of *LL-37* in HCECs, cells were infected for different duration with Sp ATCC and *LL-37* expression was checked by quantitative PCR. *LL-37* expression peaked at 4h post infection and declined by 24h post infection (**Figure 5.7B**). HCECs were exposed to *S. pneumoniae* for 3h and western blotting (**Figure 5.7C**) and immunostaining (**Figure 5.7D**) was performed, which also confirmed the protein level increase in LL-37 expression in HCECs during *S. pneumoniae* infection.

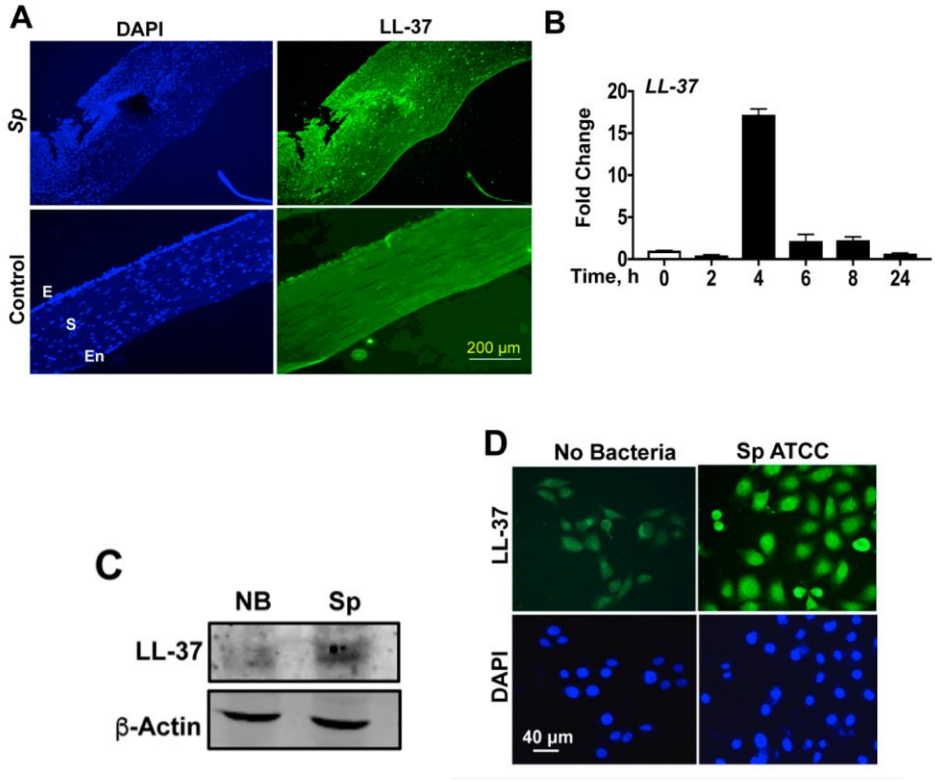


Figure 5.7 LL-37 expression in response to *S. pneumoniae* infection. (A) LL-37 expression seen in *S. pneumoniae* infected corneal sections. E= epithelium, S= stroma, En= endothelium. Imaging was done using 10X objective under fluorescent microscope. (B) *LL37* mRNA expression checked by qPCR at different time points of infection by Sp ATCC. (C) Protein level expression of LL-37 in HCECs infected by Sp ATCC. Determined by western blotting. (D) Expression and localization of LL-37 in HCECs infected with Sp ATCC for 3h and immunostained with anti LL-37 antibody. The imaging was done using fluorescent microscope using 20X objective.

5.2.5 *De novo* LL-37 expression in HCECs in response to *S. pneumoniae* infection

Unlike in the case of some proteins that are recycled, *de novo* protein synthesis depends upon mRNA transcription. Therefore, to check if LL-37 expression in HCECs during Sp ATCC infection followed *de novo* protein synthesis or not, HCECs were exposed to Sp ATCC with or without cycloheximide (CHX; 1µg/ml) for 3h and *LL-37* expression was determined by qPCR and western blotting. CHX is a well-known eukaryotic *de novo* protein-synthesis inhibitor (Lawana et al., 2014). We saw a significant reduction in the

expression of LL-37 in HCECs infected with Sp ATCC in presence of CHX both at gene (**Figure 5.8A**) and protein levels (**Figure 5.8B**) indicating that LL-37 expression in response to Sp ATCC infection is *de novo*.

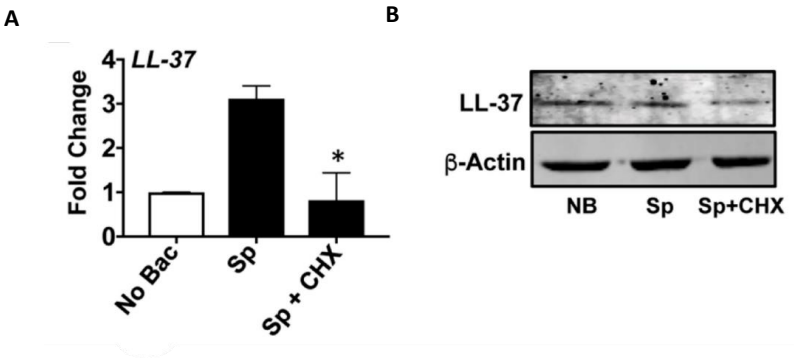


Figure 5.8 LL-37 expression in response to SP infection. (A) De novo expression of LL-37 checked by infecting the cells with SP ATCC for 3h in presence of CHX (added 30 minutes prior to infection) followed by gentamicin treatment for 1h and checking the expression of LL-37 by qPCR and (B) western blotting. GraphPad Prism software was used to calculate significance by ANOVA (* $p < 0.05$ and ** $p < 0.005$).

5.2.6 Antimicrobial activity of LL-37 against *S. pneumoniae*

LL-37 shows a broad-spectrum of antimicrobial action against many pathogens like Group A *Streptococcus, S. aureus, P. aeruginosa* etc. (Dorschner et al., 2001; Ling C. Huang et al., 2006; Kang et al., 2019; Turner et al., 1998). Therefore, we wanted to check the antimicrobial activity of LL-37 against the ATCC and clinical strains of *S. pneumoniae*. 10^4 cfu/ml of Sp ATCC or Sp CS were incubated for 4h with an increasing concentration of LL-37 (0-100ug/ml) and then serially diluted and plated on blood agar plates to check the colony-forming units. There was a decrease in the CFUs with increasing concentration of LL-37 (**Figure 5.9**).

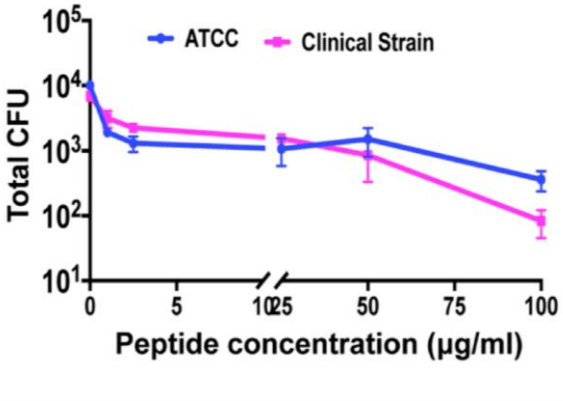


Figure 5.9 Efficiency of LL37 against *S. pneumoniae*. SP ATCC and SP CS were treated with different concentrations of LL37 for 4h and plated to count CFUs. The total CFU was plotted against the increasing LL-37 peptide concentrations and represented as an X-Y curve.

5.2.7 Wound healing activity of LL-37 in HCECs

Earlier studies have shown that LL-37 has wound healing capability by activating cell migration, proliferation and tissue vascularization in human keratinocyte cell line (HaCaT), endothelial cells, immortalized airway epithelium cell (NCI-H292) and primary airway epithelial cells, and *in vivo* mouse study (Carretero et al., 2008; Ramos et al., 2011; Shaykhiev et al., 2005). Since our focus was on the *in vitro* role of LL-37 in *S. pneumoniae* infections we further wanted to confirm the wound healing activity of LL-37 in HCECs. *In vitro* scratch assay is a simple and widely adapted method to check the cell migration during wound healing (Grada et al., 2017). Scratch was made on the monolayer of an overnight grown culture of HCECs in a serum free media, with a 10µl tip head. Thereafter the cells were incubated with media only or different concentrations of LL-37 (100ng/ml, 250ng/ml or 500ng/ml). The wound closure was observed and images were taken at different time intervals (0h, 8h, 12h, 24h, 30h) (**Figure 5.10 A, B**) and the percentage wound closure was calculated using ImageJ. The percentage wound closure was higher in the cells incubated with all concentrations of LL-37, when compared to the control (**Figure 5.10C**). The experiment was repeated twice and we saw similar result.

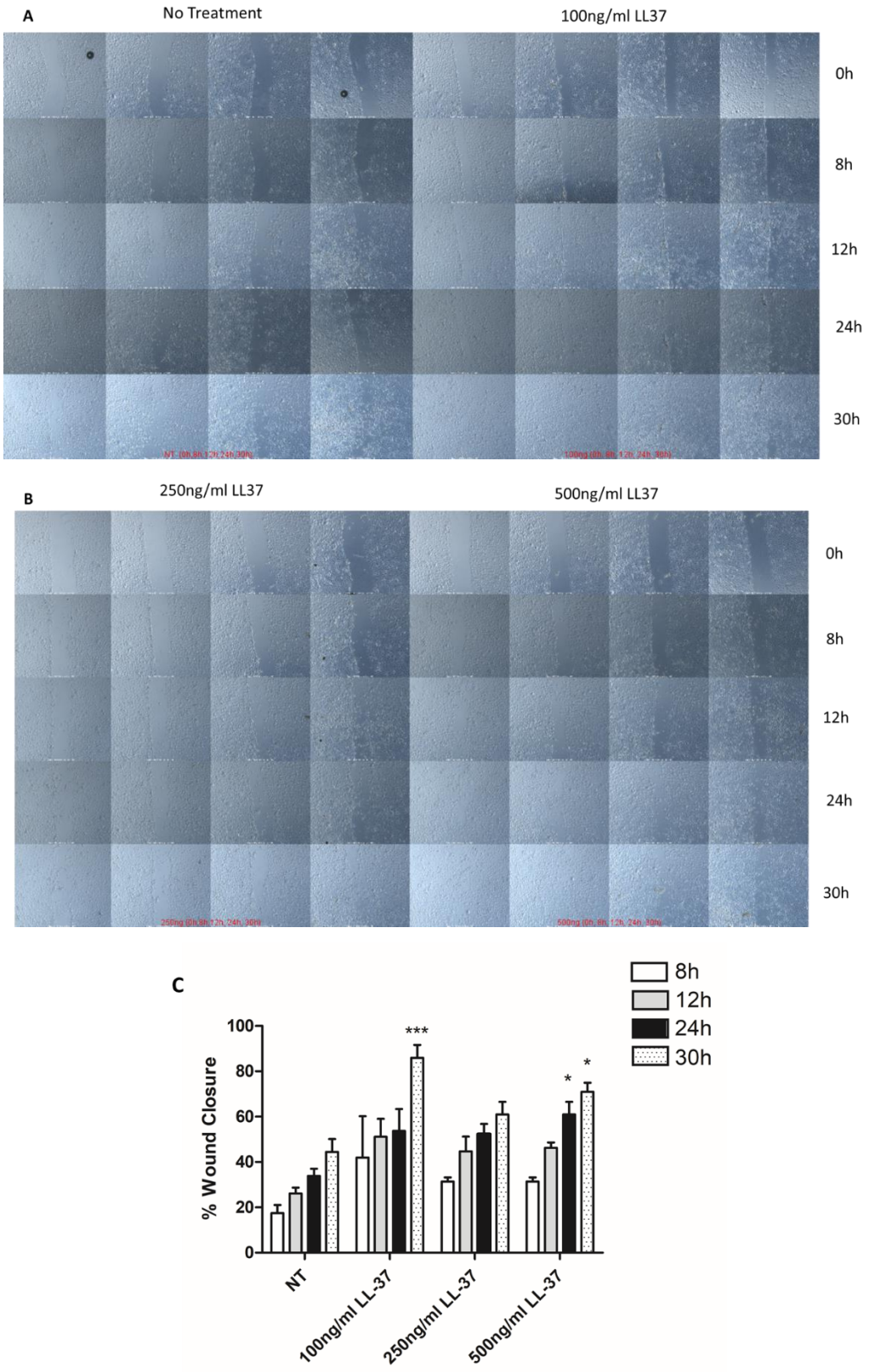


Figure 5.10 Scratch assay to determine the wound healing of LL-37 in HCECs. Scratch was made with 10µl tip on a monolayer of HCECs and incubated with serum free media containing different concentrations of LL-37. The wound was observed and images taken at 5X objective at different time points (A, B). The area of wound was calculated using ImageJ software and was plotted as percentage wound closure relative to wound area at 0h (C). *p<0.05, ***p<0.0005, compared to corresponding time point of NT. NT- No treatment.

Migration and proliferation of cells have been shown to play an important role in wound healing process (Enoch & Leaper, 2005; Schreier et al., 1993; Wallace et al., 2021). The damaged cells in the wound are known to secrete some growth factors, cytokines and chemokines, at the site of wound that mediates the migration and proliferation of cells (Barrientos et al., 2008). Therefore, to check the proliferation of HCECs in LL-37 mediated wound healing, we made a scratch wound in a monolayer of HCECs as described above and incubated with various concentrations of LL-37 for 24h. Thereafter we fixed the cells and did immunostaining for Ki-67, a proliferation marker. We did not find any marked difference in Ki-67 positive cells in the LL-37 treated and non-treated control HCECs, indicating that the wound closure till 24h was mostly the result of HCEC migration (**Figure 5.11**).

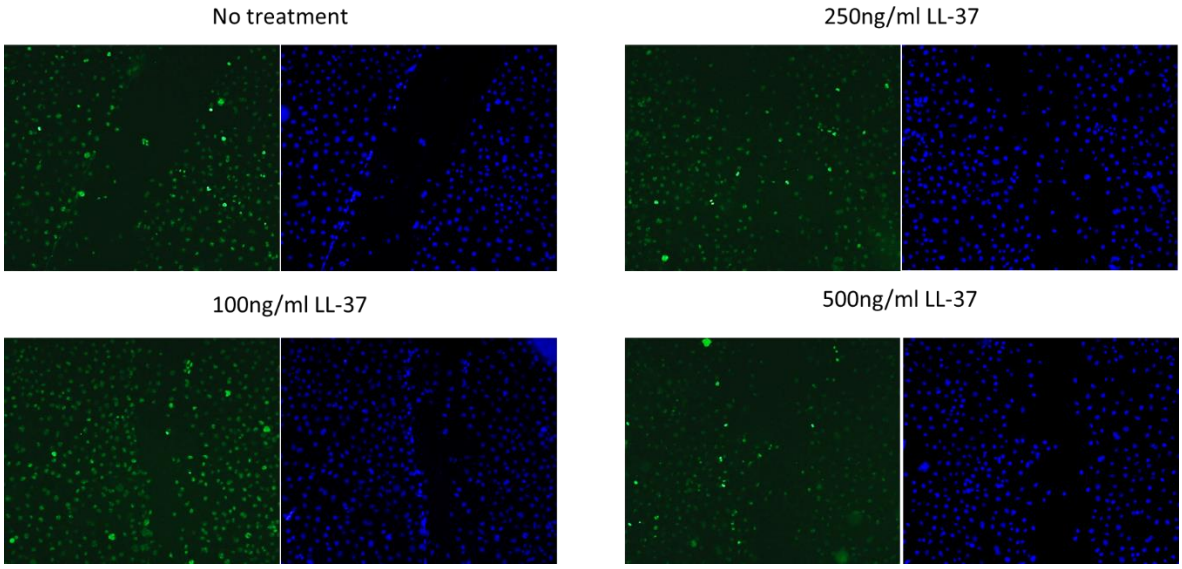


Figure 5.11 Ki67 staining to determine the proliferating HCECs. The monolayer of HCECs were scratched using a 10µl tip and immunostained with Ki67 antibody, 24h post scratch and observed under fluorescent microscope at 10X objective.

5.2.8 Activation of NF- κβ in HCECs in response to *S. pneumoniae*

The expression of AMPs like *LL-37* is known to occur via NF-κβ and/or MAPK signaling pathway in response to various stimuli including bacterial infections (Kusaka et al., 2018; Shamsuddin et al., 2011; Zhao et al., 2018). Therefore, we first wanted to determine the activation of NF-κβ signaling during *S. pneumoniae* infection. HCECs were exposed to Sp ATCC or Sp CS for different time points (30 minutes, 1h, 2h) and phosphorylation of IκB, that indicates activation of NF-κβ, was checked by Western blotting. Sp ATCC and the Sp

CS caused early phosphorylation (30 minutes) of IκB upon infection, which decreased after 1h in HCECs infected with Sp CS. (**Figure 5.12**).

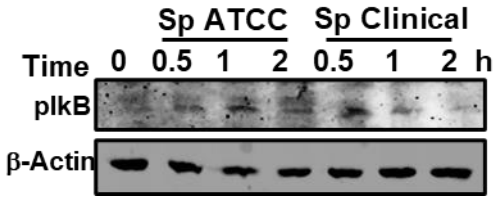


Fig 5.12 Western blotting results showing expression level of pIkB in HCECs cell lysates after infection with Sp ATCC and Sp CS at different time points.

The nuclear translocation of p65 was also checked with immunostaining. Nuclear translocation of p65 was observed in cells infected with both Sp ATCC and Sp CS by 30 minutes. There was reduction in nuclear localization of p65 by 1h in cells infected with Sp CS as compared to Sp ATCC infected cells (**Figure 5.13**).

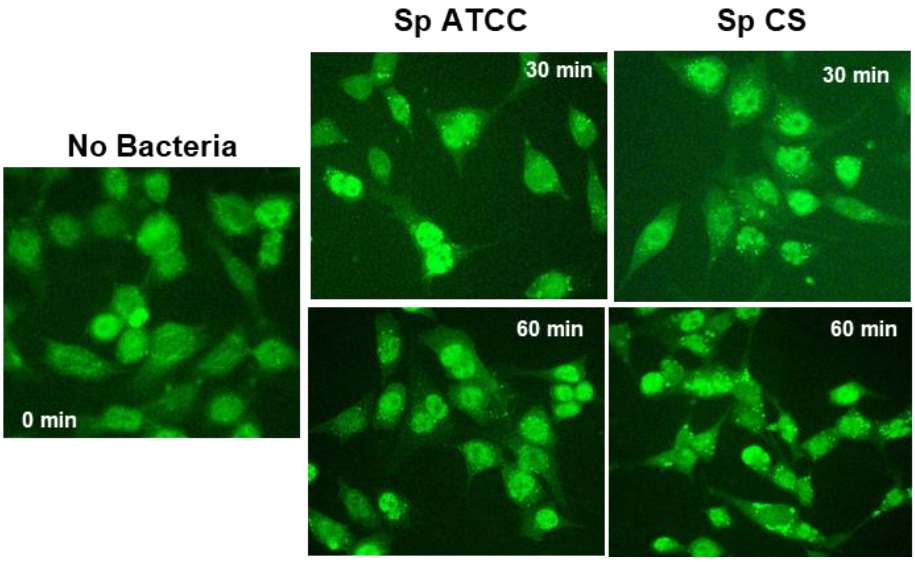


Figure 5.13 Nuclear translocation of p65 subunit of NF-kβ in HCECs on infection with Sp ATCC strain and Sp CS at different time points. HCECs were infected and immunostained with NF-kβ antibody and observed under fluorescent microscope at 40X objective.

5.2.9 Activation of MAPK Pathways in HCECs in response to *S. pneumoniae*

Further, to determine the activation of MAPK signaling pathways during *S. pneumoniae* infection, HCECs were exposed to SP ATCC or SP CS for different time points (30 minutes, 1h, 2h) and activation of p38, JNK, and ERK was checked by western blotting. Increased phosphorylation of JNK (**Figure 5.14A**), ERK (**Figure 5.14B**) and p38 (**Figure 5.14C**) was observed in HCEC during both Sp ATCC and Sp CS infection, within 30

minutes of infection. Sp CS caused reduced activation of JNK in HCECs as compared to Sp ATCC (**Figure 5.14A**). There were no differences in ERK (**Figure 5.14B**) and p38 (**Figure 5.14C**) activation in cells when infected with either of the strains of *S. pneumoniae*.

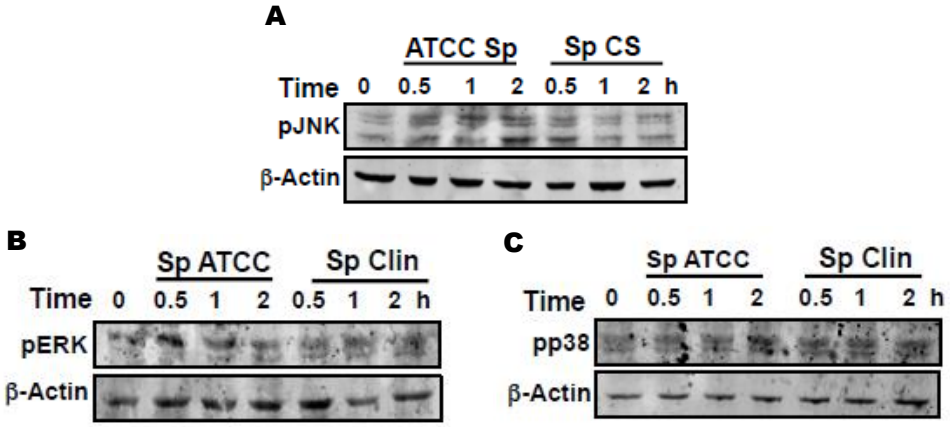


Figure 5.14 Activation of MAPK pathway. Expression of JNK (A), p38 (B) and ERK (C) in HCECs upon infection with ATCC strain and CS of Sp for 0.5h,1h & 2h.

5.2.10 *LL-37* expression in HCECs in response to *S. pneumoniae* is not mediated by NF- κβ and MAPK Pathways

To further confirm the role of the signaling pathways in the expression of *LL-37*, HCECs were infected with Sp ATCC for 3h along with the inhibitors (added 2h prior to infection) for IκB (MG132), p38 (SB203580), JNK (SP600125), and ERK (PD98059), followed by 1h gentamicin treatment. Thereafter the mRNA level expression of *LL-37* was checked by qPCR. We saw no significant change in LL37 expression (**Figure 5.15**) indicating that its expression in response to SP ATCC infection is not mediated by NF-κβ and MAPK pathways in HCECs.

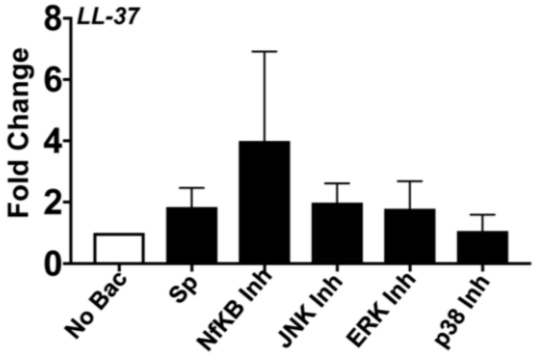


Figure 5.15 *LL-37* expression in response to *S. pneumoniae* infection is not mediated by NF-κβ and MAPK pathway. HCECs were infected with Sp ATCC alongwith NF-κB inhibitor MG132 (10 μM), JNK inhibitor SP600125 (25 μM), p38 inhibitor SB20358 (10 μM), and ERK inhibitor PD98059 (20 μM), and *LL-37* expression was determined by qPCR with GAPDH as the housekeeping gene.

5.2.11 STAT3 mediated *LL-37* expression in HCECs in response to *S. pneumoniae* infection

Since the expression of *LL-37* in HCECs was not via NF-κB and MAPK signaling and it was shown earlier that *LL-37* promoter region has two STAT3 binding sites (Miraglia et al., 2016) (**Figure 5.16A**), we checked whether STAT3 is activated in HCECs during *S. pneumoniae* infection or not. On receiving signals, STAT3 are phosphorylated, homodimerizes and translocates to the nucleus. HCECs were exposed to Sp ATCC for different duration and activation by phosphorylation of STAT3 at tyrosine 705 was checked by western blotting. We saw the phosphorylation of STAT3 from 30 minutes up to 2h post infection (**Figure 5.16B**). Total STAT3 and beta actin were used as controls. Further, to confirm that *LL-37* expression is via active or phosphorylated STAT3, HCECs were exposed to SP ATCC with or without STAT3 inhibitor, Stattic (25µM), for 4h and *LL-37* expression analyzed by qPCR. Stattic is known to inhibit the dimerization and nuclear translocation of phosphorylated STAT3 in cells (Schust et al., 2006). We observed a significant reduction of *LL-37* expression in HCECs infected in presence of Stattic as compared to only infected cells (**Figure 5.16C**). This indicates that the induction of *LL-37* expression in HCECs during *S. pneumoniae* infection is via STAT3 pathway activation.

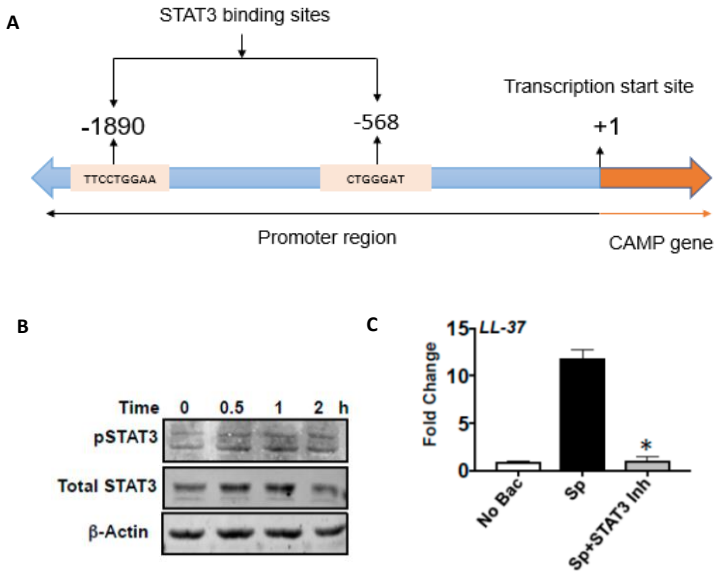


Figure 5.16 *LL-37* expression by *S. pneumoniae* is mediated by STAT3. STAT3 binding sites in *LL-37* promoter region (A). *S. pneumoniae* causes the activation of STAT3 at 30m that continues till 2h, as seen in Western blot analysis (B). HCECs were exposed to *S. pneumoniae* for 4h in the absence or presence of a STAT3 inhibitor (25µM), and expression of *LL-37* was determined by qPCR (C). GraphPad Prism software was used to calculate significance by ANOVA (* $p < 0.05$ and ** $p < 0.005$).

5.2.12 *S. pneumoniae* induces oxidative stress and reduces antioxidant response during keratitis

ROS generation and oxidative stress is a double-edged sword that can be used by the host to combat pathogens or by pathogens to exacerbate the severity of the disease. We know that *S. pneumoniae* produces H_2O_2 under aerobic conditions (Rai et al., 2015). Therefore, to check if *S. pneumoniae* induces oxidative stress during corneal infections, presence of 3-nitrotyrosine was checked in corneal tissue sections attained from patients with *S. pneumoniae* keratitis. 3-nitrotyrosine is formed due to nitration of tyrosine residues and serves as a marker for oxidative stress (Ahsan, 2013; Bandookwala & Sengupta, 2020). The infected corneal tissues used in this study were surgically removed during therapeutic penetrating keratoplasty from *S. pneumoniae* keratitis patients at LVPEI and these were obtained from the Pathology department, LVPEI. We observed specific staining for 3-nitrotyrosine in corneal sections infected with *S. pneumoniae* indicating the occurrence of oxidative stress in these tissues (**Figure 5.17Ai, ii**) whereas, the control cadaveric corneal sections showed no staining for 3-nitrotyrosine (**Figure 5.17Aiii**). Further, the corneal sections were also checked for catalase, an important enzymatic antioxidant, present in the cornea to combat oxidative stress (Álvarez-Barrios et al., 2021). We observed reduced expression of catalase in the infected corneal sections (**Figure 5.17Bi, ii**) when compared to control cadaveric corneal sections (**Figure 5.17Biii**). This indicates that *S. pneumoniae* not only induces oxidative stress but also suppresses the antioxidant responses in the host during corneal infections.

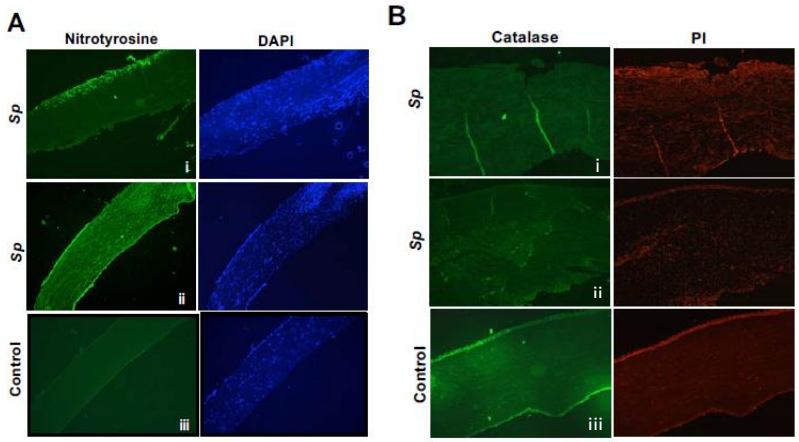


Figure 5.17 Oxidative stress during *S. pneumoniae* keratitis. Corneal tissues (n=5) obtained after penetrating keratoplasty of *S. pneumoniae* keratitis patients were immunostained for 3-nitrotyrosine (A) and catalase (B) followed by secondary Alexa fluor 488 antibody and observed under fluorescent microscope using 10X objective.

5.2.13 *S. pneumoniae* induces reactive oxygen species (ROS) in HCECs

Since we saw the presence of oxidative stress in infected corneal tissues from patients, we checked the generation of ROS *in vitro* in HCECs during *S. pneumoniae* infection. HCECs were exposed to Sp ATCC for 1h or 2h and then incubated with H_2CFDA dye for 30 minutes, as described earlier. The generation of ROS was quantitatively measured using spectrophotometer with excitation at 485 nm and emission at 525 nm. We saw a significant increase in ROS at 1h which continued till 2h (**Figure 5.18A**). We also did fluorescent microscopy and confirmed an increase in ROS generation in cells in response to *S. pneumoniae* infection for 2h (**Figure 5.18B**).

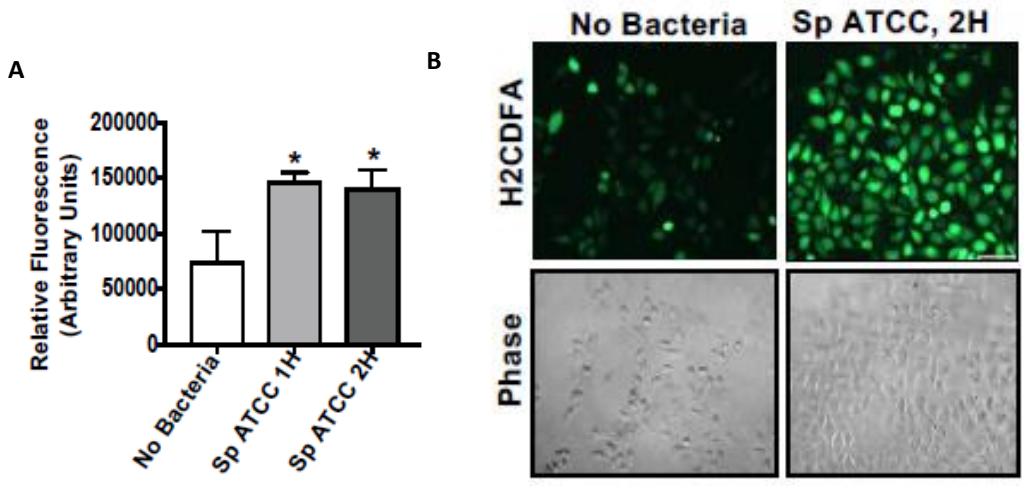


Figure 5.18 Reactive oxygen species generation during *S. pneumoniae* keratitis. HCEC were infected with *S. pneumoniae* for 1h and 2h and generation of reactive oxygen species were detected by H2CFDA (A). The cells were also imaged under fluorescent microscope using 10X objective (B). (* $p < 0.05$).

5.2.14 No significant change in *Nrf2* gene expression in cornea and HCECs during *S. pneumoniae* infection

As a protective response towards enhanced ROS generation, the host cells activate the antioxidant response elements (ARE) that consequently leads to the reduction in ROS. Nrf2 is known to regulate the expression of several ARE associated antioxidant genes (Rushmore et al., 1991; Venugopal & Jaiswal, 1996). Therefore, we further checked *Nrf2* expression in HCECs in response to *S. pneumoniae* infection. We checked the expression of *Nrf2* in corneal scrapings from patients with *S. pneumoniae* keratitis and found that there was no significant difference in the *Nrf2* gene expression in patients' samples when

compared to the control (**Figure 5.19A**). However, we saw a significant decrease in the expression of *Nrf*2 in HCECs infected with Sp ATCC for 4h (**Figure 5.19B**).

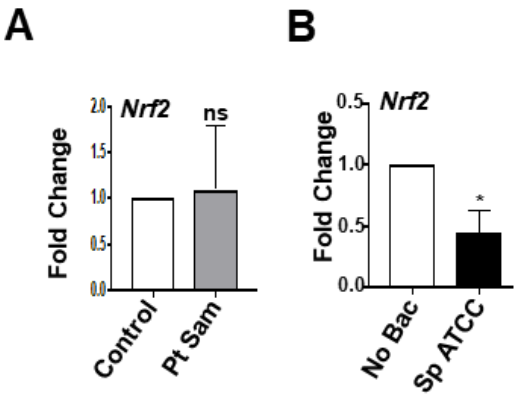


Figure 5.19 *S. pneumoniae* does not affect *Nrf*2 gene expression**.** The expression of *Nrf2* was determined by quantitative PCR from corneal scrapings obtained from *S. pneumoniae* keratitis patients (A) and HCEC infected with *S. pneumoniae* for 4h (B). (ns is not significant).

5.2.15 No nuclear translocation of Nrf2 during *S. pneumoniae* infection

Since we did not see any significant difference in the expression of *Nrf*2 gene in response to *S. pneumoniae* infection, indicating that *S. pneumoniae* does not actively affect Nrf2 expression, we further wanted to confirm that *S. pneumoniae* Nrf2 activation and nuclear translocation in corneal cells. We infected HCECs with Sp ATCC for 2h, immunostained the cells for Nrf2 and observed them under fluorescent microscope. We saw considerably less nuclear translocation of Nrf2 in control uninfected HCECs (**Figure 5.20i**) or in HCECs in response to *S. pneumoniae* (**Figure 5.20ii**) whereas there was a marked increase in the translocation of Nrf2 in the nucleus of HCECs in response to tBHQ, a known activator of Nrf2 (**Figure 5.20iii**). This indicates that *S. pneumoniae* inhibits the nuclear translocation of Nrf2 in HCECs.

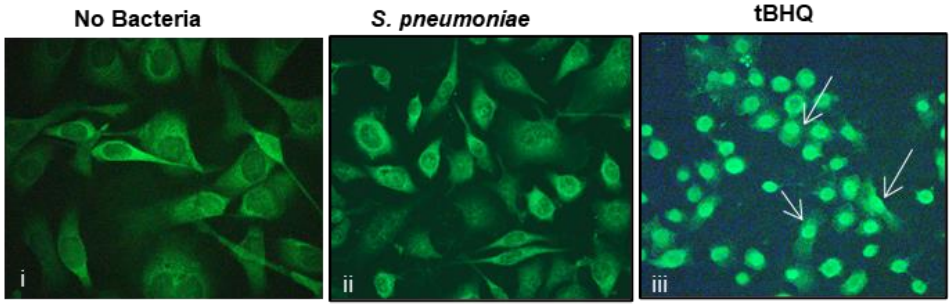


Figure 5.20 *S. pneumoniae* inhibits Nrf2 activation. The translocation of Nrf2 from cytoplasm to nucleus was determined in HCEC in response to *S. pneumoniae* (2h infection) or tBHQ by using an anti-Nrf2 antibody followed by secondary Alexa fluor 488 antibody using 40X objective.

5.2.16 Downregulation of antioxidant genes in HCECs during *S. pneumoniae* infection

Since we observed an inhibition of Nrf2 nuclear translocation in response to *S. pneumoniae* infection, we further checked its effect on the expression of Nrf2 mediated antioxidant genes. HCECs were infected with Sp ATCC for 4h, and the expression of genes like hemeoxygenase (*HO-1*), peroxiredoxin 1(*Per1*), ferritin (*FRT*) and NAD(P)H:quinone oxidoreductase (*NQO-1*) were checked by quantitative PCR. We saw a significant decrease in the expression of *FRT* and *NQO-1*. However, we did not see any significant difference in the expression of *HO-1* and *Per1* (**Figure 5.21**). This indicates that the inhibition of nuclear translocation of Nrf2 in response to *S. pneumoniae* infection consequently leads to the downregulation of some of the Nrf2 mediated antioxidant gene expression, thereby affecting efficient host antioxidant response.

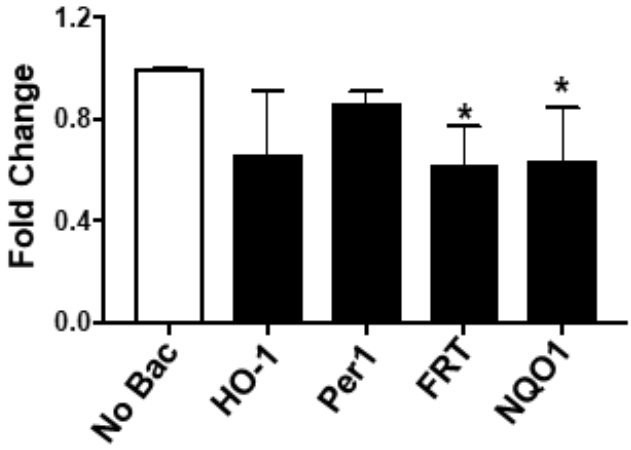


Figure 5.21 *S. pneumoniae* inhibits expression of Nrf2 mediated antioxidant genes. The expressions of antioxidant genes in HCEC in response to *S. pneumoniae* (4h) were determined by quantitative PCR. (* $p < 0.05$).

5.2.17 tBHQ upregulates expression of *Nrf2* and Nrf2 mediated antioxidant genes in HCECs during *S. pneumoniae* infection

Since tBHQ is a known inducer and activator of Nrf2, we further checked if it has any effect on *Nrf*2 gene expression, its nuclear translocation and expression of Nrf2 mediated antioxidant genes. To check the cytotoxic effect of tBHQ, we incubated HCECs in the presence of increasing concentrations of tBHQ (5µM, 25µM, 50µM and 100µM) for 6h and checked the cell viability by MTT assay as described before. We saw that there was no significant change in the cell viability in HCECs treated with 5µM, 25µM or 50µM of tBHQ, when compared to the untreated cells. However, there was a significant difference

in the percentage of cell viability of HCECs treated with 100µM of tBHQ (**Figure 5.22**). Therefore, for our further experiments we used 25µM of tBHQ, to omit any cytotoxicity related anomalies in our result.

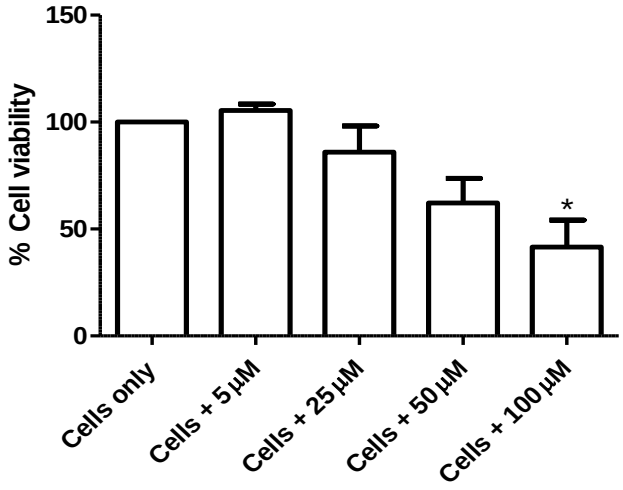


Figure 5.22 Cytotoxic effect of tBHQ. HCECs were incubated with increasing concentrations of tBHQ (5µM,25µM,50µM and 100µM) for 6h and the cell viability checked with MTT assay. (* indicates $p<0.05$ compared to cells only).

HCECs were infected with Sp ATCC for 4h in the presence or absence of tBHQ, and the expression of *Nrf*2 gene was checked by qPCR. We found that there was a significant increase in *Nrf2* gene expression in the presence of tBHQ in both infected and uninfected cells (**Figure 5.23A**). The Nrf2 mediated antioxidant genes like *HO-1* and *FRT* was also significantly upregulated in the presence of tBHQ in both infected and uninfected cells (**Figure 5.23 C, D**). We did not see much nuclear translocation of Nrf2 in response to *S. pneumoniae* infection (**Figure 5.23Bii**), similar to the uninfected HCEC (**Figure 5.23Bi**). However, we noted a marked increase in the nuclear translocation of Nrf2 in the presence of tBHQ (**Figure 5.23Biii**) as well as tBHQ only treated control (**Figure 5.23Biv**), indicating that tBHQ can reverse the *S. pneumoniae* mediated suppression of Nrf2 expression and activation, consequently leading to the induction of Nrf2 mediated antioxidant response in HCECs.

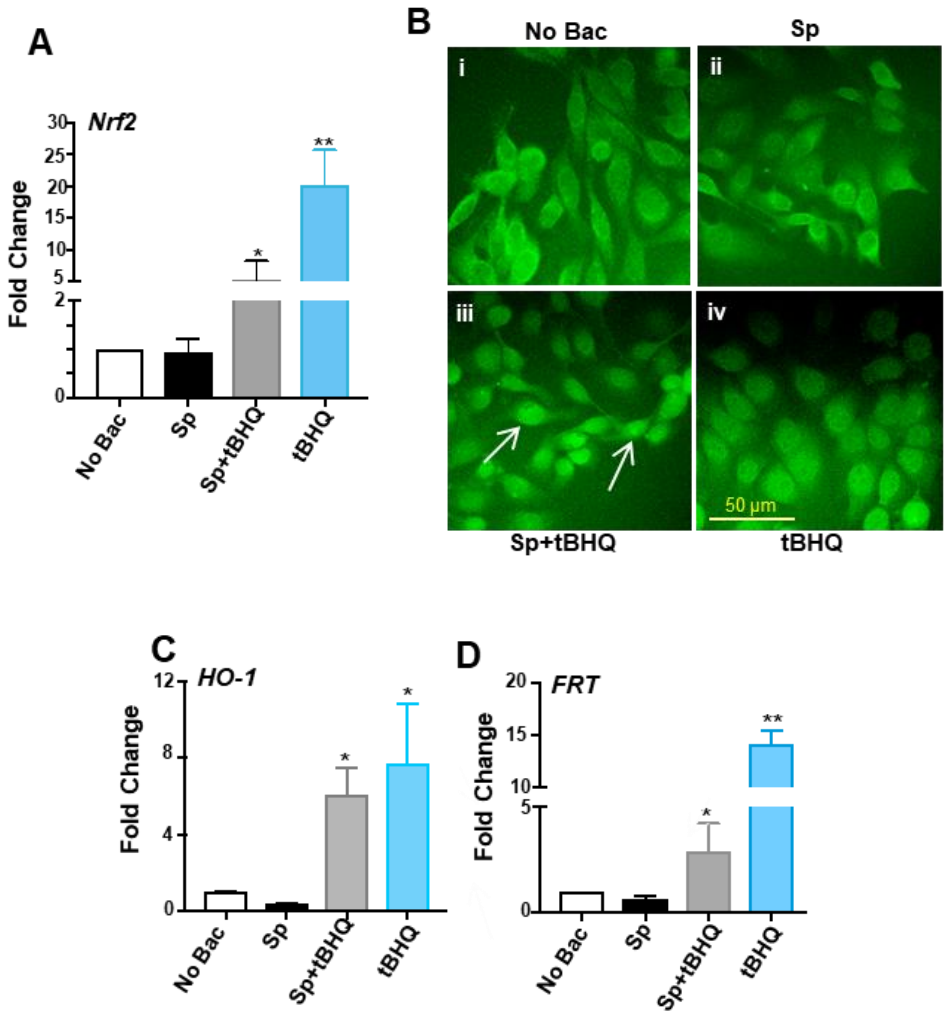


Figure 5.23 tBHQ activates Nrf2 and increases expression of Nrf2 mediated genes in HCEC. The increase in Nrf2 expression and activation in HCEC in presence of *S. pneumoniae* infection (4h) was determined. The gene expression of Nrf2 was detected by qPCR (A). Translocation of Nrf2 was determined by immunocytochemistry using anti-Nrf2 antibody followed by secondary anti-mouse Alexa fluor 488 antibody 488 and observed under fluorescent microscope with 40X objective (B). The expression of Nrf2 mediated genes, HO-1 (C) and FRT (D) in response to *S. pneumoniae* and tBHQ was also determined by qPCR. (* indicates $p < 0.05$, ** indicates $p < 0.005$ compared to no bac).

5.2.18 tBHQ decreases ROS generation in HCECs during *S. pneumoniae* infection

Since tBHQ was able to induce Nrf2 dependent antioxidant response in HCECs, we further checked if there was any effect on ROS generation in response to *S. pneumoniae* in presence of tBHQ. HCECs were infected with Sp ATCC for 2h in the presence or absence of tBHQ and incubated with H_2CFDA dye for 30 minutes for flow cytometry analysis. We observed an increase in ROS generation in response to exposure to Sp ATCC for 2h, that decreased considerably in the presence of tBHQ pretreatment (**Figure 5.24A**). The mean fluorescent intensities of the same experiment are graphically presented in **Figure 5.24B**. This indicates

that tBHQ is able to reduce excessive ROS generation in response to *S. pneumoniae* infection.

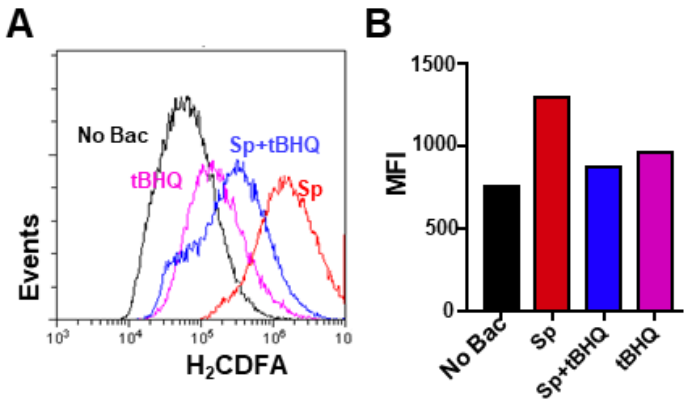


Figure 5.24 tBHQ decreases ROS generation in *S. pneumoniae* infected HCEC. The ROS generation in HCEC during *S. pneumoniae* infection for 2h in presence or absence of tBHQ was determined by flow cytometry (A). The mean fluorescent intensity (MFI) observed during flow cytometry experiment was presented graphically (B).

5.2.19 PI3K signaling mediated suppression of autophagy in *S. pneumoniae* infected HCEC

Autophagy is one of the most important innate immune responses of the host against the pathogens. It helps in the degradation and elimination of intracellular pathogens and also helps in regulating inflammatory responses and adaptive immunity (Germic et al., 2019; Junkins et al., 2013). To evade the host response some pathogens have devised mechanisms to inhibit or suppress autophagy. For instance, *P. aeruginosa* inhibits autophagy via ExoS in epithelial cells, that aids in its intracellular survival (Rao et al., 2021). *S. pneumoniae* was shown to evade host autophagy by CbpC mediated depletion of Atg14 (Shizukuishi et al., 2020). *Enterococcus faecalis* can survive intracellularly in macrophages by resisting phagosome acidification and autophagy (Zou & Shankar, 2016). It is also known that ROS mediated oxidative stress can cross-talk with autophagy. Excessive ROS generation causes oxidative damage to the intracellular molecules that may induce autophagy, which in turn results in the removal of these damaged components from the cells (L. Li et al., 2015). Since we observed that *S. pneumoniae* induces ROS generation in the host, we wanted to check if induction of ROS has any effect on autophagy signaling or not. HCECs were infected with Sp ATCC for either 2h or 6h and were immunostained for LC3B. Microtubule-associated protein 1A/1B-light chain 3 (LC3) is a soluble protein of approximately 17kDa. LC3-phosphatidylethanolamine conjugate (LC3-II) is formed

during autophagy by the conjugation of cytosolic form of LC3 (LC3-I) to phosphatidylethanolamine. LC3-II is then recruited to membrane of nascent autophagosomes where it appears as puncta. Hence, LC3 puncta in the cytoplasm of the host is an important marker that accesses the number of autophagosomes in the cells, indicating the activation of autophagy (Kabeya et al., 2000). We saw a low level of LC3B puncta in HCECs in response to Sp ATCC similar to that in the control cells. The cells that were incubated with 10μM of MG132 as a positive control, where we saw a marked increase in the LC3B puncta (**Figure 5.25A**). MG132 is a reversible proteasome inhibitor that induces the formation of protein aggregates, thereby activating autophagy (Ge et al., 2009). LC3B puncta present per cell were counted and plotted as a graph, where we can see that there is a significant decrease in LC3B puncta in Sp ATCC infected cells that significantly increased in the MG132 treated cells (**Figure 5.25B**).

Further we checked the expression of genes that are known to be induced during autophagy, by quantitative PCR (Klionsky et al., 2021). HCECs were infected for 4h with Sp ATCC and the expression of *Atg5, Atg7* and beclin1 was determined. Atg5 and Atg7 are key autophagy components that forms a part of ubiquitin-like complex 1, a crucial part of autophagosome formation (H. Y. Liu et al., 2009). ATG5 and ATG7 is crucial for autophagic vesicle formation, expansion and autophagosome-lysosome fusion (*Macroautophagy | Pathway - PubChem*, n.d.; Ye et al., 2018). Beclin1 is an important component of class III phosphatidylinositol 3-kinase (PtdIns3K) complex that plays a crucial role in vesicle trafficking during autophagy (*BECN1 Beclin 1 [Homo Sapiens (Human)] - Gene - NCBI*, n.d.; H. Y. Liu et al., 2009). These autophagy related genes were downregulated in HCECs in response to *S. pneumoniae* infection (**Figure 5.25C**). To further check which upstream signaling is involved in the downregulation of autophagy, we looked into the activation of PI3K/Akt/mTOR signaling pathway as it is one of the major pathways that regulates autophagy (Wu et al., 2009). PI3K is known to activate Akt that further activates mechanistic target of the rapamycin complex (mTOR) which leads to inhibition of autophagy (Kma & Baruah, 2021). During infection or stress, it has been shown that autophagy is activated in the host via PI3K-Akt-mTOR, that clears out the intracellular pathogens, thereby enhancing cell survival (Kapuy et al., 2014; X. Zhang et al., 2017). Therefore, to evade autophagy some pathogens can regulate the PI3K-Akt-mTOR to its advantage and inhibit autophagic responses (Ganesan et al., 2017; Lu et al., 2019). We infected HCECs with Sp ATCC for different duration and checked the activation

or phosphorylation of PI3K and Akt by western blotting. PI3K was found to be phosphorylated within 1h of infection (**Figure 5.25D**). We also saw phosphorylation of Akt that began as early as 30 minutes post infection (**Figure 5.25E**). These results indicate that *S. pneumoniae* inactivates and regulates autophagy in HCECs via PI3K/Akt signaling pathway.

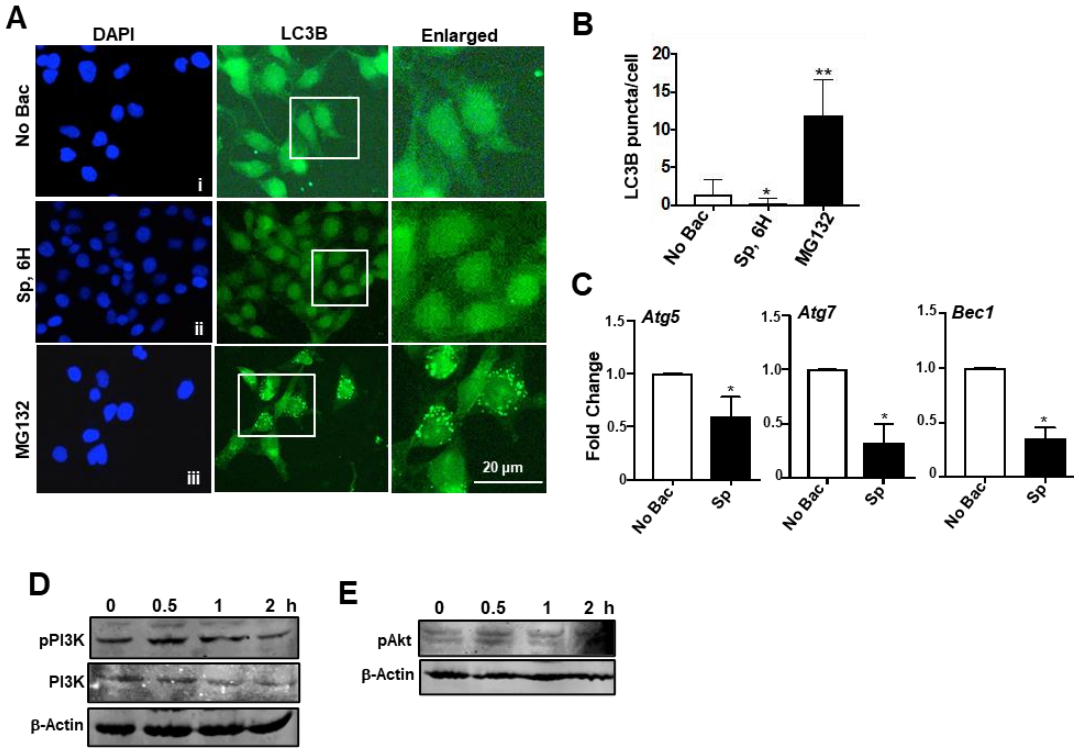


Figure 5.25 *In vitro* inhibition of autophagy by *S. pneumoniae*. HCECs were infected with Sp ATCC for 2h and 6h and immunostained with anti-LC3B antibody and counterstained with secondary Alexa fluor 488 and observed under fluorescent microscope with 40X objective (A). The number of puncta per cell was counted and plotted as a graph. At least 100 cells were counted for each condition (B). Autophagy related gene expression was checked by qPCR and statistically analyzed using Graph pad prism (C). The phosphorylation of PI3K (D) and AKT (E) was checked by western blotting with beta actin as the housekeeping control. (* denotes $p< 0.05$, *** denotes $p< 0.0005$).

5.2.20 tBHQ induces autophagy in *S. pneumoniae* infected HCECs

Since we saw a suppression of autophagy in HCECs in response to *S. pneumoniae* infection and tBHQ mediated reduction in *S. pneumoniae* induced ROS generation we further wanted to check if tBHQ is able to restore autophagy as well. HCECs were infected with Sp ATCC for 6h in presence or absence of tBHQ and cells were immunostained for LC3B and observed under fluorescent microscope. As was observed earlier, we saw diffused LC3 staining in HCECs in response to Sp ATCC infection (**Figure 5.26Aii**). However, we saw

increased number of distinctive puncta of LC3B in the presence of tBHQ (**Figure 5.26Aiii**). The number of puncta per cell were counted and plotted as a graph where we could see a significant increase in the number pf puncta per cell in the presence of tBHQ when compared to uninfected cells (**Figure 5.26B**). We also checked the expression of *Atg5, Atg7* and *beclin1* in HCECs infected with Sp ATCC in presence or absence of tBHQ. We observed an increase in the expression of these autophagy related genes in the presence of tBHQ (**Figure 5.26C**). These results indicate that tBHQ was able to induce autophagy in *S. pneumoniae* infected cells.

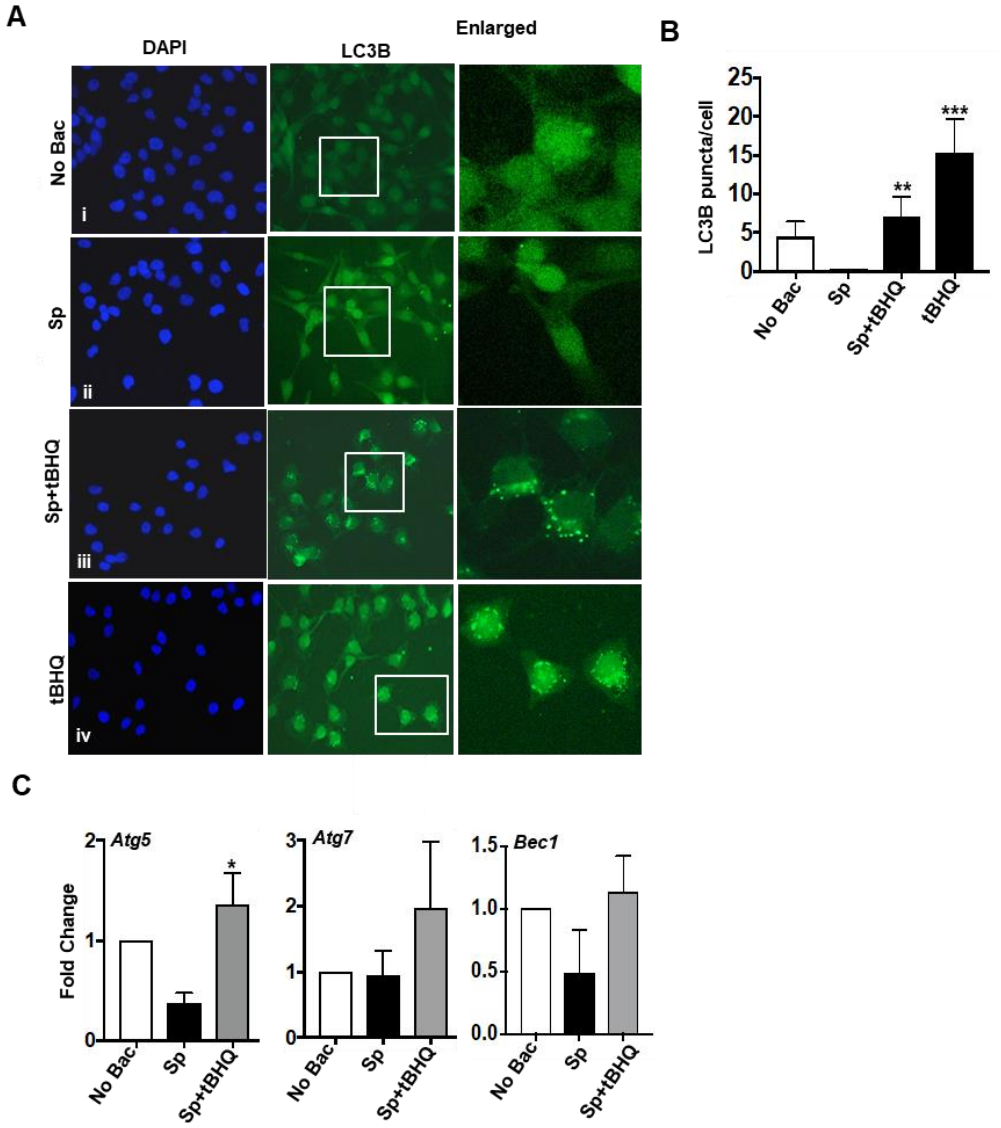


Figure 5.26 Induction of autophagy by tBHQ in *S. pneumoniae* infected HCECs. HCECs were infected with Sp ATCC for 6h in the presence or absence of tBHQ and immunostained with anti-LC3B antibody and secondary Alexa fluor 488. The cells were observed under fluorescent microscope with 40X objective (A). The number of puncta per cell were plotted graphically and analysed using Graph Pad prism (B). The expression of autophagy related genes was determined by qPCR (C). (* denotes p< 0.05, *** denotes p< 0.0005).

5.2.21 tBHQ induced autophagy is Nrf2 dependent

Since we saw the induction of autophagy in the presence of tBHQ, we speculated that this induction might be mediated by the activation of *Nrf2* by tBHQ. HCECs were transfected with 150nM of Nrf2 siRNA to transiently deplete *Nrf2* expression, and thereafter infected with Sp ATCC for 6h in the presence or absence of tBHQ. Non- transfected cells were used as control and infected similarly. As we observed earlier, there was no induction of autophagy in response to *S. pneumoniae* infection either in control siRNA (**Figure 5.27Aii**) or *Nrf2* siRNA transfected cells (**Figure 5.27Av**), whereas autophagy was induced in the presence of tBHQ in control siRNA transfected HCECs (**Figure 5.27Aiii**), as indicated by the presence of distinctive LC3B puncta. However, we did not see any LC3B puncta in Nrf2 depleted HCECs in the presence of tBHQ (**Figure 5.27Avi**), indicating that tBHQ induces autophagy in a Nrf2 dependent manner. The LC3B puncta per cell were counted and presented graphically, where we could see that there was a significant reduction in the number of puncta per cell in Nrf2 depleted HCECs, , when compared to cells transfected with control siRNA (**Figure 5.27B**).

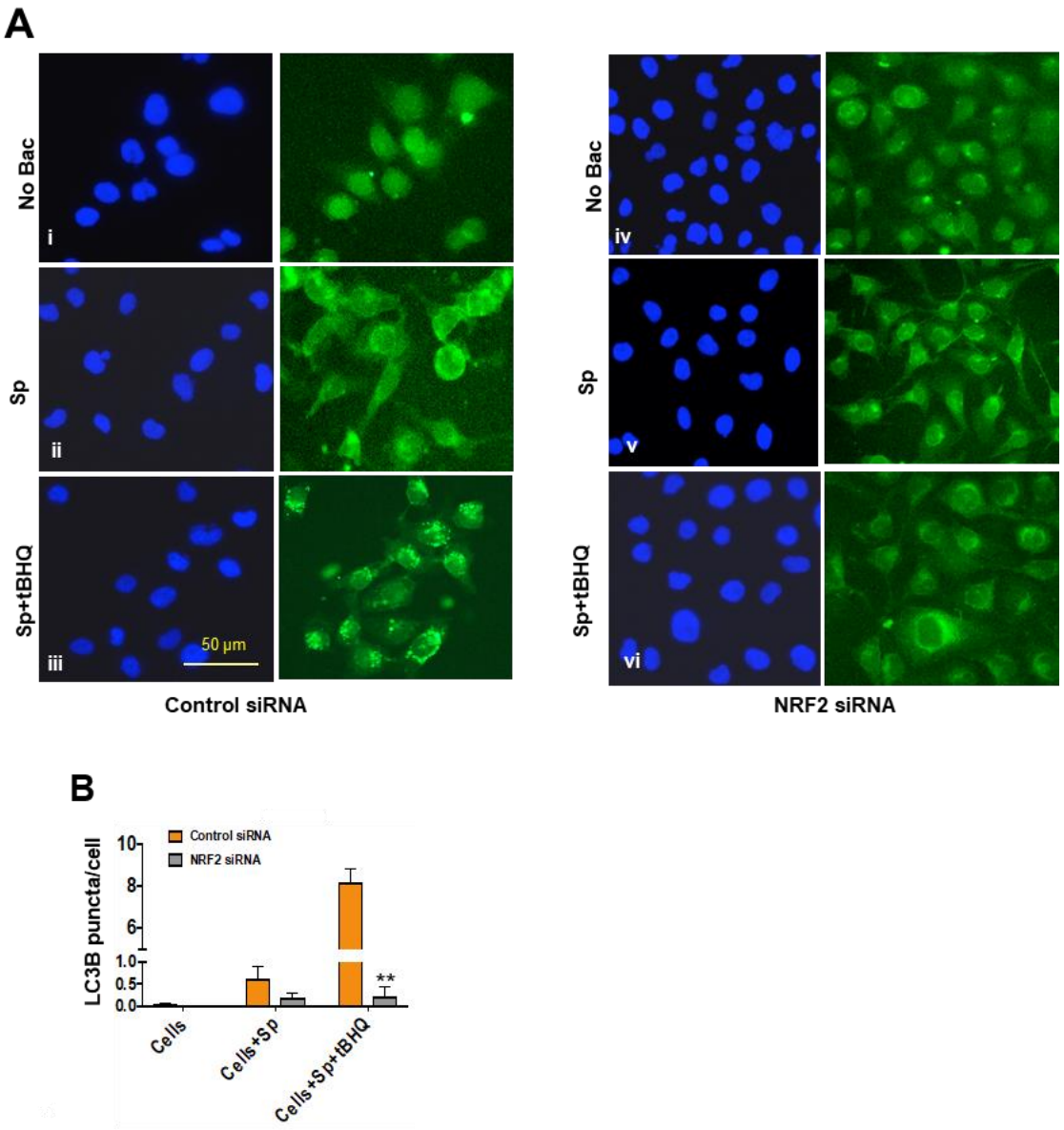


Figure 5.27 Nrf2 dependent induction of autophagy. HCECs were transfected with Nrf2 siRNA or control siRNA, and infected with Sp ATCC for 6h in the presence or absence of tBHQ. Cells were immunostained for LC3B and observed under fluorescent microscope with 40X objective (A). The number of LC3B puncta per cell was counted and plotted as a graph (B). ** indicates $p < 0.005$ compared to control siRNA transfected cell, same treatment.

5.2.22 tBHQ reduces *S. pneumoniae* mediated cytotoxicity and reduces bacterial load

Since we saw that tBHQ reduces ROS and induces autophagy in *S. pneumoniae* infected HCECs, we further wanted to check its effect on *S. pneumoniae* induced cytotoxicity. We infected HCECs with Sp ATCC for 6h in the presence or absence of tBHQ, washed twice with 1X PBS and further incubated for 16h. Thereafter, we checked the cell viability by propidium iodide (PI) staining. We saw a considerable amount of death in HCECs in

response to Sp ATCC infection whereas in presence of tBHQ there was a marked reduction in cell death, indicated by lower amount of PI staining (**Figure 5.28A**). The PI positive cells were also counted and plotted in a graph form, where we see a significant amount of cell death at 24h in the infected HCECs in the absence of tBHQ (**Figure 5.28B**). Next, we checked the effect of tBHQ on intracellular bacterial load in the cells for which HCECs were infected with Sp ATCC for 6h in the presence or absence of tBHQ, washed, lysed with 0.3% triton X-100, serially diluted and plated on blood agar medium. The CFU was counted the next day. We saw that there was a significant reduction in intracellular bacterial load in presence of tBHQ, either in cells infected with ATCC (Sp ATCC) or clinical strain (Sp CS) of *S. pneumoniae* (**Figure 5.28C**). We also plated the supernatant to check the effect of tBHQ on extracellular unattached bacteria, and found that in the presence of tBHQ there was a significant decrease in the extracellular bacterial load as well (**Figure 5.28D**). However, we did not see any direct bacteriostatic or bactericidal effect of tBHQ on *S. pneumoniae* as indicated by no significant difference in optical density (O.D.) measured at 600nm after 24h between Sp ATCC incubated with or without tBHQ (**Figure 5.28E**). All these results indicate that tBHQ inhibits the *S. pneumoniae* mediated cytotoxicity and has an inhibitory effect on intracellular and extracellular bacteria, but does not directly kill or inhibit the bacteria.

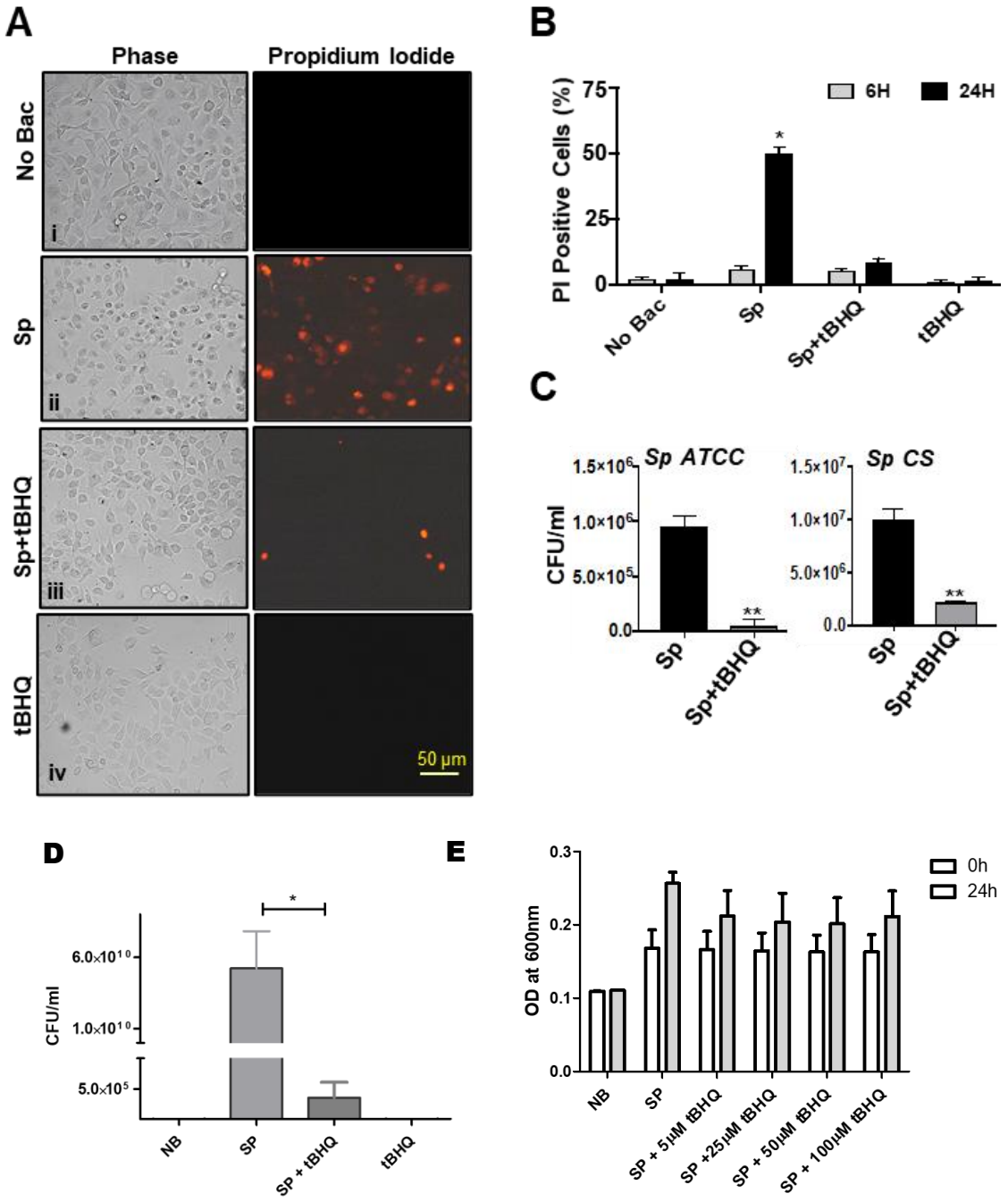


Figure 5.28 tBHQ reduces cell death and inhibits *S. pneumoniae* invasion in HCEC. Cells were infected with *S. pneumoniae* for 6 h in presence or absence of tBHQ, washed and further incubated with media containing antibiotics for 16 h. The viability of cells was determined by propidium iodide staining and observed under microscope using 20X objective (A). PI positive cells were counted from at least three different fields for each condition and represented in a graphical form (B). HCEC were infected with *S. pneumoniae* (6h) washed and lysed using triton X-100 to determine the bacterial load attached to HCEC, and plated on blood agar plate by serial dilutions (C). Supernatant was also plated after infection of HCECs with *S. pneumoniae* for 6h, to check the extracellular unattached bacteria by serial dilutions (D). *S. pneumoniae* were incubated with different concentrations of tBHQ for 24h and OD was measured at 600 nm to check the direct effect of tBHQ on *S. pneumoniae* (E) (NB means No Bacteria, * represents $p<0.05$, ** represents $p<0.005$).

5.2.23 tBHQ increases AMP expression and reduces pro-inflammatory cytokines in *S. pneumoniae* infected HCECs

Since we saw that tBHQ is able to alleviate *S. pneumoniae* mediated cell cytotoxicity and reduce intracellular and extracellular bacterial load but does not have any direct inhibitory effect on the bacteria, we speculated that this reduction in severity of infection might be mediated by tBHQ induced immunomodulatory effects in the host. Therefore, we checked the expression of AMPs and proinflammatory cytokines in HCECs infected with Sp ATCC for 4h in the presence or absence of tBHQ. We saw a significant upregulation of AMPs like *hBD-3* (~290 fold), *LL-37* (~50 fold), *S100A9* (~10 fold), *S100A12* (~70 fold), lipocalin 2 (~100 fold) and hepcidin (~35 fold) in the infected cells in the presence of tBHQ (**Figure 5.29 A-F**). We saw an increase in the expression of proinflammatory cytokines like IL-1β, IL-6 and IL-8 in response to Sp ATCC infection that was significantly decreased in the presence of tBHQ (**Figure 5.29 G-I**), when compared to control cells. ELISA was also performed to quantify the level of cytokine expression and we saw a significant decrease in pg/ml of IL-1β and IL-6 in the presence of tBHQ (**Figure 5.29 J-K**). These results indicate that tBHQ mediated reduction in cell cytotoxicity and reduction in severity of *S. pneumoniae* infection might be because of increased AMP and reduced pro-inflammatory cytokine production.

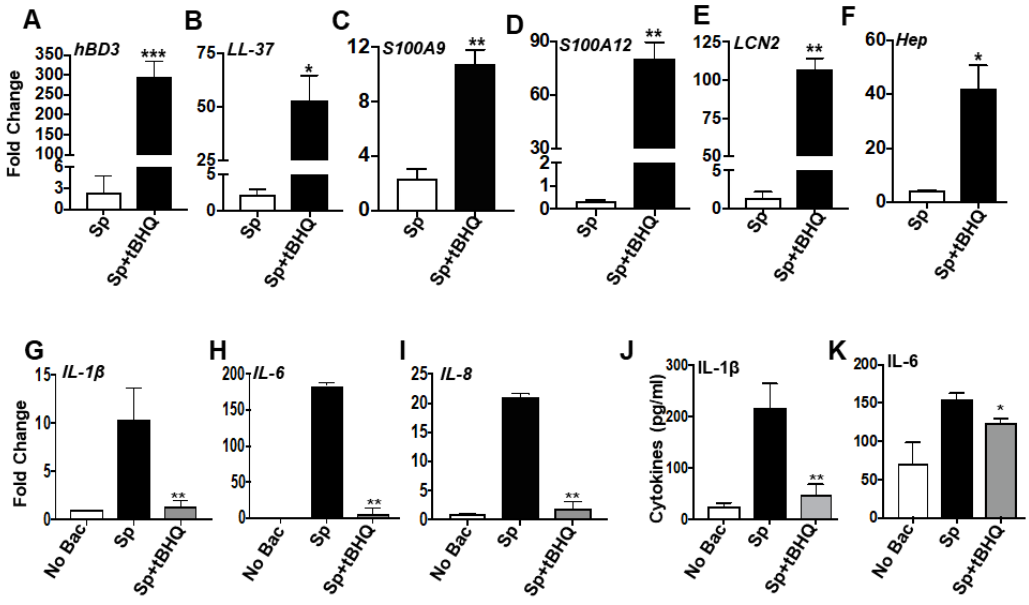


Figure 5.29 tBHQ induces AMP expression and reduces pro-inflammatory cytokine response in HCECs. Cells were infected with Sp ATCC for 4 h in presence or absence of tBHQ and expression of several AMPs (A-F) and cytokines (G-I) were determined by quantitative PCR. GAPDH was used as housekeeping gene. The protein level of IL-1b and IL-6 was determined by quantibody array of ELISA (J-K). Experiments were repeated twice in duplicates with similar results. (* indicates $p < 0.05$, **indicates $p < 0.005$).

5.2.24 LL-37 depletion increases Sp ATCC induced cytotoxicity and bacterial load in HCECs

Since we saw an increase in LL-37 expression in the corneal sections of *S. pneumoniae* keratitis patients and also in HCECs during *S. pneumoniae* infection, along with inhibition of growth of *S. pneumoniae* by LL-37 *in vitro*, we further wanted to confirm the role of LL-37 in tBHQ mediated reduction of cell cytotoxicity and intracellular bacterial load. We transiently depleted LL-37 in HCECs using 150nM LL-37 siRNA and infected with Sp ATCC for 6h in the presence or absence of tBHQ. The supernatant was collected for LDH assay to check the cytotoxicity and the cells were lysed with 0.3% triton X-100, diluted and plated on blood agar medium. We observed a significant increase in the LDH release in transfected cells when compared to the non-transfected control cells. The release of LDH was significantly less in both the transfected and non-transfected cells in the presence of tBHQ when compared to the cells in its absence (**Figure 5.30A**). Thus, the depletion of LL-37 increases *S. pneumoniae* mediated cytotoxicity in HCECs, that reduces significantly upon tBHQ treatment, thereby indicating that the reduction in cytotoxicity in presence of tBHQ is LL-37 mediated, to a certain extent. We also saw a significant increase in the bacterial load as indicated by CFU/ml in the LL-37 depleted HCECs compared to control non-transfected cells. Additionally, we observed a significant reduction in bacterial load in both transfected and non-transfected HCECs in the presence of tBHQ (**Figure 5.30B**). This indicates that tBHQ mediates reduction in bacterial load via LL-37 production. The depletion of LL-37 was also confirmed by quantitative PCR. We saw a significant decrease in the expression of LL-37 in the cells that were transfected with LL-37 siRNA when compared to the control (**Figure 5.30C**).

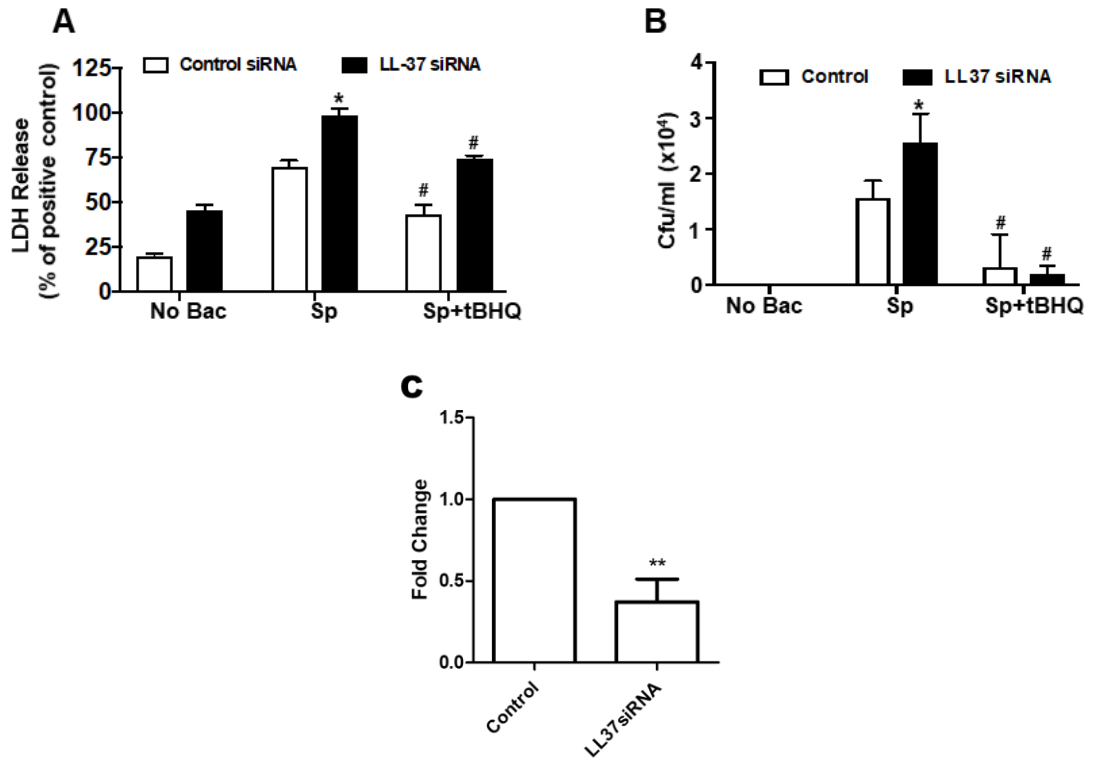


Figure 5.30 Depletion of LL-37 in host cells increases severity of *S. pneumoniae* infection. HCEC were transfected with control siRNA or LL-37 siRNA and infected with S. pneumoniae for 4 h in presence or absence of tBHQ and cell cytotoxicity was determined by lactate dehydrogenase assay (A). The bacterial viability was determined by cfu (B). Depletion of LL-37 was checked by qPCR (C). (* denotes $p < 0.05$ compared to No Bac, ** denotes $p < 0.005$ compared to control, # denotes $p < 0.05$ compared to Sp).

5.3 DISCUSSION

S. pneumoniae is a gram positive opportunistic pathogen that is known to cause community acquired pneumonia, worldwide (Dion & Ashurst, 2021). Additionally, it is also widely known to cause diseases like sinusitis, otitis media, bacteremia, osteomyelitis, septic arthritis and meningitis (Ekinci et al., 2021; Hamory et al., 1979; Murthy et al., 2015; Oordt-Speets et al., 2018; Torres et al., 1998). There are many accounts of ocular diseases caused by *S. pneumoniae* (Miller et al., 2004; Teweldemedhin et al., 2017), one of which is keratitis. *S. pneumoniae* is one of the leading causes of bacterial keratitis (Gurnani & Kaur, 2021). Since, antibiotics are used to treat pneumococcal keratitis in majority of the cases, there is a growing concern for the development of antibiotic resistance in the ocular isolates of *S. pneumoniae*. A surveillance study, Antibiotic Resistance Monitoring in Ocular MicRorganisms (ARMOR) 2009, revealed that about 9.3% of ocular *S. pneumoniae* isolates were non-susceptible to 2 or more antibacterial drug classes (Haas et al., 2011). A

more recent ARMOR study (2009-2016) reported that 31.4% of conjunctival *S pneumoniae* isolates showed *in vitro* resistance to azithromycin, 15.4% to tetracycline, 29.7% to penicillin and 8.8% to imipenem (P. A. Asbell & DeCory, 2018). Therefore, our focus in this chapter is to study the role of AMP in infections caused by *S. pneumoniae* so that we could better understand the expression of AMPs and the signaling pathways mediating their expression. As we saw there was a differential expression of AMP in patients' samples with *S. pneumoniae* keratitis (**Chapter III**), we further checked their expression *in vitro*. We saw a significant upregulation of *hBD-1*, *hBD-2*, *hBD-4*, *S100A7* and *LL-37* in immortalized HCECs during Sp ATCC infection. We also saw a significant increase in the expression of *hBD-3, S100A8, S100A9* and *LL-37* in primary HCECs in response to Sp ATCC infection. We saw suppression in the expression of some of the AMPs in HCECs infected with Sp CS when compared to HCECs infected with Sp ATCC, which was not due to *S. pneumoniae* mediated cytotoxicity. In **Chapter III**, we also saw a suppression in the expression of AMPs in response to *P. aeruginosa* infection that was T3SS dependent. Therefore, future studies on the mechanism of AMP suppression by *S. pneumoniae* is required. Since, the innate immune response like AMP expression is also known to be mediated by macrophages, we also checked AMP expression during *S. pneumoniae* infection in U937 cell line, and found that there was no significant change in the expression of AMP in response to either ATCC or clinical strain of *S. pneumoniae*.

Next, we focused on LL-37, the only AMP belonging to cathelicidin group in humans, as we saw an upregulation of *LL-37* in both patients and corneal epithelial cells *in vitro*. Earlier studies also showed *LL-37* expression in human corneal epithelium and epithelial cells (Gordon, Huang, et al., 2005; L.C. Huang et al., 2003; Ling C. Huang et al., 2006). We checked the expression of *LL-37* in corneal tissues from patients with *S. pneumoniae* keratitis and found that there was a marked increase in the expression of *LL-37* in the patients' sections when compared to the control cadaveric corneal tissues. We also checked the *in vitro* expression *LL-37* during *S. pneumoniae* infection, and found an increase in its expression both at RNA and protein level, where its RNA level expression was seen to peak at 4h post-infection. The *LL-37* expression in HCECs during *S. pneumoniae* infection was found to be *de novo*. LL-37 also showed antibacterial activity against both ATCC and clinical isolates of *S. pneumoniae*. LL-37 also promoted wound healing in HCECs at a concentration as low as 100ng/ml, that was mostly mediated by migration of cells. In one study, 1µg/ml of LL-37 was also shown to induce a 3-fold

increase in keratinocyte migration at 12h compared to the control cells (Tokumaru et al., 2005). In another study it was shown that *LL-37* expression was upregulated in regenerating human corneal epithelium and activates HCEC migration but not proliferation (Ling C. Huang et al., 2006). In yet another study it was shown that LL-37 may regulate wound healing on ocular surface by inducing cell migration and cytokine production (Petkova et al., 2006). We also saw that LL-37 induced migration rather than proliferation of HCEC, similar to the earlier study.

AMP expression is known to be mediated by various cellular signaling pathways like NF-κβ and MAPK, therefore we checked the activation of these pathways in HCECs during *S. pneumoniae* infection. We found that both Sp ATCC and Sp CS were able to activate NF-κβ and MAPK signaling but none of these pathways were involved in the expression of *LL-37*. Since there are reports of *LL-37* expression occurring via JAK-STAT3 pathway (Miraglia et al., 2016), we checked the activation of STAT3 and found that Sp ATCC was able to activate STAT3 in HCEC. We further confirmed that the inhibition of STAT3 significantly reduces the expression of *LL-37*. This is the first report that shows that *LL-37* expression in HCECs is mediated by STAT3 during *S. pneumoniae* infection.

Generation of ROS mediated oxidative stress in response to pathogen is one of the defense mechanisms of the host that helps in killing and elimination of the pathogens. However, there are some pathogens like *S. pneumoniae* that are not only able to survive and replicate in high ROS environment but capable of producing ROS themselves. *S. pneumoniae* is able to produce H_2O_2 that may exceed 1mM, while growing under aerobic conditions, which has been shown to be detrimental to the host and other bacteria, but not to itself (Pericone et al., 2003). Therefore, we first checked the occurrence of oxidative stress in patients' corneal sections. We saw a marked increase in the oxidative stress in patients' corneal tissues when compared to the control sections, with a simultaneous reduction in the expression of catalase, an enzyme produced by the corneal cells to counteract oxidative stress. We also saw the induction of ROS *in vitro* in response to *S. pneumoniae* infection. One of the pathways that is activated during oxidative stress in mammalian cells is Keap1-Nrf2-ARE pathway. Therefore, we checked the expression and activation of Nrf2 in HCECs in response to *S. pneumoniae* infection. We saw no significant differences in the expression of *Nrf2* in patients' corneal sample and Sp ATCC infected HCECs when compared to the uninfected control, indicating that *S. pneumoniae* actively

suppresses the expression of *Nrf2*. We also did not see any nuclear translocation of Nrf2 indicating that *S. pneumoniae* also suppresses the activation of Nrf2. The expression of antioxidant genes like *Per1* and *FRT* was also downregulated, indicating that suppression of Nrf2 expression and activation also simultaneously suppresses the expression of its downstream target ARE genes.

tBHQ is a known inducer of Nrf2 which is approved by Food and Drug Administration (FDA), USA, and therefore we wanted to check if its application has any effect in the host response against *S. pneumoniae*. We saw that in the presence of tBHQ there was a significant upregulation and activation of Nrf2 and Nrf2 targeted antioxidant genes like *HO-1* and *FRT* in HCECs infected with Sp ATCC. There was a subsequent decrease in ROS generation as well, in the presence of tBHQ in HCECs infected with Sp ATCC. Autophagy is one of the host responses against the pathogen where it helps in elimination of the pathogen and pathogen induced damaged biomolecules. However, some pathogens like *S. pneumoniae* evades the autophagy response of the host by several mechanisms. Recently it was shown that intracellular *S. pneumoniae* survives within the host by hijacking host's autophagy response by suppression of autophagosome-lysosome fusion (Shizukuishi et al., 2020). Therefore, we checked the effect of *S. pneumoniae* infection in autophagy response of HCECs. We found that *S. pneumoniae* suppressed autophagy in HCECs as evident by the presence of diffused LC3B staining in cells and reduced expression of autophagy related genes like *Atg5*, *Atg7* and *Bec1*. This suppression of autophagy was found to be mediated by activation of PI3K/AKT pathway as we saw an increased phosphorylation of PI3K and AKT, in response to *S. pneumoniae* infection. Since tBHQ reduced oxidative stress in Sp ATCC infected HCECs via Nrf2, we also checked the effect of tBHQ in autophagy. There are some reports that highlighted a cross-talk between oxidative stress, Nrf2 and autophagy. For instance, it has been shown that autophagy activates Keap1-Nrf2 pathway via p62 and ROS regulates autophagy via P13K-Akt pathway (Ichimura et al., 2013; Kma & Baruah, 2021). We saw that tBHQ induced autophagy in Sp ATCC infected HCECs, as evident by the presence of punctate LC3B in the cells and consequently upregulated the expression of *Atg5*, *Atg7* and *Bec1* that indicates that the activation of autophagy is Nrf2 mediated. It was further confirmed when we saw a reduction in the quantity of punctate LC3B in cells transiently transfected with *Nrf2* siRNA and infected with Sp ATCC in the presence of tBHQ. tBHQ was also shown to reduce *S. pneumoniae* mediated cell cytotoxicity and bacterial load in HCECs. However, we did not

see any direct bacteriostatic or bactericidal effect of tBHQ on *S. pneumoniae*. tBHQ also significantly upregulated the expression of AMPs like *hBD-3*, *LL-37*, *S100A9*, *S100A12*, *LCN* and hepcidin, and downregulated the expression of pro-inflammatory cytokines like IL-1β, IL-6 and IL-8. To check if the upregulation of *LL-37* is playing a part in reducing the severity of the infection, we transiently depleted LL-37 in HCECs and checked the effect of tBHQ on cell cytotoxicity and bacterial load. We found that in LL-37 depleted cells that were infected with Sp ATCC, there was an increase in cell cytotoxicity and bacterial load, that significantly reduced in the presence of tBHQ.

To conclude (**Figure 5.31**), we found that *S. pneumoniae* exerts oxidative stress and induces the expression of *LL-37* via activation of STAT3. We also saw that *S. pneumoniae* suppresses autophagy in the corneal cells. Further we saw that tBHQ reduces *S. pneumoniae* mediated oxidative stress by activating Nrf2 and Nrf2 mediated antioxidant responses. tBHQ also upregulates AMP expression, downregulates pro-inflammatory cytokine expression and activates autophagy, thereby reducing the cytotoxicity and bacterial load *in vitro*. Hence, tBHQ can be studied further as an alternative to antibiotics in treating *S. pneumoniae* related ocular infections.

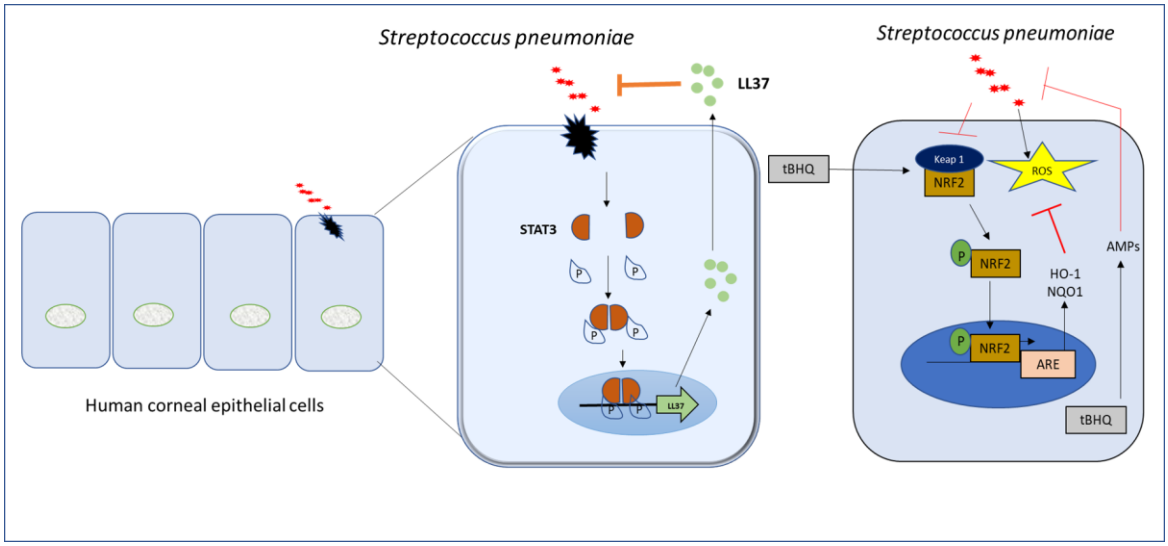


Figure 5.31 Representative image summarizing the findings of the work. *S. pneumoniae* induces *LL-37* expression via activation of STAT3. tBHQ activates Nrf2 and Nrf2 mediated antioxidant responses in HCECs. tBHQ also induces the expression of *LL-37* along with other AMPs that help to combat *S. pneumoniae* infection. Hence, tBHQ helps to reduce the severity of pneumococcal infection in HCECs.

*** A part of this chapter has been published in the journal *Pathogens* (Sharma et al., 2019). The citations are given below and the publications are attached.**

Sharma, P., Sharma, N., Mishra, P., Joseph, J., Mishra, D. K., Garg, P., & Roy, S. (2019). Differential Expression of Antimicrobial Peptides in *Streptococcus pneumoniae* Keratitis and STAT3-Dependent Expression of LL-37 by *Streptococcus pneumoniae* in Human Corneal Epithelial Cells. Pathogens (Basel, Switzerland), 8(1), 31. https://doi.org/10.3390/pathogens8010031

CHAPTER VI

SUMMARY

In this study our focus was to decipher the role of AMPs in bacterial keratitis. We reported differential expression of AMPs in patients with *P. aeruginosa* keratitis. We saw an upregulation of some AMPs like *LL-37* and *S100A12* that might play a role in *P. aeruginosa* infection. We also checked the expression of the same group of AMPs in corneal cells (immortalised HCECs) and macrophages (U937) in response to *P. aeruginosa*. We found that T3SS dependent suppression of AMPs in HCECs and U937 in response to *P. aeruginosa* infection. However, there was no T3SS dependent suppression of AMPs seen in HCECs infected with *P. aeruginosa* for a longer time period (24h) indicating a role of T3SS in the expression of AMPs and host response during the initial stages of infection. We also found that *P. aeruginosa* supresses the activation of signaling pathways like ERK-MAPK, p38-MAPK and NF-κβ in a T3SS dependent manner. The generation of ROS was also supressed in a T3SS dependent manner by *P. aeruginosa.* Further, we saw that the chemical inhibition of T3SS by INP0341, a salicylidene acylhydrazide derivative, activates the innate immune response in HCECs in response to *P. aeruginosa* infection. Inhibition of T3SS by INP0341 resulted in the upregulation of AMPs and increase ROS generation in HCECs. We also saw a decrease in *P. aeruginosa* mediated cytotoxicity in the presence of T3SS inhibitor in HCECs. In our *in vivo* study on C57BL/6 mice, INP0341 was also found to relatively alleviate the condition in mice eyes when compared to eyes infected with wild type bacteria only, having an intact T3SS. We saw lesser opacity, reduced colony forming units per eye (CFU/eye), lesser cellular infiltration in cornea and increased expression of AMPs in infected murine eyes treated with INP0341 as compared to untreated infected eyes. The eyes infected with T3SS mutant *P. aeruginosa* showed similar results as the eyes infected with wild type bacteria along with INP0341. This indicates that T3SS plays a role in suppressing AMP expression in host and contributes to the severity of keratitis.

We also reported differential expression of AMPs in patients with *S pneumoniae* corneal infections and we found an upregulation of several AMPs including *LL-37*. However, the expression profile of AMPs in response to *S. pneumoniae* was different from that in response to *P. aeruginosa* infection indicating to the unique host response specific towards different pathogens. We also checked the expression of the same group of AMPs in corneal cells (primary and immortalised HCECs) and macrophages (U937) in response to *S. pneumoniae*. We saw significant upregulation of some AMPs in HCECs infected by laboratory strain of *S. pneumoniae* (SP ATCC). However, the expression of AMPs in HCECs infected by ocular clinical isolate of *S. pneumoniae* (SP CS) was significantly

reduced that was not related to cell death. We also saw significant upregulation of AMPs like *hBD-3*, *S100A8*, *S100A9* and *LL-37* in primary HCECs infected with SP ATCC. There was a significant increase in the expression of *S100A9* in primary HCECs infected with clinical strain of *S. pneumoniae*. There was no significant change in expression of AMPs in monocyte derived cell line, U937 when either infected with ATCC strain or clinical strain of *S. pneumoniae*. We also found that *S. pneumoniae* activates signaling pathways like ERK-MAPK, p38-MAPK, JNK-MAPK and NF-κβ in HCECs. We saw an upregulated expression of *LL-37*, the only human cathelicidin, in the patients' corneal scrapings, patients' tissues and HCECs. The expression of *LL-37* was found to be mediated by the activation of STAT3. We further studied the occurrence of oxidative stress in human cornea and HCECs in response to *S. pneumoniae* as the bacteria is known to produce excessive H_2O_2. We reported the increase in oxidative stress and suppression of antioxidant response in patients' corneal tissues and HCECs. We also, confirmed the suppression of autophagic response in the corneal cells in response to *S. pneumoniae* infection which we found was mediated via Nrf2. Therefore, we carried out further experiments in HCECs using tBHQ, a known inducer of Nrf2. We found that in the presence of tBHQ there was a decrease in *S. pneumoniae* induced oxidative stress and activation of antioxidant responses in HCECs. We also saw an activation of autophagy, reduction in cell cytotoxicity and bacterial load in *S. pneumoniae* infected HCECs in the presence of tBHQ. tBHQ also upregulated the expression of AMPs and decrease in expression of pro-inflammatory cytokines. The reduction in cell cytotoxicity and bacterial load was found to be partially mediated by LL-37 expression.

To our knowledge, our study provides the first report on differential expression of AMPs in patients with *P. aeruginosa* and *S. pneumoniae* keratitis. We also reported T3SS mediated suppression of host innate immune response in epithelial cells for the first time. We also show for the first time that STAT3 mediates the expression of *LL-37* in HCECs in response to *S. pneumoniae* infection. The added knowledge about the expression pattern of AMPs during bacterial keratitis might help in selection of putative AMPs to be used for therapeutic intervention. The knowledge about signaling pathways involved in AMP expression is also crucial to better understand the underlying mechanism of AMP expression. The use of T3SS inhibitor also holds promise as an alternative therapeutic, because we saw that it leads to the activation of host responses like ROS generation and AMP expression. Further, the tBHQ can also be used as a therapeutic as we saw that it is

able to alleviate the severity of *S. pneumoniae* infection by reducing cell cytotoxicity and bacterial load, by reduction in ROS generation, activation of autophagy and AMP expression.

However, there are certain limitations of this study. Though we see that AMP have a good potential to be used as an alternative to currently used antibiotics, the translation of AMP usage from bench to bedside has many hurdles. The *in vivo* stability and efficacy of AMP needs to be studied further for it to be applied clinically. Also, further studies need to be done on signaling involved in the expression of other AMPs too, so that we can better understand the pathways that can be targeted to modulate the AMP expression according to the therapeutic need. Also, we did not look into the mechanism of resistance against AMP that the bacteria might develop. There are several studies pointing out the various resistance mechanisms that bacteria employ against the AMPs. Therefore, all these aspects need to be addressed in future.

Our study has provided an insight into the role of AMPs in bacterial keratitis, caused by both gram negative, *P. aeruginosa* and gram positive, *S. pneumoniae*, that might give a basis to future studies on AMPs. This knowledge will be immensely helpful in selection of AMPs for therapeutic usage. AMPs can not only be synthesised and applied externally on the infection sites but can also be upregulated intrinsically by using inhibitors and chemicals that either supress bacterial virulence or enhance AMP expression. Thus, our study also provides a background on the usage of other alternatives therapeutics like T3SS inhibitor, INP0341 and Nrf2 activator, tBHQ.

REFERENCES

Acharya, M., Farooqui, J. H., Jain, S. & Mathur, U. (2019). Pearls and paradigms in Infective Keratitis. *Romanian Journal of Ophthalmology*, *63*(2), 119–127. http://www.ncbi.nlm.nih.gov/pubmed/31334389

Acharya, M., Farooqui, J., Singh, A., Gandhi, A. & Mathur, U. (2019). Bacterial isolates in microbial keratitis: Three-year trend analysis from North India. In *Indian Journal of Ophthalmology* (Vol. 67, Issue 9, pp. 1508–1509). Wolters Kluwer Medknow Publications. https://doi.org/10.4103/ijo.IJO_678_19

Ageitos, J. M., Sánchez-Pérez, A., Calo-Mata, P. & Villa, T. G. (2017). Antimicrobial peptides (AMPs): Ancient compounds that represent novel weapons in the fight against bacteria. In *Biochemical Pharmacology* (Vol. 133, pp. 117–138). Elsevier Inc. https://doi.org/10.1016/j.bcp.2016.09.018

Ahsan, H. (2013). 3-Nitrotyrosine: A biomarker of nitrogen free radical species modified proteins in systemic autoimmunogenic conditions. *Human Immunology*, *74*(10), 1392–1399. https://doi.org/10.1016/J.HUMIMM.2013.06.009

Akpek, E. K. & Gottsch, J. D. (2003). Immune defense at the ocular surface. *Eye 2003 17:8*, *17*(8), 949–956. https://doi.org/10.1038/sj.eye.6700617

Alhazmi, A. (2018). NOD-like receptor(s) and host immune responses with Pseudomonas aeruginosa infection. *Inflammation Research 2018 67:6*, *67*(6), 479–493. https://doi.org/10.1007/S00011-018-1132-0

Alto, N. M. & Orth, K. (2012). Subversion of Cell Signaling by Pathogens. *Cold Spring Harbor Perspectives in Biology*, *4*(9). https://doi.org/10.1101/CSHPERSPECT.A006114

Álvarez-Barrios, A., Álvarez, L., García, M., Artime, E., Pereiro, R. & González-Iglesias, H. (2021). Antioxidant Defenses in the Human Eye: A Focus on Metallothioneins. *Antioxidants 2021, Vol. 10, Page 89*, *10*(1), 89. https://doi.org/10.3390/ANTIOX10010089

Amiel, E., Lovewell, R. R., O'Toole, G. A., Hogan, D. A. & Berwin, B. (2010). Pseudomonas aeruginosa evasion of phagocytosis is mediated by loss of swimming motility and is independent of flagellum expression. *Infection and Immunity*, *78*(7), 2937–2945. https://doi.org/10.1128/IAI.00144-10

Aminov, R. I. (2010). A brief history of the antibiotic era: Lessons learned and challenges for the future. *Frontiers in Microbiology*, *1*(DEC), 134. https://doi.org/10.3389/fmicb.2010.00134

Anantharajah, A., Mingeot-Leclercq, M. P. & Van Bambeke, F. (2016). Targeting the Type Three Secretion System in Pseudomonas aeruginosa. In *Trends in Pharmacological Sciences* (Vol. 37, Issue 9, pp. 734–749). Elsevier Ltd. https://doi.org/10.1016/j.tips.2016.05.011

Andersson, D. I. & Hughes, D. (2011). Persistence of antibiotic resistance in bacterial populations. In *FEMS Microbiology Reviews* (Vol. 35, Issue 5, pp. 901–911). Oxford Academic. https://doi.org/10.1111/j.1574-6976.2011.00289.x

Angus, A. A., Evans, D. J., Barbieri, J. T. & Fleiszig, S. M. J. (2010). The ADP-ribosylation domain of Pseudomonas aeruginosa ExoS is required for membrane bleb niche formation and bacterial survival within epithelial cells. *Infection and Immunity*, *78*(11), 4500–4510. https://doi.org/10.1128/IAI.00417-10

Anunthawan, T., De La Fuente-Núñez, C., Hancock, R. E. W. & Klaynongsruang, S. (2015). Cationic amphipathic peptides KT2 and RT2 are taken up into bacterial cells and kill planktonic and biofilm bacteria. *Biochimica et Biophysica Acta - Biomembranes*, *1848*(6), 1352–1358. https://doi.org/10.1016/j.bbamem.2015.02.021

Asadi Karam, M. R., Habibi, M. & Bouzari, S. (2019). Urinary tract infection: Pathogenicity,

antibiotic resistance and development of effective vaccines against Uropathogenic Escherichia coli. *Molecular Immunology*, *108*, 56–67. https://doi.org/10.1016/J.MOLIMM.2019.02.007

Asbell, P. A. & DeCory, H. H. (2018). Antibiotic resistance among bacterial conjunctival pathogens collected in the Antibiotic Resistance Monitoring in Ocular Microorganisms (ARMOR) surveillance study. *PLOS ONE*, *13*(10), e0205814. https://doi.org/10.1371/JOURNAL.PONE.0205814

Asbell, P. & Brocks, D. (2010). Cornea Overview. *Encyclopedia of the Eye*, 522–531. https://doi.org/10.1016/B978-0-12-374203-2.00058-0

Bajracharya, L., Bade, A. R., Gurung, R. & Dhakhwa, K. (2020). Demography, risk factors, and clinical and microbiological features of microbial keratitis at a tertiary eye hospital in nepal. *Clinical Ophthalmology*, *14*, 3219–3226. https://doi.org/10.2147/OPTH.S266218

Balasubramanian, D., Schneper, L., Kumari, H. & Mathee, K. (2013). A dynamic and intricate regulatory network determines Pseudomonas aeruginosa virulence. In *Nucleic Acids Research* (Vol. 41, Issue 1, pp. 1–20). https://doi.org/10.1093/nar/gks1039

Balczon, R., Prasain, N., Ochoa, C., Prater, J., Zhu, B., Alexeyev, M., Sayner, S., Frank, D. W. & Stevens, T. (2013). Pseudomonas aeruginosa Exotoxin Y-Mediated Tau Hyperphosphorylation Impairs Microtubule Assembly in Pulmonary Microvascular Endothelial Cells. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0074343

Bandookwala, M. & Sengupta, P. (2020). 3-Nitrotyrosine: a versatile oxidative stress biomarker for major neurodegenerative diseases. *The International Journal of Neuroscience*, *130*(10), 1047–1062. https://doi.org/10.1080/00207454.2020.1713776

Barbieri, J. T. & Sun, J. (2004). Pseudomonas aeruginosa ExoS and ExoT. *Reviews of Physiology, Biochemistry and Pharmacology*, *152*, 79–92. https://doi.org/10.1007/s10254-004-0031-7

Antimicrobial resistance, 4 (testimony of N. Barg, F. C. Tenover, & J. E. McGowan). Retrieved July 17, 2020, from https://www.who.int/news-room/fact-sheets/detail/antimicrobial-resistance

Barrientos, S., Stojadinovic, O., Golinko, M. S., Brem, H. & Tomic-Canic, M. (2008). Growth factors and cytokines in wound healing. *Wound Repair and Regeneration : Official Publication of the Wound Healing Society [and] the European Tissue Repair Society*, *16*(5), 585–601. https://doi.org/10.1111/J.1524-475X.2008.00410.X

Bauer, B., Wex, T., Kuester, D., Meyer, T. & Malfertheiner, P. (2013). Differential expression of human beta defensin 2 and 3 in gastric mucosa of helicobacter pylori-infected individuals. *Helicobacter*, *18*(1), 6–12. https://doi.org/10.1111/hel.12000

Bayir, H. (2005). Reactive oxygen species. *Critical Care Medicine*, *33*(12 SUPPL.). https://doi.org/10.1097/01.CCM.0000186787.64500.12

Bayroodi, E. & Jalal, R. (2016). Modulation of antibiotic resistance in Pseudomonas aeruginosa by ZnO nanoparticles. *Iranian Journal of Microbiology*, *8*(2), 85. /pmc/articles/PMC4906724/

Bechinger, B. (2015). The SMART model: Soft membranes adapt and respond, also Transiently, in the presence of antimicrobial peptides. In *Journal of Peptide Science* (Vol. 21, Issue 5, pp. 346–355). John Wiley and Sons Ltd. https://doi.org/10.1002/psc.2729

BECN1 beclin 1 [Homo sapiens (human)] - Gene - NCBI. (n.d.). Retrieved December 27, 2021, from https://www.ncbi.nlm.nih.gov/gene/8678

Behrens, A., Sibilia, M. & Wagner, E. F. (1999). Amino-terminal phosphorylation of c-Jun regulates stress-induced apoptosis and cellular proliferation. *Nature Genetics*, *21*(3), 326–329. https://doi.org/10.1038/6854

Bell, M. (2014). Antibiotic Misuse: A Global Crisis. *JAMA Internal Medicine*, *174*(12), 1920–1921. https://doi.org/10.1001/JAMAINTERNMED.2014.3289

Bharathi, M. J., Ramakrishnan, R., Vasu, S., Meenakshi, R., Shivkumar, C. & Palaniappan, R. (2003). EPIDEMIOLOGY OF BACTERIAL KERATITIS IN A REFERRAL CENTRE IN SOUTH INDIA. *Indian Journal of Medical Microbiology*, *21*(4), 239–245. https://doi.org/10.1016/S0255-0857(21)03006-1

Biggest Threats and Data | Antibiotic/Antimicrobial Resistance | CDC. (n.d.). Retrieved November 7, 2021, from https://www.cdc.gov/drugresistance/biggest-threats.html

Blair, J. M. A., Webber, M. A., Baylay, A. J., Ogbolu, D. O. & Piddock, L. J. V. (2015). Molecular mechanisms of antibiotic resistance. In *Nature Reviews Microbiology* (Vol. 13, Issue 1, pp. 42–51). https://doi.org/10.1038/nrmicro3380

Bleves, S., Viarre, V., Salacha, R., Michel, G. P. F., Filloux, A. & Voulhoux, R. (2010). Protein secretion systems in Pseudomonas aeruginosa: A wealth of pathogenic weapons. *International Journal of Medical Microbiology*, *300*(8), 534–543. https://doi.org/10.1016/J.IJMM.2010.08.005

Blindness and vision impairment. (n.d.). Retrieved November 7, 2021, from https://www.who.int/news-room/fact-sheets/detail/blindness-and-visual-impairment

Boman, H. G., Steiner, H., Hultmark, D., Engström, Å. & Bennich, H. (2020). *Insect Two Antibacterial Proteins Involved in Pillars Article: Sequence and Specificity of*. http://www.jimmunol.org/content/182/11/6635.citation

Borkar, D. S., Fleiszig, S. M. J., Leong, C., Lalitha, P., Srinivasan, M., Ghanekar, A. A., Tam, C., Li, W. Y., Zegans, M. E., McLeod, S. D., Lietman, T. M. & Acharya, N. R. (2013). Association Between Cytotoxic and Invasive Pseudomonas aeruginosa and Clinical Outcomes in Bacterial Keratitis. *JAMA Ophthalmology*, *131*(2), 147–153. https://doi.org/10.1001/JAMAOPHTHALMOL.2013.778

Bourcier, T., Thomas, F., Borderie, V., Chaumeil, C. & Laroche, L. (2003). Bacterial keratitis: Predisposing factors, clinical and microbiological review of 300 cases. *British Journal of Ophthalmology*, *87*(7), 834–838. https://doi.org/10.1136/bjo.87.7.834

Breustedt, D. A., Korndörfer, I. P., Redl, B. & Skerra, A. (2005). The 1.8-Å crystal structure of human tear lipocalin reveals an extended branched cavity with capacity for multiple ligands. *Journal of Biological Chemistry*, *280*(1), 484–493. https://doi.org/10.1074/JBC.M410466200

Brogden, K. A. (2005). Antimicrobial peptides: Pore formers or metabolic inhibitors in bacteria? In *Nature Reviews Microbiology* (Vol. 3, Issue 3, pp. 238–250). https://doi.org/10.1038/nrmicro1098

Brooks, L. R. K. & Mias, G. I. (2018). Streptococcus pneumoniae's Virulence and Host Immunity: Aging, Diagnostics, and Prevention. *Frontiers in Immunology*, *0*(JUN), 1366. https://doi.org/10.3389/FIMMU.2018.01366

Brown, K. L., Poon, G. F. T., Birkenhead, D., Pena, O. M., Falsafi, R., Dahlgren, C., Karlsson, A., Bylund, J., Hancock, R. E. W. & Johnson, P. (2011). Host Defense Peptide LL-37 Selectively Reduces Proinflammatory Macrophage Responses. *The Journal of Immunology*, *186*(9), 5497–5505. https://doi.org/10.4049/JIMMUNOL.1002508

Byfield, F. J., Kowalski, M., Cruz, K., Leszczyńska, K., Namiot, A., Savage, P. B., Bucki, R. & Janmey, P. A. (2011). Cathelicidin LL-37 Increases Lung Epithelial Cell Stiffness, Decreases Transepithelial Permeability, and Prevents Epithelial Invasion by Pseudomonas aeruginosa. *The Journal of Immunology*, *187*(12), 6402–6409. https://doi.org/10.4049/jimmunol.1102185

Carretero, M., Escámez, M. J., García, M., Duarte, B., Holguín, A., Retamosa, L., Jorcano, J. L., Río, M. Del & Larcher, F. (2008). In vitro and In vivo Wound Healing-Promoting Activities of Human Cathelicidin LL-37. *Journal of Investigative Dermatology*, *128*(1), 223–236. https://doi.org/10.1038/SJ.JID.5701043

Causes of Antimicrobial (Drug) Resistance | NIH: National Institute of Allergy and Infectious Diseases. (n.d.). Retrieved November 20, 2021, from https://www.niaid.nih.gov/research/antimicrobial-resistance-causes

Chai, W., Zhang, J., Duan, Y., Pan, D., Liu, W., Li, Y., Yan, X. & Chen, B. (2014). Pseudomonas pyocyanin stimulates IL-8 expression through MAPK and NF-κB pathways in differentiated U937 cells. *BMC Microbiology 2014 14:1*, *14*(1), 1–12. https://doi.org/10.1186/1471-2180-14-26

Charani, E., McKee, M., Ahmad, R., Balasegaram, M., Bonaconsa, C., Merrett, G. B., Busse, R., Carter, V., Castro-Sanchez, E., Franklin, B. D., Georgiou, P., Hill-Cawthorne, K., Hope, W., Imanaka, Y., Kambugu, A., Leather, A. J., Mbamalu, O., McLeod, M., Mendelson, M., … Holmes, A. H. (2021). Optimising antimicrobial use in humans – review of current evidence and an interdisciplinary consensus on key priorities for research. *The Lancet Regional Health – Europe*, *7*. https://doi.org/10.1016/J.LANEPE.2021.100161

Chidambaram, J. D., Venkatesh Prajna, N., Srikanthi, P., Lanjewar, S., Shah, M., Elakkiya, S., Lalitha, P. & Burton, M. J. (2018). Epidemiology, risk factors, and clinical outcomes in severe microbial keratitis in South India. *Ophthalmic Epidemiology*, *25*(4), 297–305. https://doi.org/10.1080/09286586.2018.1454964

Chongsiriwatana, N. P., Lin, J. S., Kapoor, R., Wetzler, M., Rea, J. A. C., Didwania, M. K., Contag, C. H. & Barron, A. E. (2017). Intracellular biomass flocculation as a key mechanism of rapid bacterial killing by cationic, amphipathic antimicrobial peptides and peptoids. *Scientific Reports*, *7*(1). https://doi.org/10.1038/s41598-017-16180-0

Colonne, P. M., Winchell, C. G. & Voth, D. E. (2016). Hijacking host cell highways: Manipulation of the host actin cytoskeleton by obligate intracellular bacterial pathogens. *Frontiers in Cellular and Infection Microbiology*, *6*(SEP), 107. https://doi.org/10.3389/FCIMB.2016.00107/BIBTEX

Cornelis, G. R. (2006). The type III secretion injectisome. In *Nature Reviews Microbiology* (Vol. 4, Issue 11, pp. 811–825). Nature Publishing Group. https://doi.org/10.1038/nrmicro1526

Cote, C. K., Blanco, I. I., Hunter, M., Shoe, J. L., Klimko, C. P., Panchal, R. G. & Welkos, S. L. (2020). Combinations of early generation antibiotics and antimicrobial peptides are effective against a broad spectrum of bacterial biothreat agents. *Microbial Pathogenesis*, 104050. https://doi.org/10.1016/j.micpath.2020.104050

Cowell, B. A., Evans, D. J. & Fleiszig, S. M. J. (2005). Actin cytoskeleton disruption by ExoY and its effects on Pseudomonas aeruginosa invasion. *FEMS Microbiology Letters*, *250*(1), 71–76. https://doi.org/10.1016/J.FEMSLE.2005.06.044

Cunliffe, R. N. & Mahida, Y. R. (2004). Expression and regulation of antimicrobial peptides in the gastrointestinal tract. *Journal of Leukocyte Biology*, *75*(1), 49–58. https://doi.org/10.1189/JLB.0503249

Dacheux, D., Attree, I., Schneider, C. & Toussaint, B. (1999). Cell death of human polymorphonuclear neutrophils induced by a Pseudomonas aeruginosa cystic fibrosis isolate requires a functional type III secretion system. *Infection and Immunity*, *67*(11), 6164–6167. https://doi.org/10.1128/IAI.67.11.6164-6167.1999

Dacheux, Denis, Toussaint, B., Richard, M., Brochier, G., Croize, J. & Attree, I. (2000). Pseudomonas aeruginosa cystic fibrosis isolates induce rapid, type III secretion-dependent, but ExoU-independent, oncosis of macrophages and polymorphonuclear neutrophils. *Infection and Immunity*, *68*(5), 2916–2924. https://doi.org/10.1128/IAI.68.5.2916-2924.2000

Das, S., Samantaray, R., Mallick, A., Sahu, S. K. & Sharma, S. (2019). Types of organisms and in-vitro susceptibility of bacterial isolates from patients with microbial keratitis: A trend analysis of 8 years. *Indian Journal of Ophthalmology*, *67*(1), 49–53. https://doi.org/10.4103/ijo.IJO_500_18

Dcosta, V. M., King, C. E., Kalan, L., Morar, M., Sung, W. W. L., Schwarz, C., Froese, D., Zazula, G., Calmels, F., Debruyne, R., Golding, G. B., Poinar, H. N. & Wright, G. D. (2011). Antibiotic resistance is ancient. In *Nature* (Vol. 477, Issue 7365, pp. 457–461). Nature Publishing Group. https://doi.org/10.1038/nature10388

Denardi, L. B., de Arruda Trindade, P., Weiblen, C., Ianiski, L. B., Stibbe, P. C., Pinto, S. C. & Santurio, J. M. (2021). In vitro activity of the antimicrobial peptides h-Lf1-11, MSI-78, LL-37, fengycin 2B, and magainin-2 against clinically important bacteria. *Brazilian Journal of Microbiology : [Publication of the Brazilian Society for Microbiology]*. https://doi.org/10.1007/S42770-021-00645-6

Deorukhkar, S., Katiyar, R. & Saini, S. (2012). Epidemiological features and laboratory results of bacterial and fungal keratitis: A five-year study at a rural tertiary-care hospital in western Maharashtra, India. *Singapore Medical Journal*, *53*(4), 264–267. https://pubmed.ncbi.nlm.nih.gov/22511050/

Dérijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M. & Davis, R. J. (1994). JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain. *Cell*, *76*(6), 1025–1037. https://doi.org/10.1016/0092-8674(94)90380-8

DiMango, E., Ratner, A. J., Bryan, R., Tabibi, S. & Prince, A. (1998). Activation of NF-kappaB by adherent Pseudomonas aeruginosa in normal and cystic fibrosis respiratory epithelial cells. *Journal of Clinical Investigation*, *101*(11), 2598. https://doi.org/10.1172/JCI2865

Dimatatac, E. L., Alejandria, M. M., Montalban, C., Pineda, C., Ang, C. & Delino, R. (2003). Clinical Outcomes and Costs of Care of Antibiotic Resistant Pseudomonas aeruginosa Infections. *Philipp. J. Microbiol. Infect. Dis.*, *32*, 159–167. http://citeseerx.ist.psu.edu/viewdoc/download?doi=10.1.1.558.1840&rep=rep1&type=pdf

Dion, C. F. & Ashurst, J. V. (2021). Streptococcus Pneumoniae. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK470537/

Donato, R. (1999). Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1450*(3), 191–231. https://doi.org/10.1016/S0167-4889(99)00058-0

Dong, J., Wang, J., He, Y., Li, C., Zhou, A., Cui, J., Xu, W., Zhong, L., Yin, Y., Zhang, X. & Wang, H. (2014). GHIP in Streptococcus pneumoniae is involved in antibacterial resistance and elicits a strong innate immune response through TLR2 and JNK/p38MAPK. *The FEBS Journal*, *281*(17), 3803–3815. https://doi.org/10.1111/FEBS.12903

Dorschner, R. A., Pestonjamasp, V. K., Tamakuwala, S., Ohtake, T., Rudisill, J., Nizet, V.,

Agerberth, B., Gudmundsson, G. H. & Gallo, R. L. (2001). Cutaneous Injury Induces the Release of Cathelicidin Anti-Microbial Peptides Active Against Group A Streptococcus. *Journal of Investigative Dermatology*, *117*(1), 91–97. https://doi.org/10.1046/J.1523-1747.2001.01340.X

Dressel, S., Harder, J., Cordes, J., Wittersheim, M., Meyer-Hoffert, U., Sunderkötter, C. & Gläser, R. (2010). Differential expression of antimicrobial peptides in margins of chronic wounds. *Experimental Dermatology*, *19*(7), 628–632. https://doi.org/10.1111/J.1600-0625.2009.01030.X

Du, X., Youle, R. J., FitzGerald, D. J. & Pastan, I. (2010). Pseudomonas Exotoxin A-Mediated Apoptosis Is Bak Dependent and Preceded by the Degradation of Mcl-1. *Molecular and Cellular Biology*, *30*(14), 3444–3452. https://doi.org/10.1128/mcb.00813-09

Dua, H. S., Otri, A. M., Hopkinson, A. & Mohammed, I. (2014). In vitro studies on the antimicrobial peptide human beta-defensin 9 (HBD9): Signalling pathways and pathogen-related response (an American ophthalmological society thesis). *Transactions of the American Ophthalmological Society*, *112*, 50–73. http://www.ncbi.nlm.nih.gov/guide/all/

Dudal, S., Turriere, C., Bessoles, S., Fontes, P., Sanchez, F., Liautard, J., Liautard, J.-P. & Lafont, V. (2006). Release of LL-37 by activated human Vgamma9Vdelta2 T cells: a microbicidal weapon against Brucella suis. *Journal of Immunology (Baltimore, Md. : 1950)*, *177*(8), 5533–5539. https://doi.org/10.4049/JIMMUNOL.177.8.5533

Duncan, M. C., Linington, R. G. & Auerbuch, V. (2012). *Chemical Inhibitors of the Type Three Secretion System: Disarming Bacterial Pathogens*. https://doi.org/10.1128/AAC.00975-12

Dürr, U. H. N., Sudheendra, U. S. & Ramamoorthy, A. (2006). LL-37, the only human member of the cathelicidin family of antimicrobial peptides. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1758*(9), 1408–1425. https://doi.org/10.1016/J.BBAMEM.2006.03.030

Dutta, N. K., Annadurai, S., Mazumdar, K., Dastidar, S. G., Kristiansen, J. E., Molnar, J., Martins, M. & Amaral, L. (2007). Potential management of resistant microbial infections with a novel non-antibiotic: the anti-inflammatory drug diclofenac sodium. *International Journal of Antimicrobial Agents*, *30*(3), 242–249. https://doi.org/10.1016/J.IJANTIMICAG.2007.04.018

Eghrari, A. O., Riazuddin, S. A. & Gottsch, J. D. (2015). Overview of the Cornea: Structure, Function, and Development. *Progress in Molecular Biology and Translational Science*, *134*, 7–23. https://doi.org/10.1016/BS.PMBTS.2015.04.001

Ekinci, E., Desmet, S., Van Heirstraeten, L., Mertens, C., Wouters, I., Beutels, P., Verhaegen, J., Malhotra-Kumar, S. & Theeten, H. (2021). Streptococcus pneumoniae Serotypes Carried by Young Children and Their Association With Acute Otitis Media During the Period 2016–2019. *Frontiers in Pediatrics*, *9*, 650. https://doi.org/10.3389/FPED.2021.664083/BIBTEX

Enoch, S. & Leaper, D. J. (2005). Basic science of wound healing. *Surgery (Oxford)*, *23*(2), 37–42. https://doi.org/10.1383/SURG.23.2.37.60352

Erdem, H., Tetik, A., Arun, O., Besirbellioglu, B. A., Coskun, O. & Eyigun, C. P. (2011). War and infection in the pre-antibiotic era: The Third Ottoman Army in 1915. *Scandinavian Journal of Infectious Diseases*, *43*(9), 690–695. https://doi.org/10.3109/00365548.2011.577801

Estimated Burden of Keratitis — United States, 2010. (n.d.). Retrieved March 6, 2020, from https://www.cdc.gov/mmwr/preview/mmwrhtml/mm6345a3.htm

Evans, D. J. & Fleiszig, S. M. J. (2013). Why does the healthy cornea resist pseudomonas aeruginosa infection? *American Journal of Ophthalmology*, *155*(6).

https://doi.org/10.1016/J.AJO.2013.03.001

Eze, M. O. (1991). Avoidance, and inactivation of reactive oxygen species: Novel microbial immune evasion strategies. *Medical Hypotheses*, *34*(3), 252–255. https://doi.org/10.1016/0306-9877(91)90219-O

Fahlgren, A., Hammarström, S. & Danielsson, Å. (2003). Increased expression of antimicrobial peptides and lysozyme in colonic epithelial cells of patients with ulcerative colitis. *Clinical and Experimental Immunology*, *131*(1), 90–101. https://doi.org/10.1046/j.1365-2249.2003.02035.x

Faure, E., Mear, J. B., Faure, K., Normand, S., Couturier-Maillard, A., Grandjean, T., Balloy, V., Ryffel, B., Dessein, R., Chignard, M., Uyttenhove, C., Guery, B., Gosset, P., Chamaillard, M. & Kipnis, E. (2014). Pseudomonas aeruginosa type-3 secretion system dampens host defense by exploiting the NLRC4-coupled inflammasome. *American Journal of Respiratory and Critical Care Medicine*, *189*(7), 799–811. https://doi.org/10.1164/rccm.201307-1358OC

Feng, C., Huang, Y., He, W., Cheng, X., Liu, H., Huang, Y., Ma, B., Zhang, W., Liao, C., Wu, W., Shao, Y., Xu, D., Su, Z. & Lu, W. (2019). Tanshinones: First-in-Class Inhibitors of the Biogenesis of the Type 3 Secretion System Needle of Pseudomonas aeruginosa for Antibiotic Therapy. *ACS Central Science*, *5*(7), 1278–1288. https://doi.org/10.1021/ACSCENTSCI.9B00452/SUPPL_FILE/OC9B00452_SI_001.PDF

Fernandes, M., Vira, D., Medikonda, R. & Kumar, N. (2016). Extensively and pan-drug resistant Pseudomonas aeruginosa keratitis: clinical features, risk factors, and outcome. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *254*(2), 315–322. https://doi.org/10.1007/s00417-015-3208-7

Filloux, A. (2011). Protein secretion systems in Pseudomonas aeruginosa: An essay on diversity, evolution, and function. *Frontiers in Microbiology*, *2*(JULY), 155. https://doi.org/10.3389/FMICB.2011.00155/BIBTEX

Flaxman, S. R., Bourne, R. R. A., Resnikoff, S., Ackland, P., Braithwaite, T., Cicinelli, M. V., Das, A., Jonas, J. B., Keeffe, J., Kempen, J., Leasher, J., Limburg, H., Naidoo, K., Pesudovs, K., Silvester, A., Stevens, G. A., Tahhan, N., Wong, T., Taylor, H., … Zheng, Y. (2017). Global causes of blindness and distance vision impairment 1990–2020: a systematic review and meta-analysis. *The Lancet Global Health*, *5*(12), e1221–e1234. https://doi.org/10.1016/S2214-109X(17)30393-5

Fleiszig, S. M. J. & Evans, D. J. (2002). The pathogenesis of bacterial keratitis: Studies with Pseudomonas aeruginosa. In *Clinical and Experimental Optometry* (Vol. 85, Issue 5, pp. 271–278). https://doi.org/10.1111/j.1444-0938.2002.tb03082.x

Fleiszig, S. M. J., Zaidi, T. S., Preston, M. J., Grout, M., Evans, D. J. & Pier, G. B. (1996). Relationship between cytotoxicity and corneal epithelial cell invasion by clinical isolates of Pseudomonas aeruginosa. *Infection and Immunity*, *64*(6), 2288–2294. https://doi.org/10.1128/iai.64.6.2288-2294.1996

Fong, C. F., Hu, F. R., Tseng, C. H., Wang, I. J., Chen, W. L. & Hou, Y. C. (2007). Antibiotic Susceptibility of Bacterial Isolates from Bacterial Keratitis Cases in a University Hospital in Taiwan. *American Journal of Ophthalmology*, *144*(5), 682-689.e1. https://doi.org/10.1016/J.AJO.2007.06.038

Forrester, J. V., Dick, A. D., McMenamin, P. G., Roberts, F. & Pearlman, E. (2016). Anatomy of the eye and orbit. *The Eye*, 1-102.e2. https://doi.org/10.1016/B978-0-7020-5554-6.00001-0

Frank, D. W. (1997). The exoenzyme S regulon of Pseudomonas aeruginosa . *Molecular Microbiology*, *26*(4), 621–629. https://doi.org/10.1046/j.1365-2958.1997.6251991.x

Galdiero, S., Falanga, A., Berisio, R., Grieco, P., Morelli, G. & Galdiero, M. (2015). Antimicrobial peptides as an opportunity against bacterial diseases. *Current Medicinal Chemistry*, *22*(14), 1665–1677. https://doi.org/10.2174/0929867322666150311145632

Galle, M., Carpentier, I. & Beyaert, R. (2012). *Structure and Function of the Type III Secretion System of Pseudomonas aeruginosa*.

Gallo, R. L. & Hooper, L. V. (2012). Epithelial antimicrobial defence of the skin and intestine. *Nature Reviews. Immunology*, *12*(7), 503. https://doi.org/10.1038/NRI3228

Ganesan, R., Hos, N. J., Gutierrez, S., Fischer, J., Stepek, J. M., Daglidu, E., Krönke, M. & Robinson, N. (2017). Salmonella Typhimurium disrupts Sirt1/AMPK checkpoint control of mTOR to impair autophagy. *PLOS Pathogens*, *13*(2), e1006227. https://doi.org/10.1371/JOURNAL.PPAT.1006227

Ganz, T. (2003). Hepcidin, a key regulator of iron metabolism and mediator of anemia of inflammation. *Blood*, *102*(3), 783–788. https://doi.org/10.1182/BLOOD-2003-03-0672

Garreis, F., Gottschalt, M., Schlorf, T., Gläser, R., Harder, J., Worlitzsch, D. & Paulsen, F. P. (2011). Expression and regulation of antimicrobial peptide psoriasin (S100A7) at the ocular surface and in the lacrimal apparatus. *Investigative Ophthalmology and Visual Science*, *52*(7), 4914–4922. https://doi.org/10.1167/iovs.10-6598

Garrity-Ryan, L., Kazmierczak, B., Kowal, R., Comolli, J., Hauser, A. & Engel, J. N. (2000). The arginine finger domain of ExoT contributes to actin cytoskeleton disruption and inhibition of internalization of Pseudomonas aeruginosa by epithelial cells and macrophages. *Infection and Immunity*, *68*(12), 7100–7113. https://doi.org/10.1128/IAI.68.12.7100-7113.2000

Ge, P. F., Zhang, J. Z., Wang, X. F., Meng, F. K., Li, W. C., Luan, Y. X., Ling, F. & Luo, Y. N. (2009). Inhibition of autophagy induced by proteasome inhibition increases cell death in human SHG-44 glioma cells. *Acta Pharmacologica Sinica 2009 30:7*, *30*(7), 1046–1052. https://doi.org/10.1038/aps.2009.71

Gellatly, S. L. & Hancock, R. E. W. (2013). Pseudomonas aeruginosa : new insights into pathogenesis and host defenses. *Pathogens and Disease*, *67*(3), 159–173. https://doi.org/10.1111/2049-632X.12033

Germic, N., Frangez, Z., Yousefi, S. & Simon, H. U. (2019). Regulation of the innate immune system by autophagy: monocytes, macrophages, dendritic cells and antigen presentation. *Cell Death & Differentiation 2019 26:4*, *26*(4), 715–727. https://doi.org/10.1038/s41418-019-0297-6

Goldstein, M. H., Kowalski, R. P. & Gordon, Y. J. (1999). Emerging fluoroquinolone resistance in bacterial keratitis: A 5-year review. In *Ophthalmology* (Vol. 106, Issue 7, pp. 1213–1318). Elsevier Inc. https://doi.org/10.1016/s0161-6420(99)00716-2

Gollwitzer, H., Dombrowski, Y., Prodinger, P. M., Peric, M., Summer, B., Hapfelmeier, A., Saldamli, B., Pankow, F., Von Eisenhart-Rothe, R., Imhoff, A. B., Schauber, J., Thomas, P., Burgkart, R. & Banke, I. J. (2013). Antimicrobial peptides and proinflammatory cytokines in periprosthetic joint infection. *Journal of Bone and Joint Surgery - Series A*, *95*(7), 644–651. https://doi.org/10.2106/JBJS.L.00205

Gordon, Y. J., Huang, L. C., Romanowski, E. G., Yates, K. A., Proske, R. J. & McDermott, A. M. (2005). Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Current Eye Research*, *30*(5), 385–394. https://doi.org/10.1080/02713680590934111

Gordon, Y. J., Romanowski, E. G. & McDermott, A. M. (2005). Mini review: A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. In *Current Eye*

Research (Vol. 30, Issue 7, pp. 505–515). NIH Public Access. https://doi.org/10.1080/02713680590968637

Gottsch, J. D., Li, Q., Ashraf, M. F., O'Brien, T. P., Stark, W. J. & Liu, S. H. (1998). Defensin gene expression in the cornea. *Current Eye Research*, *17*(11), 1082–1086. https://doi.org/10.1076/CEYR.17.11.1082.5235

Grada, A., Otero-Vinas, M., Prieto-Castrillo, F., Obagi, Z. & Falanga, V. (2017). Research Techniques Made Simple: Analysis of Collective Cell Migration Using the Wound Healing Assay. *Journal of Investigative Dermatology*, *137*(2), e11–e16. https://doi.org/10.1016/J.JID.2016.11.020

Grier, M. C., Garrity-Ryan, L. K., Bartlett, V. J., Klausner, K. A., Donovan, P. J., Dudley, C., Alekshun, M. N., Ken Tanaka, S., Draper, M. P., Levy, S. B. & Kim, O. K. (2010). N-Hydroxybenzimidazole inhibitors of ExsA MAR transcription factor in Pseudomonas aeruginosa: In vitro anti-virulence activity and metabolic stability. *Bioorganic & Medicinal Chemistry Letters*, *20*(11), 3380–3383. https://doi.org/10.1016/J.BMCL.2010.04.014

Guo, Y., Pan, W., Liu, S., Shen, Z., Xu, Y. & Hu, L. (2020). ERK/MAPK signalling pathway and tumorigenesis (Review). *Experimental and Therapeutic Medicine*, *19*(3), 1997–2007. https://doi.org/10.3892/ETM.2020.8454

Gurnani, B. & Kaur, K. (2021). Bacterial Keratitis. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK574509/

Haas, W., Pillar, C. M., Torres, M., Morris, T. W. & Sahm, D. F. (2011). Monitoring Antibiotic Resistance in Ocular Microorganisms: Results From the Antibiotic Resistance Monitoring in Ocular MicRorganisms (ARMOR) 2009 Surveillance Study. *American Journal of Ophthalmology*, *152*(4), 567-574.e3. https://doi.org/10.1016/J.AJO.2011.03.010

Hamory, B. H., Sande, M. A., Sydnor, A., Seale, D. L. & Gwaltney, J. M. (1979). Etiology and antimicrobial therapy of acute maxillary sinusitis. *The Journal of Infectious Diseases*, *139*(2), 197–202. https://doi.org/10.1093/INFDIS/139.2.197

Haneda, T., Ishii, Y., Shimizu, H., Ohshima, K., Iida, N., Danbara, H. & Okada, N. (2012). Salmonella type III effector SpvC, a phosphothreonine lyase, contributes to reduction in inflammatory response during intestinal phase of infection. *Cellular Microbiology*, *14*(4), 485–499. https://doi.org/10.1111/j.1462-5822.2011.01733.x

Harder, J. & Schröder, J. M. (2002). RNase 7, a novel innate immune defense antimicrobial protein of healthy human skin. *Journal of Biological Chemistry*, *277*(48), 46779–46784. https://doi.org/10.1074/jbc.M207587200

Hase, K., Murakami, M., Iimura, M., Cole, S. P., Horibe, Y., Ohtake, T., Obonyo, M., Gallo, R. L., Eckmann, L. & Kagnoff, M. F. (2003). Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori. *Gastroenterology*, *125*(6), 1613–1625. https://doi.org/10.1053/J.GASTRO.2003.08.028

Hauck, D., Joachim, I., Frommeyer, B., Varrot, A., Philipp, B., Möller, H. M., Imberty, A., Exner, T. E. & Titz, A. (2013). Discovery of Two Classes of Potent Glycomimetic Inhibitors of Pseudomonas aeruginosa LecB with Distinct Binding Modes. *ACS Chemical Biology*, *8*(8), 1775–1784. https://doi.org/10.1021/CB400371R

Hauser, A. R. (2009). The type III secretion system of Pseudomonas aeruginosa: infection by injection. *Nature Reviews Microbiology 2009 7:9*, *7*(9), 654–665. https://doi.org/10.1038/nrmicro2199

Haynes, R. J., Tighe, P. J. & Dua, H. S. (1999). Antimicrobial defensin peptides of the human ocular surface. *British Journal of Ophthalmology*, *83*(6), 737–741.

https://doi.org/10.1136/bjo.83.6.737

Heidari, H., Hadadi, M., Sedigh Ebrahim-Saraie, H., Mirzaei, A., Taji, A., Hosseini, S. R. & Motamedifar, M. (2018). Characterization of virulence factors, antimicrobial resistance patterns and biofilm formation of Pseudomonas aeruginosa and Staphylococcus spp. strains isolated from corneal infection. *Journal Francais d'Ophtalmologie*, *41*(9), 823–829. https://doi.org/10.1016/j.jfo.2018.01.012

Heimer, S. R., Evans, D. J., Stern, M. E., Barbieri, J. T., Yahr, T. & Fleiszig, S. M. J. (2013). Pseudomonas aeruginosa Utilizes the Type III Secreted Toxin ExoS to Avoid Acidified Compartments within Epithelial Cells. *PLoS ONE*, *8*(9). https://doi.org/10.1371/journal.pone.0073111

Henningham, A., Döhrmann, S., Nizet, V. & Cole, J. N. (2015). Mechanisms of group A Streptococcus resistance to reactive oxygen species. *FEMS Microbiology Reviews*, *39*(4), 488–508. https://doi.org/10.1093/FEMSRE/FUU009

Hilchie, A. L., Wuerth, K. & Hancock, R. E. W. (2013). Immune modulation by multifaceted cationic host defense (antimicrobial) peptides. *Nature Chemical Biology 2013 9:12*, *9*(12), 761–768. https://doi.org/10.1038/nchembio.1393

Hilliam, Y., Kaye, S. & Winstanley, C. (2020). Pseudomonas aeruginosa and microbial keratitis. In *Journal of Medical Microbiology* (Vol. 69, Issue 1, pp. 3–13). Microbiology Society. https://doi.org/10.1099/jmm.0.001110

Hippenstiel, S., Soeth, S., Kellas, B., Fuhrmann, O., Seybold, J., Krüll, M., Eichel-Streiber, C. v., Goebeler, M., Ludwig, S. & Suttorp, N. (2000). Rho proteins and the p38-MAPK pathway are important mediators for LPS-induced interleukin-8 expression in human endothelial cells. *Blood*, *95*(10), 3044–3051. https://doi.org/10.1182/BLOOD.V95.10.3044

Hommes, T. J., Hoogendijk, A. J., Dessing, M. C., Van'T Veer, C., Florquin, S., Colonna, M., De Vos, A. F. & Van Der Poll, T. (2014). Triggering receptor expressed on myeloid cells-1 (TREM-1) improves host defence in pneumococcal pneumonia. *Journal of Pathology*, *233*(4), 357–367. https://doi.org/10.1002/path.4361

Hoover, D. M., Chertov, O. & Lubkowski, J. (2001). The structure of human β-defensin-1. New insights into structural properties of β-defensins. *Journal of Biological Chemistry*, *276*(42), 39021–39026. https://doi.org/10.1074/JBC.M103830200

Hop, H. T., Reyes, A. W. B., Huy, T. X. N., Arayan, L. T., Min, W. G., Lee, H. J., Rhee, M. H., Chang, H. H. & Kim, S. (2017). Activation of NF-κB-mediated TNF-induced antimicrobial immunity is required for the efficient Brucella abortus clearance in RAW 264.7 cells. *Frontiers in Cellular and Infection Microbiology*, *7*(OCT), 437. https://doi.org/10.3389/FCIMB.2017.00437/BIBTEX

Hossain, F., Dohra, H. & Yamazaki, M. (2021). Effect of membrane potential on entry of lactoferricin B-derived 6-residue antimicrobial peptide into single *Escherichia coli* cells and lipid vesicles. *Journal of Bacteriology*. https://doi.org/10.1128/JB.00021-21

Hossain, Z. (2014). Bacteria: Streptococcus. *Encyclopedia of Food Safety*, *1*, 535–545. https://doi.org/10.1016/B978-0-12-378612-8.00116-5

Hotinger, J. A. & May, A. E. (2020). Antibodies Inhibiting the Type III Secretion System of Gram-Negative Pathogenic Bacteria. *Antibodies*, *9*(3), 35. https://doi.org/10.3390/antib9030035

Hotinger, J. A., Pendergrass, H. A. & May, A. E. (2021). Molecular Targets and Strategies for Inhibition of the Bacterial Type III Secretion System (T3SS); Inhibitors Directly Binding to T3SS Components. *Biomolecules*, *11*(2), 1–35. https://doi.org/10.3390/BIOM11020316

Hritonenko, V., Mun, J. J., Tam, C., Simon, N. C., Barbieri, J. T., Evans, D. J. & Fleiszig, S. M. J. (2011). Adenylate cyclase activity of Pseudomonas aeruginosa ExoY can mediate blebniche formation in epithelial cells and contributes to virulence. *Microbial Pathogenesis*, *51*(5), 305–312. https://doi.org/10.1016/j.micpath.2011.08.001

Huang, J., Canadien, V., Lam, G. Y., Steinberg, B. E., Dinauer, M. C., Magalhaes, M. A. O., Glogauer, M., Grinstein, S. & Brumell, J. H. (2009). Activation of antibacterial autophagy by NADPH oxidases. *Proceedings of the National Academy of Sciences*, *106*(15), 6226–6231. https://doi.org/10.1073/PNAS.0811045106

Huang, L.C., Proske, R. J. & McDermott, A. M. (2003). Expression of the Peptide Antibiotic LL-37/hCAP18 (Cathelicidin) by Human Corneal Epithelial Cells. *Investigative Ophthalmology & Visual Science*, *44*(13), 1335–1335.

Huang, Ling C., Jean, D., Proske, R. J., Reins, R. Y. & McDermott, A. M. (2009). Ocular Surface Expression and In Vitro Activity of Antimicrobial Peptides. *Http://Dx.Doi.Org/10.1080/02713680701446653*, *32*(7–8), 595–609. https://doi.org/10.1080/02713680701446653

Huang, Ling C., Petkova, T. D., Reins, R. Y., Proske, R. J. & McDermott, A. M. (2006). Multifunctional Roles of Human Cathelicidin (LL-37) at the Ocular Surface. *Investigative Ophthalmology & Visual Science*, *47*(6), 2369–2380. https://doi.org/10.1167/IOVS.05-1649

Hudson, D. L., Layton, A. N., Field, T. R., Bowen, A. J., Wolf-Watz, H., Elofsson, M., Stevens, M. P. & Galyov, E. E. (2007). Inhibition of type III secretion in Salmonella enterica serovar typhimurium by small-molecule inhibitors. *Antimicrobial Agents and Chemotherapy*, *51*(7), 2631–2635. https://doi.org/10.1128/AAC.01492-06/ASSET/5C412F21-9928-4566-99BD-D2B9021F62B9/ASSETS/GRAPHIC/ZAC0070766460004.JPEG

Hung, K. W., Hsu, C. C. & Yu, C. (2013). Solution structure of human Ca2+-bound S100A12. *Journal of Biomolecular NMR*, *57*(3), 313–318. https://doi.org/10.1007/S10858-013-9781-3

Hyams, C., Camberlein, E., Cohen, J. M., Bax, K. & Brown, J. S. (2010). The Streptococcus pneumoniae capsule inhibits complement activity and neutrophil phagocytosis by multiple mechanisms. *Infection and Immunity*, *78*(2), 704–715. https://doi.org/10.1128/IAI.00881-09

Ibrahim, D., Jabbour, J.-F. & Kanj, S. S. (2020). Current choices of antibiotic treatment for Pseudomonas aeruginosa infections. *Current Opinion in Infectious Diseases*, *33*(6), 464–473. https://doi.org/10.1097/QCO.0000000000000677

Ichimura, Y., Waguri, S., Sou, Y. shin, Kageyama, S., Hasegawa, J., Ishimura, R., Saito, T., Yang, Y., Kouno, T., Fukutomi, T., Hoshii, T., Hirao, A., Takagi, K., Mizushima, T., Motohashi, H., Lee, M. S., Yoshimori, T., Tanaka, K., Yamamoto, M. & Komatsu, M. (2013). Phosphorylation of p62 Activates the Keap1-Nrf2 Pathway during Selective Autophagy. *Molecular Cell*, *51*(5), 618–631. https://doi.org/10.1016/J.MOLCEL.2013.08.003/ATTACHMENT/E599D2D0-D409-4115-8390-F356E151A408/MMC1.PDF

Imbert, P. R., Louche, A., Luizet, J.-B., Grandjean, T., Bigot, S., Wood, T. E., Gagné, S., Blanco, A., Wunderley, L., Terradot, L., Woodman, P., Garvis, S., Filloux, A., Guery, B. & Salcedo, S. P. (2017). A Pseudomonas aeruginosa TIR effector mediates immune evasion by targeting UBAP1 and TLR adaptors. *The EMBO Journal*, *36*(13), 1869–1887. https://doi.org/10.15252/EMBJ.201695343

Itoh, K., Chiba, T., Takahashi, S., Ishii, T., Igarashi, K., Katoh, Y., Oyake, T., Hayashi, N., Satoh, K., Hatayama, I., Yamamoto, M. & Nabeshima, Y. ichi. (1997). An Nrf2/Small Maf Heterodimer Mediates the Induction of Phase II Detoxifying Enzyme Genes through Antioxidant Response Elements. *Biochemical and Biophysical Research Communications*,

236(2), 313–322. https://doi.org/10.1006/BBRC.1997.6943

Jaiswal, A. K. (2004). Nrf2 signaling in coordinated activation of antioxidant gene expression. *Free Radical Biology & Medicine*, *36*(10), 1199–1207. https://doi.org/10.1016/J.FREERADBIOMED.2004.02.074

Joyce, E. A., Popper, S. J. & Falkow, S. (2009). Streptococcus pneumoniae nasopharyngeal colonization induces type I interferons and interferon-induced gene expression. *BMC Genomics 2009 10:1*, *10*(1), 1–16. https://doi.org/10.1186/1471-2164-10-404

Juhas, M. (2015). Type IV secretion systems and genomic islands-mediated horizontal gene transfer in Pseudomonas and Haemophilus. *Microbiological Research*, *170*, 10–17. https://doi.org/10.1016/J.MICRES.2014.06.007

Jung, M., Weigert, A., Tausendschön, M., Mora, J., Ören, B., Sola, A., Hotter, G., Muta, T. & Brüne, B. (2012). Interleukin-10-Induced Neutrophil Gelatinase-Associated Lipocalin Production in Macrophages with Consequences for Tumor Growth. *Molecular and Cellular Biology*, *32*(19), 3938–3948. https://doi.org/10.1128/MCB.00413-12/ASSET/3394BB5E-14CD-4D0D-94B6-4586695A1029/ASSETS/GRAPHIC/ZMB9991096640006.JPEG

Junkins, R. D., Shen, A., Rosen, K., McCormick, C. & Lin, T. J. (2013). Autophagy Enhances Bacterial Clearance during P. aeruginosa Lung Infection. *PLoS ONE*, *8*(8), 72263. https://doi.org/10.1371/journal.pone.0072263

Jurado-Martín, I., Sainz-Mejías, M. & McClean, S. (2021). Pseudomonas aeruginosa: An audacious pathogen with an adaptable arsenal of virulence factors. In *International Journal of Molecular Sciences* (Vol. 22, Issue 6, pp. 1–37). Int J Mol Sci. https://doi.org/10.3390/ijms22063128

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., Kominami, E., Ohsumi, Y. & Yoshimori, T. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO Journal*, *19*(21), 5720–5728. https://doi.org/10.1093/EMBOJ/19.21.5720

Kaliamurthy, J., Kalavathy, C. M., Parmar, P., Nelson Jesudasan, C. A. & Thomas, P. A. (2013). Spectrum of bacterial keratitis at a tertiary eye care centre in India. *BioMed Research International*, *2013*, 181564–181564. https://doi.org/10.1155/2013/181564

Kang, J., Dietz, M. J. & Li, B. (2019). Antimicrobial peptide LL-37 is bactericidal against Staphylococcus aureus biofilms. *PLOS ONE*, *14*(6), e0216676. https://doi.org/10.1371/JOURNAL.PONE.0216676

Kanthawong, S., Nazmi, K., Wongratanacheewin, S., Bolscher, J. G. M., Wuthiekanun, V. & Taweechaisupapong, S. (2009). In vitro susceptibility of Burkholderia pseudomallei to antimicrobial peptides. *International Journal of Antimicrobial Agents*, *34*(4), 309–314. https://doi.org/10.1016/J.IJANTIMICAG.2009.05.012

Kapuy, O., Vinod, P. K. & Bánhegyi, G. (2014). mTOR inhibition increases cell viability via autophagy induction during endoplasmic reticulum stress – An experimental and modeling study. *FEBS Open Bio*, *4*, 704–713. https://doi.org/10.1016/J.FOB.2014.07.006

Karthikeyan, R. S. G., Priya, J. L., Leal, S. M., Toska, J., Rietsch, A., Prajna, V., Pearlman, E. & Lalitha, P. (2013). Host Response and Bacterial Virulence Factor Expression in Pseudomonas aeruginosa and Streptococcus pneumoniae Corneal Ulcers. *PLoS ONE*, *8*(6), 64867. https://doi.org/10.1371/journal.pone.0064867

Kaufman, M. R., Jia, J., Zeng, L., Ha, U., Chow, M. & Jin, S. (2000). Pseudomonas aeruginosa mediated apoptosis requires the ADP-ribosylating activity of ExoS. *Microbiology*, *146*(10), 2531–2541. https://doi.org/10.1099/00221287-146-10-2531

Keay, L., Edwards, K., Naduvilath, T., Taylor, H. R., Snibson, G. R., Forde, K. & Stapleton, F. (2006). Microbial keratitis: Predisposing factors and morbidity. *Ophthalmology*, *113*(1), 109–116. https://doi.org/10.1016/j.ophtha.2005.08.013

Keyer, K., Strohmeier Gort, A. & Imlay, J. A. (1995). Superoxide and the Production of Oxidative DNA Damage. *JOURNAL OF BACTERIOLOGY*, *177*(23), 6782–6790.

Keyser, P., Elofsson, M., Rosell, S. & Wolf-Watz, H. (2008). Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria. *Journal of Internal Medicine*, *264*(1), 17–29. https://doi.org/10.1111/J.1365-2796.2008.01941.X

Khalifa, L., Brosh, Y., Gelman, D., Coppenhagen-Glazer, S., Beyth, S., Poradosu-Cohen, R., Que, Y.-A., Beyth, N. & Hazan, R. (2015). Targeting Enterococcus faecalis Biofilms with Phage Therapy. *Applied and Environmental Microbiology*, *81*(8), 2696–2705. https://doi.org/10.1128/AEM.00096-15

Kharazmi, A. (1991). Mechanisms involved in the evasion of the host defence by Pseudomonas aeruginosa. *Immunology Letters*, *30*(2), 201–205. https://doi.org/10.1016/0165-2478(91)90026-7

Khoo, P., Cabrera-Aguas, M. P., Nguyen, V., Lahra, M. M. & Watson, S. L. (2020). Microbial keratitis in Sydney, Australia: risk factors, patient outcomes, and seasonal variation. *Graefe's Archive for Clinical and Experimental Ophthalmology*, *258*(8), 1745–1755. https://doi.org/10.1007/s00417-020-04681-0

Kierstan Boyd, D. T. and R. H. J. J. M. (2018). *Parts of the Eye - American Academy of Ophthalmology*. American Academy of Ophtalmology (Online). https://www.aao.org/eye-health/anatomy/parts-of-eye

Kim, J., Lee, H. W., Rhee, D. K., Paton, J. C. & Pyo, S. (2017). Pneumolysin-induced autophagy contributes to inhibition of osteoblast differentiation through downregulation of Sp1 in human osteosarcoma cells. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1861*(11), 2663–2673. https://doi.org/10.1016/J.BBAGEN.2017.07.008

Kim, K. M. & Ki, S. H. (2017). Nrf2: A Key Regulator of Redox Signaling in Liver Diseases. *Liver Pathophysiology: Therapies and Antioxidants*, 355–374. https://doi.org/10.1016/B978-0-12-804274-8.00028-X

Kim, Y. J., Shin, H. S., Lee, J. H., Jung, Y. W., Kim, H. B. & Ha, U. H. (2013). Pneumolysin-mediated expression of β-defensin 2 is coordinated by p38 MAP kinase-MKP1 in human airway cells. *Journal of Microbiology*, *51*(2), 194–199. https://doi.org/10.1007/S12275-013-2579-X

Kimura, K., Iwatsuki, M., Nagai, T., Matsumoto, A., Takahashi, Y., Shiomi, K., Omura, S. & Abe, A. (2010). A small-molecule inhibitor of the bacterial type III secretion system protects against in vivo infection with Citrobacter rodentium. *The Journal of Antibiotics 2011 64:2*, *64*(2), 197–203. https://doi.org/10.1038/ja.2010.155

Klionsky, D. J., Abdel-Aziz, A. K., Abdelfatah, S., Abdellatif, M., Abdoli, A., Abel, S., Abeliovich, H., Abildgaard, M. H., Abudu, Y. P., Acevedo-Arozena, A., Adamopoulos, I. E., Adeli, K., Adolph, T. E., Adornetto, A., Aflaki, E., Agam, G., Agarwal, A., Aggarwal, B. B., Agnello, M., … Tong, C. K. (2021). Guidelines for the use and interpretation of assays for monitoring autophagy (4th edition) 1. *Autophagy*, *17*(1), 1–382. https://doi.org/10.1080/15548627.2020.1797280

Kloth, C., Schirmer, B., Munder, A., Stelzer, T., Rothschuh, J. & Seifert, R. (2018). The Role of Pseudomonas aeruginosa ExoY in an Acute Mouse Lung Infection Model. *Toxins*, *10*(5). https://doi.org/10.3390/TOXINS10050185

Kma, L. & Baruah, T. J. (2021). The interplay of ROS and the PI3K/Akt pathway in autophagy regulation. *Biotechnology and Applied Biochemistry*. https://doi.org/10.1002/BAB.2104

Koeninger, L., Armbruster, N. S., Brinch, K. S., Kjaerulf, S., Andersen, B., Langnau, C., Autenrieth, S. E., Schneidawind, D., Stange, E. F., Malek, N. P., Nordkild, P., Jensen, B. A. H. & Wehkamp, J. (2020). Human β-Defensin 2 Mediated Immune Modulation as Treatment for Experimental Colitis. *Frontiers in Immunology*, *11*, 93. https://doi.org/10.3389/fimmu.2020.00093

Kolář, M., Urbánek, K. & Látal, T. (2001). Antibiotic selective pressure and development of bacterial resistance. *International Journal of Antimicrobial Agents*, *17*(5), 357–363. https://doi.org/10.1016/S0924-8579(01)00317-X

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y. S., Ueno, I., Sakamoto, A., Tong, K. I., Kim, M., Nishito, Y., Iemura, S. I., Natsume, T., Ueno, T., Kominami, E., Motohashi, H., Tanaka, K. & Yamamoto, M. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nature Cell Biology 2010 12:3*, *12*(3), 213–223. https://doi.org/10.1038/ncb2021

Krachler, A. M., Woolery, A. R. & Orth, K. (2011). Host–pathogen interactions: Manipulation of kinase signaling by bacterial pathogens. *The Journal of Cell Biology*, *195*(7), 1083. https://doi.org/10.1083/JCB.201107132

Krall, R., Sun, J., Pederson, K. J. & Barbieri, J. T. (2002). In Vivo Rho GTPase-Activating Protein Activity of Pseudomonas aeruginosa Cytotoxin ExoS. *Infection and Immunity*, *70*(1), 360. https://doi.org/10.1128/IAI.70.1.360-367.2002

Krisanaprakornkit, S., Kimball, J. R. & Dale, B. A. (2002). Regulation of Human β-Defensin-2 in Gingival Epithelial Cells: The Involvement of Mitogen-Activated Protein Kinase Pathways, But Not the NF-κB Transcription Factor Family. *The Journal of Immunology*, *168*(1), 316–324. https://doi.org/10.4049/JIMMUNOL.168.1.316

Krumova, K. & Cosa, G. (2016). Overview of Reactive Oxygen Species. In *Comprehensive Series in Photochemical & Photobiological Sciences* (Vol. 1, pp. 1–21). Royal Society of Chemistry. https://doi.org/10.1039/9781782622208-00001

Kumar, A., Zhang, J. & Yu, F. S. X. (2004). Innate immune response of corneal epithelial cells to Staphylococcus aureus infection: Role of peptidoglycan in stimulating proinflammatory cytokine secretion. *Investigative Ophthalmology and Visual Science*, *45*(10), 3513–3522. https://doi.org/10.1167/iovs.04-0467

Kumar, A., Zhang, J. & Yu, F. S. X. (2006). Toll-like receptor 2-mediated expression of β-defensin-2 in human corneal epithelial cells. *Microbes and Infection*, *8*(2), 380–389. https://doi.org/10.1016/j.micinf.2005.07.006

Kunimoto, D. Y., Sharma, S., Garg, P., Gopinathan, U., Miller, D. & Rao, G. N. (2000). Corneal ulceration in the elderly in Hyderabad, south India. *British Journal of Ophthalmology*, *84*(1), 54–59. https://doi.org/10.1136/BJO.84.1.54

Kusaka, S., Nishida, A., Takahashi, K., Bamba, S., Yasui, H., Kawahara, M., Inatomi, O., Sugimoto, M. & Andoh, A. (2018). Expression of human cathelicidin peptide LL-37 in inflammatory bowel disease. *Clinical & Experimental Immunology*, *191*(1), 96–106. https://doi.org/10.1111/CEI.13047

Kwon, J. H. & Powderly, W. G. (2021). The post-antibiotic era is here. *Science*, *373*(6554), 471. https://doi.org/10.1126/SCIENCE.ABL5997

Lakhundi, S., Siddiqui, R. & Khan, N. A. (2017). Pathogenesis of microbial keratitis. In

Microbial Pathogenesis (Vol. 104, pp. 97–109). Academic Press. https://doi.org/10.1016/j.micpath.2016.12.013

Lambert, P. A. (2002). Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. *Journal of the Royal Society of Medicine, Supplement*, *95*(41), 22–26. http://www.cmdr.ubc.ca/bobh/oprfmodel.htm].

Lap-Ki Ng, A., Kai-Wang To, K., Chi-Lai Choi, C., Hsu Yuen, L., Yim, S.-M. M., Shun-Kit Chan, K., Shiu-Ming Lai, J., Yat-Hin Wong, I., Ng, A. L. K., To, K. K. W., Choi, C. C. L., Yuen, L. H., Yim, S.-M. M., Chan, K. S. K., Lai, J. S. M. & Wong, I. Y. H. (2015). Predisposing Factors, Microbial Characteristics, and Clinical Outcome of Microbial Keratitis in a Tertiary Centre in Hong Kong: A 10-Year Experience. *Journal of Ophthalmology*, *2015*. https://doi.org/10.1155/2015/769436

Lau, A., Wang, X.-J., Zhao, F., Villeneuve, N. F., Wu, T., Jiang, T., Sun, Z., White, E. & Zhang, D. D. (2010). A Noncanonical Mechanism of Nrf2 Activation by Autophagy Deficiency: Direct Interaction between Keap1 and p62. *Molecular and Cellular Biology*, *30*(13), 3275–3285. https://doi.org/10.1128/MCB.00248-10/ASSET/F8BD7F3D-F1D1-4B45-B0CD-C8ED45012A1E/ASSETS/GRAPHIC/ZMB9991086610005.JPEG

Lawana, V., Korrapati, M. C. & Mehendale, H. M. (2014). Cycloheximide. *Encyclopedia of Toxicology: Third Edition*, 1103–1105. https://doi.org/10.1016/B978-0-12-386454-3.00298-0

Le, C. F., Fang, C. M. & Sekaran, S. D. (2017). Intracellular Targeting Mechanisms by Antimicrobial Peptides. *Antimicrobial Agents and Chemotherapy*, *61*(4). https://doi.org/10.1128/AAC.02340-16

Lee, A. E., Niruttan, K., Rawson, T. M. & Moore, L. S. P. (2019). Antibacterial resistance in ophthalmic infections: A multi-centre analysis across UK care settings. *BMC Infectious Diseases*, *19*(1), 1–8. https://doi.org/10.1186/s12879-019-4418-0

Lee, D. G., Urbach, J. M., Wu, G., Liberati, N. T., Feinbaum, R. L., Miyata, S., Diggins, L. T., He, J., Saucier, M., Déziel, E., Friedman, L., Li, L., Grills, G., Montgomery, K., Kucherlapati, R., Rahme, L. G. & Ausubel, F. M. (2006). Genomic analysis reveals that Pseudomonas aeruginosa virulence is combinatorial. *Genome Biology*, *7*(10), 1–14. https://doi.org/10.1186/gb-2006-7-10-r90

Lee, J. M. & Johnson, J. A. (2004). An important role of Nrf2-ARE pathway in the cellular defense mechanism. *Journal of Biochemistry and Molecular Biology*, *37*(2), 139–143. https://doi.org/10.5483/BMBREP.2004.37.2.139

Lehmann, J., Retz, M., Harder, J., Krams, M., Kellner, U., Hartmann, J., Hohgräwe, K., Raffenberg, U., Gerber, M., Loch, T., Weichert-Jacobsen, K. & Stöckle, M. (2002). Expression of human beta-defensins 1 and 2 kidneys with chronic bacterial infection. *BMC Infectious Diseases*, *2*(1), 1–10. https://doi.org/10.1186/1471-2334-2-20/FIGURES/4

Lehmann, O. J., Hussain, I. R. & Watt, P. J. (2000). Investigation of β defensin gene expression in the ocular anterior segment by semiquantitative RT-PCR. *British Journal of Ophthalmology*, *84*(5), 523–526. https://doi.org/10.1136/BJO.84.5.523

Lewies, A., Du Plessis, L. H. & Wentzel, J. F. (2019). Antimicrobial Peptides: the Achilles' Heel of Antibiotic Resistance? *Probiotics and Antimicrobial Proteins*, *11*(2), 370–381. https://doi.org/10.1007/s12602-018-9465-0

Li, B., Xi, P., Wang, Z., Han, X., Xu, Y., Zhang, Y. & Miao, J. (2018). PI3K/Akt/mTOR signaling pathway participates in Streptococcus uberis-induced inflammation in mammary epithelial cells in concert with the classical TLRs/NF-κB pathway. *Veterinary Microbiology*,

227, 103–111. https://doi.org/10.1016/j.vetmic.2018.10.031

Li, G., Domenico, J., Jia, Y., Lucas, J. J. & Gelfand, E. W. (2009). NF-κB-dependent induction of cathelicidin-related antimicrobial peptide in murine mast cells by lipopolysaccharide. *International Archives of Allergy and Immunology*, *150*(2), 122–132. https://doi.org/10.1159/000218115

Li, H., Zhou, X., Huang, Y., Liao, B., Cheng, L. & Ren, B. (2021). Reactive Oxygen Species in Pathogen Clearance: The Killing Mechanisms, the Adaption Response, and the Side Effects. *Frontiers in Microbiology*, *11*, 3610. https://doi.org/10.3389/FMICB.2020.622534/BIBTEX

Li, L., Tan, J., Miao, Y., Lei, P. & Zhang, Q. (2015). ROS and Autophagy: Interactions and Molecular Regulatory Mechanisms. *Cellular and Molecular Neurobiology 2015 35:5*, *35*(5), 615–621. https://doi.org/10.1007/S10571-015-0166-X

Li, P., Shi, J., He, Q., Hu, Q., Wang, Y. Y., Zhang, L. J., Chan, W. T. & Chen, W. X. (2015). Streptococcus pneumoniae induces autophagy through the inhibition of the PI3K-I/Akt/mTOR pathway and ROS hypergeneration in A549 cells. *PLoS ONE*, *10*(3), e0122753. https://doi.org/10.1371/journal.pone.0122753

Li, Q., Kumar, A., Gui, J. F. & Yu, F. S. X. (2008). Staphylococcus aureus lipoproteins trigger human corneal epithelial innate response through toll-like receptor-2. *Microbial Pathogenesis*, *44*(5), 426–434. https://doi.org/10.1016/j.micpath.2007.11.006

Lima, C. D. M. de, Calegari-Silva, T. C., Pereira, R. M. S., Santos, S. A. de O. L., Lopes, U. G., Plotkowski, M.-C. M. & Saliba, A. M. (2012). ExoU Activates NF-κB and Increases IL-8/KC Secretion during Pseudomonas aeruginosa Infection. *PLOS ONE*, *7*(7), e41772. https://doi.org/10.1371/JOURNAL.PONE.0041772

Lima, P. G., Oliveira, J. T. A., Amaral, J. L., Freitas, C. D. T. & Souza, P. F. N. (2021). Synthetic antimicrobial peptides: Characteristics, design, and potential as alternative molecules to overcome microbial resistance. *Life Sciences*, *278*. https://doi.org/10.1016/J.LFS.2021.119647

Lin, A., Rhee, M. K., Akpek, E. K., Amescua, G., Farid, M., Garcia-Ferrer, F. J., Varu, D. M., Musch, D. C., Dunn, S. P. & Mah, F. S. (2019). Bacterial Keratitis Preferred Practice Pattern®. *Ophthalmology*, *126*(1), P1–P55. https://doi.org/10.1016/j.ophtha.2018.10.018

Liu, G. Y., Essex, A., Buchanan, J. T., Datta, V., Hoffman, H. M., Bastian, J. F., Fierer, J. & Nizet, V. (2005). Staphylococcus aureus golden pigment impairs neutrophil killing and promotes virulence through its antioxidant activity. *The Journal of Experimental Medicine*, *202*(2), 209–215. https://doi.org/10.1084/JEM.20050846

Liu, H. Y., Han, J., Cao, S. Y., Hong, T., Zhuo, D., Shi, J., Liu, Z. & Cao, W. (2009). Hepatic autophagy is suppressed in the presence of insulin resistance and hyperinsulinemia. Inhibition of FoxO1-dependent expression of key autophagy genes by insulin. *Journal of Biological Chemistry*, *284*(45), 31484–31492. https://doi.org/10.1074/JBC.M109.033936/ATTACHMENT/738D5E64-7CE8-4E75-AC9C-6817C46EC529/MMC1.PDF

Livermore, D. M. (1995). β-lactamases in laboratory and clinical resistance. In *Clinical Microbiology Reviews* (Vol. 8, Issue 4, pp. 557–584). American Society for Microbiology. https://doi.org/10.1128/cmr.8.4.557-584.1995

Lu, P., Wang, S., Lu, Y., Neculai, D., Sun, Q. & Van Der Veen, S. (2019). A Subpopulation of Intracellular Neisseria gonorrhoeae Escapes Autophagy-Mediated Killing Inside Epithelial Cells. *The Journal of Infectious Diseases*, *219*(1), 133–144. https://doi.org/10.1093/INFDIS/JIY237

Luo, Y. & Song, Y. (2021). Mechanism of Antimicrobial Peptides: Antimicrobial, Anti-Inflammatory and Antibiofilm Activities. *International Journal of Molecular Sciences*, *22*(21). https://doi.org/10.3390/IJMS222111401

Lyczak, J. B., Cannon, C. L. & Pier, G. B. (2000). Establishment of Pseudomonas aeruginosa infection: Lessons from a versatile opportunist. In *Microbes and Infection* (Vol. 2, Issue 9, pp. 1051–1060). Elsevier Masson SAS. https://doi.org/10.1016/S1286-4579(00)01259-4

Ma, Q. (2013). Role of Nrf2 in Oxidative Stress and Toxicity. *Annual Review of Pharmacology and Toxicology*, *53*, 401. https://doi.org/10.1146/ANNUREV-PHARMTOX-011112-140320

Macroautophagy | Pathway - PubChem. (n.d.). Retrieved December 27, 2021, from https://pubchem.ncbi.nlm.nih.gov/pathway/Reactome:R-HSA-1632852

Magrone, T., Russo, M. A. & Jirillo, E. (2018). Antimicrobial Peptides in Human Disease: Therapeutic Approaches. Second of Two Parts. *Current Pharmaceutical Design*, *24*(10), 1148–1156. https://doi.org/10.2174/1381612824666180327155230

Mah, F. S. & Baum, J. (2015). Keratitis. In *Clinical Infectious Disease, Second Edition* (pp. 88–96). StatPearls Publishing. https://doi.org/10.1017/CBO9781139855952.016

Malanovic, N. & Lohner, K. (2016). Gram-positive bacterial cell envelopes: The impact on the activity of antimicrobial peptides. *Biochimica et Biophysica Acta - Biomembranes*, *1858*(5), 936–946. https://doi.org/10.1016/j.bbamem.2015.11.004

Malley, R., Henneke, P., Morse, S. C., Cieslewicz, M. J., Lipsitch, M., Thompson, C. M., Kurt-Jones, E., Paton, J. C., Wessels, M. R. & Golenbock, D. T. (2003). Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(4), 1966–1971. https://doi.org/10.1073/PNAS.0435928100

Mancl, J. M., Suarez, C., Liang, W. G., Kovar, D. R. & Tang, W. J. (2020). Pseudomonas aeruginosa exoenzyme Y directly bundles actin filaments. *Journal of Biological Chemistry*, *295*(11), 3506–3517. https://doi.org/10.1074/jbc.RA119.012320

Mannis, M. J. (2002). The use of antimicrobial peptides in ophthalmology: An experimental study in corneal preservation and the management of bacterial keratitis. *Transactions of the American Ophthalmological Society*, *100*, 243–271. /pmc/articles/PMC1358966/?report=abstract

Manrique-Moreno, M., Suwalsky, M., Patiño-González, E., Fandiño-Devia, E., MałgorzataJemioła-Rzemińska & Strzałka, K. (2020). Interaction of the antimicrobial peptide ΔM3 with the Staphylococcus aureus membrane and molecular models. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 183498. https://doi.org/https://doi.org/10.1016/j.bbamem.2020.183498

Martineau, A. R., Wilkinson, K. A., Newton, S. M., Floto, R. A., Norman, A. W., Skolimowska, K., Davidson, R. N., Sørensen, O. E., Kampmann, B., Griffiths, C. J. & Wilkinson, R. J. (2007). IFN-gamma- and TNF-independent vitamin D-inducible human suppression of mycobacteria: the role of cathelicidin LL-37. *Journal of Immunology (Baltimore, Md. : 1950)*, *178*(11), 7190–7198. https://doi.org/10.4049/JIMMUNOL.178.11.7190

Maurice, D. M. (1957). The structure and transparency of the cornea. *The Journal of Physiology*, *136*(2), 263–286. https://doi.org/10.1113/jphysiol.1957.sp005758

Mazel, D. & Davies, J. (1999). Antibiotic resistance in microbes. *Cellular and Molecular Life Sciences*, *56*(9–10), 742–754. https://doi.org/10.1007/s000180050021

McCaa, C. S. (1982). The eye and visual nervous system: anatomy, physiology and toxicology.

Environmental Health Perspectives, *Vol. 44*, 1–8. https://doi.org/10.1289/ehp.82441

McCaffrey, R. L. & Allen, L.-A. H. (2006). Francisella tularensis LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. *Journal of Leukocyte Biology*, *80*(6), 1224–1230. https://doi.org/10.1189/JLB.0406287

McDermott, A. M. (2009). The Role of Antimicrobial Peptides at the Ocular Surface. *Ophthalmic Research*, *41*(2), 60. https://doi.org/10.1159/000187622

McDermott, A. M., Redfern, R. L., Zhang, B., Pei, Y., Huang, L. & Proske, R. J. (2003). Defensin expression by the cornea: Multiple signalling pathways mediate IL-1β stimulation of hBD-2 expression by human corneal epithelial cells. *Investigative Ophthalmology and Visual Science*, *44*(5), 1859–1865. https://doi.org/10.1167/iovs.02-0787

McDevitt, E., Khan, F., Scasny, A., Thompson, C. D., Eichenbaum, Z., McDaniel, L. S. & Vidal, J. E. (2020). Hydrogen Peroxide Production by Streptococcus pneumoniae Results in Alpha-hemolysis by Oxidation of Oxy-hemoglobin to Met-hemoglobin. *MSphere*, *5*(6). https://doi.org/10.1128/MSPHERE.01117-20

McIntosh, R. S., Cade, J. E., Al-Abed, M., Shanmuganathan, V., Gupta, R., Bhan, A., Tighe, P. J. & Dua, H. S. (2005). The spectrum of antimicrobial peptide expression at the ocular surface. *Investigative Ophthalmology and Visual Science*, *46*(4), 1379–1385. https://doi.org/10.1167/iovs.04-0607

McIsaac, S. M., Stadnyk, A. W. & Lin, T.-J. (2012). Toll-like receptors in the host defense against *Pseudomonas aeruginosa* respiratory infection and cystic fibrosis. *Journal of Leukocyte Biology*, *92*(5), 977–985. https://doi.org/10.1189/jlb.0811410

McNamara, N. A., Van, R., Tuchin, O. S. & Fleiszig, S. M. J. (1999). Ocular Surface Epithelia Express mRNA for Human Beta Defensin-2. *Experimental Eye Research*, *69*(5), 483–490. https://doi.org/10.1006/EXER.1999.0722

Méndez-Samperio, P., Miranda, E. & Trejo, A. (2008). Expression and secretion of cathelicidin LL-37 in human epithelial cells after infection by Mycobacterium bovis Bacillus Calmette-Guérin. *Clinical and Vaccine Immunology : CVI*, *15*(9), 1450–1455. https://doi.org/10.1128/CVI.00178-08

Menendez, A. & Brett Finlay, B. (2007). Defensins in the immunology of bacterial infections. In *Current Opinion in Immunology* (Vol. 19, Issue 4, pp. 385–391). https://doi.org/10.1016/j.coi.2007.06.008

Merriman, J. A., Nemeth, K. A. & Schlievert, P. M. (2014). Novel antimicrobial peptides that inhibit gram positive bacterial exotoxin synthesis. *PLoS ONE*, *9*(4), 95661. https://doi.org/10.1371/journal.pone.0095661

Miller, J. J., Scott, I. U., Flynn, H. W., Smiddy, W. E., Corey, R. P. & Miller, D. (2004). Endophthalmitis caused by streptococcus pneumoniae. *American Journal of Ophthalmology*, *138*(2), 231–236. https://doi.org/10.1016/J.AJO.2004.03.008

Miraglia, E., Nylén, F., Johansson, K., Arnér, E., Cebula, M., Farmand, S., Ottosson, H., Strömberg, R., Gudmundsson, G. H., Agerberth, B. & Bergman, P. (2016). Entinostat up-regulates the CAMP gene encoding LL-37 via activation of STAT3 and HIF-1α transcription factors. *Scientific Reports 2016 6:1*, *6*(1), 1–12. https://doi.org/10.1038/srep33274

Mittal, R., Lisi, C. V., Kumari, H., Grati, M., Blackwelder, P., Yan, D., Jain, C., Mathee, K., Weckwerth, P. H. & Liu, X. Z. (2016). Otopathogenic Pseudomonas aeruginosa Enters and Survives Inside Macrophages. *Frontiers in Microbiology*, *7*(NOV), 1828. https://doi.org/10.3389/FMICB.2016.01828

Mohammadpour, M., Mohajernezhadfard, Z., Khodabande, A. & Vahedi, P. (2011). Antibiotic susceptibility patterns of pseudomonas corneal ulcers in contact lens wearers. *Middle East African Journal of Ophthalmology*, *18*(3), 228–231. https://doi.org/10.4103/0974-9233.84053

Mohammed, I., Said, D. G. & Dua, H. S. (2017). Human antimicrobial peptides in ocular surface defense. *Progress in Retinal and Eye Research*, *61*, 1–22. https://doi.org/10.1016/J.PRETEYERES.2017.03.004

Mohammed, I., Suleman, H., Otri, A. M., Kulkarni, B. B., Chen, P., Hopkinson, A. & Dua, H. S. (2010). Localization and gene expression of human β-defensin 9 at the human ocular surface epithelium. *Investigative Ophthalmology and Visual Science*, *51*(9), 4677–4682. https://doi.org/10.1167/iovs.10-5334

Mohammed, I., Yeung, A., Abedin, A., Hopkinson, A. & Dua, H. S. (2011). Signalling pathways involved in ribonuclease-7 expression. *Cellular and Molecular Life Sciences*, *68*(11), 1941–1952. https://doi.org/10.1007/S00018-010-0540-2

Mukherjee, P. K. (2019). Evaluation of Herbal Drugs for Antimicrobial and Parasiticidal Effects. In *Quality Control and Evaluation of Herbal Drugs* (pp. 573–598). Elsevier. https://doi.org/10.1016/b978-0-12-813374-3.00015-6

Mukherjee, S. & Hooper, L. V. (2015). Antimicrobial defense of the intestine. *Immunity*, *42*(1), 28–39. https://doi.org/10.1016/J.IMMUNI.2014.12.028

Mun, Y., Kim, M. K. & Oh, J. Y. (2019). Ten-year analysis of microbiological profile and antibiotic sensitivity for bacterial keratitis in Korea. *PLOS ONE*, *14*(3), e0213103. https://doi.org/10.1371/JOURNAL.PONE.0213103

Murphy, M. P. (2009). How mitochondria produce reactive oxygen species. *Biochemical Journal*, *417*(1), 1–13. https://doi.org/10.1042/BJ20081386

Murthy, R., Petrescu, D. & Salit, I. E. (2015). Osteomyelitis with a twist: Streptococcus pneumoniae causing sternoclavicular septic arthritis. *The Canadian Journal of Infectious Diseases & Medical Microbiology*, *26*(5), 251. https://doi.org/10.1155/2015/426704

N'Guessan, P. D., Hippenstiel, S., Etouem, M. O., Zahlten, J., Beermann, W., Lindner, D., Opitz, B., Witzenrath, M., Rosseau, S., Suttorp, N. & Schmeck, B. (2006). Streptococcus pneumoniae induced p38 MAPK- and NF-κB-dependent COX-2 expression in human lung epithelium. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *290*(6), 1131–1138. https://doi.org/10.1152/AJPLUNG.00383.2005/ASSET/IMAGES/LARGE/ZH50060645500008.JPEG

Naito, Y., Moriyama, K. & Sawa, T. (2017). Anti-PcrV Immunization for Pseudomonas aeruginosa Pneumonia in Cystic Fibrosis. *Progress in Understanding Cystic Fibrosis*. https://doi.org/10.5772/INTECHOPEN.69767

Newman, J. W., Floyd, R. V. & Fothergill, J. L. (2017). The contribution of Pseudomonas aeruginosa virulence factors and host factors in the establishment of urinary tract infections. *FEMS Microbiology Letters*, *364*(15), 124. https://doi.org/10.1093/FEMSLE/FNX124

Nguyen, G. T., Green, E. R. & Mecsas, J. (2017). Neutrophils to the ROScue: Mechanisms of NADPH Oxidase Activation and Bacterial Resistance. *Frontiers in Cellular and Infection Microbiology*, *7*(AUG), 373. https://doi.org/10.3389/FCIMB.2017.00373

Nielsen, B. S., Borregaard, N., Bundgaard, J. R., Timshel, S., Sehested, M. & Kjeldsen, L. (1996). Induction of NGAL synthesis in epithelial cells of human colorectal neoplasia and inflammatory bowel diseases. *Gut.Bmj.Com*, *38*, 414–420.

https://doi.org/10.1136/gut.38.3.414

Niu, W., Sun, B., Li, M., Cui, J., Huang, J. & Zhang, L. (2018). TLR-4/microRNA-125a/NF-κB signaling modulates the immune response to Mycobacterium tuberculosis infection. *Cell Cycle*, *17*(15), 1931–1945. https://doi.org/10.1080/15384101.2018.1509636

Niyonsaba, F., Ushio, H., Nakano, N., Ng, W., Sayama, K., Hashimoto, K., Nagaoka, I., Okumura, K. & Ogawa, H. (2007). Antimicrobial Peptides Human β-Defensins Stimulate Epidermal Keratinocyte Migration, Proliferation and Production of Proinflammatory Cytokines and Chemokines. *Journal of Investigative Dermatology*, *127*(3), 594–604. https://doi.org/10.1038/SJ.JID.5700599

Noore, J., Noore, A. & Li, B. (2013). Cationic antimicrobial peptide ll-37 is effective against both extraand intracellular staphylococcus aureus. *Antimicrobial Agents and Chemotherapy*, *57*(3), 1283–1290. https://doi.org/10.1128/AAC.01650-12/ASSET/E90C48E2-6AA2-4EB7-8A6E-A41A03164CE2/ASSETS/GRAPHIC/ZAC9991016370007.JPEG

Norcross, E. W., Sanders, M. E., Moore, Q. C., III & Marquart, M. E. (2011). Pathogenesis of A Clinical Ocular Strain of Streptococcus pneumoniae and the Interaction of Pneumolysin with Corneal Cells. *Journal of Bacteriology & Parasitology*, *2*(2), 108. https://doi.org/10.4172/2155-9597.1000108

Nordfelth, R., Kauppi, A. M., Norberg, H. A., Wolf-Watz, H. & Elofsson, M. (2005). Small-Molecule Inhibitors Specifically Targeting Type III Secretion. *Infection and Immunity*, *73*(5), 3104. https://doi.org/10.1128/IAI.73.5.3104-3114.2005

Nuti, R., Goud, N. S., Saraswati, A. P., Alvala, R. & Alvala, M. (2017). Antimicrobial Peptides: A Promising Therapeutic Strategy in Tackling Antimicrobial Resistance. *Current Medicinal Chemistry*, *24*(38). https://doi.org/10.2174/0929867324666170815102441

O'Brien, T. P. (2003). Management of bacterial keratitis: Beyond exorcism towards consideration of organism and host factors. In *Eye* (Vol. 17, Issue 8, pp. 957–974). Nature Publishing Group. https://doi.org/10.1038/sj.eye.6700635

Okonkwo, A. C. O., Siah, W. F., Hogg, H. D. J., Anwar, H. & Figueiredo, F. C. (2018). Microbial keratitis in corneal grafts: Predisposing factors and outcomes. *Eye (Basingstoke)*, *32*(4), 775–781. https://doi.org/10.1038/eye.2017.310

Oliveira, I. M., Borges, A., Borges, F. & Simões, M. (2019). Repurposing ibuprofen to control Staphylococcus aureus biofilms. *European Journal of Medicinal Chemistry*, *166*, 197–205. https://doi.org/10.1016/J.EJMECH.2019.01.046

Ong, P. Y., Ohtake, T., Brandt, C., Strickland, I., Boguniewicz, M., Ganz, T., Gallo, R. L. & Leung, D. Y. M. (2002). Endogenous Antimicrobial Peptides and Skin Infections in Atopic Dermatitis. *New England Journal of Medicine*, *347*(15), 1151–1160. https://doi.org/10.1056/nejmoa021481

Oordt-Speets, A. M., Bolijn, R., Van Hoorn, R. C., Bhavsar, A. & Kyaw, M. H. (2018). Global etiology of bacterial meningitis: A systematic review and meta-analysis. *PLoS ONE*, *13*(6). https://doi.org/10.1371/JOURNAL.PONE.0198772

Opitz, B., Püschel, A., Schmeck, B., Hocke, A. C., Rosseau, S., Hammerschmidt, S., Schumann, R. R., Suttorp, N. & Hippenstiel, S. (2004). Nucleotide-binding oligomerization domain proteins are innate immune receptors for internalized Streptococcus pneumoniae. *The Journal of Biological Chemistry*, *279*(35), 36426–36432. https://doi.org/10.1074/JBC.M403861200

Organización Mundial de la Salud. (2015). *Global Antimicrobial Resistance and Use Surveillance System (GLASS)*. Who. https://www.who.int/data/gho/data/themes/topics/global-

antimicrobial-resistance-surveillance-system-(glass)

Otri, A. M., Mohammed, I., Al-Aqaba, M. A., Fares, U., Peng, C., Hopkinson, A. & Dua, H. S. (2012). Variable expression of human beta defensins 3 and 9 at the human ocular surface in infectious keratitis. *Investigative Ophthalmology and Visual Science*, *53*(2), 757–761. https://doi.org/10.1167/iovs.11-8467

Pan, Z., Chen, Y., Zhang, W., Jie, Y., Li, N. & Wu, Y. (2003). Rat Corneal Allograft Survival Prolonged by the Superantigen Staphylococcal Enterotoxin B. *Investigative Ophthalmology & Visual Science*, *44*(8), 3346–3351. https://doi.org/10.1167/IOVS.02-0845

Paranjape, S. M., Lauer, T. W., Montelaro, R. C., Mietzner, T. A. & Vij, N. (2013). Modulation of proinflammatory activity by the engineered cationic antimicrobial peptide WLBU-2. *F1000Research*, *2*, 36. https://doi.org/10.12688/F1000RESEARCH.2-36.V1

Park, J., Min, J. S., Kim, B., Chae, U. Bin, Yun, J. W., Choi, M. S., Kong, I. K., Chang, K. T. & Lee, D. S. (2015). Mitochondrial ROS govern the LPS-induced pro-inflammatory response in microglia cells by regulating MAPK and NF-κB pathways. *Neuroscience Letters*, *584*, 191–196. https://doi.org/10.1016/J.NEULET.2014.10.016

Pearlman, E., Sun, Y., Roy, S., Karmakar, M., Hise, A. G., Szczotka-Flynn, L., Ghannoum, M., Chinnery, H. R., McMenamin, P. G. & Rietsch, A. (2013). Host Defense at the Ocular Surface. *International Reviews of Immunology*, *32*(1), 4. https://doi.org/10.3109/08830185.2012.749400

Pendleton, J. N., Gorman, S. P. & Gilmore, B. F. (2013). Clinical relevance of the ESKAPE pathogens. In *Expert Review of Anti-Infective Therapy* (Vol. 11, Issue 3, pp. 297–308). Expert Rev Anti Infect Ther. https://doi.org/10.1586/eri.13.12

Peng, M. Y., Cevallos, V., McLeod, S. D., Lietman, T. M. & Rose-Nussbaumer, J. (2018). Bacterial Keratitis. *Cornea*, *37*(1), 84–87. https://doi.org/10.1097/ICO.0000000000001417

Pericone, C. D., Park, S., Imlay, J. A. & Weiser, J. N. (2003). Factors contributing to hydrogen peroxide resistance in Streptococcus pneumoniae include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the fenton reaction. *Journal of Bacteriology*, *185*(23), 6815–6825. https://doi.org/10.1128/JB.185.23.6815-6825.2003

Peter, G. & Klein, J. O. (2008). Streptococcus pneumoniae. *Principles and Practice of Pediatric Infectious Disease*, 725–733. https://doi.org/10.1016/B978-0-7020-3468-8.50129-2

Petkova, T. D., Huang, L. C., Reins, R. Y. & McDermott, A. M. (2006). Effect of LL–37 (Cathelicidin) on Human Corneal Epithelial Cell Migration and Cytokine Secretion. *Investigative Ophthalmology & Visual Science*, *47*(13), 5019–5019.

Piacenza, L., Trujillo, M. & Radi, R. (2019). Reactive species and pathogen antioxidant networks during phagocytosis. *Journal of Experimental Medicine*, *216*(3), 501–516. https://doi.org/10.1084/JEM.20181886

Pittet, L. A., Quinton, L. J., Yamamoto, K., Robson, B. E., Ferrari, J. D., Algül, H., Schmid, R. M. & Mizgerd, J. P. (2011). Earliest innate immune responses require macrophage RelA during pneumococcal pneumonia. *American Journal of Respiratory Cell and Molecular Biology*, *45*(3), 573–581. https://doi.org/10.1165/rcmb.2010-0210OC

Plüddemann, A., Mukhopadhyay, S., Gordon, S. & William, S. (2011). Innate immunity to intracellular pathogens: macrophage receptors and responses to microbial entry. *Immunological Reviews*, *240*, 11–24.

Ponsoda, X., Jover, R., Castell, J. V. & Gómez-Lechón, M. J. (1991). Measurement of intracellular LDH activity in 96-well cultures: A rapid and automated assay for cytotoxicity

studies. *Journal of Tissue Culture Methods 1991 13:1*, *13*(1), 21–24. https://doi.org/10.1007/BF02388199

Poole, K. (2011). Pseudomonas aeruginosa: Resistance to the max. *Frontiers in Microbiology*, *2*(APR), 65. https://doi.org/10.3389/fmicb.2011.00065

Prasad, S. V., Fiedoruk, K., Daniluk, T., Piktel, E. & Bucki, R. (2019). Expression and Function of Host Defense Peptides at Inflammation Sites. *International Journal of Molecular Sciences*, *21*(1). https://doi.org/10.3390/IJMS21010104

Puig, M., Weiss, M., Salinas, R., A Johnson, D. & Kheirkhah, A. (2020). Etiology and Risk Factors for Infectious Keratitis in South Texas. *Journal of Ophthalmic and Vision Research*, *15*(2), 128. https://doi.org/10.18502/jovr.v15i2.6729

Pütsep, K. & Faye, I. (2009). Hans G Boman (1924-2008): pioneer in peptide-mediated innate immune defence. *Scandinavian Journal of Immunology*, *70*(3), 317–319. https://doi.org/10.1111/j.1365-3083.2009.02293.x

Qiu, D.-H., Huang, Z.-L., Zhou, T., Shen, C. & Hider, R. C. (2011). In vitro inhibition of bacterial growth by iron chelators. *FEMS Microbiology Letters*, *314*(2), 107–111. https://doi.org/10.1111/J.1574-6968.2010.02153.X

Rafii, S., Roda, D., Geuna, E., Jimenez, B., Rihawi, K., Capelan, M., Yap, T. A., Molife, L. R., Kaye, S. B., De Bono, J. S. & Banerji, U. (2015). Higher risk of infections with PI3K-AKT-mTOR pathway inhibitors in patients with advanced solid tumors on phase I clinical trials. *Clinical Cancer Research*, *21*(8), 1869–1876. https://doi.org/10.1158/1078-0432.CCR-14-2424

Rai, P., Parrish, M., Tay, I. J. J., Li, N., Ackerman, S., He, F., Kwang, J., Chow, V. T. & Engelward, B. P. (2015). Streptococcus pneumoniae secretes hydrogen peroxide leading to DNA damage and apoptosis in lung cells. *Proceedings of the National Academy of Sciences*, *112*(26), E3421–E3430. https://doi.org/10.1073/PNAS.1424144112

Ramos, R., Silva, J. P., Rodrigues, A. C., Costa, R., Guardão, L., Schmitt, F., Soares, R., Vilanova, M., Domingues, L. & Gama, M. (2011). Wound healing activity of the human antimicrobial peptide LL37. *Peptides*, *32*(7), 1469–1476. https://doi.org/10.1016/J.PEPTIDES.2011.06.005

Rao, L., De La Rosa, I., Xu, Y., Sha, Y., Bhattacharya, A., Holtzman, M. J., Gilbert, B. E. & Eissa, N. T. (2021). Pseudomonas aeruginosa survives in epithelia by ExoS-mediated inhibition of autophagy and mTOR. *EMBO Reports*, *22*(2), e50613. https://doi.org/10.15252/embr.202050613

Raval, Y. S., Mohamed, A., Zmuda, H. M., Patel, R. & Beyenal, H. (2019). Antibiotic Resistance: Hydrogen-Peroxide-Generating Electrochemical Scaffold Eradicates Methicillin-Resistant Staphylococcus aureus Biofilms (Global Challenges 6/2019). *Global Challenges*, *3*(6), 1970061. https://doi.org/10.1002/GCH2.201970061

Ray, P. D., Huang, B. W. & Tsuji, Y. (2012). Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular Signalling*, *24*(5), 981. https://doi.org/10.1016/J.CELLSIG.2012.01.008

Redfern, R. L., Reins, R. Y. & McDermott, A. M. (2011). Toll-like receptor activation modulates antimicrobial peptide expression by ocular surface cells. *Experimental Eye Research*, *92*(3), 209–220. https://doi.org/10.1016/J.EXER.2010.12.005

Rivas-Santiago, B., Schwander, S. K., Sarabia, C., Diamond, G., Klein-Patel, M. E., Hernandez-Pando, R., Ellner, J. J. & Sada, E. (2005). Human β-defensin 2 is expressed and associated with Mycobacterium tuberculosis during infection of human alveolar epithelial cells.

Infection and Immunity, *73*(8), 4505–4511. https://doi.org/10.1128/IAI.73.8.4505-4511.2005/ASSET/A3794F67-0A9B-498F-A953-BEDD742D4349/ASSETS/GRAPHIC/ZII0080550320006.JPEG

Roy, S., Karmakar, M. & Pearlman, E. (2014). CD14 mediates Toll-like receptor 4 (TLR4) endocytosis and spleen tyrosine kinase (Syk) and interferon regulatory transcription factor 3 (IRF3) activation in epithelial cells and impairs neutrophil infiltration and Pseudomonas aeruginosa killing in vivo. *The Journal of Biological Chemistry*, *289*(2), 1174–1182. https://doi.org/10.1074/JBC.M113.523167

Roy, S., Marla, S. & Praneetha, D. C. (2015). Recognition of Corynebacterium pseudodiphtheriticum by toll-like receptors and up-regulation of antimicrobial peptides in human corneal epithelial cells. *Virulence*, *6*(7), 716–721. https://doi.org/10.1080/21505594.2015.1066063

Roy, S., Sun, Y. & Pearlman, E. (2011). Interferon-γ-induced MD-2 Protein Expression and Lipopolysaccharide (LPS) Responsiveness in Corneal Epithelial Cells Is Mediated by Janus Tyrosine Kinase-2 Activation and Direct Binding of STAT1 Protein to the MD-2 Promoter. *Journal of Biological Chemistry*, *286*(27), 23753–23762. https://doi.org/10.1074/jbc.M111.219345

Rushmore, T. H., Morton, M. R. & Pickett, C. B. (1991). The antioxidant responsive element: Activation by oxidative stress and identification of the DNA consensus sequence required for functional activity. *Journal of Biological Chemistry*, *266*(18), 11632–11639. https://doi.org/10.1016/s0021-9258(18)99004-6

Sagerfors, S., Ejdervik-Lindblad, B. & Söderquist, B. Infectious keratitis: isolated microbes and their antibiotic susceptibility pattern during 2004–2014 in Region Örebro County, Sweden. *Acta Ophthalmologica*, *98*(3), 255–260. https://doi.org/10.1111/aos.14256

Sani, M. A. & Separovic, F. (2016). How Membrane-Active Peptides Get into Lipid Membranes. *Accounts of Chemical Research*, *49*(6), 1130–1138. https://doi.org/10.1021/acs.accounts.6b00074

Sareila, O., Kelkka, T., Pizzolla, A., Hultqvist, M. & Holmdahl, R. (2011). NOX2 complex-derived ROS as immune regulators. *Antioxidants & Redox Signaling*, *15*(8), 2197–2208. https://doi.org/10.1089/ARS.2010.3635

Sato, H. & Frank, D. W. (2004). ExoU is a potent intracellular phospholipase. In *Molecular Microbiology* (Vol. 53, Issue 5, pp. 1279–1290). https://doi.org/10.1111/j.1365-2958.2004.04194.x

Sauer, A., Greth, M., Letsch, J., Becmeur, P.-H., Borderie, V., Daien, V., Bron, A., Creuzot-Garcher, C., Kodjikian, L., Burillon, C., Robert, P.-Y., Mouriaux, F., Muraine, M., Gueudry, J., Malecaze, F., Cochener, B., Chiquet, C., Labetoulle, M. & Bourcier, T. (2020). Contact Lenses and Infectious Keratitis. *Cornea*, *39*(6), 769–774. https://doi.org/10.1097/ICO.0000000000002248

Sawa, T. (2014). The molecular mechanism of acute lung injury caused by Pseudomonas aeruginosa: from bacterial pathogenesis to host response. *Journal of Intensive Care 2014 2:1*, *2*(1), 1–11. https://doi.org/10.1186/2052-0492-2-10

Saxton, R. A. & Sabatini, D. M. (2017). mTOR Signaling in Growth, Metabolism, and Disease. In *Cell* (Vol. 168, Issue 6, pp. 960–976). Cell Press. https://doi.org/10.1016/j.cell.2017.02.004

Schaefer, F., Bruttin, O., Zografos, L. & Guex-Crosier, Y. (2001). Bacterial keratitis: A prospective clinical and microbiological study. *British Journal of Ophthalmology*, *85*(7), 842–847. https://doi.org/10.1136/bjo.85.7.842

Scharf, S., Hippenstiel, S., Flieger, A., Suttorp, N. & N'Guessan, P. D. (2010). Induction of human β-defensin-2 in pulmonary epithelial cells by Legionella pneumophila: Involvement of TLR2 and TLR5, p38 MAPK, JNK, NF-κB, and AP-1. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *298*(5), 687–695. https://doi.org/10.1152/AJPLUNG.00365.2009/ASSET/IMAGES/LARGE/ZH50051056570007.JPEG

Schauber, J., Svanholm, C., Termén, S., Iffland, K., Menzel, T., Scheppach, W., Melcher, R., Agerberth, B., Lührs, H. & Gudmundsson, G. H. (2003). Expression of the cathelicidin LL-37 is modulated by short chain fatty acids in colonocytes: Relevance of signalling pathways. *Gut*, *52*(5), 735–741. https://doi.org/10.1136/gut.52.5.735

Scheenstra, M. R., van Harten, R. M., Veldhuizen, E. J. A., Haagsman, H. P. & Coorens, M. (2020). Cathelicidins Modulate TLR-Activation and Inflammation. In *Frontiers in Immunology* (Vol. 11, p. 1137). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2020.01137

Schmeck, B., Huber, S., Moog, K., Zahlten, J., Hocke, A. C., Opitz, B., Hammerschmidt, S., Mitchell, T. J., Kracht, M., Rosseau, S., Suttorp, N. & Hippenstiel, S. (2006). Pneumococci induced TLR- and Rac1-dependent NF-kappaB-recruitment to the IL-8 promoter in lung epithelial cells. *American Journal of Physiology. Lung Cellular and Molecular Physiology*, *290*(4). https://doi.org/10.1152/AJPLUNG.00271.2005

Schmeck, B., Moog, K., Zahlten, J., van Laak, V., N'Guessan, P. D., Opitz, B., Rosseau, S., Suttorp, N. & Hippenstiel, S. (2006). Streptococcus pneumoniae induced c-Jun-N-terminal kinase- and AP-I -dependent IL-8 release by lung epithelial BEAS-2B cells. *Respiratory Research*, *7*(1), 1–9. https://doi.org/10.1186/1465-9921-7-98/FIGURES/4_489

Schmeck, B., Zahlten, J., Moog, K., Van Laak, V., Huber, S., Hocke, A. C., Opitz, B., Hoffmann, E., Kracht, M., Zerrahn, J., Hammerschmidt, S., Rosseau, S., Suttorp, N. & Hippenstiel, S. (2004). Streptococcus pneumoniae-induced p38 MAPK-dependent Phosphorylation of RelA at the Interleukin-8 Promotor *. *Journal of Biological Chemistry*, *279*(51), 53241–53247. https://doi.org/10.1074/JBC.M313702200

Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods*, *9*(7), 671–675. https://doi.org/10.1038/NMETH.2089

Schreier, T., Degen, E. & Baschong, W. (1993). Fibroblast migration and proliferation during in vitro wound healing. *Research in Experimental Medicine 1993 193:1*, *193*(1), 195–205. https://doi.org/10.1007/BF02576227

Schröder, N. W. J., Morath, S., Alexander, C., Hamann, L., Hartung, T., Zähringer, U., Göbel, U. B., Weber, J. R. & Schumann, R. R. (2003). Lipoteichoic acid (LTA) of Streptococcus pneumoniae and Staphylococcus aureus activates immune cells via Toll-like receptor (TLR)-2, lipopolysaccharide-binding protein (LBP), and CD14, whereas TLR-4 and MD-2 are not involved. *The Journal of Biological Chemistry*, *278*(18), 15587–15594. https://doi.org/10.1074/JBC.M212829200

Schubert, F., Sekundo, W. & Paul, C. (2020). Treatment refractory multidrug-resistant bacterial keratitis. In *Ophthalmologe* (pp. 1–4). Springer Medizin. https://doi.org/10.1007/s00347-020-01129-y

Schust, J., Sperl, B., Hollis, A., Mayer, T. U. & Berg, T. (2006). Stattic: A Small-Molecule Inhibitor of STAT3 Activation and Dimerization. *Chemistry & Biology*, *13*(11), 1235–1242. https://doi.org/10.1016/J.CHEMBIOL.2006.09.018

Seil, M., Nagant, C., Dehaye, J. P., Vandenbranden, M. & Lensink, M. F. (2010). Spotlight on Human LL-37, an Immunomodulatory Peptide with Promising Cell-Penetrating Properties.

Pharmaceuticals, *3*(11), 3435. https://doi.org/10.3390/PH3113435

Semple, F. & Dorin, J. R. (2012). β-Defensins: Multifunctional Modulators of Infection, Inflammation and More? *Journal of Innate Immunity*, *4*(4), 337–348. https://doi.org/10.1159/000336619

Shamsuddin, N., Blair, J. & Kumar, A. (2011). Toll Like Receptor 2 Mediates The Innate Immune Response Of Retinal Muller Glia To Staphylococcus Aureus. *Investigative Ophthalmology & Visual Science*, *52*(14), 2959–2959.

Sharma, A. (2021). *Indian Priority Pathogen List TO GUIDE RESEARCH, DISCOVERY AND DEVELOPMENT OF NEW ANTIBIOTICS IN INDIA*.

Shaykhiev, R., Beißwenger, C., Kändler, K., Senske, J., Püchner, A., Damm, T., Behr, J. & Bals, R. (2005). Human endogenous antibiotic LL-37 stimulates airway epithelial cell proliferation and wound closure. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *289*(5 33-5), 842–848. https://doi.org/10.1152/AJPLUNG.00286.2004/ASSET/IMAGES/LARGE/ZH50110543680005.JPEG

Shen, E. P., Hsieh, Y.-T., Chu, H.-S., Chang, S.-C. & Hu, F.-R. (2015). Correlation of Pseudomonas aeruginosa Genotype With Antibiotic Susceptibility and Clinical Features of Induced Central Keratitis. *Investigative Ophthalmology & Visual Science*, *56*(1), 365–371. https://doi.org/10.1167/IOVS.14-15241

Sheremet, A. B., Nesterenko, L. N. & Zigangirova, N. A. (2020). The Type Three Secretion System of Pseudomonas aeruginosa as a Target for Development of Antivirulence Drugs. In *Molecular Genetics, Microbiology and Virology* (Vol. 35, Issue 1, pp. 1–13). Pleiades Publishing. https://doi.org/10.3103/S0891416820010073

Shibata, W., Hirata, Y., Yoshida, H., Otsuka, M., Hoshida, Y., Ogura, K., Maeda, S., Ohmae, T., Yanai, A., Mitsuno, Y., Seki, N., Kawabe, T. & Omata, M. (2005). NF-kB and ERK-signaling pathways contribute to the gene expression induced by cag PAI-positive-Helicobacter pylori infection. *World Journal of Gastroenterology : WJG*, *11*(39), 6134. https://doi.org/10.3748/WJG.V11.I39.6134

Shizukuishi, S., Ogawa, M., Matsunaga, S., Tomokiyo, M., Ikebe, T., Fushinobu, S., Ryo, A. & Ohnishi, M. (2020). Streptococcus pneumoniae hijacks host autophagy by deploying CbpC as a decoy for Atg14 depletion. *EMBO Reports*, *21*(5). https://doi.org/10.15252/EMBR.201949232

Sigurdsson, S., Erlendsdóttir, H., Quirk, S. J., Kristjánsson, J., Hauksson, K., Andrésdóttir, B. D. I., Jónsson, A. J., Halldórsson, K. H., Sæmundsson, Á., Ólason, Ó. H., Hrafnkelsson, B., Kristinsson, K. G. & Haraldsson, Á. (2017). Pneumococcal vaccination: Direct and herd effect on carriage of vaccine types and antibiotic resistance in Icelandic children. *Vaccine*, *35*(39), 5242–5248. https://doi.org/10.1016/J.VACCINE.2017.08.020

Sikora, A. & Zahra, F. (2021). Nosocomial Infections. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK559312/

Simonetti, O., Cirioni, O., Goteri, G., Lucarini, G., Kamysz, E., Kamysz, W., Orlando, F., Rizzetto, G., Molinelli, E., Morroni, G., Ghiselli, R., Provinciali, M., Giacometti, A. & Offidani, A. (2021). Efficacy of Cathelicidin LL-37 in an MRSA Wound Infection Mouse Model. *Antibiotics (Basel, Switzerland)*, *10*(10). https://doi.org/10.3390/ANTIBIOTICS10101210

Singh, M., Gour, A., Gandhi, A., Mathur, U. & Farooqui, J. H. (2020). Demographic details, risk factors, microbiological profile, and clinical outcomes of pediatric infectious keratitis cases

in North India. *Indian Journal of Ophthalmology*, *68*(3), 434–440. https://doi.org/10.4103/ijo.IJO_928_19

Skerrett, S. J., Wilson, C. B., Liggitt, H. D. & Hajjar, A. M. (2007). Redundant Toll-like receptor signaling in the pulmonary host response to Pseudomonas aeruginosa. *Https://Doi.Org/10.1152/Ajplung.00250.2006*, *292*(1). https://doi.org/10.1152/AJPLUNG.00250.2006

Sommer, M. O. A., Dantas, G. & Church, G. M. (2009). Functional characterization of the antibiotic resistance reservoir in the human microflora. *Science*, *325*(5944), 1128–1131. https://doi.org/10.1126/science.1176950

Sørensen, O. E., Thapa, D. R., Rosenthal, A., Liu, L., Roberts, A. A. & Ganz, T. (2005). Differential Regulation of β-Defensin Expression in Human Skin by Microbial Stimuli. *The Journal of Immunology*, *174*(8), 4870–4879. https://doi.org/10.4049/JIMMUNOL.174.8.4870

Spencer, J. D., Schwaderer, A. L., Wang, H., Bartz, J., Kline, J., Eichler, T., Desouza, K. R., Sims-Lucas, S., Baker, P. & Hains, D. S. (2013). Ribonuclease 7, an antimicrobial peptide upregulated during infection, contributes to microbial defense of the human urinary tract. *Kidney International*, *83*(4), 615–625. https://doi.org/10.1038/KI.2012.410

Spohn, R., Daruka, L., Lázár, V., Martins, A., Vidovics, F., Grézal, G., Méhi, O., Kintses, B., Számel, M., Jangir, P. K., Csörgő, B., Györkei, Á., Bódi, Z., Faragó, A., Bodai, L., Földesi, I., Kata, D., Maróti, G., Pap, B., … Pál, C. (2019). Integrated evolutionary analysis reveals antimicrobial peptides with limited resistance. *Nature Communications*, *10*(1), 1–13. https://doi.org/10.1038/s41467-019-12364-6

Sridhar, M. S. (2018). Anatomy of cornea and ocular surface. *Indian Journal of Ophthalmology*. https://doi.org/10.4103/ijo.IJO_646_17

Srinivasan, M., Gonzales, C. A., George, C., Cevallos, V., Mascarenhas, J. M., Asokan, B., Wilkins, J., Smolin, G. & Whitcher, J. P. (1997). Epidemiology and aetiological diagnosis of corneal ulceration in Madurai, south India. *British Journal of Ophthalmology*, *81*(11), 965–971. https://doi.org/10.1136/BJO.81.11.965

Steiner, H., Hultmark, D., Engström, Å., Bennich, H. & Boman, H. G. (1981). Sequence and specificity of two antibacterial proteins involved in insect immunity. *Nature*, *292*(5820), 246–248. https://doi.org/10.1038/292246a0

Stevens, J. M., Galyov, E. E. & Stevens, M. P. (2006). Actin-dependent movement of bacterial pathogens. In *Nature Reviews Microbiology* (Vol. 4, Issue 2, pp. 91–101). Nature Publishing Group. https://doi.org/10.1038/nrmicro1320

Stevens, T. C., Ochoa, C. D., Morrow, K. A., Robson, M. J., Prasain, N., Zhou, C., Alvarez, D. F., Frank, D. W., Balczon, R. & Stevens, T. (2014). The Pseudomonas aeruginosa exoenzyme Y impairs endothelial cell proliferation and vascular repair following lung injury. *American Journal of Physiology - Lung Cellular and Molecular Physiology*, *306*(10). https://doi.org/10.1152/ajplung.00135.2013

Streptococcus pneumoniae: For Clinicians | CDC. (n.d.). Retrieved December 11, 2021, from https://www.cdc.gov/pneumococcal/clinicians/streptococcus-pneumoniae.html

Stryjewski, M. E., Hall, R. P., Chu, V. H., Kanafani, Z. A., O'Riordan, W. D., Weinstock, M. S., Stienecker, R. S., Streilein, R., Dorschner, R. A., Fowler, Jr., V. G., Corey, G. R. & Gallo, R. L. (2007). Expression of Antimicrobial Peptides in the Normal and Involved Skin of Patients with Infective Cellulitis. *The Journal of Infectious Diseases*, *196*(9), 1425–1430. https://doi.org/10.1086/522630

Subedi, D., Vijay, A. K. & Willcox, M. (2018). Overview of mechanisms of antibiotic resistance in *Pseudomonas aeruginosa* : an ocular perspective. *Clinical and Experimental Optometry*, *101*(2), 162–171. https://doi.org/10.1111/cxo.12621

Sultana, S. T., Atci, E., Babauta, J. T., Mohamed Falghoush, A., Snekvik, K. R., Call, D. R. & Beyenal, H. (2015). Electrochemical scaffold generates localized, low concentration of hydrogen peroxide that inhibits bacterial pathogens and biofilms. *Scientific Reports 2015 5:1*, *5*(1), 1–10. https://doi.org/10.1038/srep14908

Sun, Y., Karmakar, M., Taylor, P. R., Rietsch, A. & Pearlman, E. (2012). ExoS and ExoT ADP Ribosyltransferase Activities Mediate Pseudomonas aeruginosa Keratitis by Promoting Neutrophil Apoptosis and Bacterial Survival. *The Journal of Immunology*, *188*(4), 1884–1895. https://doi.org/10.4049/jimmunol.1102148

Suwal, S., Bhandari, D., Thapa, P., Shrestha, M. K. & Amatya, J. (2016). Microbiological profile of corneal ulcer cases diagnosed in a tertiary care ophthalmological institute in Nepal. *BMC Ophthalmology*, *16*(1), 1–6. https://doi.org/10.1186/S12886-016-0388-9/TABLES/3

Tan, S. Y. & Tatsumura, Y. (2015). Alexander Fleming (1881–1955): Discoverer of penicillin. *Singapore Medical Journal*, *56*(7), 366. https://doi.org/10.11622/SMEDJ.2015105

Tang, A., Caballero, A. R., Marquart, M. E., Bierdeman, M. A. & O'callaghan, R. J. (2018). Mechanism of pseudomonas aeruginosa small protease (PASP), a corneal virulence factor. *Investigative Ophthalmology and Visual Science*, *59*(15), 5993–6002. https://doi.org/10.1167/iovs.18-25834

Teweldemedhin, M., Gebreyesus, H., Atsbaha, A. H., Asgedom, S. W. & Saravanan, M. (2017). Bacterial profile of ocular infections: A systematic review. *BMC Ophthalmology*, *17*(1), 1–9. https://doi.org/10.1186/S12886-017-0612-2/TABLES/3

Thanabalasuriar, A., Scott, B. N. V., Peiseler, M., Willson, M. E., Zeng, Z., Warrener, P., Keller, A. E., Surewaard, B. G. J., Dozier, E. A., Korhonen, J. T., Cheng, L. I. tin., Gadjeva, M., Stover, C. K., DiGiandomenico, A. & Kubes, P. (2019). Neutrophil Extracellular Traps Confine Pseudomonas aeruginosa Ocular Biofilms and Restrict Brain Invasion. *Cell Host and Microbe*, *25*(4), 526-536.e4. https://doi.org/10.1016/j.chom.2019.02.007

Thomas-Virnig, C. L., Centanni, J. M., Johnston, C. E., He, L. K., Schlosser, S. J., Van Winkle, K. F., Chen, R., Gibson, A. L., Szilagyi, A., Li, L., Shankar, R. & Allen-Hoffmann, B. L. (2009). Inhibition of Multidrug-resistant Acinetobacter baumannii by Nonviral Expression of hCAP-18 in a Bioengineered Human Skin Tissue. *Molecular Therapy: The Journal of the American Society of Gene Therapy*, *17*(3), 562. https://doi.org/10.1038/MT.2008.289

Ting, D. S. J., Ho, C. S., Cairns, J., Elsahn, A., Al-Aqaba, M., Boswell, T., Said, D. G. & Dua, H. S. (2021). 12-year analysis of incidence, microbiological profiles and in vitro antimicrobial susceptibility of infectious keratitis: The Nottingham Infectious Keratitis Study. *British Journal of Ophthalmology*, *105*(3), 328–333. https://doi.org/10.1136/bjophthalmol-2020-316128

Ting, D. S. J., Ho, C. S., Deshmukh, R., Said, D. G. & Dua, H. S. (2021). Infectious keratitis: an update on epidemiology, causative microorganisms, risk factors, and antimicrobial resistance. In *Eye (Basingstoke)* (Vol. 35, Issue 4, pp. 1084–1101). Springer Nature. https://doi.org/10.1038/s41433-020-01339-3

Tokumaru, S., Sayama, K., Shirakata, Y., Komatsuzawa, H., Ouhara, K., Hanakawa, Y., Yahata, Y., Dai, X., Tohyama, M., Nagai, H., Yang, L., Higashiyama, S., Yoshimura, A., Sugai, M. & Hashimoto, K. (2005). Induction of Keratinocyte Migration via Transactivation of the Epidermal Growth Factor Receptor by the Antimicrobial Peptide LL-37. *The Journal of Immunology*, *175*(7), 4662–4668. https://doi.org/10.4049/JIMMUNOL.175.7.4662

Tomlinson, G., Chimalapati, S., Pollard, T., Lapp, T., Cohen, J., Camberlein, E., Stafford, S., Periselneris, J., Aldridge, C., Vollmer, W., Picard, C., Casanova, J.-L., Noursadeghi, M. & Brown, J. (2014). TLR-Mediated Inflammatory Responses to Streptococcus pneumoniae Are Highly Dependent on Surface Expression of Bacterial Lipoproteins. *The Journal of Immunology Author Choice*, *193*(7), 3736. https://doi.org/10.4049/JIMMUNOL.1401413

Torres, J. M., Cardenas, O., Vasquez, A. & Schlossberg, D. (1998). Streptococcus pneumoniae Bacteremia in a Community Hospital. *Chest*, *113*(2), 387–390. https://doi.org/10.1378/CHEST.113.2.387

Travassos, L. H., Carneiro, L. A. M., Girardin, S. E., Boneca, I. G., Lemos, R., Bozza, M. T., Domingues, R. C. P., Coyle, A. J., Bertin, J., Philpott, D. J. & Plotkowski, M. C. (2005). Nod1 Participates in the Innate Immune Response to Pseudomonas aeruginosa*. *Journal of Biological Chemistry*, *280*(44), 36714–36718. https://doi.org/10.1074/JBC.M501649200

Tsan, M. F. (2006). OXYGEN TOXICITY. *Encyclopedia of Respiratory Medicine, Four-Volume Set*, 282–289. https://doi.org/10.1016/B0-12-370879-6/00287-8

Tullos, N. A., Thompson, H. W., Taylor, S. D., Sanders, M., Norcross, E. W., Tolo, I., Moore, Q. & Marquart, M. E. (2013). Modulation of immune signaling, bacterial clearance, and corneal integrity by toll-like receptors during streptococcus pneumoniae keratitis. *Current Eye Research*, *38*(10), 1036–1048. https://doi.org/10.3109/02713683.2013.804094

Türkoğlu, O., Kandiloğlu, G., Berdeli, A., Emingil, G. & Atilla, G. (2011). Antimicrobial peptide hCAP-18/LL-37 protein and mRNA expressions in different periodontal diseases. *Oral Diseases*, *17*(1), 60–67. https://doi.org/10.1111/J.1601-0825.2010.01704.X

Turner, J., Cho, Y., Dinh, N. N., Waring, A. J. & Lehrer, R. I. (1998). Activities of LL-37, a cathelin-associated antimicrobial peptide of human neutrophils. *Antimicrobial Agents and Chemotherapy*, *42*(9), 2206–2214. https://doi.org/10.1128/AAC.42.9.2206

Ung, L., Bispo, P. J. M., Shanbhag, S. S., Gilmore, M. S. & Chodosh, J. (2019). The persistent dilemma of microbial keratitis: Global burden, diagnosis, and antimicrobial resistance. In *Survey of Ophthalmology* (Vol. 64, Issue 3, pp. 255–271). Elsevier USA. https://doi.org/10.1016/j.survophthal.2018.12.003

Uribe-Quero, E. & Rosales, C. (2017). Control of phagocytosis by microbial pathogens. *Frontiers in Immunology*, *8*(OCT), 1368. https://doi.org/10.3389/FIMMU.2017.01368/BIBTEX

Urwin, L., Okurowska, K., Crowther, G., Roy, S., Garg, P., Karunakaran, E., MacNeil, S., Partridge, L. J., Green, L. R. & Monk, P. N. (2020). Corneal Infection Models: Tools to Investigate the Role of Biofilms in Bacterial Keratitis. *Cells 2020, Vol. 9, Page 2450*, *9*(11), 2450. https://doi.org/10.3390/CELLS9112450

Usher, L. R., Lawson, R. A., Geary, I., Taylor, C. J., Bingle, C. D., Taylor, G. W. & Whyte, M. K. B. (2002). Induction of Neutrophil Apoptosis by the Pseudomonas aeruginosa Exotoxin Pyocyanin: A Potential Mechanism of Persistent Infection. *The Journal of Immunology*, *168*(4), 1861–1868. https://doi.org/10.4049/jimmunol.168.4.1861

Uusitalo, P., Hägglund, U., Rhöös, E., Scherman Norberg, H., Elofsson, M. & Sundin, C. (2017). The salicylidene acylhydrazide INP0341 attenuates Pseudomonas aeruginosa virulence in vitro and in vivo. *The Journal of Antibiotics 2017 70:9*, *70*(9), 937–943. https://doi.org/10.1038/ja.2017.64

Vareechon, C., Zmina, S. E., Karmakar, M., Pearlman, E. & Rietsch, A. (2017). Pseudomonas aeruginosa Effector ExoS Inhibits ROS Production in Human Neutrophils. *Cell Host and Microbe*. https://doi.org/10.1016/j.chom.2017.04.001

Varriale, L., Dipineto, L., Russo, T. P., Borrelli, L., Romano, V., D'Orazio, S., Pace, A., Menna,

L. F., Fioretti, A. & Santaniello, A. (2020). Antimicrobial Resistance of Escherichia coli and Pseudomonas aeruginosa from Companion Birds. *Antibiotics*, *9*(11), 780. https://doi.org/10.3390/antibiotics9110780

Vasil, M. L. (1986). Pseudomonas aeruginosa: Biology, mechanisms of virulence, epidemiology. *The Journal of Pediatrics*, *108*(5), 800–805. https://doi.org/10.1016/S0022-3476(86)80748-X

Vazirani, J., Wurity, S. & Ali, M. H. (2015). Multidrug-resistant Pseudomonas aeruginosa keratitis: Risk factors, clinical characteristics, and outcomes. *Ophthalmology*, *122*(10), 2110–2114. https://doi.org/10.1016/j.ophtha.2015.06.007

Venugopal, R. & Jaiswal, A. K. (1996). Nrf1 and Nrf2 positively and c-Fos and Fra1 negatively regulate the human antioxidant response element-mediated expression of NAD(P)H:quinone oxidoreductase1 gene. *Proceedings of the National Academy of Sciences of the United States of America*, *93*(25), 14960–14965. https://doi.org/10.1073/PNAS.93.25.14960

Vos, A. F. de, Dessing, M. C., Lammers, A. J. J., Porto, A. P. N. A. de, Florquin, S., Boer, O. J. de, Beer, R. de, Terpstra, S., Bootsma, H. J., Hermans, P. W., Veer, C. van 't & Poll, T. van der. (2015). The Polysaccharide Capsule of Streptococcus pneumonia Partially Impedes MyD88-Mediated Immunity during Pneumonia in Mice. *PLOS ONE*, *10*(2), e0118181. https://doi.org/10.1371/JOURNAL.PONE.0118181

Wallace, H. A., Basehore, B. M. & Zito, P. M. (2021). Wound Healing Phases. *StatPearls*. https://www.ncbi.nlm.nih.gov/books/NBK470443/

Wang, Guan, Fu, Y., Ma, K., Liu, J. & Liu, X. (2020). NOD2 regulates microglial inflammation through the TAK1-NF-κB pathway and autophagy activation in murine pneumococcal meningitis. *Brain Research Bulletin*, *158*, 20–30. https://doi.org/10.1016/J.BRAINRESBULL.2020.02.006

Wang, Guangshun, Epand, R. F., Mishra, B., Lushnikova, T., Thomas, V. C., Bayles, K. W. & Epand, R. M. (2012). Decoding the functional roles of cationic side chains of the major antimicrobial region of human cathelicidin LL-37. *Antimicrobial Agents and Chemotherapy*, *56*(2), 845–856. https://doi.org/10.1128/AAC.05637-11

Wang, L., Zhang, X., Wu, G., Qi, Y., Zhang, J., Yang, J., Wang, H. & Xu, W. (2020). Streptococcus pneumoniae aminopeptidase N contributes to bacterial virulence and elicits a strong innate immune response through MAPK and PI3K/AKT signaling. *Journal of Microbiology 2020 58:4*, *58*(4), 330–339. https://doi.org/10.1007/S12275-020-9538-0

Wanke, I., Steffen, H., Christ, C., Krismer, B., Götz, F., Peschel, A., Schaller, M. & Schittek, B. (2011). Skin Commensals Amplify the Innate Immune Response to Pathogens by Activation of Distinct Signaling Pathways. *Journal of Investigative Dermatology*, *131*(2), 382–390. https://doi.org/10.1038/JID.2010.328

Watson, S. L., Gatus, B. J., Cabrera-Aguas, M., Armstrong, B. H., George, R., Khoo, P. & Lahra, M. M. (2020). *Bacterial Ocular Surveillance System (BOSS) Sydney, Australia 2017-2018*. https://doi.org/10.33321/cdi.2020.44.86

Wei, Z. & Liu, H. T. (2002). MAPK signal pathways in the regulation of cell proliferation in mammalian cells. *Cell Research 2002 12:1*, *12*(1), 9–18. https://doi.org/10.1038/sj.cr.7290105

Weichhart, T., Hengstschläger, M. & Linke, M. (2015). Regulation of innate immune cell function by mTOR. In *Nature Reviews Immunology* (Vol. 15, Issue 10, pp. 599–614). Nature Publishing Group. https://doi.org/10.1038/nri3901

WHO publishes list of bacteria for which new antibiotics are urgently needed. (n.d.). Retrieved

July 23, 2020, from https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Wilson, S. E. (2020). Bowman's layer in the cornea– structure and function and regeneration. *Experimental Eye Research*, *195*. https://doi.org/10.1016/j.exer.2020.108033

World Health Organization (WHO). (2020). GLASS whole-genome sequencing for surveillance of antimicrobial resistance: Global Antimicrobial Resistance and Use Surveillance System (GLASS). In *Who*. https://apps.who.int/iris/bitstream/handle/10665/332081/9789240005587-eng.pdf?ua=1

Wu, Y. T., Tan, H. L., Huang, Q., Ong, C. N. & Shen, H. M. (2009). Activation of the PI3K-Akt-mTOR signaling pathway promotes necrotic cell death via suppression of autophagy. *Autophagy*, *5*(6), 824–834. https://doi.org/10.4161/auto.9099

Yahr, T. L., Vallis, A. J., Hancock, M. K., Barbieri, J. T. & Frank, D. W. (1998). ExoY, an adenylate cyclase secreted by the Pseudomonas aeruginosa type III system. In *National Acad Sciences* (Vol. 95). www.pnas.org.

Yang, J., Wise, L. & Fukuchi, K. I. (2020). TLR4 Cross-Talk With NLRP3 Inflammasome and Complement Signaling Pathways in Alzheimer's Disease. In *Frontiers in Immunology* (Vol. 11, p. 724). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2020.00724

Yang, Z. J., Chee, C. E., Huang, S. & Sinicrope, F. A. (2011). The Role of Autophagy in Cancer: Therapeutic Implications. *Molecular Cancer Therapeutics*, *10*(9), 1533–1541. https://doi.org/10.1158/1535-7163.MCT-11-0047

Yazici, A., Ortucu, S., Taskin, M. & Marinelli, L. (2018). Natural-based Antibiofilm and Antimicrobial Peptides from Microorganisms. *Current Topics in Medicinal Chemistry*, *18*(24), 2102–2107. https://doi.org/10.2174/1568026618666181112143351

Ye, X., Zhou, X. J. & Zhang, H. (2018). Exploring the role of autophagy-related gene 5 (ATG5) yields important insights into autophagy in autoimmune/autoinflammatory diseases. In *Frontiers in Immunology* (Vol. 9, Issue OCT, p. 2334). Frontiers Media S.A. https://doi.org/10.3389/fimmu.2018.02334

Yesilkaya, H., Farshchi Andisi, V., Andrew, P. W. & Bijlsma, J. J. E. (2013). Streptococcus pneumoniae and reactive oxygen species: an unusual approach to living with radicals. *Trends in Microbiology*, *21*, 187–195. https://doi.org/10.1016/j.tim.2013.01.004

Zaiou, M., Nizet, V. & Gallo, R. L. (2003). Antimicrobial and protease inhibitory functions of the human cathelicidin (hCAP18/LL-37) prosequence. *The Journal of Investigative Dermatology*, *120*(5), 810–816. https://doi.org/10.1046/J.1523-1747.2003.12132.X

Zarubin, T. & Han, J. (2005). Activation and signaling of the p38 MAP kinase pathway. *Cell Research*, *15*(1), 11–18. https://doi.org/10.1038/SJ.CR.7290257

Zhang, J., Wu, X. Y. & Yu, F. S. X. (2005). Inflammatory responses of corneal epithelial cells to Pseudomonas aeruginosa infection. *Current Eye Research*, *30*(7), 527–534. https://doi.org/10.1080/02713680590968150

Zhang, Q. Y., Yan, Z. Bin, Meng, Y. M., Hong, X. Y., Shao, G., Ma, J. J., Cheng, X. R., Liu, J., Kang, J. & Fu, C. Y. (2021). Antimicrobial peptides: mechanism of action, activity and clinical potential. *Military Medical Research*, *8*(1). https://doi.org/10.1186/S40779-021-00343-2

Zhang, X., Huang, T., Wu, Y., Peng, W., Xie, H., Pan, M., Zhou, H., Cai, B. & Wu, Y. (2017). Inhibition of the PI3K-Akt-mTOR signaling pathway in T lymphocytes in patients with active tuberculosis. *International Journal of Infectious Diseases*, *59*, 110–117.

https://doi.org/10.1016/j.ijid.2017.04.004

Zhao, L., Tan, S., Zhang, H., Liu, P., Tan, Y. Z., Li, J. C., Jia, D. & Shen, X. F. (2018). Astragalus polysaccharides exerts anti-infective activity by inducing human cathelicidin antimicrobial peptide LL-37 in respiratory epithelial cells. *Phytotherapy Research*, *32*(8), 1521–1529. https://doi.org/10.1002/PTR.6080

Zigangirova, N. A., Nesterenko, L. N., Sheremet, A. B., Soloveva, A. V., Luyksaar, S. I., Zayakin, E. S., Balunets, D. V. & Gintsburg, A. L. (2021). Fluorothiazinon, a small-molecular inhibitor of T3SS, suppresses salmonella oral infection in mice. *The Journal of Antibiotics 2021 74:4*, *74*(4), 244–254. https://doi.org/10.1038/s41429-020-00396-w

Zolfaghar, I., Evans, D. J. & Fleiszig, S. M. J. (2003). Twitching motility contributes to the role of pili in corneal infection caused by Pseudomonas aeruginosa. *Infection and Immunity*, *71*(9), 5389–5393. https://doi.org/10.1128/IAI.71.9.5389-5393.2003

Zou, J. & Shankar, N. (2016). The opportunistic pathogen Enterococcus faecalis resists phagosome acidification and autophagy to promote intracellular survival in macrophages. *Cellular Microbiology*, *18*(6), 831–843. https://doi.org/10.1111/CMI.12556

Annexure 1 Primer sequences

Gene Sequence (5'→3')		
	Forward	**Reverse**
hBD-1	GCCTCCAAAGGAGCCAGCCT	CTTCTGGTCACTCCCAGCTCA
hBD-2	CAGCCATCAGCCATGAGG	TGGCTTTTTGCAGCATTTT
hBD-3	TCTCAGCGTGGGGTGAAGC	CGGCCGCCTCTGACTCTG
hBD-4	AGACTTGTGCTGCTATTAGCCG	GGGCAGTCCCATAACCACATA
S100A7	CGATCTTTCGGCAATACAGTG	GTTCACCAGACCTGGCATGT
S100A8	CCGAGTGTCCTCAGTATATA	GCCCATCTTTATCACCAGAAT
S100A9	TGGCTCCTCGGCTTTGG	CGACATTTTGCAAGTCATCGTC
S100A12	AGCATCTGGAGGGAATTGTCA	GCAATGGCTACCAGGGATATGAA
LL-37	TCGGATGCTAACCTCTACCG	ACAGGCTTTGGCGTGTCT
Rnase7	GAACACCAAGCGCAAAGC	CAGCAGAAGCAGCGAAGG
Hepcidin	CTGACCAGTGGCTCTGTTTTC	GAAGTGGGTGTCTCCCCTC
Lipocalin 2	TGAAGGCTTTCAGGGCAACT	GCTGCCGGTATCAGCACATAA
NRF2	CGGTATGCAACAGGACATTG	ACTGGTTGGGGTCTTCTGTG
HO-1	AAGACTGCGTTCCTGCTCAAC	AAAGCCCTACAGCAACTGTCG
Per-1	CCACGGAGATCATTGCTTTCA	AGGTGTATTGACCCATGCTAGAT
Ferritin	CCCCCATTTGTGTGACTTCAT	GCCCGAGGCTTAGCTTTCATT
NQO-1	GAAGCCGCAGACCTTGTGAT	CTGCCTTCTTACTCCGGAAGG
ATG-7	ATGATCCCTGTAACTTAGCCCA	CACGGAAGCAAACAACTTCAAC
ATG-5	AAGATGTGCTTCGAGATGTGT	CACTTTGTCAGTTACCAACGTCA
Bec 1	GGTGTCTCTCGCAGATTCATC	TCAGTCTTCGGCTGAGGTTCT
IL-1β	CCACAGACCTTCCAGGAGAA	GTGCAGTTCAGTGATCGTACAG
IL-6	CTCACCTCTTCAGAACGAATTG	CCATCTTTGGAAGGTTCAGGT
IL-8	GCAGTTTTGCCAAGGAGTGCT	GCATCTGGCAACCCTACAAC
GAPDH	GATCCCTCCAAAATCAAGTG	GGCAGAGATGATGACCCTTTT
mBD-1	CCAGATGGAGCCAGGTGTTG	AGCTGGAGCGGAGACAGAATCC
mBD-2	AAGTATTGGATACGAAGCAG	TGGCAGAAGGAGGACAAATG
mS100A9	ATACTCTAGGAAGGAAGGACACC	TCCATGATGTCATTTATGAGGGC
CRAMP	GTCTTGGGAACCATGCAGTT	TGGTTGAAGTCATCCACAGC
mLipocalin2	GCAGGTGGTACGTTGTGGG	CTCTTGTAGCTCATAGATGGTGC
mGAPDH	AGGTCGGTGTGAACGGATTTG	GTAGACCATGTAGTTGAGGTCA

Annexure 2 Chemicals and Materials used in the study

Sl	Chemicals and Materials	Company	Cat./Ref. No.
SDS-PAGE and Western Blotting			
1	Tris Base	G-Biosciences	RC1217
2	SDS (Sodium dodecyl Sulfate)	G-Biosciences	RC1184
3	Glycine	G-Biosciences	RC1094
4	Sodium Hydroxide	Sisco Research Laboratories (SRL)	68151
5	Sodium Chloride	SRL	41721
6	TEMED	BioRad	161-0801
7	Ponceau	Sigma Aldrich	P3504
8	Acrylamide/Bis-acrylamide-30% soln.	Sigma	A3574
9	Ammonium persulphate (APS)	Thermo Fisher	15055
10	2-Mercaptoethanol	SRL	69892
11	Pierce BCA Protein Assay Kit	Thermo Scientific	23225
12	Methanol	Qualigens	Q43637G
13	Blotting-grade Blocker	BioRad	1706404
14	Nitrocellulose membrane (0.45uM)	G-Biosciences	GS-NCM-304
15	Semi-dry transfer Unit	Amersham Biosciences	80-6210-34
16	Hoefer dual gel caster	Amersham	NA
17	Vertical electrophoresis unit	Hoefer/ Tarsons	NA
18	Ceramic plate for electrophoresis	Tarsons	7082
19	Glass plate for vertical electrophoresis	Tarsons	7083
20	Mini trans- blot filter paper	BioRad	1703932
21	Cell Lysis Buffer (10X)	Cell Signalling Technology	9803S
22	3-color pre-stained protein ladder	Puregene	PG-PMT2922
23	Rectangular glass plates 10*10.5cm (5)	Cytiva	SE262P-5
RNA isolation, cDNA synthesis, qPCR			
1	Ethylenediamine tetraacetic acid (EDTA)	Qualigens	Q23254
2	Agarose	Lonza	50004
3	Ultrapure distilled water Dnase/Rnase free	Invitrogen	10977-035
4	Chloroform	Qualigens	Q12305
5	Propan-2-ol	Fisher Scientific	13825
6	Ethanol/Stenol	China	0002
7	Optical sheets	Thermo Fisher	4311971
8	qPCR plates	Thermo Fisher/Labcon	AB1400/3979-520
9	TRIzol reagent	Invitrogen	15596026
10	Sybr Green PCR master mix	Thermo Fisher	F-416L

Sl	**Chemicals and Materials**	**Company**	**Cat./Ref. No.**
11	cDNA kit	Thermo Fisher	AB1453B
12	1 kb DNA ladder	Puregene	PG010-50 0DI
13	6x DNA loading dye	Thermo Fisher	R0611
14	100 bp DNA Ladder	BR Biochem Life Sciences	BM001-R500
15	Nanodrop	Biochrom	28956057
Cell Culture			
1	Trypsin EDTA 1X	Hyclone/HiMedia	SH40003.01/TCL007
2	EGF	Thermo Fisher	PHG0311
3	INSULIN	Thermo Fisher/ HiMedia	12585014/ TCL035
4	Keratinocyte-SFM (1X) medium	Thermo Fisher Scientific	17005042
5	DMEMf12	HyClone	SH30023.01
6	DMEMF12	Lonza	12-719F
7	RPMI-1640 Medium	HyClone	SH30027.01
8	OptiMEM	Gibco	51985-034
9	DMSO	Sigma	D8418
10	FBS	Gibco/Biowest	10270106/ S1810
11	Penicillin-Streptomycin 100x solution	HyClone	SV30010
12	Gentamicin (80mg/2ml) injection I.P.	Abaris Healthcare	NA
13	Dispase II	Gibco	17105041
14	T25 flasks	Nunc	156367
15	Lipofectamine	Thermo Fischer	L3000-001
Bacterial culture			
1	disposable loops	Tarsons	39269099
2	PBS tablets	Thermo Fisher	003002
3	L- shaped Cell spreaders	Tarsons	920082
4	Disposable Cuvetts	G-bioscience	786009A
5	LB broth	HiMedia	M1245
6	LB agar	HiMedia	M1151
7	Todd Hewitt Broth	Sigma	T1438
8	Agar powder bacteriological	HiMedia	GRM02
9	Blood Agar Plates	Chromogenic	PL090-16
10	Sterile cotton swabs	HiMedia	PW005
11	90mm Petriplates	Tarsons	460091

Sl	Chemicals and Materials	Company	Cat./Ref. No.
Antibodies / Dyes			
1	Anti-Rabbit Alexa fluor 488	Molecular Probes- Invitrogen	NA
2	Anti-mouse Alexa fluor 488	Molecular Probes- Invitrogen	NA
3	IRDye® 680RD Goat anti-Mouse IgG Secondary Antibody	LI-COR Biosciences	925-68070
4	IRDye® 680RD Donkey anti-Rabbit IgG Secondary Antibody	LI-COR Biosciences	925-68073
5	Phospho-PI3kinase p85 alpha (Tyr607)	Elabscience	E-AB-21218
6	pI3K p85β	Novus-R&D systems	MAB6777
7	Phospho-AKT	Novus-R&D systems	MAB887
8	P-p44/42 MAPK (T202/Y204)/ Phospho-ERK1/ERK2	Cell Signalling Technology /Novus-R&D systems	4376/ AF1018/ MAB1018
9	Total ERK	Cell Signalling Technology	4696
10	P-p38 MAPK (T180/Y182)	Cell Signalling Technology	9216
11	Total p38	Cell Signalling Technology	9212
12	pJNK/ pJNK(T183/Y185) (clone 1006A)	Cell Signalling Technology / Novus-R&D Systems	9255/ MAB1205
13	Total SAPK/JNK	Cell Signalling Technology	9252
14	RelA/ NFkβ-p65 antibody	Novus-R&D Systems	MAB5078
15	Phospho-IKβα (Ser32/36) antibody	Cell Signalling Technology / Novus-R&D Systems	9246/ AF4809
16	β-Actin	Cell Signalling Technology	4967
17	LC3B Antibody	Cell Signalling Technology	2775
18	LL-37 antibody	Cell Signalling Technology / Biolegend/ Novus-R&D Systems	NA/ 650302/ AF7497
19	p-STAT3 (Y705) (D3A7) XP-R	Cell Signalling Technology	91456
20	Total STAT3	Cell Signalling Technology	9132
21	Nrf2 (Clone383727) antibody	Novus-R&D Systems	MAB3925
22	Nitrotyrosine MAb (Clone 306507) antibody	Novus-R&D systems	MAB3248
23	Catalase antibody	Novus-R&D systems	NBP2-24916SS
24	Ki67 polyclonal antibody	Elabscience	E-AB-66986
25	Alexa flour-Phalloidin 488	Thermo Fisher	A12379
26	MTT	Molecular Probes	M6494
27	Propidium Iodide (PI)	Invitrogen	P3566
28	DAPI	Vector Laboratories/ ABCAM	H-1200/ ab228549
29	H2DCFDA dye	Invitrogen	D399

Sl	Chemicals and Materials	Company	Cat./Ref. No.
Peptides/Chemicals/Inhibitors/Solvents			
1	LL-37 peptide	Elabscience	E-PP-1466
2	Tert-Butylhydroquinone	Sigma-Aldrich	112941
3	Triton-X-100	SRL	2024271
4	Glycerol	Sigma	G5516
5	Paraformaldehyde	Avra	ASP2680
6	p38 MAP Kinase Inhibitor (SB203580)	InvivoGen	tlrl-sb20
7	JNK Inhibitor (SP600125)	InvivoGen	tlrl-sp60
8	MEK1 and MEK2 Inhibitor (PD98059)	InvivoGen	tlrl-pd98
Consumables/ Plasticwares			
1	1.5 ml microcentrifuge tubes (MCTs)	Tarsons	500010
2	2ml MCTs	Tarsons	500020
3	0.2mL PCR tubes	Tarsons	510051
4	0.5ml MCTs	Tarsons	500000
5	Microtips 1ml	Tarsons	521020
6	Microtips 200ul	Tarsons	521010
7	Microtips 10ul	Tarsons	521000
8	6 well Multidish	Thermo Fisher	140675
9	12 well Multidish	Thermo Fisher	150628
10	24 well Multidish	Thermo Fisher	142475
11	96 well Multidish	Thermo Fisher	167008
12	15 ml falcons	Tarsons	546021
13	50 ml falcons	Tarsons	546041
14	frosted micro slides	Blue Star	70179012
15	Cover Slips Round 18*18MM	Blue star	000871
16	Coverslip rectangle	Blue star	000895
17	Cryotubes 2ml	Thermo Fisher	375418
18	Nalgene syringe filters,22micron	Nalgene	725-2520
Miscellaneous			
1	CytoTox 96 cytotoxicity assay	Promega	G1781
2	Quantibody® Human Inflammation Array 1 Kit	RayBiotech	QAH-INF-1-1

* NA- Not available

List of presentations

i. Attended ARVO-IERG, 2016 held at L.V. Prasad Eye Institute, Hyderabad, India.

ii. Presented a poster entitled “Role of antimicrobial peptides in *Pseudomonas* keratitis” in IMMUNOCON, 2017, held at GITAM UNIVERSITY, Vishakhapatnam, India.

iii. Presented a poster entitled “Host defense mediated by antimicrobial peptides in *Streptococcus pneumoniae* keratitis” in ARVO-IERG, 2018 held at L.V. Prasad Eye Institute, Hyderabad, India.

iv. Presented a poster entitled “Role of type III secretion system inhibitor in *Pseudomonas* keratitis” in ARVO-IERG, 2019 held at Sankara Nethralaya, Chennai, India.

v. Presented thesis in a 3-minute thesis competition held at LVPEI, Hyderabad, on 28 February, 2019. Won 2nd Prize.

List of publications

- **Sharma, P**., Guha, S., Garg, P., & Roy, S. (2018). Differential expression of antimicrobial peptides in corneal infection and regulation of antimicrobial peptides and reactive oxygen species by type III secretion system of *Pseudomonas aeruginosa*. *Pathogens and Disease*, 76(1), 10.1093/femspd/fty001.
- **Sharma, P**., Sharma, N., Mishra, P., Joseph, J., Mishra, D. K., Garg, P., & Roy, S. (2019). Differential Expression of Antimicrobial Peptides in *Streptococcus pneumoniae* Keratitis and STAT3-Dependent Expression of LL-37 by *Streptococcus pneumoniae* in Human Corneal Epithelial Cells. *Pathogens* (Basel, Switzerland), 8(1),31.
- **Sharma, P**., Elofsson, M., & Roy, S. (2020). Attenuation of *Pseudomonas aeruginosa* infection by INP0341, a salicylidene acylhydrazide, in a murine model of keratitis. *Virulence*, 11(1),795–804.
- Jadi, P. K., **Sharma, P**., Bhogapurapu, B., & Roy, S. (2021). Alternative Therapeutic Interventions: Antimicrobial Peptides and Small Molecules to Treat Microbial Keratitis. Frontiers in Chemistry, 9, 694998. https://doi.org/10.3389/fchem.2021.694998
- **Sharma, P**. & Roy, S. Tert-Butylhydroquinone alleviates oxidative stress, induces autophagy in a Nrf2 dependent manner, and mediates protection against *Streptococcus pneumoniae* by induction of antimicrobial peptides in human corneal epithelial cells. (MANUSCRIPT UNDER REVIEW)
- Mallela, L. S., **Sharma, P**., Rao, T., & Roy, S. (2021). Recombinant IL-22 promotes protection in a murine model of *Aspergillus flavus* keratitis and mediates host immune responses in human corneal epithelial cells. Cellular Microbiology, 23(9), e13367. https://doi.org/10.1111/cmi.13367

Pathogens and Disease, 76, 2018, fty001

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Research Article

RESEARCH ARTICLE

Differential expression of antimicrobial peptides in corneal infection and regulation of antimicrobial peptides and reactive oxygen species by type III secretion system of *Pseudomonas aeruginosa*

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One sentence summary: *Pseudomonas aeruginosa* causes differential expression of antimicrobial peptides in patients with corneal infection and subverts host immune response *in vitro*.

Editor: Ake Forsberg

ABSTRACT

Pseudomonas aeruginosa is an opportunistic pathogen and is the major cause of corneal infection worldwide that secret several virulent toxins through its type III secretion system (T3SS). In defense against pathogenic insults, epithelial cells and macrophages express antimicrobial peptides (AMPs) that are essential components of host immune response. In this study, we have determined the expression of several AMPs in patients with *P. aeruginosa* corneal infection. We also used an *in vitro* model of infection using human corneal epithelial cells and macrophages to determine the gene expression of AMPs and cellular response to wild-type and T3SS mutant *P. aeruginosa*. We found differential expression of several AMPs in patient samples and also found that *P. aeruginosa* repress AMP expression in both epithelial cells and macrophages by its T3SS *in vitro*. It dampens AMP expression by causing delay in NF-κB, p38 and ERK activation and inhibits reactive oxygen species generation in these cells by its T3SS. Our study show the profile of AMPs expressed during *P. aeruginosa* keratitis and suggest the pivotal role of the T3SS in epithelial cells and macrophages during *P. aeruginosa* infection.

Keywords: antimicrobial peptides; type III secretion system; *Pseudomonas*; reactive oxygen species; corneal epithelial cells; MAPK activation

INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium, ubiquitous in nature and a major opportunistic human pathogen. It causes acute or chronic pulmonary infections (Lyczak, Cannon and Pier 2002) and is the leading cause of corneal infection worldwide associated with both trauma and contact lens use (Stapleton and Carnt 2012). It is responsible for 8%–21% of cases of keratitis in South India (Gopinathan *et al.* 2009) and up to 39% in the United States (Varaprasathan *et al.* 2004). Studies have

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demonstrated that Toll-like receptor (TLR) activation following *P. aeruginosa* infection results in rapid cytokines and chemokines production, recruitment of neutrophils and macrophages to cornea and development of corneal haze (Sun *et al.* 2010). Central to the pathogenicity of *P. aeruginosa* is the function of type III secretion system (T3SS) that consists of a needle-like apparatus that function in a highly regulated manner to transport bacterial toxins and other proteins into host cells (Hauser 2009). PscC is a family of secretin protein that oligomerizes to form a channel that spans through bacterial outer membrane and is an essential component of the T3SS, which functions as multicomponent export system for effector proteins (exoenzymes) (Hauser 2009; Galle, Carpentier and Beyaert 2012). Like many other Gram-negative bacteria, *P. aeruginosa* manipulates host cells by using its T3SS. Injection of effector proteins by T3SS is a common strategy employed by many human pathogens to infect and subvert cellular signaling and host responses (Kim *et al.* 2008; Sham *et al.* 2011; Reddick and Alto 2014).

The corneal epithelium provides the first line of defense against invading bacteria (Evans and Fleiszig 2013), and the host immune response to *P. aeruginosa* in corneal infection is majorly regulated by TLR4-MD-2 and TLR5 that leads to elevated expression of chemokines and proinflammatory cytokines (Sun *et al.* 2010; Roy, Sun and Pearlman 2011; Karthikeyan *et al.* 2013). Antimicrobial peptides (AMPs) play an important role in immune response against a wide range of bacteria, but there are no detailed studies of AMP expression in response to *P. aeruginosa*. AMPs are small cationic peptides and secreted by almost all living organisms and constitute an ancient form of innate immunity. In addition to having effective antibacterial activity, AMPs have several other advantages over traditional antibiotics in terms of possessing broad spectrum of activity, ability to modulate the host immune response and plays a role in wound healing (Sorensen *et al.* 2003; Yang *et al.* 2004). In mammals, including humans, defensins, cathelicidin and S100A proteins are the major classes of AMPs. There are several reports of expression of AMPs like defensins and cathelicidin in infectious keratitis (McDermott 2004; Mohammed *et al.* 2010; Otri *et al.* 2010; Otri *et al.* 2012) and also in regeneration and wound healing of corneal epithelium (Griffith *et al.* 2014). AMPs in recent years have shown noteworthy promise as an alternative therapeutic agent to treat infections particularly in the case of multidrug resistant pathogens (Seo *et al.* 2012).

In this study, we have determined the endogenous expression of several AMPs from patients with *P. aeruginosa* infection of the cornea and investigated their expression *in vitro* by epithelial cells and macrophages using wild-type *P. aeruginosa* (PAO1) and a mutant that lacks essential T3SS outer membrane component PscC (Δ*pscC*) and therefore cannot assemble functional T3SS. Our findings indicate that the T3SS of PAO1 suppresses the expression of AMPs in both epithelial cells and macrophages and subverts immune responses in these cells by causing delay in the activation of NF-κB, p38 and p42/44 (ERK) pathways and inhibiting the production of reactive oxygen species (ROS). However, further studies to better understand the mechanism of suppression of AMP gene expression and immune responses are warranted.

MATERIALS AND METHODS

Corneal scrapings

Corneal scrapings were collected from patients with bacterial keratitis after informed consent was taken. The protocol for obtaining scrapings was approved by Institutional Review Board of Hyderabad Eye Research Foundation, LVPEI, India, and the research followed the tenets of the Declaration of Helsinki. For controls, cadaveric corneas unsuitable for transplant purposes were procured from Ramayamma International Eye Bank, LVPEI, India. The clinical characteristics of the patients are present in Table 1.

Table 1. Clinical characteristics.

Characteristics	
Age	2 to 81
Mean (S.D.)	36.692 (19.011)
Sex	
Male (%)	46
Female (%)	54
Hypopyon	
Yes (%)	62
No (%)	38
Occupation	
House wife (%)	31
Agriculture/Manual labor (%)	31
Desk jobs (%)	23
Unspecified (%)	15
Size of ulcer	
<5 mm (%)	53.84
5–15 mm (%)	46.15
>15 mm (%)	0

Bacterial culture

PAO1 (Bleves *et al.* 2005) along with the mutant strain Δ*pscC* (Cisz, Lee and Rietsch 2008) has been used in this study. Both the parent and mutant PAO1 strain of *P. aeruginosa* were grown in Luria Hiveg Broth (HiMedia Laboratories, Kelton, PA) overnight at 37°C as described earlier (Roy, Bonfield and Tartakoff 2013). In brief, bacteria were subculture from overnight culture, washed twice in 1X PBS, centrifuged at 10 000 rpm for 10 min, and resuspended in 1X PBS. Dilutions of the bacterial culture were done with 1X PBS for the final inoculums.

Culture of HCEC and U937

Immortalized human corneal epithelial cells (HCEC) 10.014 pRSV-T (Roy, Marla and Praneetha 2015) were maintained in keratinocyte serum-free media containing bovine pituitary extract and recombinant human epidermal growth factors (Invitrogen, Carlsbad, CA) at 37°C and 5% CO_2. HCEC were grown overnight in 12 well plates (1×10^5 cells/well) and either incubated with bacteria for 3 h, washed with 1X PBS, cultured further in gentamycin containing media for 1 h (total 4 h) or exposed to bacteria for 6 h, washed, cultured further in gentamycin containing media for 1 h and grown overnight (total 24 h) before proceeding to RNA isolation. Human monocyte U937 was cultured in RPMI containing 5% FBS and penicillin and streptomycin under similar conditions. It was maintained in antibiotics-free media two days prior and during the course of infection.

Table 2. Oligonucleotide sequences.

Gene	Sequence (5′→3′)	
BD-1	FWD:GCCTCCAAAGGAGCCAGCCT	REV:CTTCTGGTCACTCCCAGCTCA
BD-2	FWD:CAGCCATCAGCCATGAGG	REV:TGGCTTTTTGCAGCATTTT
BD-3	FWD:TCTCAGCGTGGGGTGAAGC	REV:CGGCCGCCTCTGACTCTG
BD-4	FWD:AGACTTGTGCTGCTATTAGCCG	REV:GGGCAGTCCCATAACCACATA
S100A7	FWD:CGATCTTTCGGCAATACAGTG	REV:GTTCACCAGACCTGGCATGT
S100A8	FWD:CCGAGTGTCCTCAGTATATA	REV:GCCCATCTTTATCACCAGAAT
S100A9	FWD:TGGCTCCTCGGCTTTGG	REV:CGACATTTTGCAAGTCATCGTC
S100A12	FWD:AGCATCTGGAGGGAATTGTCA	REV:GCAATGGCTACCAGGGATATGAA
LL-37	FWD:TCGGATGCTAACCTCTACCG	REV:ACAGGCTTTGGCGTGTCT
Rnase7	FWD:GAACACCAAGCGCAAAGC	REV:CAGCAGAAGCAGCGAAGG
Hepcidin	FWD:CTGACCAGTGGCTCTGTTTTC	REV:GAAGTGGGTGTCTCCCCTC
Lipocalin 2	FWD:TGAAGGCTTTCAGGGCAACT	REV:GCTGCCGGTATCAGCACATAA
GAPDH	FWD:GATCCCTCCAAAATCAAGTG	REV:GGCAGAGATGATGACCCTTTT

RNA isolation, cDNA synthesis and quantitative PCR analysis

Quantitative real-time PCR was used to determine mRNA expression of different genes in corneal scrapings and immortalized cell lines. Total RNA was isolated using Trizol (Invitrogen), and was reverse transcribed using Reverse Transcriptase Kit (Eurogentec, Belgium) according to the manufacturer's protocol. Quantitative PCR was performed on ABI PRISM 7000HT Sequence Detection System (Applied Biosystems, Grand Island, NY) using the SYBR Green PCR Master Mix (Kapa Biosystems, Wilmington, MA). The primer sequences are shown in Table 2. Relative quantities of mRNA expression of respective genes were normalized using the $2^{-\Delta\Delta ct}$ method using GAPDH as the housekeeping gene.

Assay for ROS

1×10^4 cells per well were cultured overnight in 96 wells plate and infected with both wild-type and Δ*pscC* mutant PAO1 for 2 h at MOI 10:1 (bacteria to cells). After washing with 1X PBS, cells were incubated with 2′7′-dichlorodihydrofluorescein diacetate dye containing media (H_2CFDA; Invitrogen) for 30 min. The cells were further washed, media were added and observed under fluorescent microscope (Olympus IX73, Zeiss, Germany) using 10X objective. In separate experiments, cells were exposed to bacteria as mentioned above, and the fluorescence intensity of the cells was measured quantitatively after incubating with H_2CFDA by SpectraMax M3 (Softmax Pro 6.3).

Immunostaining

5×10^4 cells of HCEC or U937were cultured overnight on coverslips and infected with wild-type or PAO1Δ*pscC* at MOI 10:1 (bacteria to cells) for the defined time points and processed as mentioned earlier (Roy, Bonfield and Tartakoff 2013). In brief, the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100 and incubated with anti-p65 antibody (1:200; Novus Biologicals, Littleton, CO) followed by incubation with Alexafluor 488 labeled secondary antibody (1:500; Molecular probes). Images were captured on a fluorescent microscope (Olympus IX73, Zeiss) using 20X objective.

Western blot

HCEC and U937 were exposed to bacteria at MOI 10:1 (bacteria to cells) for the indicated time points. Endogenous phospho-p38α, phospho-ERK and phospho-IκB protein expression level was validated in both human cell types by western blot. Cells were washed with PBS and lysates were prepared in 1X lysis buffer (CST, Danvers, MA) and total protein was estimated by the BCA method as described in the manufacture's protocol (Thermo scientific, CA). Proteins were separated using 12% SDS-PAGE, transferred to nitrocellulose membrane and probed with p-p38, p-ERK, p-IκB and β-actin (raised in rabbit) and total p38 and ERK (raised in mouse) (1:2000 dilution; CST, MA). Subsequently blots were incubated with IRDye-680 secondary antibody (1:6000 dilution; LI-COR Biotechnology, Lincoln, NE) and were developed on Odyssey CLx Imaging System (LI-COR Biotechnology, NE). The densities of the bands were determined by Image J software (Schneider, Rasband and Eliceiri 2012).

Statistical analysis

Bar graphs represent mean and error bars represent SEM. Statistical analysis was performed using an either one-way ANOVA or unpaired *t*-test (Prism; GraphPad Software). *P*-values less than 0.05 were considered significant.

RESULTS

Differential expressions of host AMPs during *P. aeruginosa* keratitis

Although cornea is known to express several AMPs in response to microbial pathogens that mediate protection against bacterial infection (McDermott *et al.* 2003; McDermott 2009; Augustin *et al.* 2011), there are no reports on AMPs elicited in patients during *P. aeruginosa* infection. To determine the expression of these genes during corneal infections with *P. aeruginosa*, corneal scrapings were collected from patients, and expression of AMPs belonging to diverse groups was evaluated by the quantitative PCR method. As shown in Fig. 1, AMPs from these groups were differentially expressed in patients and is presented as log of relative gene expression, log (RQ). Four peptides from the human β defensins (hBD) group were assessed, and we observed a significant increase in expression of hBD1 (>21-fold), hBD2 (>5-fold) and hBD3 (>10-fold) in corneal ulcers obtained from *P. aeruginosa* keratitis patients. However, hBD4 expression was not detected in any of the samples of corneal scrapings. Expression of four S100A proteins like S100A7, S100A8, S100A9 and S100A12 was significantly up regulated in the corneal ulcers from patients infected with *P. aeruginosa*. The maximum

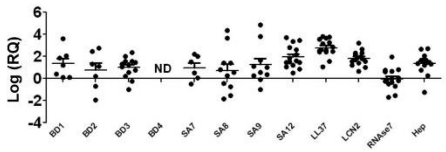

Figure 1. Differential expression of antimicrobial peptides in patients with *P. aeruginosa* keratitis. Expression of antimicrobial peptides was determined from corneal scrapings of patients infected with *P. aeruginosa* by quantitative PCR and the values are represented as log of relative gene expression (log (RQ)) compared to uninfected donor corneas. Data points represent individual patients. There was a significant difference in expressions of the AMPs in infected corneas from the uninfected control corneas as calculated by analysis of variance with a P-value of <0.001 using GraphPad Prism software.

increase in expression was observed for S100A12 (>90-fold). We also checked expression of four other AMPs, LL-37, the lone member of human cathelicidin group, hepcidin, RNAse7 and lipocalin 2. While there was reduced expression of RNAse7 in most of the patients, expression of LL-37 was significantly induced (>600-fold), along with lipocalin 2 (>60-fold) and hepcidin (>20-fold).

Subversion of AMP expression by *P. aeruginosa* is T3SS dependent

Although the expression of pro-inflammatory cytokines by HCEC or U937 in response to PAO1 has been studied earlier (Chai *et al.* 2014; Roy, Karmakar and Pearlman 2014), not much has been reported about the expression of AMPs in response to PAO1. We studied the expression of the AMPs detected in patients and further looked into the effect of T3SS of PAO1 on AMP expression *in vitro*. HCEC were infected with wild-type *P. aeruginosa* or a mutant strain that lack PscC (Δ*pscC*) at MOI 10 for two different time periods as described in methods, to determine the expressions of AMPs both at early (4 h) and late phase of infection (24 h). As shown in Fig. 2, there was considerable increase in expression of AMPs by HCEC following infection with PAO1Δ*pscC* compared to the wild type by 4h. Significant increased expressions of hBD1 (>3-fold), hBD2 (>8-fold) and hBD3 (>8-fold) were observed (Fig. 2A); however, expression of hBD4 was not detected in corneal epithelial cells similar to what was observed with patient samples (data not shown). Increased expressions of hepcidin (>5-fold), RNAse7 (>15-fold) and LL37 (>60-fold) by mutant bacteria were also observed (Fig. 2B). Similarly, there was increased expression of S100A7 (>2-fold), S100A8 (>50-fold), S100A9 (>25-fold) and S100A12 (>40-fold) in response to PAO1Δ*pscC* compared to the wild type (Fig. 2C). Conversely, there were no significant differences in the expression of the AMPs in response to wild-type or mutant strain after the prolonged exposure.

To analyze if the suppression of the expression of AMPs by T3SS is confined to epithelial cells, U937 cells were infected with either PAO1 or PAO1Δ*pscC*, and gene expression was analyzed for early phase of infection. Similar to the results obtained with epithelial cells, T3SS suppressed the expression of AMPs in U937 cells. We observed a decrease in the expression of hBD2, and hBD3 in cells infected with PAO1 compared to the mutant (Fig. 3A). The expression of LL37, Rnase7 and hepcidin were also dampened significantly by PAO1 (Fig. 3B). Comparable

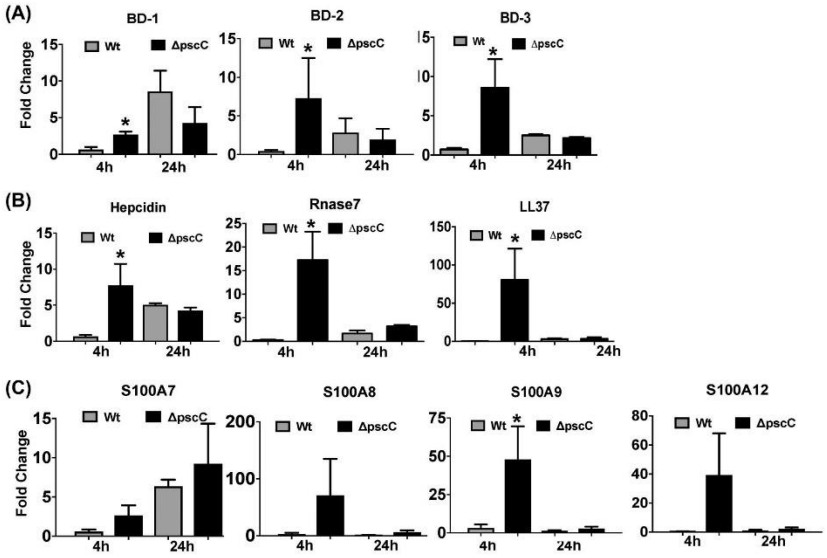

Figure 2. Expression of antimicrobial peptides in HCEC *in vitro*. Human corneal epithelial cells were exposed to PAO1 or PAO1Δ*pscC* and expression of different antimicrobial peptides were determined after 4 or 24 h by real-time PCR. GAPDH was used as housekeeping gene. Experiments were repeated twice in duplicates with similar results. (* indicates $P < 0.05$, **indicates $P < 0.005$)

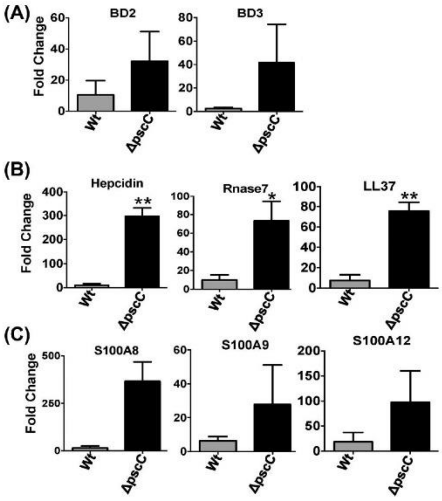

Figure 3. Expression of antimicrobial peptides in U937 *in vitro*. U937 cells were exposed to PAO1 or PAO1Δ*pscC* and expression of different antimicrobial peptides were quantitated after 4 h by real-time PCR. GAPDH was used as housekeeping gene. Experiments were repeated twice in duplicates with similar results. (* indicates $P < 0.05$, **indicates $P < 0.005$)

reductions in the expressions for S100A8, S100A9 and S100A12 (Fig. 3C) were obtained in PAO1Δ*pscC* infected cells. But we could not detect the expression of hBD1, hBD4 and S100A7 in these cells. Similar to the epithelial cells, there was no significant difference in the expression of AMPs by these cells infected with wild-type or mutant bacteria after prolonged exposure (Fig. S1, Supporting Information).

PAO1 T3SS subdues NF-κB signaling in HCEC and U937

The activation of NF-κB signaling pathway is a critical host response and several T3SS effectors of bacteria are known to interfere with different proteins of this pathway (Nadler *et al.* 2010; Newton *et al.* 2010; Rolhion *et al.* 2016). Since T3SS suppressed the AMP expression, we wanted to further see its effect on signaling pathways in these cells. First, we determined the phosphorylation of IκBα in HCEC and U937 infected with PAO1 or PAO1Δ*pscC* for different time points by western blot. As shown in Fig. 4A, increased phosphorylation of IκBα was detected as early as 30 min in PAO1Δ*pscC*-infected HCEC compared to PAO1-infected cells and was present for extended time periods. Similarly, in U937, enhanced phosphorylation of IκBα was observed within 30 min of infection of PAO1Δ*pscC* strains (Fig. 4B). Next, we looked into the nuclear translocation of p65 in cells infected with PAO1or PAO1Δ*pscC* by immunofluorescence microscopy. The increased translocation of p65 into the nucleus starts at earlier time point (30 min) in PAO1Δ*pscC* relative to PAO1-infected epithelial cells that further becomes significant by later time points (Fig. 4C). Similar observations were made for U937 cells with increased p65 translocation starting at earlier time point in cells infected with PAO1Δ*pscC* compared to the wild type (Fig. 4D). The numbers of cells with p65 staining in the nucleus were quantitated, and significantly more cells with p65 positive nucleus were detected in cells infected with PAO1Δ*pscC* for both the cell types (Fig. 4E and F).

PAO1 T3SS causes reduced activation of p38 and ERK in HCEC and U937

Aside from NF-κB, several bacterial pathogens are also known to target MAPK signaling pathways. Both HCEC and U937 were infected for defined time points and activation of MAPK was detected. The phosphorylation of p38 was detected within 30 min in HCEC infected with PAO1Δ*pscC* and remains activated for 2 h. However, there was delay in p38 activation in cells infected with PAO1 (Fig. 5A). Similarly, higher phosphorylation of p38 was observed that was more prominent by 2 h in U937 infected with PAO1Δ*pscC* compared to cells infected with PAO1 (Fig. 5C). Significant increase in ERK activation was observed in both HCEC and U937 infected with PAO1Δ*pscC* by 30 min compared to PAO1 infection (Fig. 5B and D). However, the Δ*pscC* mutant and wild-type PAO1 caused activation of JNK in both HCEC and U937 in an indistinguishable manner (data not shown).

PAO1 T3SS subverts ROS generation in HCEC and U937

One of the initial host responses to pathogens is ROS generation in response to bacterial infection not only to kill the invading pathogen, but also to mediate immune signaling cascades. It is now known that several bacteria have the ability to subvert ROS production in neutrophils and macrophages (Carlyon *et al.* 2004; Criss and Seifert 2008; Flannagan, Heit and Heinrichs 2015), thus increasing the potential for persistent infection. To determine if T3SS has any effect on ROS production in HCEC or U937, cells were infected with both wild-type and Δ*pscC* mutant PAO1 and ROS level was measured. HCEC infected with bacteria were incubated with membrane permeant fluorescent probe H_2CDFA and observed under microscope. There was an increased ROS generation in PAO1Δ*pscC* infected cells that was evident from the increased fluorescence as shown in Fig. 6A. The ROS generation was also quantitatively measured by fluorimetry, and significantly increased level of ROS was detected in cells infected with PAO1Δ*pscC* (Fig. 6B). The cells infected with *Escherichia coli* DH5α served as a positive control and resulted in a significant level of ROS generation similar to PAO1Δ*pscC*. Similar results were obtained in U937 cells where ROS generation was subverted by PAO1 in a T3SS-dependent manner (Fig. 6C and D). Here also significantly increase ROS generation was observed in cells infected with PAO1Δ*pscC* or *E. coli* DH5α compared to wild-type PAO1. Very recently Vareechon *et al.* (2017) have shown that PAO1 directly inhibits ROS production in human neutrophils via the effectors ExoS and ExoT toxins. Also, streptolysin O, a pore forming toxin has been reported to reduce ROS generation in *S. pyogenes*-infected neutrophils (Uchiyama *et al.* 2015). The reduced ROS generation by PAO1 in both the cell lines were however not due to induction of cell death as there was no marked difference in cell viability between cells infected with wild-type or mutant bacteria (Fig. S2, Supporting Information). Together, these data suggests that PAO1 inhibits ROS generation, by its type III secretory machinery, in both epithelial cells and macrophages, thereby avoiding killing by these cells.

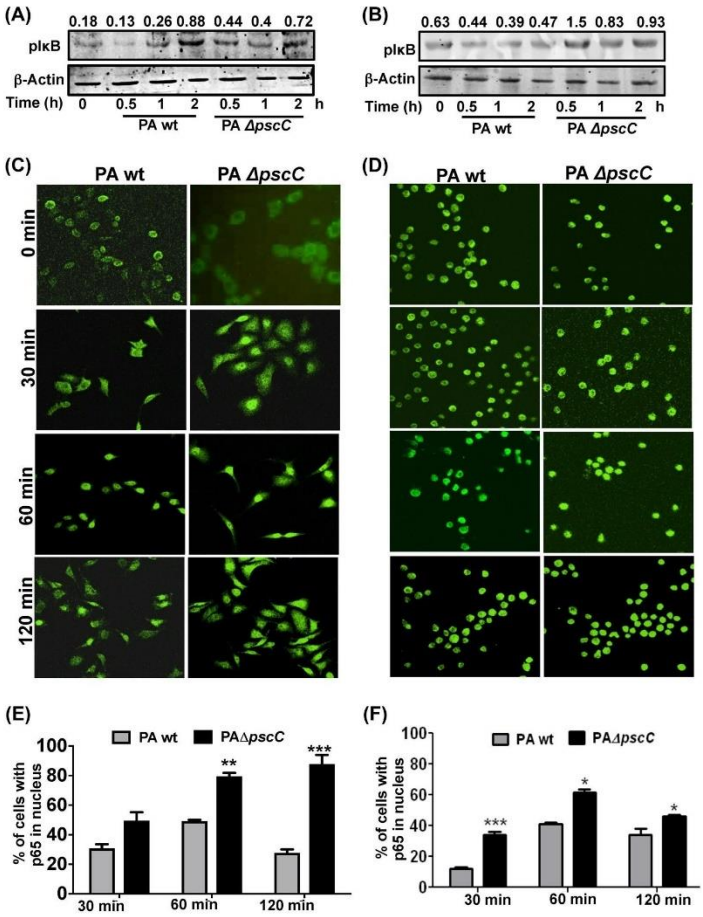

Figure 4. NF-κB signaling in HCEC and U937 in response to *P. aeruginosa*. HCEC (**A**) or U937 (**B**) was exposed to PAO1 or PAO1Δ*pscC* for 0.5, 1 and 2 h and expression of pIκBα and β-actin was analyzed by western blot analysis. The density of the bands was measured and the numbers represent the ratio of pIκBα to β-actin. Translocation of p65 subunit of NF-κB at 0.5, 1 and 2 h after incubation with PAO1 or PAO1ΔpscC in HCEC (**C**) or U937 (**D**) was observed by fluorescent microscope. Quantification of cells with p65 in nucleus in HCEC (**E**) or U937 (**F**) was done. At least one hundred cells were quantitated for each time point.

DISCUSSION

Several studies over the last decade have reported the expression of different cationic AMPs by ocular surface cells like corneal and conjunctival epithelium. While some have constitutive mode of expression, the expression of others is induced by infection, inflammation and in response to various bacterial products (McDermott 2004; Gordon, Romanowski and McDermott 2005). A very extensive study by McIntosh *et al.* (2005) has reported a spectrum of AMPs expressed at the ocular surface. Although there are plenty of reports on antimicrobial expression in *ex vivo* (Lee *et al.* 2005), *in vivo* (Nizet *et al.* 2001) and *in vitro* (Bals 2000) models of infection, there have been no reports of host endogenous AMP expression in patients with *P. aeruginosa* corneal infection. The main goal of the present study was to determine the expression profile of AMPs in corneal ulcers from patients infected with *P. aeruginosa*. We have also found that PAO1 dampens the expression of AMPs, subverts cellular signaling and ROS generation *in vitro*. Overall, the results generated in the present study augments our understanding of the pathogenesis of this blinding disease by demonstrating that T3SS sabotages host immune response in both epithelial cells and macrophages.

We assessed the expression of host AMPs in response to *P. aeruginosa* infection of the cornea and have seen significant increase in the expression of hBD 1, 2 and 3, LL-37, S100A7, S100A8, S100A9, S100A12, lipocalin 2 and hepcidin. There was

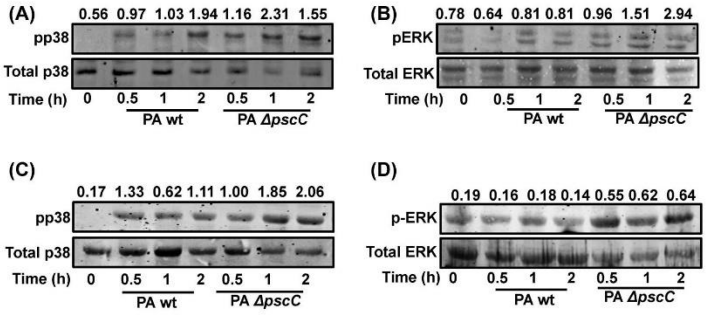

Figure 5. Role of PAO1 T3SS in MAPK activation. HCEC were infected with PAO1 or PAO1Δ*pscC* for 0.5, 1 and 2 h and expression of phospho-p38 (**A**) and phospho-ERK (**B**) was analyzed by western blot analysis. Expression of phospho-p38 (**C**) and phospho-ERK (**D**) for U937 was done in a similar way. The total proteins for p38 and ERK were also analyzed. The density of the bands was measured, and the numbers represent the ratio of the phosphorylated proteins to total proteins.

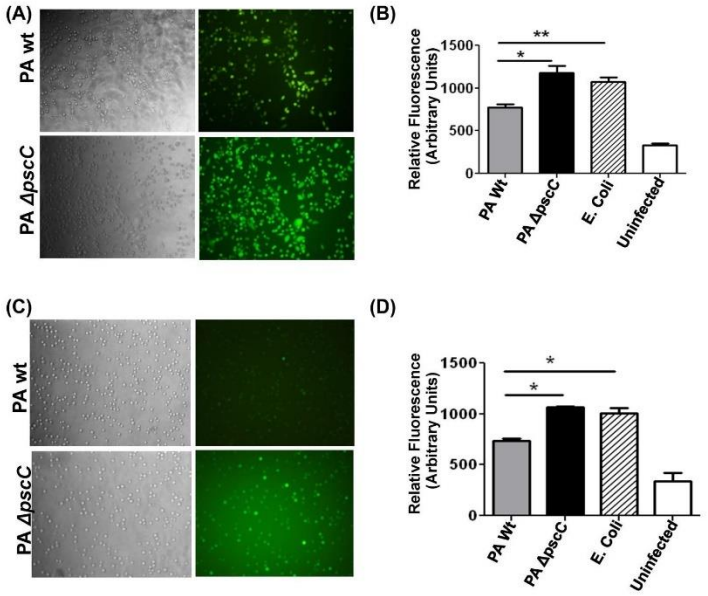

Figure 6. ROS activation in response to *P. aeruginosa*. HCEC was exposed to PAO1 or PAO1Δ*pscC* for 2 h and ROS generation was observed under microscope using H_2CFDA dye (**A**); ROS was also quantitated using the fluorescence plate reader in cells infected with PAO1 or PAO1Δ*pscC* or *E. coli* (**B**); similar experiments were done with U937 (**C** and **D**). These experiments were repeated thrice with similar results. (* indicates $P < 0.05$, **indicates $P < 0.005$).

significant decrease in expression of Rnase7, and we could not detect any hBD4 expressions in any of the patients. Although *P. aeruginosa* induced expression of AMPs in patients, we found that it subverts AMP expression during early time of infection in both epithelial cells and macrophages *in vitro*. This down regulation is mediated by the T3SS, since infection with T3SS null Δ*pscC* mutant caused a significant increase in the expression of AMPs in both the cell types. A study by Vos *et al.* (2005) describes the limited expression of AMPs in bronchial epithelial cells exposed to PAO1. Although they concluded that AMPs were not playing a role in the early host defense, but this could be due to the suppression of their expression by PAO1. The down regulation of AMP expression has also been reported in THP-1 cells infected with *Ureaplasma*, but the molecular mechanism is not known (Xiao *et al.* 2014). The difference in expression of AMPs is evident during the early infection period and therefore is only

manifested *in vitro*. Sun *et al.* also reported the subversion of the host immune response in the corneal stroma of C57BL/6 mice by the PAO1 T3SS. Increased bacterial load was observed in murine eyes infected with wild-type PAO1 compared to that infected with T3SS deficient strain. It was clear from the study that type III secretion targeted the antibacterial activity of the neutrophils (Sun *et al.* 2012).

Several bacterial effectors have earlier been reported to interfere with NF-κB signaling (Sanada *et al.* 2012). Usually the degradation of IκBα or release of p65 is affected by various effector toxins of bacteria. Elevated expression of TLRs, cytokines and inflammasomes components has been reported to be expressed during *P. aeruginosa* keratitis (Karthikeyan *et al.* 2013). Abrogation of p65 translocation has also been reported in the case of enteropathogenic *E. coli* (Sham *et al.* 2011) and *S. flexeneri* (de Jong *et al.* 2016). *Escherichia coli* has also been reported to inhibit NF-κB signaling by its type III effector proteins NleE and NleB in human epithelial (Newton *et al.* 2010) and dendritic cells (Vossenkamper *et al.* 2010). We also found that PAO1Δ*pscC* affects the kinetics of phosphorylation of IκBα and causes rapid translocation of p65 into the nucleus. PAO1 T3SS also suppressed the activation of both p38 and ERK signaling but not JNK in both the cell types. There also has been report of decreased MAPK activation by type III effector proteins of enteropathogenic *E. coli* (Sham *et al.* 2011). Internalization of PAO1 by corneal epithelial cells has earlier found to be dependent on MEK and ERK (Evans *et al.* 2002).

ROS generation by host cells play an important role and contribute to the antimicrobial capacity of the cells. Inhibition of ROS production by *F. tularensis* (McCaffrey and Allen 2006), *H. pyroli* (Allen *et al.* 2005), *C. burnetti* (Siemsen *et al.* 2009) and others in neutrophils and macrophages in T3SS-dependent and independent methods has been studied. We have also seen that PAO1 inhibits ROS generation in corneal epithelial cells and macrophages in a T3SS-dependent manner and dampens the immune response. Only very recently, Vareechon *et al.* (2017) have reported similar occurrence in neutrophils by PAO1 and have decipher the molecular mechanism behind it showing that ExoS of PAO1 interferes with the signaling pathways that leads to the assembly of NADPH oxidase. They have also shown that both ExoS and ExoT independently inhibit ROS production, whereas there is no role for ExoY. Whether it is similar in case of epithelial cells or macrophages needs further evaluation.

It is clear from this study that PAO1 suppresses the expression of AMPs in epithelial cells and macrophages and dampens innate immune response of the host cells with delayed NF-κB and MAPK activation and decreased ROS generation. Further research on AMPs is essential to combat the increase in antibiotic resistance developing among bacteria. It will be extremely relevant in future to see if inhibition of T3SS can induce AMP expression and heighten the host immune response to effectively disarm the bacteria and eventually kill them.

SUPPLEMENTARY DATA

Supplementary data are available at *FEMSPD* online.

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Conflicts of interest. None declared.

REFERENCES

Allen LA, Beecher BR, Lynch JT *et al.* Helicobacter pylori disrupts NADPH oxidase targeting in human neutrophils to induce extracellular superoxide release. *J Immunol* 2005;**174**:3658–67.

Augustin DK, Heimer SR, Tam C *et al.* Role of defensins in corneal epithelial barrier function against *Pseudomonas aeruginosa* traversal. *Infect Immun* 2011;**79**:595–605.

Bals R. Epithelial antimicrobial peptides in host defense against infection. *Respir Res* 2000;**1**:141–50.

Bleves S, Soscia C, Nogueira-Orlandi P *et al.* Quorum sensing negatively controls type III secretion regulon expression in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* 2005;**187**:3898–902.

Carlyon JA, Abdel-Latif D, Pypaert M *et al.* Anaplasma phagocytophilum utilizes multiple host evasion mechanisms to thwart NADPH oxidase-mediated killing during neutrophil infection. *Infect Immun* 2004;**72**:4772–83.

Chai W, Zhang J, Duan Y *et al.* Pseudomonas pyocyanin stimulates IL-8 expression through MAPK and NF-kappaB pathways in differentiated U937 cells. *BMC Microbiol* 2014;**14**:26.

Cisz M, Lee PC, Rietsch A. ExoS controls the cell contact-mediated switch to effector secretion in *Pseudomonas aeruginosa*. *J Bacteriol* 2008;**190**:2726–38.

Criss AK, Seifert HS. Neisseria gonorrhoeae suppresses the oxidative burst of human polymorphonuclear leukocytes. *Cell Microbiol* 2008;**10**:2257–70.

de Jong MF, Liu Z, Chen D *et al.* Shigella flexneri suppresses NF-kappaB activation by inhibiting linear ubiquitin chain ligation. *Nat Microbiol* 2016;**1**:16084.

Evans DJ, Fleiszig SM. Why does the healthy cornea resist *Pseudomonas aeruginosa* infection? *Am J Ophthalmol* 2013;**155**:961–70. e962.

Evans DJ, Maltseva IA, Wu J *et al.* *Pseudomonas aeruginosa* internalization by corneal epithelial cells involves MEK and ERK signal transduction proteins. *FEMS Microbiol Lett* 2002;**213**:73–9.

Flannagan RS, Heit B, Heinrichs DE. Antimicrobial mechanisms of macrophages and the immune evasion strategies of *Staphylococcus aureus*. *Pathogens* 2015;**4**:826–68.

Galle M, Carpentier I, Beyaert R. Structure and function of the type III secretion system of *Pseudomonas aeruginosa*. *Curr Protein Pept Sci* 2012;**13**:831–42.

Gopinathan U, Sharma S, Garg P *et al.* Review of epidemiological features, microbiological diagnosis and treatment outcome of microbial keratitis: experience of over a decade. *Indian J Ophthalmol* 2009;**57**:273–9.

Gordon YJ, Romanowski EG, McDermott AM. A review of antimicrobial peptides and their therapeutic potential as anti-infective drugs. *Curr Eye Res* 2005;**30**:505–15.

Griffith GL, Kasus-Jacobi A, Lerner MR *et al.* Corneal wound healing, a newly identified function of CAP37, is mediated by protein kinase C delta (PKCdelta). *Invest Ophthalmol Vis Sci* 2014;**55**:4886–95.

Hauser AR. The type III secretion system of *Pseudomonas aeruginosa*: infection by injection. *Nat Rev Microbiol* 2009;**7**:654–65.

Karthikeyan RS, Priya JL, Leal SM, Jr. *et al.* Host response and bacterial virulence factor expression in *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* corneal ulcers. *PLoS One* 2013;**8**:e64867.

Kim DW, Chu H, Joo DH *et al.* OspF directly attenuates the activity of extracellular signal-regulated kinase during invasion by *Shigella flexneri* in human dendritic cells. *Mol Immunol* 2008;**45**:3295–301.

Lee PH, Ohtake T, Zaiou M *et al.* Expression of an additional cathelicidin antimicrobial peptide protects against bacterial skin infection. *Proc Natl Acad Sci USA* 2005;**102**:3750–5.

Lyczak JB, Cannon CL, Pier GB. Lung infections associated with cystic fibrosis. *Clin Microbiol Rev* 2002;**15**:194–222.

McCaffrey RL, Allen LA. *Francisella tularensis* LVS evades killing by human neutrophils via inhibition of the respiratory burst and phagosome escape. *J Leukoc Biol* 2006;**80**:1224–30.

McDermott AM. Defensins and other antimicrobial peptides at the ocular surface. *Ocul Surf* 2004;**2**:229–47.

McDermott AM. The role of antimicrobial peptides at the ocular surface. *Ophthalmic Res* 2009;**41**:60–75.

McDermott AM, Redfern RL, Zhang B *et al.* Defensin expression by the cornea: multiple signalling pathways mediate IL-1beta stimulation of hBD-2 expression by human corneal epithelial cells. *Invest Ophthalmol Vis Sci* 2003;**44**:1859–65.

McIntosh RS, Cade JE, Al-Abed M *et al.* The spectrum of antimicrobial peptide expression at the ocular surface. *Invest Ophthalmol Vis Sci* 2005;**46**:1379–85.

Mohammed I, Suleman H, Otri AM *et al.* Localization and gene expression of human beta-defensin 9 at the human ocular surface epithelium. *Invest Ophthalmol Vis Sci* 2010;**51**:4677–82.

Nadler C, Baruch K, Kobi S *et al.* The type III secretion effector NleE inhibits NF-kappaB activation. *PLoS Pathog* 2010;**6**:e1000743.

Newton HJ, Pearson JS, Badea L *et al.* The type III effectors NleE and NleB from enteropathogenic *E. coli* and OspZ from *Shigella* block nuclear translocation of NF-kappaB p65. *PLoS Pathog* 2010;**6**:e1000898.

Nizet V, Ohtake T, Lauth X *et al.* Innate antimicrobial peptide protects the skin from invasive bacterial infection. *Nature* 2001;**414**:454–7.

Otri AM, Mohammed I, Abedin A *et al.* Antimicrobial peptides expression by ocular surface cells in response to *Acanthamoeba castellanii*: an in vitro study. *Br J Ophthalmol* 2010;**94**:1523–7.

Otri AM, Mohammed I, Al-Aqaba MA *et al.* Variable expression of human beta defensins 3 and 9 at the human ocular surface in infectious keratitis. *Invest Ophthalmol Vis Sci* 2012;**53**:757–61.

Reddick LE, Alto NM. Bacteria fighting back: how pathogens target and subvert the host innate immune system. *Mol Cell* 2014;**54**:321–8.

Rolhion N, Furniss RC, Grabe G *et al.* Inhibition of nuclear transport of NF-kB p65 by the *Salmonella* type III secretion system effector SpvD. *PLoS Pathog* 2016;**12**:e1005653.

Roy S, Sun Y, Pearlman E. Interferon-gamma-induced MD-2 protein expression and lipopolysaccharide (LPS) responsiveness in corneal epithelial cells is mediated by Janus tyrosine kinase-2 activation and direct binding of STAT1 protein to the MD-2 promoter. *J Biol Chem* 2011;**286**:23753–62.

Roy S, Bonfield T, Tartakoff AM. Non-apoptotic toxicity of *Pseudomonas aeruginosa* toward murine cells. *PLoS One* 2013;**8**:e54245.

Roy S, Karmakar M, Pearlman E. CD14 mediates Toll-like receptor 4 (TLR4) endocytosis and spleen tyrosine kinase (Syk) and interferon regulatory transcription factor 3 (IRF3) activation in epithelial cells and impairs neutrophil infiltration and *Pseudomonas aeruginosa* killing in vivo. *J Biol Chem* 2014;**289**:1174–82.

Roy S, Marla S, Praneetha DC. Recognition of *Corynebacterium pseudodiphtheriticum* by Toll-like receptors and up-regulation of antimicrobial peptides in human corneal epithelial cells. *Virulence* 2015;**6**:716–21.

Sanada T, Kim M, Mimuro H *et al.* The *Shigella flexneri* effector OspI deamidates UBC13 to dampen the inflammatory response. *Nature* 2012;**483**:623–6.

Schneider CA, Rasband WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. *Nat Methods* 2012;**9**:671–5.

Seo MD, Won HS, Kim JH *et al.* Antimicrobial peptides for therapeutic applications: a review. *Molecules* 2012;**17**:12276–86.

Sham HP, Shames SR, Croxen MA *et al.* Attaching and effacing bacterial effector NleC suppresses epithelial inflammatory responses by inhibiting NF-kappaB and p38 mitogen-activated protein kinase activation. *Infect Immun* 2011;**79**:3552–62.

Siemsen DW, Kirpotina LN, Jutila MA *et al.* Inhibition of the human neutrophil NADPH oxidase by *Coxiella burnetii*. *Microbes Infect* 2009;**11**:671–9.

Sorensen OE, Cowland JB, Theilgaard-Monch K *et al.* Wound healing and expression of antimicrobial peptides/polypeptides in human keratinocytes, a consequence of common growth factors. *J Immunol* 2003;**170**:5583–9.

Stapleton F, Carnt N. Contact lens-related microbial keratitis: how have epidemiology and genetics helped us with pathogenesis and prophylaxis. *Eye (Lond)* 2012;**26**:185–93.

Sun Y, Karmakar M, Taylor PR *et al.* ExoS and ExoT ADP ribosyltransferase activities mediate *Pseudomonas aeruginosa* keratitis by promoting neutrophil apoptosis and bacterial survival. *J Immunol* 2012;**188**:1884–95.

Sun Y, Karmakar M, Roy S *et al.* TLR4 and TLR5 on corneal macrophages regulate *Pseudomonas aeruginosa* keratitis by signaling through MyD88-dependent and -independent pathways. *J Immunol* 2010;**185**:4272–83.

Uchiyama S, Dohrmann S, Timmer AM *et al.* Streptolysin O rapidly impairs neutrophil oxidative burst and antibacterial responses to group a streptococcus. *Front Immunol* 2015;**6**:581.

Varaprasathan G, Miller K, Lietman T *et al.* Trends in the etiology of infectious corneal ulcers at the F. I. Proctor Foundation. *Cornea* 2004;**23**:360–4.

Vareechon C, Zmina SE, Karmakar M *et al.* *Pseudomonas aeruginosa* effector ExoS inhibits ROS production in human neutrophils. *Cell Host Microbe* 2017;**21**:611–8. e615.

Vos JB, van Sterkenburg MA, Rabe KF *et al.* Transcriptional response of bronchial epithelial cells to *Pseudomonas aeruginosa*: identification of early mediators of host defense. *Physiol Genom* 2005;**21**:324–36.

Vossenkamper A, Marches O, Fairclough PD *et al.* Inhibition of NF-kappaB signaling in human dendritic cells by the enteropathogenic *Escherichia coli* effector protein NleE. *J Immunol* 2010;**185**:4118–27.

Xiao L, Crabb DM, Dai Y *et al.* Suppression of antimicrobial peptide expression by ureaplasma species. *Infect Immun* 2014;**82**:1657–65.

Yang D, Biragyn A, Hoover DM *et al.* Multiple roles of antimicrobial defensins, cathelicidins, and eosinophil-derived neurotoxin in host defense. *Annu Rev Immunol* 2004;**22**:181–215.

pathogens

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Differential Expression of Antimicrobial Peptides in *Streptococcus pneumoniae* Keratitis and STAT3-Dependent Expression of LL-37 by *Streptococcus pneumoniae* in Human Corneal Epithelial Cells

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Abstract: *Streptococcus pneumoniae* is the leading cause of bacterial keratitis in the developing world with a growing trend of acquiring resistance against various antibiotics. In the current study, we determined the expression of different antimicrobial peptides (AMPs) in response to *S. pneumoniae* in patients, as well as in primary and immortalized human corneal epithelial cells. We further focused on LL-37 and determined its expression in human cornea infected with *S. pneumoniae* and studied the killing ability of LL-37 against *S. pneumoniae*. The expression of AMPs was determined by quantitative PCR and the phosphorylation of signaling proteins was evaluated by immunoblot analysis. LL-37 expression was also determined by immunofluorescence and Western blot method and the killing ability of LL-37 against *S. pneumoniae* was determined by colony-forming units. Differential expression of antimicrobial peptides was observed in patients with *S. pneumoniae* keratitis. Although *S. pneumoniae* induced expression of the AMPs in human corneal epithelial cells (HCEC), it did not induce AMP expression in U937, a human monocyte cell line. *S. pneumoniae* also caused activation of nuclear factor kappa-light-chain enhancer of activated B cells (NF-κB)and mitogen activated protein kinase (MAPK) pathways in corneal epithelial cells. LL-37 was found to be effective against both laboratory and clinical strains of *S. pneumoniae*. LL-37 induction by *S. pneumoniae* in human corneal epithelial cells was mediated by signal transducer and activator of transcription 3 (STAT3) activation, and inhibition of STAT3 activation significantly reduced LL-37 expression. Our study determines an extensive profile of AMPs expressed in the human cornea during *S. pneumoniae* infection, and suggests the potential of LL-37 to be developed as an alternative therapeutic intervention to fight increasing antibiotic resistance among bacteria.

Keywords: antimicrobial peptides; LL-37; STAT3; corneal epithelial cells; *Streptococcus pneumoniae*

1. Introduction

Bacterial keratitis is a major cause of visual impairment and blindness worldwide[1]. It is characterized by severe pain, inflammation, and corneal opacity. *Streptococcus pneumoniae*, a Gram-

positive invasive pathogen, is one of the leading causes of keratitis in India and globally, and it is associated with both trauma and the use of contact lenses[2]. It is also widely responsible for pneumonia, meningitis[3], septicemia[4], and otitis media [5]. Central to the pathogenicity of *S. pneumonia* are pneumolysin [6,7], a pore-forming toxin, autolysin [8], and components of the cell wall [9].

Corneal infections due to *S. pneumoniae* are very aggressive irrespective of antibiotic sensitivity. A recent 10-year trend analysis in antimicrobial resistance among respiratory isolates of *S. pneumoniae* showed an increase in prevalence genotypically [10]. Furthermore, there are recent reports on infections caused by antibiotic resistant strains of *S. pneumonia* [11]. Reports also indicate the growing trend of resistance toward various classes of antibiotics including fluoroquinolones, levofloxacin, and ofloxacin in *S. pneumonia* [12,13]. The increase in antibiotic resistance in ocular isolates of *S. pneumoniae* was also recently reported [14]. This growing prevalence of antibiotic-resistant *S. pneumoniae* is an increasing concern worldwide and, thus, more research for alternative antimicrobial agents is highly required.

The corneal epithelium offers the first line of defense against invading pathogens [15] and mediates innate immune responses by secreting cytokines, chemokines, and antimicrobial peptides (AMPs) through different signaling pathways [16,17]. AMPs are central to the immune responses against pathogens and are mainly secreted by epithelial cells and immune cells. Since AMPs have a broad spectrum of activity and kill pathogens rapidly, it is mostly difficult for the pathogens to develop resistance against them [18,19]. Different classes of AMPs like defensins, cathelicidin, and S100A proteins are known to be expressed in mammals, including humans [20]. Of the several AMPs, we focused on LL-37, the only human cathelicidin, which also has multifunctional abilities including the induction of cytokines, wound healing, and autophagy in cells [21,22]. LL-37, encoded by the *CAMP* gene, is widely expressed by epithelial cells, neutrophils, macrophages, and lymphocytes[23]. It is induced mainly during bacterial infection and inflammation, and it is known to exhibit microbicidal activity, along with ability to influence several innate inflammatory processes and adaptive immune responses [24,25]. *CRAMP*, a mouse homolog to LL-37, deficient mice are reported to be more susceptible to keratitis caused by *Pseudomonas aeruginosa* [26]. Enhanced *Salmonella typhimurium* survival was reported in macrophages obtained from *CRAMP*-deficient mice [27], and these mice were more susceptible to group A *Streptococcus* skin infection [28]. Overall, these accentuate the importance of further studies to understand the role of LL-37 during infections.

In this study, we determined the expression pattern of several AMPs from patients with *S. pneumoniae* corneal infection for the first time, and also *in vitro* using both primary and immortalized human corneal epithelial cells in response to this pathogen. In addition, we infected U937 cells and checked the expression of AMPs in these cells, since immune cells migrate to the site of infection and secrete AMPs and cytokines. We also found that *S.pneumoniae* causes NF-κB, MAPK, and STAT3 activation in corneal epithelial cells. We further studied the expression of LL-37, the sole member of the human cathelicidin group, by HCEC in response to *S. pneumoniae*, and found that LL-37 expression is mediated by STAT3. We additionally examined the killing effect of LL-37 against laboratory and clinical strains of *S. pneumoniae*. Since antibiotic resistance is on rise, further studies are highly needed to ascertain other therapeutic interventions, and LL-37 might have the potential to be considered as an alternative to combat *S. pneumoniae* infections.

2. Materials and Methods

2.1. Corneal Scrapings

Corneal scrapings were collected from patients with bacterial keratitis with informed consent. The protocol for obtaining scrapings was approved by the Institutional Review Board of Hyderabad Eye Research Foundation, LV Prasad Eye Institute (LVPEI), India and the research followed the tenets of the Declaration of Helsinki. Cadaveric corneas unsuitable for transplant purposes were used for controls and were obtained from Ramayamma International Eye Bank, LVPEI, India. The

corneal tissue sections of *S. pneumoniae* patients were obtained from the Pathology Department, LVPEI.

2.2. Identification of Bacterial Strains

Corneal ulcer materials collected aseptically were investigated for bacterial identification at Jhaveri Microbiology Centre, LVPEI, following institute protocol. Briefly, ulcer materials were placed on glass slides for Gram staining and were inoculated in different specific media for bacterial cultures. The pure homogeneous culture was then subjected to Vitek 2 compact (bioMerieux Inc, Durham, NC, USA) analysis for the identification of the bacterium along with Gram staining and a series of biochemical tests.

2.3. Bacterial Culture

For bacterial infection experiments, *Streptococcus pneumoniae* American Type Culture Collection (ATCC) 49619 (Sp ATCC) and an ocular clinical isolate (Sp Clinical Strain), obtained from LV Prasad Eye Institute, were used in this study. Both the strains were grown in Todd Hewitt broth (THB) (Sigma-Aldrich, St. Louis, MO, USA) overnight at 37 °C in the presence of 5% CO_2. Bacteria were grown to mid-exponential phase containing 10^8 colony-forming units (cfu)/mL, centrifuged at 10,000 rpm for 10 min, and suspended in 1× phosphate buffer saline (PBS) to desired concentration for in vitro experiments.

2.4. Cell Culture

Immortalized human corneal epithelial cells 10.014 pRSV-T[29] were maintained in keratinocyte serum free (KSFM) media containing bovine pituitary extract and recombinant human epidermal growth factors (Invitrogen, Carlsbad, CA, USA) at 37°C and 5% CO_2[30]. HCECs were grown overnight in 12-well plates (1× 10^5 cells/well) and incubated with bacteria usually at a multiplicity of infection (MOI) of 1:10 (cells: bacteria) or as mentioned in the text. The cells were then washed with 1× PBS and processed further. Primary human corneal epithelial cells were isolated as described before[31] from donor corneas obtained from Ramayamma International Eye Bank, LVPEI, India. In brief, bulbar conjunctival tissues were removed, and the cornea was incubated in Hanks buffer solution containing dispase and antibiotics at 4 °C. Corneal epithelium was collected by gentle scraping, before being trypsinized, centrifuged, and grown in KSFM containing required growth factors. Cells from passage two were used for experiments. Human monocytes U937 were cultured under similar conditions in RPMI containing 10% fetal bovine serum (FBS), and were infected at an MOI of 1:10, unless mentioned otherwise. Cells were incubated with STAT3 inhibitor, Stattic (Abcam, Cambridge, MA, USA), during some experiments as described in the text.

2.5. RNA Isolation, Complementary DNA (cDNA) Synthesis and Quantitative PCR Analysis

The expression of different genes from corneal scrapings, and primary and immortalized cell lines were determined by quantitative PCR. Total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA, USA), and reverse transcription was done using a Verso cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's protocol. Quantitative PCR was done on an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems, Grand Island, NY, USA) using the SYBR Green PCR Master Mix (Thermo Scientific, MA, USA). The primers used were described in an earlier study[30]. Relative quantities of messenger RNA (mRNA) expression of respective genes were normalized using the $2^{-\Delta\Delta ct}$ method using *GAPDH* as the housekeeping gene.

2.6. Western Blot

HCECs were incubated with *S. pneumoniae* for indicated timepoints at an MOI of 1:10 (cells to bacteria). Endogenous phospho-Iκβ, phospho-p38, phospho-ERK, phospho-JNK, phospho-STAT3,

and LL-37 protein expression levels were validated by Western blot. The cells were washed with 1× PBS post infection and lysates were prepared using 1× lysis buffer (CST, Danvers, MA, USA); total protein was estimated by the bicinchoninic acid (BCA) method as described in the manufacturer's protocol (Thermo Scientific, CA, USA). Proteins were separated using 10% or 12% SDS-PAGE, transferred to a nitrocellulose membrane, and probed with p-p38, p-ERK, p-Iκβ, p-JNK, and p-STAT3 (raised in rabbit), and β-actin and LL-37 (raised in mouse) (1:2000 dilution; CST, MA). Subsequently, blots were incubated with IRDye-680 secondary antibody (1:6000 dilution; LI-COR Biotechnology, Lincoln, NE, USA) and were developed on an Odyssey CLx Imaging System (LI-COR Biotechnology, NE).

2.7. Immunostaining

HCECs were cultured overnight on coverslips and were infected with *S. pneumoniae* at an MOI of 1:10 (cells to bacteria) for the defined time points, before being processed as mentioned earlier[32]. In brief, the cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.2% Triton X-100, and incubated with anti-LL-37 antibody (1:100; BioLegend, San Diego, CA, USA), followed by incubation with Alexafluor-488-labeled secondary antibody (1:500; Molecular probes). Images were captured on a fluorescent microscope (Olympus IX73, Zeiss, Germany) using the 20× objective.

2.8. Immunohistochemistry

Tissue sections (5 µm) of paraffin-embedded corneas diagnosed with *S. pneumoniae* keratitis were obtained along with control cadaveric corneas found unsuitable for transplantation. These sections were deparaffinized and stained with anti LL-37 antibody (1:100; Novus BioLegend, San Diego, CA,USA) as described earlier[33] and were then counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Abcam, Cambridge) before being observed under fluorescent microscope (Olympus IX73, Zeiss, Germany) using the 20× objective and imaged using Olympus DP71 camera.

2.9. In Vitro Susceptibility Test of S. pneumoniae *with LL-37*

Both the ATCC and the clinical strain of *S. pneumoniae* were grown in THB medium, in a CO_2 incubator at 37°C until an optical density at 600 nm of 0.28 was obtained. Bacteria were centrifuged at 10,000 rpm for 10 min and were further diluted to 10^4 cfu/mL and were incubated with increasing concentration of LL-37 (Invivogen, San Diego, CA, USA) in THB medium at 37 °C for 4 h. Serial dilutions of the culture were then plated in blood agar plates and bacterial viability was quantitated by cfu after overnight incubation.

2.10. Statistical Analysis

Bar graphs represent means and error bars represent standard error of the mean (SEM). Statistical analysis was performed using either one-way ANOVA or an unpaired *t*-test using Prism7 (GraphPad Software, La Jolla, CA, USA); *p*-values less than 0.05 were considered significant.

3. Results

3.1. Differential Expressions of Antimicrobial Peptides during S. pneumoniae *Keratitis*

Expressions of AMPs were reported in several diseases caused by various pathogens like *H. pylori* [34], *P. aeruginosa*[35], mycobacterium [36], and *S. pyogenes* [37]. We also earlier reported the expression of AMPs from patients with corneal infections caused by *P. aeruginosa*[30] and *C. pseudodiphtheriticum*[38]. Here, we report the expression of several AMPs in patients with *S. pneumoniae* corneal infections as determined by quantitative PCR. As shown in Figure 1, AMPs are differentially expressed in patients with *S. pneumoniae* keratitis, presented here as the log of relative gene expression, log (RQ). We obtained corneal scrapings from 12 patients detected with *S. pneumoniae* keratitis, and eight cadaveric corneas were used for controls. The clinical characteristics

of the patients are detailed in Table 1. Four members of human beta-defensin (hBD) group were checked, and we observed a significant increase in the expression of *hBD2*, *hBD3*, and *hBD4*. However, the expression of *hBD1* was significantly reduced. The expressions of four AMPs belonging to the group of S100A proteins, *S100A7*, *S100A8*, *S100A9*, and *S100A12*, were also significantly upregulated in these patients, whereby the most increased expression was seen with *S100A8*. There was a significant increase in expression of other AMPs including *LL-37*, hepcidin, lipocalin 2, and Rnase7.

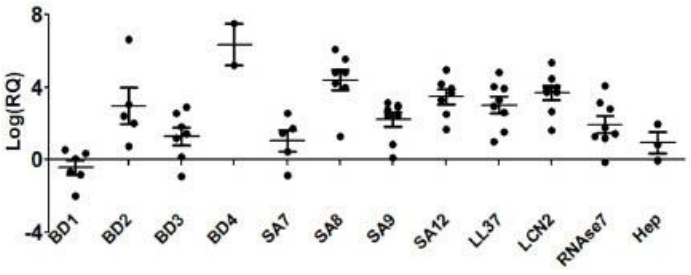

Figure 1. Differential expression of antimicrobial peptides in patients with *Streptococcus pneumoniae* keratitis. Expression of antimicrobial peptides was determined from corneal scrapings of patients infected with *S. pneumoniae* by quantitative PCR, and the values are represented as the log of relative gene expression (log (RQ)) compared to uninfected cadaveric corneas ($p<0.0001$). Data points represent individual patients.

Table 1. Clinical characteristics of patients.

Characteristics	*Streptococcus pneumoniae*
Age	25 to 90
Mean (SD)	52.25 (20.176)
Sex	
Male (%)	75
Female (%)	25
Hypopyon	
Yes (%)	25
No (%)	75
Occupation	
Agriculture/Manual Labor (%)	75
Desk Jobs (%)	25
Size of Ulcer	
<5 mm (%)	37.5
5–15 mm (%)	25
>15 mm (%)	37.5

3.2. Expression of Antimicrobial Peptides in Immortalized and Primary HCECs and U937 in Response to S. pneumoniae

Although there are few reports regarding activation of Toll-like receptors (TLRs) and inflammasomes, and the production of proinflammatory cytokines by human corneal epithelial cells in response to *S. pneumoniae*[39,40], the expression of AMPs remains to be explored. As we saw differential expression of AMPs in patients with *S. pneumoniae* corneal infections, our aim was to see if *S. pneumonia* infection causes AMP secretion *in vitro*. HCECs were exposed to the bacteria for 3 h

followed by 1 h of incubation in gentamycin-containing media to kill any external adhered bacteria; the expressions of AMPs were determined by qPCR. Furthermore, to see if there were any differences in AMP expression in response to clinical strain, we infected HCECs with an ocular clinical strain of *S. pneumoniae* (Sp CS) in addition to the laboratory ATCC strain. Sp CS was positive for pneumolysin, similar to the ATCC strain (data not shown), and was sensitive to all regular antibiotics, except amikacin. As shown in Figure 2, there was significantly increased expression of all the β-defensins studied, particularly that of *hBD2* (30-fold) and *hBD4* (50-fold). The antimicrobial peptides belonging to the S100A group were also expressed by HCECs in response to *S. pneumoniae*. There was more than a 10-fold increase of *S100A7*, and more than a six-fold increase of *S100A8* and *S100A9*. Surprisingly, S100A12, which was significantly increased in patients, was not expressed by HCECs in response to either Sp ATCC or Sp CS. Increased expressions of *LL-37* (six-fold) and hepcidin (five-fold) were also observed in HCECs in response to *S. pneumoniae*. In contrast, the Sp CS infection caused significantly reduced expression of all AMPs compared to the Sp ATCC. The reduced AMP expressions by Sp CS were, however, not due to the induction of cell death, as there was no significant difference in cell viability between cells infected with Sp CS or Sp ATCC (Figures S1A,B).

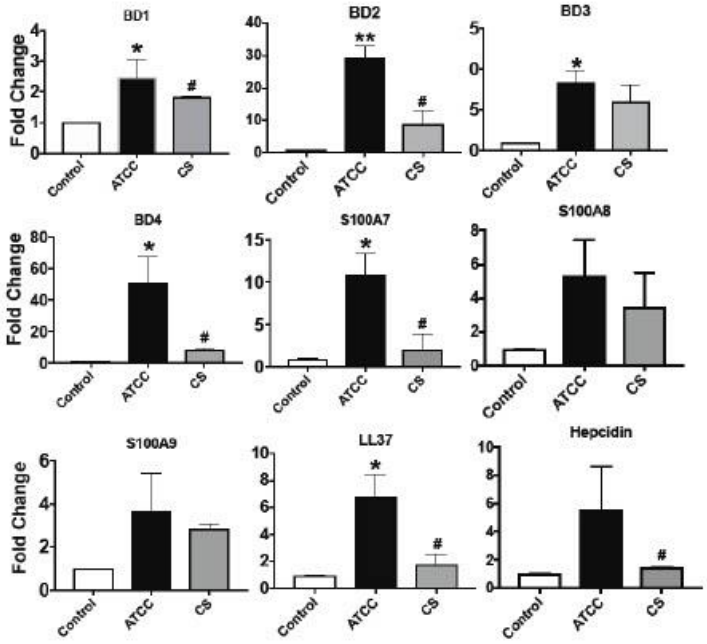

Figure 2. Expression of antimicrobial peptides *in vitro*. HCECs were exposed to *S. pneumoniae* at a multiplicity of infection (MOI) of 10:1 (bacteria:cells), both laboratory and clinical isolates, and expressions of antimicrobial peptides were determined after 4 h by real-time PCR. Uninfected cells were used as a control and *GAPDH* was used as the housekeeping gene for qPCR. The experiment was performed in technical duplicates and was repeated twice. (*$p < 0.05$, **$p < 0.005$ compared to control; #$p < 0.05$ compared to ATCC).

We next checked the expression of these AMPs in response to *S. pneumoniae* in primary corneal epithelial cells obtained from donor corneas, as primary cells closely resemble the tissue from which they are derived. Among the β-defensins, expression of only *hBD3* could be detected, which increased significantly in response to Sp ATCC. There were increased expressions of *S100A8* and

S100A9; however, changes in *S100A12* expression were not significant. Increased LL-37 expression was also observed in primary human corneal epithelial cells in response to Sp ATCC. While there was an insignificant difference in the expression of RNAse7, the expression of hepcidin was not detected in these primary cells in response to *S. pneumoniae*. Except for S100A9, there were no significant changes in the expression of other AMPs by primary corneal epithelial cells in response to clinical isolate Sp CS (Figure 3).

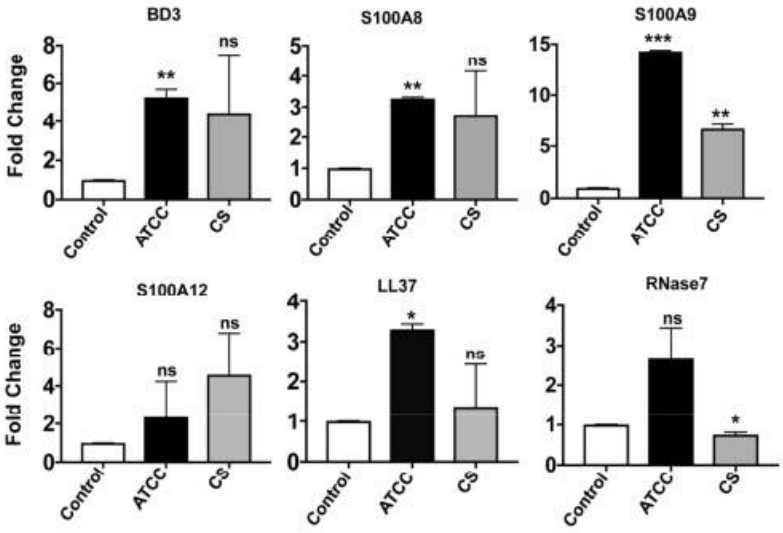

Figure 3. Expression of antimicrobial peptides in primary corneal epithelial cells *in vitro*. Primary human corneal epithelial cells were cultured from donor corneas and exposed to *S. pneumoniae* at an MOI of 10:1 (bacteria:cells), and expressions of antimicrobial peptides were determined after 4 h by real-time PCR. *GAPDH* was used as the housekeeping gene. The experiment was performed in technical duplicates and was repeated three times. (*$p < 0.05$, ** $p < 0.005$, ns is not significant).

While epithelial cells are the first line of defense, neutrophils and macrophages are prominent immune cells that combat pathogens during ocular infections [41]. Therefore, along with epithelial cells, the expression of AMPs by a human monocyte cell line, U937, was also determined. As shown in Figure 4, there was no significant increase or changes in expression of any of the AMPs by U937 in response to Sp ATCC or Sp CS.

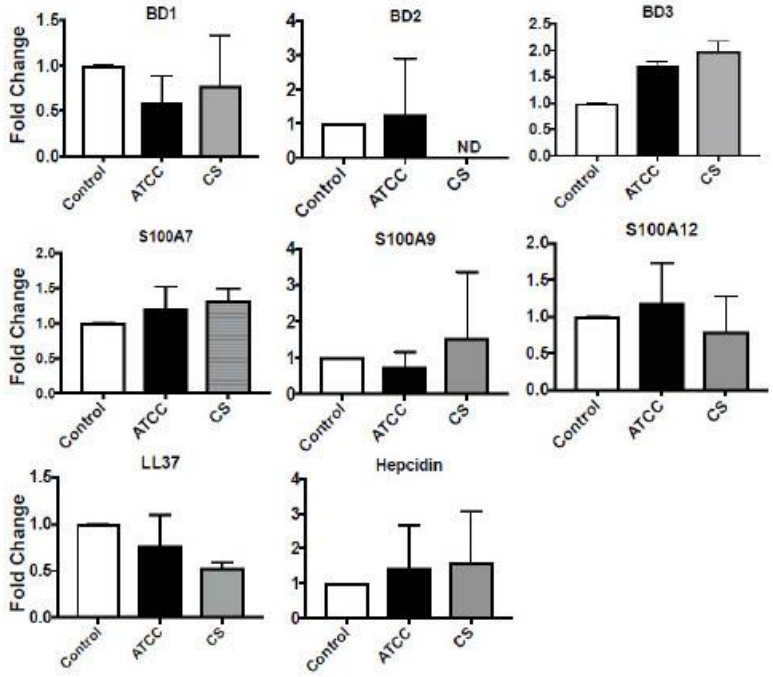

Figure 4. Expression of antimicrobial peptides in U937 *in vitro*. U937 cells were exposed to *S. pneumoniae* at an MOI of 10:1 (bacteria:cells), and expressions of antimicrobial peptides were determined after 4 h by real-time PCR. *GAPDH* was used as the housekeeping gene.

3.3. S. pneumoniae *Induces LL-37 Expression in Human Corneal Epithelial Cells*

LL-37 is the sole member of the human cathelicidin group; thus, we further investigated LL-37 expression during *S. pneumoniae* infection. We already detected increased LL-37 expression from corneal scrapings of *S. pneumonia* keratitis patients by quantitative PCR (Figure 1). To determine LL-37 expression at the protein level, tissue specimens were obtained from keratitis patients (n=4) who underwent therapeutic penetrating keratoplasty at our institute. As shown in Figure 5A, specific staining for LL-37 was observed in these sections of *S. pneumonia*-infected corneas in both the epithelium and stroma. Cadaveric corneas devoid of any infection were used as the control for the staining experiment. To study the expression of LL-37 in the immortalized corneal epithelial cell line in response to *S. pneumonia*, HCECs were infected with *S. pneumoniae* for different time periods, and the expression of LL-37 was determined by qPCR. The increase in expression of LL-37 was maximum at 4 h post infection, after which there was a decline in the expression, reaching minimum by 24 h post infection (Figure 5B). The expression of LL-37 in response to *S. pneumoniae* was also checked by immunostaining and Western blot analysis, which showed increased expression of LL-37 by *S. pneumoniae* in HCECs (Figures 5C,D). To determine if LL-37 expression by *S. pneumoniae* requires de novo protein synthesis, cells were treated with cycloheximide, a potent translation inhibitor, and LL-37 expression was checked both by qPCR and Western blot. Treatment of HCECs with cycloheximide (CHX) attributed significantly reduced LL-37 gene expression (Figure 5E) and protein levels (Figure 5F) in *S. pneumoniae* infected cells.

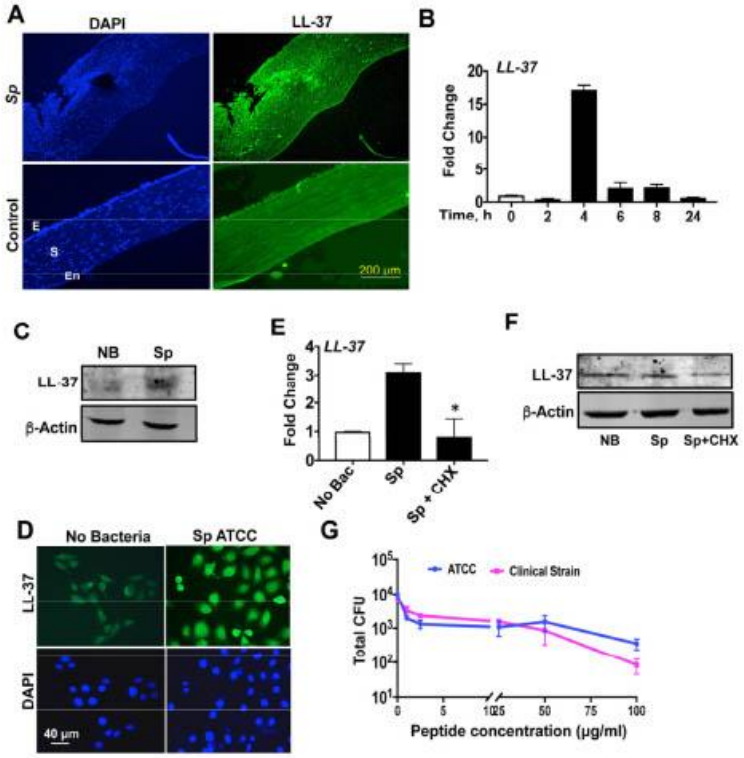

Figure 5. *S. pneumoniae* induces LL-37 expression in human corneal epithelium. Tissue sections from control cadaveric and *S. pneumoniae*-infected corneas were stained with anti-LL-37 antibody followed by Alexafluor 488 secondary antibody and was counterstained with DAPI. The sections were imaged under a fluorescence microscope using the 10× objective. The parts are labeled as follows: E = epithelium, S = stroma, En = endothelium (**A**). Human corneal epithelial cells were exposed to *S. pneumoniae* for defined time periods, and LL-37 expression was determined by quantitative PCR (**B**). LL-37 expression by HCECs in response to *S. pneumoniae* was further determined by Western blot assay using anti-LL-37 antibody (**C**). The expression of LL-37 was also determined by immunostaining. Cells were grown on coverslips and exposed to bacteria at a multiplicity of infection of 10:1 (bacteria:cells) and stained using anti-LL-37 antibody followed by Alexafluor 488 (**D**). *De novo* protein synthesis by HCECs in response to *S. pneumoniae* was inhibited in the presence of cycloheximide (1µg/mL) and the expression of LL-37 was determined by quantitative PCR (**E**) and Western blot (**F**). Laboratory and clinical strains of *S. pneumoniae* were exposed to different concentrations of LL-37 for 4 h and killing efficiency of LL-37 was determined by the colony-forming unit assay (**G**).

3.4. LL-37 Exhibits Antimicrobial Activity against S. pneumoniae

Although LL-37 was tested against various clinical isolates[26] of different bacteria, we did not come across any reports involving *S. pneumoniae*. To determine the antimicrobial properties of LL-37 against *S. pneumoniae*, LL-37 was tested against both the ATCC and clinical strains, with concentrations ranging from 0 to 100 µg/mL by colony-forming unit (cfu) assay. Both strains (10^4 cfu/mL) were incubated with different concentrations of LL-37 for 4 h at 37°C and were plated in

serial dilutions and incubated overnight. LL-37 resulted in a significant decrease in bacterial survival and was effective against both the strains (Figure 5G).

3.5. NF-κB and MAPK Pathways Are Not Responsible for LL-37 Expression by S. pneumoniae*in HCECs*

There are several reports regarding the expression of LL-37 mediated by NF-κB and MAPK signaling pathways in different cell lines [42,43]. Therefore, the role of NF-κB and MAPK signaling pathways in *S. pneumonia*-induced LL-37 expression by HCECs was explored using specific inhibitors for NF-κB and MAPK activation. Firstly, we checked if *S. pneumoniae* causes activation of NF-κB and MAPK pathways in human corneal epithelial cells. HCECs were exposed to *S. pneumoniae* for different time points, and the phosphorylation levels of IκB, p38, JNK, and ERK were determined by Western blot analysis. As shown in Figure 6, *S. pneumoniae* induced the phosphorylation of IkB (Figure 6A), JNK (Figure 6B), p38 (Figure 6C), and ERK (Figure 6D) in HCECs by 30 min of infection. Next, to see if any of these signaling proteins mediate LL-37 expression, HCEC were infected with *S. pneumoniae* in the presence of inhibitors for IκB (MG132), p38 (SB203580), JNK (SP600125), and ERK (PD98059). As shown in Figure 6E, there was no reduction in LL-37 expression by *S. pneumoniae* in the presence of these inhibitors, which clearly indicates that expression of LL-37 is not mediated by NF-κB and MAPK pathways in HCEC.

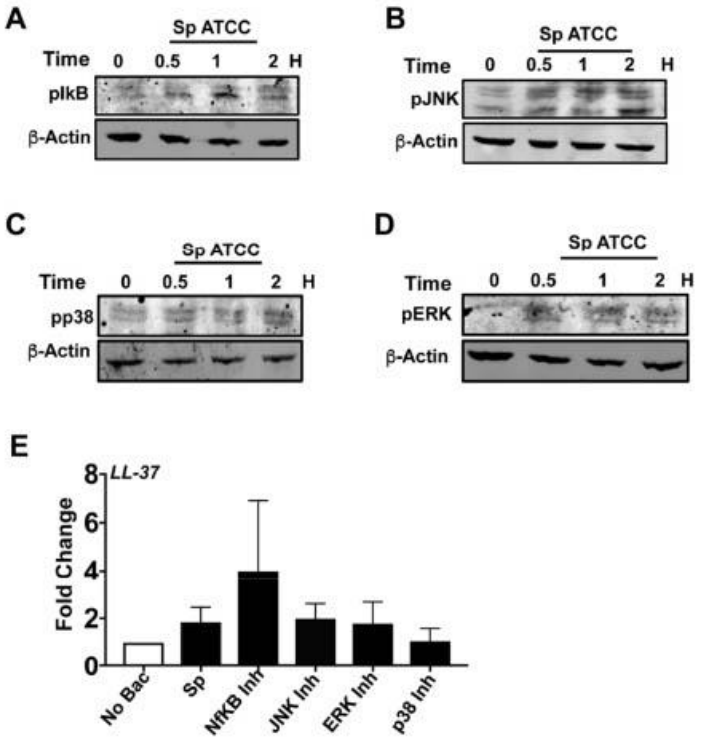

Figure 6. *S. pneumoniae* causes NF-κB and MAPK activation in human corneal epithelial cells. HCECs were exposed to *S. pneumoniae* for 0.5, 1, and 2 h, and expressions of phospho-IκB (**A**), phospho-JNK (**B**), phospho-p38 (**C**), and phospho-ERK (**D**) were determined by immunoblot assay. Immunoblot of β-actin was used as a loading control. HCECs were exposed to *S. pneumoniae* in the presence of NF-κB inhibitor MG132 (10 μM), JNK inhibitor SP600125 (25 μM), p38 inhibitor SB20358 (10 μM), and ERK inhibitor PD98059 (20 μM), and LL-37 expression was determined by qPCR (**E**).

3.6. LL-37 Expression by S. pneumoniae*in HCECsIsMediated by STAT3*

As shown in Figure 6, *S. pneumonia*-induced LL-37 expression in HCECs was not mediated by NF-κB and MAPK signaling pathways. Further bioinformatics analysis of the promoter region of LL-37 revealed STAT3-binding sites among other transcription factors. Miraglia et al. recently showed that entinostat-induced LL-37 expression was mediated by STAT3 [44]. Likewise, Gombart et al. also previously described different STAT3-binding sites in the LL-37 promoter region [45]. We, therefore, checked if activation of STAT3 might be responsible for LL-37 expression by *S. pneumoniae*. Firstly, we determined the activation of STAT3 by *S. pneumoniae* in corneal epithelial cells. HCECs were exposed to *S. pneumoniae* for 0.5, 1, and 2 h, and phosphorylation of STAT3 was evaluated. Increased phosphorylation of STAT3 was observed within 30 min of exposure to *S. pneumoniae* that continued up to 2 h (Figure 7A).Total STAT3 was also detected, and β-actin was used as a loading control. To confirm that LL-37 induction is mediated by STAT3, HCECs were exposed to *S. pneumoniae* in the presence of a selective inhibitor of STAT3, Stattic. Stattic acts by inhibiting the dimerization and nuclear translocation of STAT3 in cells [46]. We found that the STAT3 inhibitor effectively reduced the expression of LL-37 by *S. pneumoniae* (Figure7B), indicating that *S. pneumoniae*-induced LL-37 expression is mediated by STAT3. These results, therefore, indicate that *S. pneumoniae* causes phosphorylation of STAT3, which translocates to the nucleus and induces the expression of LL-37 (Figure7C) in human corneal epithelial cells.

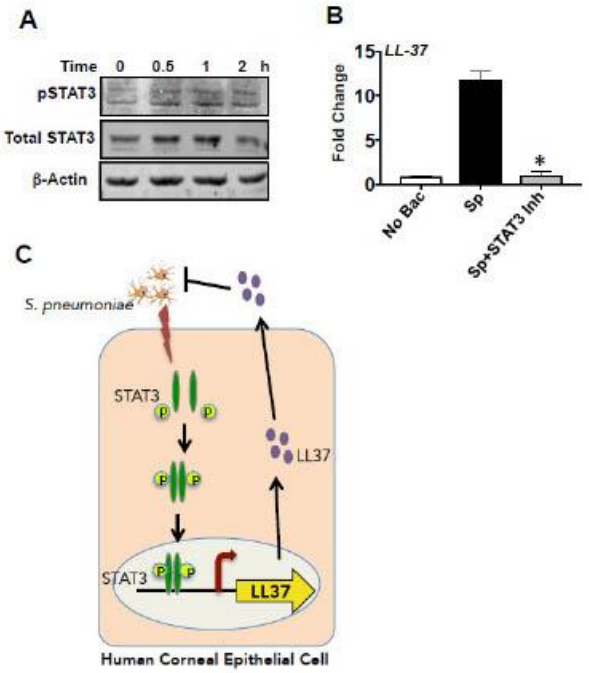

Figure 7. LL-37 expression by *S. pneumoniae* is mediated by STAT3. *S. pneumoniae* causes the activation of STAT3 in a time-dependent manner, which is evident from the phosphorylation of STAT3 in HCECs by Western blot analysis (**A**). HCECs were exposed to *S. pneumoniae* for 4 h in the absence or presence of a STAT3 inhibitor (25 μM), and expression of LL-37 was determined by quantitative PCR (**B**). Representation of proposed mechanism of STAT3 mediated expression of LL-37 by *S. pneumoniae* (**C**).

4. Discussion

S. pneumoniae is the second most leading Gram-positive bacteria causing corneal infections [47] in India. The increase in drug-resistant strains causing corneal infections[48] and the significant economic burden arising from corneal blindness make it essential to identify alternative therapeutic interventions to combat bacterial keratitis. Antimicrobial peptides are one of the most critical candidates that can be developed to overcome antimicrobial resistance among pathogens. Our group earlier reported the expression profile of AMPs in patients with *P. aeruginosa* keratitis [30] and *C. pseudodiphtheriticum* keratitis [38]. In the present study, we have determined the expression profile of endogenous AMPs in corneal ulcers from patients infected with *S. pneumoniae*. Moreover, we showed that *S. pneumoniae* induces LL-37 in a STAT3-dependent manner and that LL-37 is efficient in killing *S. pneumoniae*.

We determined the expression pattern of AMPs elicited in corneal infections caused by *S. pneumoniae* in patients for the first time, and we found significantly increased expressions of hBD2 and 3, S100A8, S100A9, S100A12, LL-37, lipocalin, Rnase7, and hepcidin. Interestingly, the profile elicited by Gram-positive *S. pneumoniae* is markedly distinct from that induced by *P. aeruginosa*[30]. While *P. aeruginosa* caused a significant decrease in Rnase7 expression, there was increased expression of the same by *S. pneumoniae*. The expression of hBD4 was not detected in any patient samples or *in vitro* during *P. aeruginosa* infection; on the contrary, expression of hBD4 was found in both patient samples and in HCECs in response to *S. pneumoniae*. Decreased expression of hBD1 was also seen in *S. pneumoniae* corneal infections compared to elevated expression of the same during *P. aeruginosa* keratitis. These findings are interesting and can probably be exploited in the future to distinguish between Gram-positive and Gram-negative bacterial infection. McIntosh et al. vividly reported the expression spectrum of AMPs by ocular surface cells, such as limbal explant, conjunctival, and corneal epithelial cells [49]. We further checked the expression of the AMPs using primary and immortalized human corneal epithelial cells *in vitro* in response to both laboratory and clinical strains. Increased expressions of AMPs were detected in both primary and immortalized cells in response to *S. pneumoniae*. There are earlier reports of expression of hBD2 by HCECs in response to *S. aureus* in a TLR2-dependent manner [50]. We also previously determined the expression of AMPs in HCECs in response to *P. aeruginosa* [30] and *C. pseudodiphtheriticum* [38], and, while the wild-type PAO1 mostly subverted the expression of AMPs, *C. pseudodiphtheriticum* caused increased expression of S100A peptides and LL-37, but no significant changes in the expression of defensins were detected. However, increased expression of defensins is observed in case of *S. pneumoniae* corneal infections. Corneal limbal epithelial cells were also shown to express several AMPs including hBD3 and RNase 7 in response to *A. castellanii* [51]. Thus, the response of the host cells toward pathogens seems to be very specific for organisms and varies significantly according to the type of insult. In contrast to the increased expression by Sp ATCC, infection by the clinical strain caused reduced expressions of the AMPs in HCECs. A similar observation was determined in primary cells, where increased expressions of AMPs were detected in response to Sp ATCC compared to Sp CS. Several pathogens including *P. aeruginosa* [30], *N. gonorrhoeae* [52], and *S. flexeneri* [53] are known to cause downregulation in the expression of various AMPs. This seems to be the case with the clinical strain of *S. pneumoniae* used in this study. Furthermore, there were no significant changes in the expression of AMPs in response to both the laboratory and the clinical strains in U937.

Although we found that *S. pneumoniae* induced upregulation of expression of several AMPs, we focused on LL-37 for further studies. LL-37 is known to be expressed by ocular surface epithelia including regenerating corneal epithelium, lung, and gastric epithelial cells [42,54,55] in response to several pathogens. *S. pneumoniae* induced LL-37 expression by lung mast cells [56] similar to what we found with human corneal epithelial cells. Increased LL-37 was also observed in corneal sections obtained from patients with *S. pneumoniae* keratitis in this study. Kinetics of LL-37 expression by HCEC was determined, showing expression peaks around 4 h after which the expression level was lowered. LL-37 was earlier reported to be expressed in response to *M. bovis* in A549 epithelial cells

[42] and in soft tissue infections by *S. pyogenes* [37]. Increased LL-37 was also detected and found to be protective in a murine model of *P. aeruginosa* keratitis [57].

The antimicrobial activity of LL-37 was studied in detail against various ocular pathogens like *P. aeruginosa, S. aureus,* and *S. epidermidis* [58]. However, there are no reports of LL-37 activity against ocular strains of *S. pneumoniae*. In this study, the potential bacteria-killing activities of LL-37 were examined against a laboratory and clinical strain of *S. pneumoniae*. Our study indicated that LL-37 is potent against *S. pneumoniae,* both laboratory and clinical strains. LL-37 was also shown to be effective in *S. pneumoniae* meningitis in the presence of ceftaroline [59]. However, in contrast to several reports describing the killing efficiency of LL-37 against various pathogens, Zahner et al. showed that LL-37 can also cause macrolide resistance in *S. pneumoniae* [60].

There are several varying reports regarding the regulation of LL-37 expression, perhaps due to different cell types and in response to varied stimuli. Several studies in the past showed that LL-37 expression is regulated by MAPK signaling pathways. Sayama et al. reported reduced LL-37 expression in the inhibition of the p38 kinase pathway in human keratinocytes. In contrast, Schauber et al. reported the regulation of LL-37 expression by ERK pathways and not by p38 in response to sodium butyrate [61]. Another group demonstrated that LL-37 expression can be regulated by both ERK and p38 activation in response to *M. bovis* in human lung epithelial cells [42]. In the current study, we observed that, although *S. pneumoniae* causes activation of NF-κB and MAPK pathways, LL-37 expression was not mediated by NF-κB, p38, ERK, or JNK. Interestingly, we found that LL-37 expression in HCEC in response to *S. pneumoniae* was mediated by STAT3. This is similar to a recent findings by Miraglia et al., where they showed that entinostat upregulates LL-37 via STAT3 and HIF-1α and not via MAPK pathways in human macrophages [44]. STAT3 was earlier reported to mediate Reg3γ expression in response to methicillin-resistant *S. aureus*[62]. STAT3 is also involved in the expression of hepcidin by interleukin 6 in HepG2 cells [63]. STAT3 was further reported to upregulate β-defensin 2 expression by IL-22 in lung epithelial cells [64].

5. Conclusions

In summary, we showed that *S. pneumoniae* elicits a wide array of AMP expression during corneal infections in humans, and induces expression of AMPs in primary and immortalized human corneal epithelial cells. We also observed that LL-37 is effective against *S. pneumoniae,* and that LL-37 expression by *S. pneumoniae* is mediated by the activation of STAT3 and not via MAPK pathways. Taken together, LL-37 might have the potential to be developed as an alternative therapeutic intervention to combat antibiotic resistance and treat bacterial keratitis.

Supplementary Materials: The following are available online at www.mdpi.com/xxx/s1: Figure S1: Determination of cell viability of HCEC after *S. pneumoniae* infection.

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References

1. Gupta, N.; Tandon, R.; Gupta, S.K.; Sreenivas, V.; Vashist, P. Burden of corneal blindness in India. *Indian J. Community Med.* **2013**, *38*, 198–206, doi:10.4103/0970-0218.120153.

2. Lin, C.C.; Lalitha, P.; Srinivasan, M.; Prajna, N.V.; McLeod, S.D.; Acharya, N.R.; Lietman, T.M.; Porco, T.C. Seasonal trends of microbial keratitis in South India. *Cornea* **2012**, *31*, 1123–1127, doi:10.1097/ICO.0b013e31825694d3.
3. Short, W.R.; Tunkel, A.R. Changing Epidemiology of Bacterial Meningitis in the United States. *Curr. Infect. Dis. Rep.* **2000**, *2*, 327–331.
4. Tomasz, A. Antibiotic resistance in Streptococcus pneumoniae. *Clin. Infect. Dis.* **1997**, *24* (Suppl. 1), S85–S88.
5. Heikkinen, T.; Chonmaitree, T. Importance of respiratory viruses in acute otitis media. *Clin. Microbiol. Rev.* **2003**, *16*, 230–241.
6. Hirst, R.A.; Kadioglu, A.; O'Callaghan, C.; Andrew, P.W. The role of pneumolysin in pneumococcal pneumonia and meningitis. *Clin. Exp. Immunol.* **2004**, *138*, 195–201, doi:10.1111/j.1365-2249.2004.02611.x.
7. Tweten, R.K. Cholesterol-dependent cytolysins, a family of versatile pore-forming toxins. *Infect. Immun.* **2005**, *73*, 6199–6209, doi:10.1128/IAI.73.10.6199-6209.2005.
8. Berry, A.M.; Lock, R.A.; Hansman, D.; Paton, J.C. Contribution of autolysin to virulence of Streptococcus pneumoniae. *Infect. Immun.* **1989**, *57*, 2324–2330.
9. Jedrzejas, M.J. Pneumococcal virulence factors: Structure and function. *Microbiol. Mol. Biol. Rev.* **2001**, *65*, 187–207, doi:10.1128/MMBR.65.2.187-207.2001.
10. Hagiwara, E.; Baba, T.; Shinohara, T.; Kitamura, H.; Sekine, A.; Komatsu, S.; Ogura, T. Ten-Year Trends and Clinical Relevance of the Antimicrobial Resistance Genotype in Respiratory Isolates of Streptococcus pneumoniae. *Chemotherapy* **2017**, *62*, 256–261, doi:10.1159/000470828.
11. Cherazard, R.; Epstein, M.; Doan, T.L.; Salim, T.; Bharti, S.; Smith, M.A. Antimicrobial Resistant Streptococcus pneumoniae: Prevalence, Mechanisms, and Clinical Implications. *Am. J. Ther.* **2017**, *24*, e361–e369, doi:10.1097/MJT.0000000000000551.
12. Goldstein, M.H.; Kowalski, R.P.; Gordon, Y.J. Emerging fluoroquinolone resistance in bacterial keratitis: A 5-year review. *Ophthalmology* **1999**, *106*, 1313–1318.
13. Snyder, M.E.; Katz, H.R. Ciprofloxacin-resistant bacterial keratitis. *Am. J. Ophthalmol.* **1992**, *114*, 336–338.
14. Lalitha, P.; Manoharan, G.; Karpagam, R.; Prajna, N.V.; Srinivasan, M.; Mascarenhas, J.; Das, M.; Porco, T.C.; Lietman, T.M.; Cevallos, V.; et al. Trends in antibiotic resistance in bacterial keratitis isolates from South India. *Br. J. Ophthalmol.* **2017**, *101*, 108–113, doi:10.1136/bjophthalmol-2016-308487.
15. Evans, D.J.; Fleiszig, S.M. Why does the healthy cornea resist Pseudomonas aeruginosa infection? *Am. J. Ophthalmol.* **2013**, *155*, 961–970e962, doi:10.1016/j.ajo.2013.03.001.
16. Adhikary, G.; Sun, Y.; Pearlman, E. C-Jun NH2 terminal kinase (JNK) is an essential mediator of Toll-like receptor 2-induced corneal inflammation. *J. Leukoc. Biol.* **2008**, *83*, 991–997, doi:10.1189/jlb.1107783.
17. Roy, S.; Karmakar, M.; Pearlman, E. CD14 mediates Toll-like receptor 4 (TLR4) endocytosis and spleen tyrosine kinase (Syk) and interferon regulatory transcription factor 3 (IRF3) activation in epithelial cells and impairs neutrophil infiltration and Pseudomonas aeruginosa killing in vivo. *J. Biol. Chem.* **2014**, *289*, 1174–1182, doi:10.1074/jbc.M113.523167.
18. Peters, B.M.; Shirtliff, M.E.; Jabra-Rizk, M.A. Antimicrobial peptides: Primeval molecules or future drugs? *PLoS Pathog.* **2010**, *6*, e1001067, doi:10.1371/journal.ppat.1001067.
19. Zasloff, M. Antimicrobial peptides of multicellular organisms. *Nature* **2002**, *415*, 389–395, doi:10.1038/415389a415389a.
20. Yan, H.; Hancock, R.E. Synergistic interactions between mammalian antimicrobial defense peptides. *Antimicrob. Agents Chemother.* **2001**, *45*, 1558–1560, doi:10.1128/AAC.45.5.1558-1560.2001.
21. Yuk, J.M.; Shin, D.M.; Lee, H.M.; Yang, C.S.; Jin, H.S.; Kim, K.K.; Lee, Z.W.; Lee, S.H.; Kim, J.M.; Jo, E.K. Vitamin D3 induces autophagy in human monocytes/macrophages via cathelicidin. *Cell Host Microbe* **2009**, *6*, 231–243, doi:10.1016/j.chom.2009.08.004.
22. Nijnik, A.; Pistolic, J.; Filewod, N.C.; Hancock, R.E. Signaling pathways mediating chemokine induction in keratinocytes by cathelicidin LL-37 and flagellin. *J. Innate Immun.* **2012**, *4*, 377–386, doi:10.1159/000335901.
23. Wong, J.H.; Ye, X.J.; Ng, T.B. Cathelicidins: Peptides with antimicrobial, immunomodulatory, anti-inflammatory, angiogenic, anticancer and procancer activities. *Curr. Protein Pept. Sci.* **2013**, *14*, 504–514.
24. Dorschner, R.A.; Pestonjamasp, V.K.; Tamakuwala, S.; Ohtake, T.; Rudisill, J.; Nizet, V.; Agerberth, B.; Gudmundsson, G.H.; Gallo, R.L. Cutaneous injury induces the release of cathelicidin anti-microbial

peptides active against group A Streptococcus. *J. Investig. Dermatol.* **2001**, *117*, 91–97, doi:10.1046/j.1523-1747.2001.01340.x.

25. Ishida, W.; Harada, Y.; Fukuda, K.; Fukushima, A. Inhibition by the Antimicrobial Peptide LL37 of Lipopolysaccharide-Induced Innate Immune Responses in Human Corneal Fibroblasts. *Invest Ophthalmol Vis Sci* **2016**, *57*, 30–39, doi:10.1167/iovs.15-17652.
26. Huang, L.C.; Petkova, T.D.; Reins, R.Y.; Proske, R.J.; McDermott, A.M. Multifunctional roles of human cathelicidin (LL-37) at the ocular surface. *Investig. Ophthalmol. Vis. Sci.* **2006**, *47*, 2369–2380, doi:10.1167/iovs.05-1649.
27. Strandberg, K.L.; Richards, S.M.; Gunn, J.S. Cathelicidin antimicrobial peptide expression is not induced or required for bacterial clearance during salmonella enterica infection of human monocyte-derived macrophages. *Infect. Immun.* **2012**, *80*, 3930–3938, doi:10.1128/IAI.00672-12.
28. LaRock, C.N.; Dohrmann, S.; Todd, J.; Corriden, R.; Olson, J.; Johannssen, T.; Lepenies, B.; Gallo, R.L.; Ghosh, P.; Nizet, V. Group A Streptococcal M1 Protein Sequesters Cathelicidin to Evade Innate Immune Killing. *Cell Host Microbe* **2015**, *18*, 471–477, doi:10.1016/j.chom.2015.09.004.
29. Araki-Sasaki, K.; Ohashi, Y.; Sasabe, T.; Hayashi, K.; Watanabe, H.; Tano, Y.; Handa, H. An SV40-immortalized human corneal epithelial cell line and its characterization. *Investig. Ophthalmol. Vis. Sci.* **1995**, *36*, 614–621.
30. Sharma, P.; Guha, S.; Garg, P.; Roy, S. Differential expression of antimicrobial peptides in corneal infection and regulation of antimicrobial peptides and reactive oxygen species by type III secretion system of Pseudomonas aeruginosa. *Pathog. Dis.* **2018**, *76*, doi:10.1093/femspd/fty001.
31. Roy, S.; Sun, Y.; Pearlman, E. Interferon-gamma-induced MD-2 protein expression and lipopolysaccharide (LPS) responsiveness in corneal epithelial cells is mediated by Janus tyrosine kinase-2 activation and direct binding of STAT1 protein to the MD-2 promoter. *J. Biol. Chem.* **2011**, *286*, 23753–23762, doi:10.1074/jbc.M111.219.
32. Roy, S.; Bonfield, T.; Tartakoff, A.M. Non-apoptotic toxicity of Pseudomonas aeruginosa toward murine cells. *PLoS ONE* **2013**, *8*, e54245, doi:10.1371/journal.pone.0054245.
33. Guha, S.; Chaurasia, S.; Ramachandran, C.; Roy, S. SLC4A11 depletion impairs NRF2 mediated antioxidant signaling and increases reactive oxygen species in human corneal endothelial cells during oxidative stress. *Sci. Rep.* **2017**, *7*, 4074, doi:10.1038/s41598-017-03654-4.
34. Mustapha, P.; Paris, I.; Garcia, M.; Tran, C.T.; Cremniter, J.; Garnier, M.; Faure, J.P.; Barthes, T.; Boneca, I.G.; Morel, F.; et al. Chemokines and antimicrobial peptides have a cag-dependent early response to Helicobacter pylori infection in primary human gastric epithelial cells. *Infect. Immun.* **2014**, *82*, 2881–2889, doi:10.1128/IAI.01517-13.
35. Berger, E.A.; McClellan, S.A.; Vistisen, K.S.; Hazlett, L.D. HIF-1alpha is essential for effective PMN bacterial killing, antimicrobial peptide production and apoptosis in Pseudomonas aeruginosa keratitis. *PLoS Pathog.* **2013**, *9*, e1003457, doi:10.1371/journal.ppat.1003457.
36. Rivas-Santiago, B.; Hernandez-Pando, R.; Carranza, C.; Juarez, E.; Contreras, J.L.; Aguilar-Leon, D.; Torres, M.; Sada, E. Expression of cathelicidin LL-37 during Mycobacterium tuberculosis infection in human alveolar macrophages, monocytes, neutrophils, and epithelial cells. *Infect. Immun.* **2008**, *76*, 935–941, doi:10.1128/IAI.01218-07.
37. Johansson, L.; Thulin, P.; Sendi, P.; Hertzen, E.; Linder, A.; Akesson, P.; Low, D.E.; Agerberth, B.; Norrby-Teglund, A. Cathelicidin LL-37 in severe Streptococcus pyogenes soft tissue infections in humans. *Infect. Immun.* **2008**, *76*, 3399–3404, doi:10.1128/IAI.01392-07.
38. Roy, S.; Marla, S.; Praneetha, D.C. Recognition of Corynebacterium pseudodiphtheriticum by Toll-like receptors and up-regulation of antimicrobial peptides in human corneal epithelial cells. *Virulence* **2015**, *6*, 716–721, doi:10.1080/21505594.2015.1066063.
39. Karthikeyan, R.S.; Priya, J.L.; Leal, S.M., Jr.; Toska, J.; Rietsch, A.; Prajna, V.; Pearlman, E.; Lalitha, P. Host response and bacterial virulence factor expression in Pseudomonas aeruginosa and Streptococcus pneumoniae corneal ulcers. *PLoS ONE* **2013**, *8*, e64867, doi:10.1371/journal.pone.0064867.
40. Karmakar, M.; Katsnelson, M.; Malak, H.A.; Greene, N.G.; Howell, S.J.; Hise, A.G.; Camilli, A.; Kadioglu, A.; Dubyak, G.R.; Pearlman, E. Neutrophil IL-1beta processing induced by pneumolysin is mediated by the NLRP3/ASC inflammasome and caspase-1 activation and is dependent on K+ efflux. *J. Immunol.* **2015**, *194*, 1763–1775, doi:10.4049/jimmunol.1401624.

41. Sun, Y.; Karmakar, M.; Roy, S.; Ramadan, R.T.; Williams, S.R.; Howell, S.; Shive, C.L.; Han, Y.; Stopford, C.M.; Rietsch, A.; et al. TLR4 and TLR5 on corneal macrophages regulate Pseudomonas aeruginosa keratitis by signaling through MyD88-dependent and -independent pathways. *J. Immunol.* **2010**, *185*, 4272–4283, doi:10.4049/jimmunol.1000874.
42. Mendez-Samperio, P.; Miranda, E.; Trejo, A. Expression and secretion of cathelicidin LL-37 in human epithelial cells after infection by Mycobacterium bovis Bacillus Calmette-Guerin. *Clin. Vac. Immunol.* **2008**, *15*, 1450–1455, doi:10.1128/CVI.00178-08.
43. Li, G.; Domenico, J.; Jia, Y.; Lucas, J.J.; Gelfand, E.W. NF-kappaB-dependent induction of cathelicidin-related antimicrobial peptide in murine mast cells by lipopolysaccharide. *Int. Arch. Allergy Immunol.* **2009**, *150*, 122–132, doi:10.1159/000218115.
44. Miraglia, E.; Nylen, F.; Johansson, K.; Arner, E.; Cebula, M.; Farmand, S.; Ottosson, H.; Stromberg, R.; Gudmundsson, G.H.; Agerberth, B.; et al. Entinostat up-regulates the CAMP gene encoding LL-37 via activation of STAT3 and HIF-1alpha transcription factors. *Sci. Rep.* **2016**, *6*, 33274, doi:10.1038/srep33274.
45. Gombart, A.F.; Borregaard, N.; Koeffler, H.P. Human cathelicidin antimicrobial peptide (CAMP) gene is a direct target of the vitamin D receptor and is strongly up-regulated in myeloid cells by 1,25-dihydroxyvitamin D3. *FASEB J.* **2005**, *19*, 1067–1077, doi:10.1096/fj.04-3284com.
46. Schust, J.; Sperl, B.; Hollis, A.; Mayer, T.U.; Berg, T. Stattic: A small-molecule inhibitor of STAT3 activation and dimerization. *Chem. Biol.* **2006**, *13*, 1235–1242, doi:10.1016/j.chembiol.2006.09.018.
47. Ramesh, S.; Ramakrishnan, R.; Bharathi, M.J.; Amuthan, M.; Viswanathan, S. Prevalence of bacterial pathogens causing ocular infections in South India. *Indian J. Pathol. Microbiol.* **2010**, *53*, 281–286, doi:10.4103/0377-4929.64336.
48. Chawla, K.; Gurung, B.; Mukhopadhyay, C.; Bairy, I. Reporting Emerging Resistance of Streptococcus pneumoniae from India. *J. Glob. Infect. Dis.* **2010**, *2*, 10–14, doi:10.4103/0974-777X.59245.
49. McIntosh, R.S.; Cade, J.E.; Al-Abed, M.; Shanmuganathan, V.; Gupta, R.; Bhan, A.; Tighe, P.J.; Dua, H.S. The spectrum of antimicrobial peptide expression at the ocular surface. *Investig. Ophthalmol. Vis. Sci.* **2005**, *46*, 1379–1385, doi:10.1167/iovs.04-0607.
50. Kumar, A.; Zhang, J.; Yu, F.S. Toll-like receptor 2-mediated expression of beta-defensin-2 in human corneal epithelial cells. *Microbes Infect.* **2006**, *8*, 380–389, doi:10.1016/j.micinf.2005.07.006.
51. Otri, A.M.; Mohammed, I.; Abedin, A.; Cao, Z.; Hopkinson, A.; Panjwani, N.; Dua, H.S. Antimicrobial peptides expression by ocular surface cells in response to Acanthamoeba castellanii: An in vitro study. *Br. J. Ophthalmol.* **2010**, *94*, 1523–1527, doi:10.1136/bjo.2009.178236.
52. Bergman, P.; Johansson, L.; Asp, V.; Plant, L.; Gudmundsson, G.H.; Jonsson, A.B.; Agerberth, B. Neisseria gonorrhoeae downregulates expression of the human antimicrobial peptide LL-37. *Cell. Microbiol.* **2005**, *7*, 1009–1017, doi:10.1111/j.1462-5822.2005.00530.x.
53. Sperandio, B.; Regnault, B.; Guo, J.; Zhang, Z.; Stanley, S.L., Jr.; Sansonetti, P.J.; Pedron, T. Virulent Shigella flexneri subverts the host innate immune response through manipulation of antimicrobial peptide gene expression. *J. Exp. Med.* **2008**, *205*, 1121–1132, doi:10.1084/jem.20071698.
54. Hase, K.; Murakami, M.; Iimura, M.; Cole, S.P.; Horibe, Y.; Ohtake, T.; Obonyo, M.; Gallo, R.L.; Eckmann, L.; Kagnoff, M.F. Expression of LL-37 by human gastric epithelial cells as a potential host defense mechanism against Helicobacter pylori. *Gastroenterology* **2003**, *125*, 1613–1625.
55. Sun, C.; Zhu, M.; Yang, Z.; Pan, X.; Zhang, Y.; Wang, Q.; Xiao, W. LL-37 secreted by epithelium promotes fibroblast collagen production: A potential mechanism of small airway remodeling in chronic obstructive pulmonary disease. *Lab. Investig.* **2014**, *94*, 991–1002, doi:10.1038/labinvest.2014.86.
56. Cruse, G.; Fernandes, V.E.; de Salort, J.; Pankhania, D.; Marinas, M.S.; Brewin, H.; Andrew, P.W.; Bradding, P.; Kadioglu, A. Human lung mast cells mediate pneumococcal cell death in response to activation by pneumolysin. *J. Immunol.* **2010**, *184*, 7108–7115, doi:10.4049/jimmunol.0900802.
57. Kumar, A.; Gao, N.; Standiford, T.J.; Gallo, R.L.; Yu, F.S. Topical flagellin protects the injured corneas from Pseudomonas aeruginosa infection. *Microbes Infect.* **2010**, *12*, 978–989, doi:10.1016/j.micinf.2010.06.007.
58. Gordon, Y.J.; Huang, L.C.; Romanowski, E.G.; Yates, K.A.; Proske, R.J.; McDermott, A.M. Human cathelicidin (LL-37), a multifunctional peptide, is expressed by ocular surface epithelia and has potent antibacterial and antiviral activity. *Curr. Eye Res.* **2005**, *30*, 385–394, doi:10.1080/02713680590934111.

59. Sakoulas, G.; Rose, W.; Nonejuie, P.; Olson, J.; Pogliano, J.; Humphries, R.; Nizet, V. Ceftaroline restores daptomycin activity against daptomycin-nonsusceptible vancomycin-resistant Enterococcus faecium. *Antimicrob. Agents Chemother.* **2014**, *58*, 1494–1500, doi:10.1128/AAC.02274-13.
60. Zahner, D.; Zhou, X.; Chancey, S.T.; Pohl, J.; Shafer, W.M.; Stephens, D.S. Human antimicrobial peptide LL-37 induces MefE/Mel-mediated macrolide resistance in Streptococcus pneumoniae. *Antimicrob. Agents Chemother.* **2010**, *54*, 3516–3519, doi:10.1128/AAC.01756-09.
61. Peric, M.; Koglin, S.; Kim, S.M.; Morizane, S.; Besch, R.; Prinz, J.C.; Ruzicka, T.; Gallo, R.L.; Schauber, J. IL-17A enhances vitamin D3-induced expression of cathelicidin antimicrobial peptide in human keratinocytes. *J. Immunol.* **2008**, *181*, 8504–8512.
62. Choi, S.M.; McAleer, J.P.; Zheng, M.; Pociask, D.A.; Kaplan, M.H.; Qin, S.; Reinhart, T.A.; Kolls, J.K. Innate Stat3-mediated induction of the antimicrobial protein Reg3gamma is required for host defense against MRSA pneumonia. *J. Exp. Med.* **2013**, *210*, 551–561, doi:10.1084/jem.20120260.
63. Wrighting, D.M.; Andrews, N.C. Interleukin-6 induces hepcidin expression through STAT3. *Blood* **2006**, *108*, 3204–3209, doi:10.1182/blood-2006-06-027631.
64. Gan, Y.; Cui, X.; Ma, T.; Liu, Y.; Li, A.; Huang, M. Paeoniflorin upregulates beta-defensin-2 expression in human bronchial epithelial cell through the p38 MAPK, ERK, and NF-kappaB signaling pathways. *Inflammation* **2014**, *37*, 1468–1475, doi:10.1007/s10753-014-9872-7.

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BRIEF REPORT

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Attenuation of *Pseudomonas aeruginosa* infection by INP0341, a salicylidene acylhydrazide, in a murine model of keratitis

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ABSTRACT

Pseudomonas aeruginosa: is an opportunistic pathogen and a major cause of corneal infections worldwide. The bacterium secretes several toxins through its type III secretion system (T3SS) to subvert host immune responses. In addition, it is armed with intrinsic as well as acquired antibiotic resistance mechanisms that make treatment a significant challenge and new therapeutic interventions are needed. Type III secretion inhibitors have been studied as an alternative or in accompaniment to traditional antibiotics to inhibit virulence of bacteria. In this study, INP0341, a T3SS inhibitor, inhibited cytotoxicity by *P. aeruginosa* toward human corneal epithelial cells (HCEC) at 100 µM without affecting bacterial growth in the liquid media. An increased expression of antimicrobial peptides and reactive oxygen species generation was also observed in cells exposed to *P. aeruginosa* in the presence of INP0341. Furthermore, INP0341 efficiently attenuated corneal infection by *P. aeruginosa* in an experimental model of murine keratitis as evident from corneal opacity, clinical score and bacterial load. Thus, INP0341 appears to be a promising candidate to treat corneal infection caused by *P. aeruginosa* and can be further considered as an alternative therapeutic intervention.

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Introduction

Pseudomonas aeruginosa is a gram-negative bacterium, ubiquitous in nature and a major opportunistic human pathogen. Corneal infections caused by *P. aeruginosa* are associated with both trauma and contact lens use and are a foremost cause of blindness worldwide [1]. In the cornea, *P. aeruginosa* activates the Toll like receptors (TLRs) that results in prompt production of cytokines and chemokines, recruitment of immune cells to the cornea and development of corneal opacity [2]. The corneal epithelium provides the first line of defense against invading bacteria [3] and the host immune response to *P. aeruginosa* is regulated by TLR4-MD-2 and TLR5 leading to an elevated expression of proinflammatory cytokines and antimicrobial peptides (AMPs) [2,4–6]. One of the fundamental virulence factors of *P. aeruginosa* is the type III secretion system (T3SS) which consists of a syringe-like apparatus that functions in a highly controlled manner to transport bacterial toxins and other proteins into the host cells [7] and amend different functions of the host to survive [8]. We and others have recently shown that wild-type PAO1 subverts the host immune responses including AMP expression [6] and attenuates generation of reactive oxygen species (ROS) in neutrophils and epithelial cells by its T3SS [6,9].

P. aeruginosa infections are increasingly concerning with their rise in antibiotic resistance. In contrast to other gram-negative bacteria, *P. aeruginosa* is less vulnerable to various antibiotics due to low penetrance across their outer membrane and the presence of several multi-drug efflux pumps and intrinsic β-lactamases [10,11]. To make the situation worse, *P. aeruginosa* can form biofilms that have reduced susceptibility to antibiotics [12]. Thus, it becomes important to identify and study novel therapeutic agents that are effective against *P. aeruginosa*.

T3SSs are highly conserved in several gram-negative pathogenic bacteria and play important roles by secreting toxins into the host cells and thus contributes to pathogenesis [7]. Therefore, they are considered important targets for development of new anti-infective agents. T3SS inhibitors are expected to abolish bacterial virulence without directly killing the bacteria and thus reduce selective pressure that leads to the development of resistance. To date, several small compounds have been developed as T3SS inhibitors and salicylidene

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Supplemental data for this article can be accessed here.

acylhydrazides are extensively studied among them. INP0010 [13], INP0400 [14] and INP0007 [15] are examples of salicylidene acylhydrazides that effectively inhibit the T3SSs of several pathogens including *Salmonella, Shigella* and *Yersinia*. For this study we have selected INP0341 as it was earlier shown to be effective against *P. aeruginosa* [16]. It is also known to attenuate the infectivity of *C. trachomatis* both *in vitro* and *in vivo* [17,18]. Uusitalo *et al.* have reported INP0341 to be effective in prevention of biofilms formed by *Pseudomonas* and to attenuate *P. aeruginosa* infection in a burn wound model in Balb/c mice[16].

Herein we demonstrate that INP0341 prevents cytotoxicity induced by *P. aeruginosa* in human corneal epithelial cells and causes increased expression of antimicrobial peptides and reactive oxygen species generation in response to *P. aeruginosa*. In addition, topical application of INP0341 inhibits bacterial growth and facilitates bacterial clearance in a murine model of *P. aeruginosa* keratitis.

Materials and methods

INP0341

INP0341[19] was synthesized as described previously and analytical data were in agreement with those previously reported. Stock solutions of INP0341 (25 mM) were prepared in dimethylsulfoxide (DMSO), stored under dark and dry conditions as described[16]. An intermediate 5 mM solution was made in 50% aqueous DMSO, from which the working solutions were prepared further.

Bacterial culture

PAO1[20], the mutant strain PAO1Δ*pscC*[21] and two clinical isolates of *P. aeruginosa* were used in this study. For identification of the clinical isolates, corneal ulcer materials were collected aseptically and investigated following the Institute protocol as described earlier[22]. Briefly, ulcer materials were placed on glass slides for Gram staining and were inoculated in different specific media for bacterial cultures. The pure homogenous culture was then subjected to Vitek 2 compact (bioMerieux, France) analysis for identification of the bacterium along with Gram staining and series of biochemical tests. All strains of *P. aeruginosa* were grown as described earlier [23]. In brief, bacteria were subcultured from overnight culture in Brain Heart Infusion broth (HiMedia Laboratories, West Chester, USA), washed twice in 1X phosphate buffered saline (PBS), centrifuged at 10,000 rpm for 10 min, and resuspended in 1X PBS. Dilutions of the sample were done with serum free media for the final inoculums.

Culture of HCEC

Immortalized human corneal epithelial cells (HCEC) 10.014 pRSV-T[24] were maintained in keratinocyte serum free media containing bovine pituitary extract and recombinant human epidermal growth factors (Invitrogen, Carlsbad, USA) at 37°C and 5% CO_2 and cultured as mentioned before. To study the AMP expression, HCEC were grown in 12-well plates (1 x 10^5 cells/well) and infected with PAO1 in the presence or absence of INP0341 for 4 h after which cells were processed further.

Toxicity of INP0341 against HCECs

Cytotoxicity of INP0341 toward HCEC was determined quantitatively by measuring the release of lactate dehydrogenase (LDH) into the culture media using CytoTox nonradioactive cytotoxicity assay kit (Promega, Madison, USA) following the manufacturer's protocol. Briefly, cells were grown to confluency and 50 μM (1% DMSO), 100 μM (2% DMSO), 250 μM (5% DMSO) and 500 μM (10% DMSO) of INP0341was added in triplicate and incubated for 6 h. Cells incubated with Triton X-100 were used as a positive control. The culture supernatant was used for LDH estimation by a colorimetric assay, absorbance was recorded at 490 nm[25].

Cell toxicity induced by P. aeruginosa

PAO1 or PAO1Δ*pscC* was grown overnight, and subcultured to an OD_{600} of 0.2 (10^8 colony forming units/ml, cfu/ml), centrifuged and resuspended in cell culture medium. HCECs were exposed to PAO1 in the presence or absence of INP0341 (100 μM, 2% final DMSO concentration), or PAO1Δ*pscC* at a multiplicity of infection (MOI) 10:1 (bacteria:cells) for 6 h, washed and imaged using phase contrast microscope. Cell death was measured by LDH assay using CytoTox nonradioactive cytotoxicity assay kit (Promega, Madison, USA) following the manufacturer's protocol. Cells were fixed with 4% paraformaldehyde and stained with Alexa Fluor 488 phalloidin (ThermoFisher Scientific, Waltham, USA) for 15 min and were counterstained with DAPI (Vector Laboratories, Burlingame, USA) to visualize the nucleus. Images of the cells were captured on a fluorescent microscope (Olympus IX73, Zeiss, Germany) using the 100X objective.

Assay for reactive oxygen species

1 x 10^4 cells per well were cultured overnight in 96-well plates and infected with PAO1 in the presence or absence of INP0341 (100 µM, 2% final DMSO concentration) for 2 h at a MOI of 10. Cells were also infected with Δ*pscC* mutant PAO1 for the same period of time. After washing with 1X PBS, cells were incubated with 2′7′-dichlorodihydrofluorescein diacetate dye containing media (H_2CFDA; Invitrogen, Carlsbad, USA) for 15 min. The cells were further washed, media was added and the cells were observed under a fluorescent microscope (Olympus IX73, Zeiss, Germany) using a 10X objective. In separate experiments, cells were exposed to bacteria as mentioned above and the fluorescence intensity of the H_2CFDA dye was measured quantitatively by SpectraMaxM3 (Softmax Pro 6.3).

Murine models of corneal infection

C57BL/6 mice (6–8 week old) were purchased from Cyagen Biosciences and experiments were performed at Vivo Bio Tech Ltd, Hyderabad, a Contract Research Organization. All animal experiments were approved by the Institutional Animal Ethics Committee of the test facility (VB/IAEC/09/2018/281/Mouse/C57BL/6). The animals were housed in specific pathogen free conditions in microisolator cages and were treated in accordance with the guidelines provided in the ARVO Statement for the Use of Animals in Ophthalmic and Vision research. Mice were anesthetized by intraperitoneal injection of ketamine (8.7 mg/ml) and xylazine (0.5 mg/ml) at a dose of 0.01 ml/g body weight and the corneal epithelium was abraded with three parallel 1 mm scratches using a 26 gauge needle and separated in two random groups. A 2.5 µL aliquot containing approximately 1 × 10^5 PAO1 was added topically to one eye, and 1X PBS was added to the fellow eye of one group, and mice remained in this position for 5 min. To the second group, 5 µL of 500 µM of INP0341 in 10% DMSO was added immediately after addition of PAO1 to one eye, and only INP0341 (in 10% DMSO) was added to the other eye. 5 µL of DMSO was also added to the infected group untreated with INP0341. The second dose of INP0341 was added topically 6 h post infection to the second group. Mice were euthanized and examined under a stereomicroscope for corneal opacification, ulceration, or perforation 24 h post infection. Clinical scores for the opacity were determined in a blinded fashion according to the scale earlier reported[26]. To measure cfu, whole eyes were homogenized in sterile 1X PBS using a tissue homogenizer (Genetix Biotech, Hyderabad, India) and serial dilutions were plated on LB agar plates, and cfu was counted manually.

Histology and immunohistochemistry

Eyes from control and infected mice were enucleated and placed in 10% formalin and corneal sections were prepared. Hematoxylin and eosin (H&E) staining was performed following deparaffinization of 5 µm corneal sections as described earlier[2].

RNA isolation, cDNA synthesis and quantitative PCR analysis

Quantitative real-time PCR was used to determine mRNA expression of different AMP genes from murine corneas and HCEC. The primers used are shown in Table 1 . Relative quantities of mRNA expression of respective genes were normalized using the $2^{-\Delta\Delta ct}$ method using GAPDH as the housekeeping gene.

Statistical analysis

Bar graphs represent mean and error bars represent standard error of mean (SEM). Statistical analysis was performed using either a one-way ANOVA or an unpaired *t* test (Prism; GraphPad Software). *p* values less than 0.05 were considered significant.

Table 1. Oligonucleotide sequences.

Gene	Sequence (5′→3′)
hBD-2	FWD:CAGCCATCAGCCATGAGG
	REV:TGGCTTTTTGCAGCATTTT
hBD-3	FWD:TCTCAGCGTGGGGTGAAGC
	REV:CGGCCGCCTCTGACTCTG
S100A9	FWD:TGGCTCCTCGGCTTTGG
	REV:CGACATTTTGCAAGTCATCGTC
S100A12	FWD:AGCATCTGGAGGGAATTGTCA
	REV:GCAATGGCTACCAGGGATATGAA
LL-37	FWD:TCGGATGCTAACCTCTACCG
	REV:ACAGGCTTTGGCGTGTCT
Rnase7	FWD:GAACACCAAGCGCAAAGC
	REV:CAGCAGAAGCAGCGAAGG
GAPDH	FWD:GATCCCTCCAAAATCAAGTG
	REV:GGCAGAGATGATGACCCTTTT
mBD-1	FWD:CCAGATGGAGCCAGGTGTTG
	REV:AGCTGGAGCGGAGACAGAATCC
mBD-2	FWD:AAGTATTGGATACGAAGCAG
	REV:TGGCAGAAGGAGGACAAATG
mS100A9	FWD:ATACTCTAGGAAGGAAGGACACC
	REV:TCCATGATGTCATTTATGAGGGC
CRAMP	FWD:GTCTTGGGAACCATGCAGTT
	REV:TGGTTGAAGTCATCCACAGC
mLipocalin2	FWD:GCAGGTGGTACGTTGTGGG
	REV:CTCTTGTAGCTCATAGATGGTGC
mGAPDH	FWD:AGGTCGGTGTGAACGGATTTG
	REV:GTAGACCATGTAGTTGAGGTCA

Results

INP0341 prevents cytotoxic effects of P. aeruginosa on HCECs

INP0341 (Figure 1(a)) was earlier shown to reduce cytotoxicity by *P. aeruginosa* in HeLa cells[16]. Building on these results we first investigated if INP0341 exerted any cytotoxic effect on HCEC or bacteria. HCECs were incubated with increasing concentrations of INP0341 for 6 h, and cell viability was determined by measuring the release of cytosolic enzyme lactate dehydrogenase (LDH) into the culture media[27]. Cells lysed using detergent were used as a positive control. No significant cytotoxicity was observed in INP0341 treated cells even at higher concentrations (Figure 1(b)). Toxicity toward bacteria was also determined by monitoring the growth at 6 and 24 h in the presence of increased concentrations of INP0341 and no significant difference in growth was observed in the presence of higher concentrations of

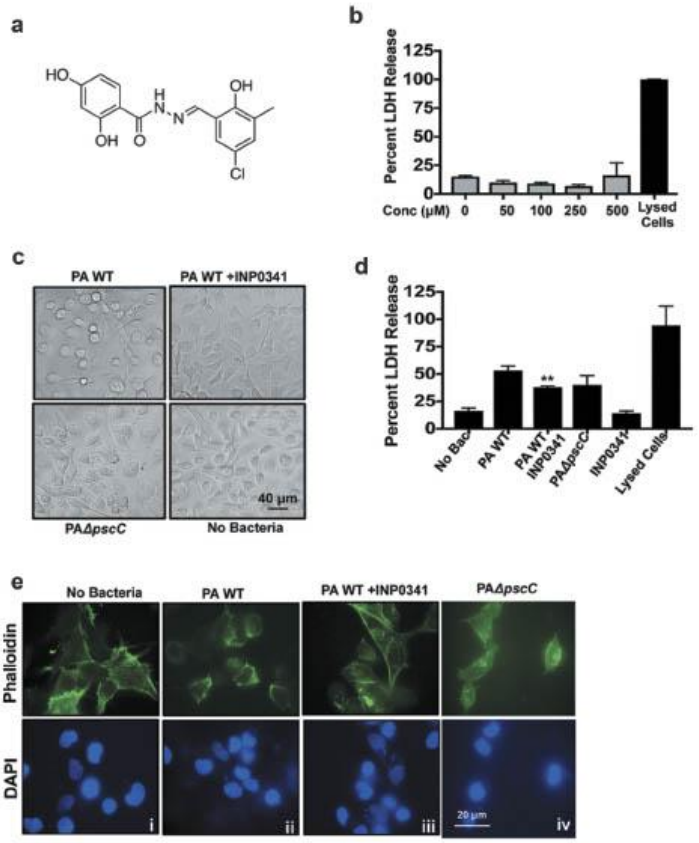

Figure 1. INP0341 impedes cytotoxic effects of *P. aeruginosa* on HCEC. Chemical structure of INP0341 (a). HCEC were exposed to different concentrations of INP0341 for 6 h to test its effect on cell viability using the lactate dehydrogenase (LDH) assay. Cells lysed with detergent were used as a positive control and cytotoxicity was measured as a percentage of total LDH (b). HCEC were infected with PAO1 in the presence or absence of INP0341 (100 μM), or PAO1Δ*pscC* for 6 h and cell morphology was imaged under a bright field microscope (c) and cell viability was determined by LDH assay (d). Cells were stained with phalloidin- Alexa Fluor 488 and imaged under a fluorescent microscope using a 100x objective and oil-immersion (e). The experiments were repeated at least three times.

the inhibitor (Fig. S1). Since INP0341 was not toxic to HCECs, we then examined the effect of INP0341 on the cytotoxicity of *P. aeruginosa* toward HCECs. PAO1 is known to cause cell cytotoxicity in airway epithelial cells[28], and murine macrophages[23] . HCECs were exposed to PAO1 in the presence or absence of INP0341 (100 μM) or PAO1Δ*pscC*, and cytotoxicity was determined after 6 h by observing the cell phenotype and LDH assay. Cells exposed to PAO1 changed morphologically and about seventy percent of the cells became rounded up. This was significantly reduced in the presence of the inhibitor, and cells were of a similar phenotype to those of uninfected cells (Figure 1(c)). In comparison to the cells completely lysed by triton-X 100, PAO1 infection caused fifty percent of LDH maximum release, which was significantly lowered in the presence of INP0341 (Figure 1(d)). HCEC treated with INP0341 only showed less than 15% LDH maximum release. Several bacteria including *P. aeruginosa* cause T3SS mediated disruption of the actin cytoskeleton of the host cells[29]. To observe if INP0341 inhibits actin cytoskeleton rearrangements in cells induced by *P. aeruginosa*, HCECs were infected with *P. aeruginosa* in the presence or absence of INP0341 and stained with phalloidin-Alexa Fluor 488. We observed redistribution of the actin cytoskeleton along with cell rounding in cells infected with *P. aeruginosa* (Figure 1(e) ii). This effect was visibly inhibited in the presence of INP0341 (Figure 1(e) iii).

Inhibition of T3SS by INP0341 enhanced AMP expression by HCEC in response to P. aeruginosa

In our earlier study we investigated the expression of AMPs from corneal scrapings of patients with *P. aeruginosa* corneal infections and saw differential expression of several AMPs, including human β defensins (hBD) 2, and 3, S100A9, S100A12 and LL-37. We also found that PAO1 subverts the expression of these AMPs in HCECs *in vitro*[6]. However, we observed that the expression of the AMPs increased significantly when cells were exposed to PAO1Δ*pscC*, a T3SS mutant that fails to transfer exotoxins to the host cells. We therefore continued to investigate if pharmacological inhibition of the T3SS has a similar effect on AMP expression. Two clinical isolates of *P. aeruginosa* along with PAO1 were included for this experiment. Both the clinical isolates were positive for *exoS, T* and *Y* genes but not *exoU*, similar to PAO1 (data not shown), and thus harbor the T3SS. HCECs were infected either with PAO1 or the clinical isolates at MOI 10 in presence or absence of 100 μM INP0341 and the expression of AMPs was determined. We found increased expression of hBD-2, hBD-3, LL-37, RNase7, S100A9 and S100A12 in HCEC in response to PAO1 in the presence of INP0341 compared to PAO1 alone (Figure 2(a)). Increased AMP expression was also observed in cells exposed to clinical isolates of *P. aeruginosa* in the presence of INP0341 compared to bacteria alone in agreement with our hypothesis (Figure 2(b,c)).

Effect of INP0341 on PAO1 induced ROS generation by HCEC

ROS generation in response to infections is not only to kill the invading pathogen but also to mediate the host immune responses. Like several other bacteria, *P. aeruginosa* has the ability to subvert ROS generation mediated by its T3SS in neutrophils[9] and epithelial cells[6], thus reducing the phagocytic killing by cells. Therefore we investigated if INP0341 could inhibit this subversion of ROS generation in host cells. HCECs were exposed to PAO1 in the presence or absence of INP0341 for 2 h and ROS generation was determined using fluorescent probe H_2CDFA. Similar to our earlier observations[6], we saw a minimum level of ROS in cells infected with PAO1 (Figure 2(d) ii). However, a significant increase in ROS generation was observed in HCECs infected with PAO1 in the presence of INP0341 (Figure 2(d) iii) compared to cells infected without INP0341 (Figure 2(d) ii). Increased ROS generation was also observed in PAO1Δ*pscC* infected cells (Figure 2(d) iv) compared to PAO1 infected cells as reported earlier[6]. The ROS generation was also quantitatively measured by fluorimeter, and significantly increased levels of ROS were detected in cells infected with PAO1 in the presence of INP0341 (Figure 2(e)) compared to infected cells alone.

INP0341 attenuates P. aeruginosa infection in a murine model of keratitis

To determine the effect of INP0341 in *P. aeruginosa* keratitis *in vivo*, we used our established murine model of keratitis[2] in which corneas of C57BL/6 mice were scratched and infected with PAO1. INP0341 (500 μM in 10% DMSO) was applied topically at 0, and 6 h post infection. Mice were also infected with PAO1Δ*pscC* as a separate group. Animals were euthanized after 24 h and corneas were imaged for opacification, and cfu in whole eyes were quantified after enucleation. Increased corneal opacification was detected in mice infected with wild-type PAO1, but significantly less opacity was observed in PAO1 infected mice treated with INP0341, or in mice infected with PAO1Δ*pscC* (Figure 3(a)). Significant differences were noted in the

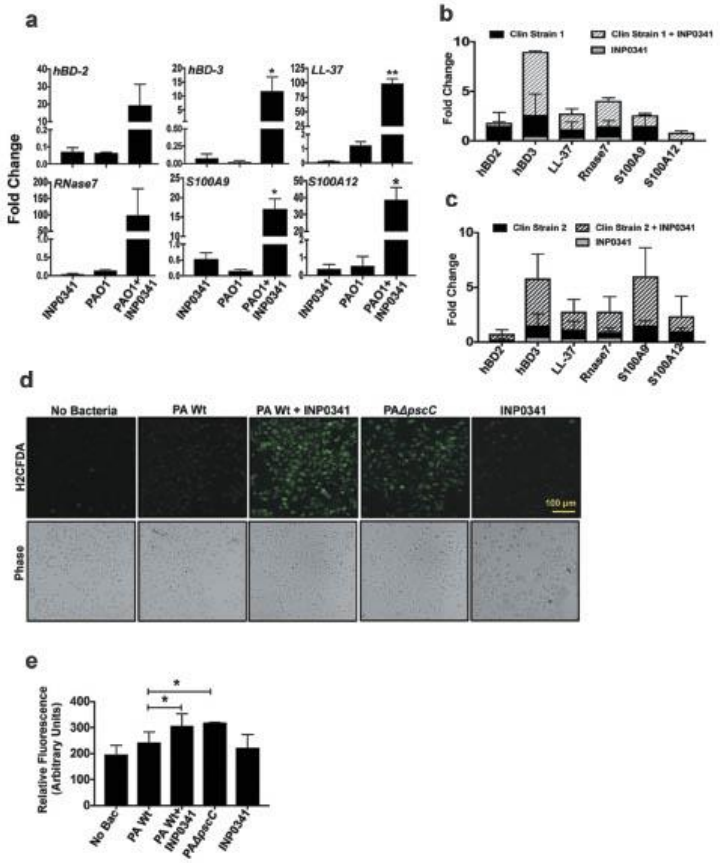

Figure 2. Increased expression of AMPs and generation of ROS in HCEC exposed to *P. aeruginosa* in presence of INP0341. HCEC were infected with PAO1 (a) or clinical isolates (b and c) in the presence or absence of INP0341 for 4 h, and AMP gene expression was determined by quantitative PCR using the $2^{-\Delta\Delta Ct}$ method. GAPDH was used as a housekeeping gene. For detection of ROS, HCEC were exposed to PAO1 in the presence or absence of INP0341 (100 µM) or PAO1Δ*pscC* for 2 h and incubated with H_2CFDA for 15 min and then observed and imaged using a fluorescent microscope (d). The generation of ROS was also quantitated using a fluorescent plate reader (e). The experiments were done in duplicate and repeated three times. (*indicates $p < 0.05$).

clinical scores of the corneal opacity between the groups (Figure 3(b)). Consistent with these data, cfu per eye obtained from INP0341 treated corneas were significantly lower by almost three log compare to untreated corneas that were infected with PAO1 ($p < 0.0219$) (Figure 3(c)). There was significantly reduced opacity and cfu in corneas infected with PAO1Δ*pscC* compared to PAO1 infected corneas as was reported earlier[30]. Hematoxylin and eosin stained sections of mice corneas showed reduced cellular infiltration in the corneal stroma of PAO1 infected mice treated with INP0341 compared to untreated infected mice (Figure 3(d)). There was also less infiltration in PAO1Δ*pscC* infected mice compared to PAO1 infected ones. Since expression of AMPs in HCECs was augmented in the presence of INP0341 in response to PAO1, we further studied the expression of AMPs in the presence of the inhibitor during corneal infections *in vivo*. Corneas were infected with PAO1 in the presence or absence of INP0341 as mentioned

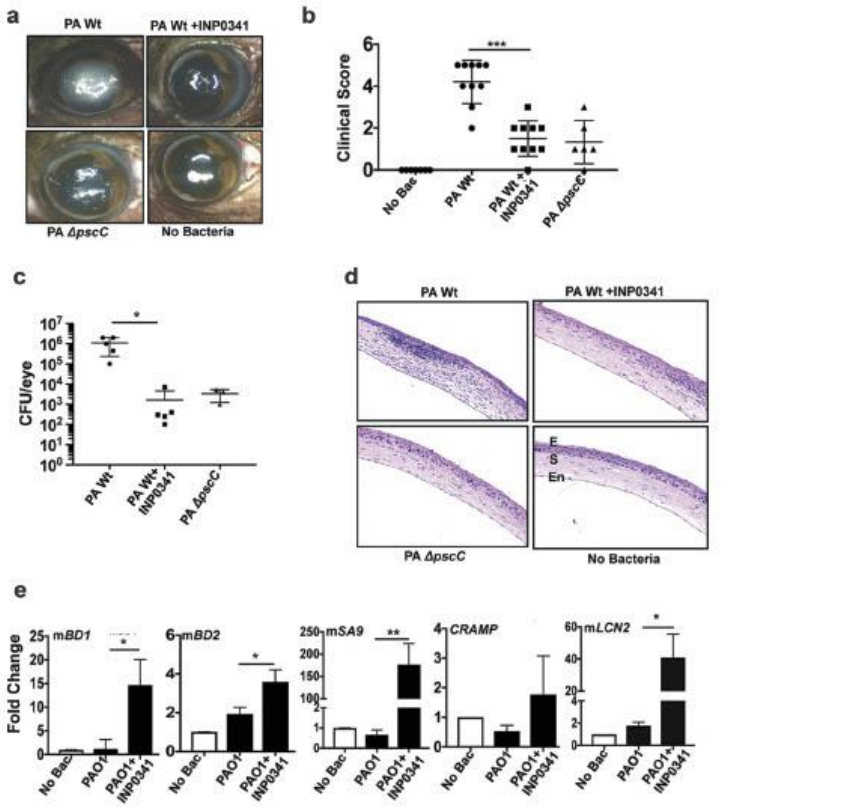

Figure 3. INP0341 attenuates *P. aeruginosa* infection and facilitates bacterial clearance in a murine model of keratitis. C57BL/6 mice were infected with *P. aeruginosa* and topically treated with INP0341 at 0, and 6 h post infection. Mice were euthanized 24 h post infection and representative images of corneal opacification (a) and their clinical score (b) were recorded. Cfu was measured from whole eye homogenates 24 h post infection (n = 5 mice). Data points represent individual infected corneas (c). Corneal sections were stained with hematoxylin and eosin to visualize cellular infiltration. E, epithelium; S, stroma; En, endothelium (d). Corneas (n = 3) were excised 24 h post infection and homogenized for RNA isolation and expression of AMPs was determined by quantitative PCR using $2^{-\Delta\Delta Ct}$method. GAPDH was used as a housekeeping gene. (*indicates $p < 0.05$; ** indicates $p < 0.005$).

earlier, and AMP expression was determined 24 h post infection by QPCR. Similar to results seen with human corneal epithelial cells *in vitro*, there was reduced expression of mBD-1, mBD-2, mS100A9, lipocalin (mLCN2) and CRAMP in PAO1 infected corneas, however AMP expression increased significantly in infected corneas treated with INP0341 (Figure 3(e)).

In a subsequent experiment, infected corneas were treated either at 0 and 6 h (group I) or at 3 and 6 h (group II) post infection with INP0341 (500 μM in 10% DMSO). Mice were euthanized and corneas were imaged 24 h post infection and reduced opacity was observed in both the group I and group II mice compared to the infected group (Fig. S2A). Significant reduction in clinical scores of the opacity was observed in mice of group I compared to the infected group ($p < 0.0014$). Although clinical scores of mice of group II were less than the infected group, the difference was not significant (Fig. S2B). Additionally, reduced cfu were observed in mice of group I but not of group II compared to the infected group (Fig. S2C). Consistent with these data, the hematoxylin and eosin staining of corneas of group I mice show reduced

cellular infiltrations in the corneal stroma compared to PAO1 infected mice (**Fig. S2D**). The infiltrations observed in the corneal sections of group II mice were more than that of group I but lower than PAO1 infected group. Taken together, these findings indicate that INP0341 is effective in controlling infection in an experimental model of *Pseudomonas* keratitis.

Discussion

In this study, we investigated the application of INP0341, a T3SS inhibitor, for the treatment of *P. aeruginosa* keratitis in a murine model. *P. aeruginosa* employs T3SS to translocate toxins into eukaryotic cells resulting in infection. It also has a unique ability to develop antibiotic resistance[31]. The increase in antibiotic resistance has generated interests in targeting T3SS to prevent or treat infection by mechanisms distinct from those of conventional antibiotics. Furthermore, since T3SSs is typically found in pathogenic bacteria, targeting this system will not affect the vast commensal population aside[32], thus reducing the likelihood of the emergence of resistant bacteria in this population. It has been reported that hydroxyquinolines effectively inhibited T3SS in *Y. pseudotuberculosis, C. trachomatis*[33], and *P. aeruginosa* mediated lung injury[34]. Phenoxyactemaide inhibitors also selectively inhibit T3SS by presumably targeting the needle protein PscF[35]. Recently, Berube *et al.* reported that several phenoxyacetamide inhibitors could inhibit abscess formation in a murine model of *P. aeruginosa* infection[36]. Aurodox, a polyketide compound, that inhibits T3SS, was also found to be effective against *C. rodentium* induced colonic hyperplasia in a mouse model[37].

The salicylidene acylhydrazides block the T3SS in several pathogens including *Y. pseudotuberculosis* [38,39], *C. trachomatis*[40], *S. enterica*, and *P. aeruginosa*. This class of compounds has been shown to inhibit the *S. flexeneri* invasion of HeLa to induce apoptosis in macrophages *in vitro*[14], and to inhibit *S. enterica* protein secretion and invasion of Madin-Darby Canine Kidney cells[13]. Of the several salicylidene acylhydrazides, INP0341 has been extensively studied and was found to be effective against several pathogens, including *P. aeruginosa* and *C. trachomatis*. The salicylidene acylhydrazides have the capacity to chelate iron and INP0341 was shown to exhibit *in vitro* activity against *N. gonorrhoeae*[17] and HIV[41] through an iron restriction mechanism. A recent study revealed that the topical application of INP0341 significantly increased the survival of mice with burn wounds infected with *P. aeruginosa*[16]. We have extended our studies to determine the effect of INP0341 on *P. aeruginosa* in corneal epithelial cells. Here we showed that INP0341 protected corneal epithelial cells from *P. aeruginosa* infection. It has previously been shown that PAO1 inhibited host immune responses both *in vivo* and *in vitro*. Increased bacterial load was observed in the corneas of C57BL/6 mice infected with PAO1 compared with those infected with a T3SS deficient strain[30]. PAO1 was also found to impede the expression of AMPs, reduce the generation of ROS, and inhibit T3SS mediated p38 and ERK signaling in HCEC [6]. However, in the present study, a significant increase in the expression of AMPs was observed when HCECs were infected with PAO1 in the presence of INP0341. Treatment with INP0341 was also found to be effective against clinical isolates of PAO1 expressing ExoS. Furthermore, in the presence of INP0341, increased ROS generation was observed in PAO1 infected HCEC. Finally, we also demonstrated that INP0341 exerted a therapeutic effect following topical application to mouse corneas infected with PAO1. Significantly reduced bacterial load and increased expression of AMPs were observed in murine corneas infected with PAO1 in the presence of INP0341 applied at the time of infection. Although administered prophylactically and in higher doses than those required *in vitro*, our data suggested that INP0341 might be used to treat *Pseudomonas* infections as it improved bacterial clearance in infected corneas. However, further research is required to develop an optimal formulation and to determine the pharmacokinetic profile of INP0341 for ocular administration of the compound that can be useful to treat these blinding corneal infections. In conclusion, this study determines the potential of INP0341 to treat corneal infections caused by *Pseudomonas* and suggests that virulence inhibitors can be utilized for therapeutic intervention to combat antibiotic resistance.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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References

[1] Stapleton F, Carnt N. Contact lens-related microbial keratitis: how have epidemiology and genetics helped us with pathogenesis and prophylaxis. Eye (Lond). 2012;26:185–193.

[2] Sun Y, Karmakar M, Roy S, et al. TLR4 and TLR5 on corneal macrophages regulate Pseudomonas aeruginosa keratitis by signaling through MyD88-dependent and -independent pathways. J Immunol. 2010;185:4272–4283.

[3] Evans DJ, Fleiszig SM. Why does the healthy cornea resist Pseudomonas aeruginosa infection? Am J Ophthalmol. 2013;155(961–70):e2.

[4] Roy S, Sun Y, Pearlman E. Interferon-gamma-induced MD-2 protein expression and lipopolysaccharide (LPS) responsiveness in corneal epithelial cells is mediated by Janus tyrosine kinase-2 activation and direct binding of STAT1 protein to the MD-2 promoter. J Biol Chem. 2011;286:23753–23762.

[5] Karthikeyan RS, Priya JL, Leal SM Jr., et al. Host response and bacterial virulence factor expression in Pseudomonas aeruginosa and streptococcus pneumoniae corneal ulcers. PLoS One. 2013;8:e64867.

[6] Sharma P, Guha S, Garg P, et al. Differential expression of antimicrobial peptides in corneal infection and regulation of antimicrobial peptides and reactive oxygen species by type III secretion system of Pseudomonas aeruginosa. Pathog Dis. 2018;76. DOI:10.1093/femspd/fty001

[7] Hauser AR. The type III secretion system of Pseudomonas aeruginosa: infection by injection. Nature Rev Microbiol. 2009;7:654–665.

[8] Sundin C, Hallberg B, Forsberg A. ADP-ribosylation by exoenzyme T of Pseudomonas aeruginosa induces an irreversible effect on the host cell cytoskeleton in vivo. FEMS Microbiol Lett. 2004;234:87–91.

[9] Vareechon C, Zmina SE, Karmakar M, et al. Pseudomonas aeruginosa effector ExoS inhibits ROS production in human neutrophils. Cell Host Microbe. 2017;21:611–8 e5.

[10] Lambert PA. Mechanisms of antibiotic resistance in Pseudomonas aeruginosa. J R Soc Med. 2002;95(Suppl 41):22–26.

[11] Rocchetta HL, Burrows LL, Lam JS. Genetics of O-antigen biosynthesis in Pseudomonas aeruginosa. Microbiol Mol Biol Rev. 1999;63:523–553.

[12] Hoiby N, Bjarnsholt T, Moser C, et al. ESCMID guideline for the diagnosis and treatment of biofilm infections 2014. Clin Microbiol Infect. 2015;21(Suppl 1):S1–25. .

[13] Negrea A, Bjur E, Ygberg SE, et al. Salicylidene acylhydrazides that affect type III protein secretion in Salmonella enterica serovar typhimurium. Antimicrob Agents Chemother. 2007;51:2867–2876.

[14] Veenendaal AK, Sundin C, Blocker AJ. Small-molecule type III secretion system inhibitors block assembly of the Shigella type III secreton. J Bacteriol. 2009;191:563–570.

[15] Kauppi AM, Nordfelth R, Uvell H, et al. Targeting bacterial virulence: inhibitors of type III secretion in Yersinia. Chem Biol. 2003;10:241–249.

[16] Uusitalo P, Hagglund U, Rhoos E, et al. The salicylidene acylhydrazide INP0341 attenuates Pseudomonas aeruginosa virulence in vitro and in vivo. J Antibiot (Tokyo). 2017;70:937–943.

[17] Chu H, Slepenkin A, Elofsson M, et al. Candidate vaginal microbicides with activity against Chlamydia trachomatis and Neisseriagonorrhoeae. Int J Antimicrob Agents. 2010;36:145–150.

[18] Pedersen C, Slepenkin A, Andersson SB, et al. Formulation of the microbicide INP0341 for in vivo protection against a vaginal challenge by Chlamydia trachomatis. PLoS One. 2014;9:e110918.

[19] Slepenkin A, Enquist PA, Hagglund U, et al. Reversal of the antichlamydial activity of putative type III secretion inhibitors by iron. Infect Immun. 2007;75:3478–3489.

[20] Bleves S, Soscia C, Nogueira-Orlandi P, et al. Quorum sensing negatively controls type III secretion regulon expression in Pseudomonas aeruginosa PAO1. J Bacteriol. 2005;187:3898–3902.

[21] Cisz M, Lee PC, Rietsch A. ExoS controls the cell contact-mediated switch to effector secretion in Pseudomonas aeruginosa. J Bacteriol. 2008;190:2726–2738.

[22] Sharma P, Sharma N, Mishra P, et al. Differential expression of antimicrobial peptides in streptococcus pneumoniae keratitis and STAT3-dependent expression of LL-37 by streptococcus pneumoniae in human corneal epithelial cells. Pathogens. 2019;8:31–48.

[23] Roy S, Bonfield T, Tartakoff AM. Non-apoptotic toxicity of Pseudomonas aeruginosa toward murine cells. PLoS One. 2013;8:e54245.

[24] Araki-Sasaki K, Ohashi Y, Sasabe T, et al. An SV40-immortalized human corneal epithelial cell line and its characterization. Invest Ophthalmol Vis Sci. 1995;36:614–621.

[25] Ponsoda X, Jover R, Castell JV, et al. Measurement of intracellular LDH activity in 96-well cultures: A rapid and automated assay for cytotoxicity studies. J Tissue Culture Methods. 1991;13:21–24.

[26] Pan Z, Chen Y, Zhang W, et al. Rat corneal allograft survival prolonged by the superantigen staphylococcal enterotoxin B. Invest Ophthalmol Vis Sci. 2003;44:3346–3351.

[27] Allen M, Millett P, Dawes E, et al. Lactate dehydrogenase activity as a rapid and sensitive test for the quantification of cell numbers in vitro. Clin Mater. 1994;16:189–194.

[28] O'Grady EP, Mulcahy H, O'Callaghan J, et al. Pseudomonas aeruginosa infection of airway epithelial cells modulates expression of Kruppel-like factors 2 and 6 via RsmA-mediated regulation of type III exoenzymes S and Y. Infect Immun. 2006;74:5893–5902.

[29] Cowell BA, Evans DJ, Fleiszig SM. Actin cytoskeleton disruption by ExoY and its effects on Pseudomonas aeruginosa invasion. FEMS Microbiol Lett. 2005;250:71–76.

[30] Sun Y, Karmakar M, Taylor PR, et al. ExoS and ExoT ADP ribosyltransferase activities mediate Pseudomonas aeruginosa keratitis by promoting neutrophil apoptosis and bacterial survival. J Immunol. 2012;188:1884–1895.

[31] Breidenstein EB, de la Fuente-nunez C, Hancock RE. Pseudomonas aeruginosa: all roads lead to resistance. Trends Microbiol. 2011;19:419–426.
[32] Gauthier A, Robertson ML, Lowden M, et al. Transcriptional inhibitor of virulence factors in enteropathogenic Escherichia coli. Antimicrob Agents Chemother. 2005;49:4101–4109.
[33] Enquist PA, Gylfe A, Hagglund U, et al. Derivatives of 8-hydroxyquinoline–antibacterial agents that target intra- and extracellular Gram-negative pathogens. Bioorg Med Chem Lett. 2012;22:3550–3553.
[34] Anantharajah A, Faure E, Buyck JM, et al. Inhibition of the injectisome and flagellar type III secretion systems by INP1855 Impairs Pseudomonas aeruginosa pathogenicity and inflammasome activation. J Infect Dis. 2016;214:1105–1116.
[35] Bowlin NO, Williams JD, Knoten CA, et al. Mutations in the Pseudomonas aeruginosa needle protein gene pscF confer resistance to phenoxyacetamide inhibitors of the type III secretion system. Antimicrob Agents Chemother. 2014;58:2211–2220.
[36] Berube BJ, Murphy KR, Torhan MC, et al. Impact of type III secretion effectors and of phenoxyacetamide inhibitors of type III secretion on abscess formation in a mouse model of Pseudomonas aeruginosa infection. Antimicrob Agents Chemother. 2017;61. DOI:10.1128/AAC.01202-17.
[37] Kimura K, Iwatsuki M, Nagai T, et al. A small-molecule inhibitor of the bacterial type III secretion system protects against in vivo infection with Citrobacter rodentium. J Antibiot (Tokyo). 2011;64:197–203.
[38] Bailey L, Gylfe A, Sundin C, et al. Small molecule inhibitors of type III secretion in Yersinia block the Chlamydia pneumoniae infection cycle. FEBS Lett. 2007;581:587–595.
[39] Nordfelth R, Kauppi AM, Norberg HA, et al. Small-molecule inhibitors specifically targeting type III secretion. Infect Immun. 2005;73:3104–3114.
[40] Slepenkin A, Chu H, Elofsson M, et al. Protection of mice from a Chlamydia trachomatis vaginal infection using a Salicylidene acylhydrazide, a potential microbicide. J Infect Dis. 2011;204:1313–1320.
[41] Forthal DN, Phan TB, Slepenkin AV, et al. In vitro anti-HIV-1 activity of salicylidene acylhydrazide compounds. Int J Antimicrob Agents. 2012;40:354–360.

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REVIEW
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Alternative Therapeutic Interventions: Antimicrobial Peptides and Small Molecules to Treat Microbial Keratitis

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Microbial keratitis is a leading cause of blindness worldwide and results in unilateral vision loss in an estimated 2 million people per year. Bacteria and fungus are two main etiological agents that cause corneal ulcers. Although antibiotics and antifungals are commonly used to treat corneal infections, a clear trend with increasing resistance to these antimicrobials is emerging at rapid pace. Extensive research has been carried out to determine alternative therapeutic interventions, and antimicrobial peptides (AMPs) are increasingly recognized for their clinical potential in treating infections. Small molecules targeted against virulence factors of the pathogens and natural compounds are also explored to meet the challenges and growing demand for therapeutic agents. Here we review the potential of AMPs, small molecules, and natural compounds as alternative therapeutic interventions for the treatment of corneal infections to combat antimicrobial resistance. Additionally, we have also discussed about the different formats of drug delivery systems for optimal administration of drugs to treat microbial keratitis.

Keywords: ocular surface, wound healing, microbial keratitis, antimicrobial pepides, small molecules, drug delivery systems

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INTRODUCTION

Microbial keratitis is a leading cause of blindness in India and globally and is a major public health issue that compromises the quality of life. The early symptoms include redness, extreme pain, light sensitivity, and reduced vision. According to the World Health Organization (WHO), corneal infections are likely to cause 1.5–2 million new cases of blindness per year (Whitcher et al., 2001). The risk factors include ocular trauma and injuries, commonly caused by vegetative matters in the developing world. A study found that almost 70% of microbial keratitis cases in South India resulted from ocular trauma and 63% of all patients were involved in agriculture (Bharathi et al., 2007). Alternatively, the increased use of contact lens and self-diagnosed use of corticosteroids in the modern urban livelihood are the contributing risk factors in the developed world. Studies conducted in France and Sweden showed contact lens wear as a major risk factor in 50% of all keratitis cases (Sagerfors et al., 2020). Bacterial and fungal infections constitute two major forms of corneal infections, consisting of 60 and 39%, respectively (Das et al., 2019). The most common bacterial species responsible for causing corneal infections are *Pseudomonas aeruginosa* and *Streptococcus* spp. along with *Staphylococcus aureus*, *S. epidermidis, Serratia marcescens*, and *Haemophilus influenza* (Bremond-Gignac et al., 2011; Paradiso et al., 2016; Chojnacki et al., 2019; Urwin et al., 2020). In contrast to bacterial keratitis, fungal keratitis occurs widely in the tropical and subtropical climates, and *Aspergillus* spp. and *Fusarium* spp. are the most common etiological agents. Fungal infections of

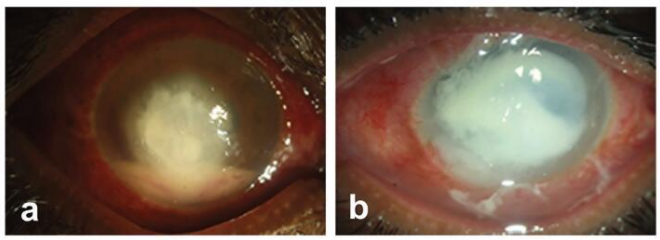

FIGURE 1 | Representative images of microbial keratitis. The pathogens causing infections penetrates deep into the stroma inducing inflammation and corneal opacity that leads to loss of vision.

the cornea need to be promptly recognized to facilitate a complete recovery as it is often difficult to treat due to penetration of the hyphae into the stroma. Infection with *Fusarium* spp. often completely destroy an eye in a few weeks, since the infection is usually severe, and perforation may supervene (Thomas, 2003). Studies have shown that almost 42–60% of corneal infections due to *Aspergillus* spp. lead to penetrating keratoplasty (Leck et al., 2002).

The representative image of corneal infections caused by *A. flavus* (**Figure 1A**) and *P. aeruginosa* (**Figure 1B**) shows increased inflammation and corneal opacity associated with microbial keratitis. Bacterial infections are commonly treated by topical, or subconjunctival administration with penicillin, fluoroquinolones, tetracyclines, and aminoglycosides (Sharma, 2011; Dubald et al., 2018). However, bacteria are gaining resistance against available antibiotics due to the misuse or prophylactic use of antibiotics as well as adaptation of bacteria to the available therapeutics. Similarly, a parallel rise in the number of drug-resistant fungal species is also reported lately. Therefore, researchers are trying to develop new therapeutics or exploring the natural compound to inhibit microbial infections and combat antimicrobial resistance (AMR).

In this review, we have discussed the important role of antimicrobial peptides (AMPs), natural compounds, and small molecules in combating corneal infections. Additionally, we have explored the importance of drug delivery systems in delivering the therapeutics for the treatment of microbial keratitis.

METHODS

We used online literature databases like PubMed and Scopus for searching relevant research articles based on the specific keywords. The keywords used for search included "keratitis", "antimicrobial resistance", "antimicrobial peptides", "small molecules", "drug delivery systems for delivering antimicrobial drugs," and "wound healing". The results were narrowed down by selecting articles relevant to the current review. The Food and Drug Administration (FDA) website was searched for the list of approved antimicrobial peptides in clinical use.

ANTIMICROBIAL RESISTANCE AND LIMITATIONS OF ANTIMICROBIAL DRUGS

Currently, ocular infections due to bacteria are managed using antibiotics like fluoroquinolones, vancomycin, cefazoline, bacitracin, sulfamethoxazole, erythromycin, chloramphenicol, and aminoglycosides (Lin et al., 2019). However, ever since the discovery of the first antibiotic penicillin, bacteria have shown to develop resistance against them in no time (Fairbrother, 1956). The Antibiotic Resistance Monitoring in Ocular Microorganisms (ARMOR) surveillance program, in its recent antibiotic resistance reports on 4,829 isolates, showed that *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA), are resistant to moxifloxacin (Thomas et al., 2019). In a recent report it was found that isolates of *S. aureus* and *S. pneumoniae* causing infectious keratitis show reduced susceptibility to fluoroquinolones (Sagerfors et al., 2020). Other reports found that *Corynebacterium* spp. present on the ocular surface were resistant to quinolones (Aoki et al., 2021) and macrolides in addition to fluoroquinolones (Hoshi et al., 2020). According to the World Health Organization (WHO), several bacteria like *Klebsiella pneumoniae*, *E. coli*, *S. aureus*, and *Neisseria gonorrhoeae* show resistance toward antibiotics like ciprofloxacin, carbapenem, fluoroquinolone, methicillin, third generation cephalosporins, sulphonamides, penicillin, tetracyclines, macrolides, fluoroquinolones, and early generation cephalosporins. Earlier, the WHO had published a list of pathogens developing resistance that require immediate attention for the development of new antimicrobials against them and *Acinetobacter baumannii* and *P. aeruginosa* were listed as "critical" priority targets (Shrivastava et al., 2018). It is predicted that *P. aeruginosa* causing ocular infections can develop resistance against beta-lactam, aminoglycoside, and fluoroquinolones. Genetic mutations and inter/intra-species transfer of mobile genetic elements was found to be the major cause of antibiotic resistance spread (Subedi et al., 2018). In another study, 42.9% of *P. aeruginosa* and 57.1% of *Staphylococcus spp.* isolated from corneal ulcers exhibited multidrug (MDR) resistance (Heidari et al., 2018). It was also found that MDR and MRSA isolates are increasing in serious ocular infections (Asbell et al., 2008). Ciprofloxacin and

levofloxacin resistance among methicillin-sensitive *S. aureus* was also found frequently in corneal and conjunctival isolates (Marangon et al., 2004). Isolates of *P. aeruginosa* and *S. aureus* were also found to be resistant to ciprofloxacin, gentamicin, and cephalosporins (Willcox, 2011). Currently for management of MDR bacteria, antibiotics like linezolid, colistin, and imipenem are used (Egrilmez and Yildirim-Theveny, 2020). However from what we know about earlier antibiotics, the development of resistance against these antibiotics in bacteria may occur anytime soon. Similarly, the repertoire of effective antifungal agents remains very limited, with only three classes of drugs available for systemic therapy, polyenes (e.g. amphotericin B), triazoles (e.g. fluconazole), and echinocandins (e.g. caspofungin) (Nami et al., 2019). Fluconazole and ketoconazole show better intraocular penetration and acts against keratitis with deep lesions and are considered effective against both *Candida* and filamentous fungi (Chang and Chodosh, 2011). Natamycin is effective when it is topically administered; however, only 2% of the total drug is bioavailable in the cornea (O'day et al., 1986). *Scedosporium* spp. show resistance against amphotericin *in vitro* and both amphotericin B and miconazole show variable activity against *Fusarium* spp. (Klepser, 2011). Ketoconazole and itraconazole have poor *in vitro* activity against *Aspergillus* and *Fusarium* spp. (Pearce et al., 2009). Hence, several alternative therapeutic approaches are explored for the management of ocular microbial infections, some of them include corneal collagen cross-linking (Price and Price, 2016; Naranjo et al., 2019), photodynamic antimicrobial therapy (PDAT), and the use of antimicrobial peptides (AMPs), including host defense peptides and synthetic peptides. Alternative strategies are also required to develop antifungals as the development of drug resistance and limited availability of antifungals represent the main concerns for the current antifungal treatments. The major advantages of AMPs as an alternative are their broad-spectrum activity, rapid killing, anti-biofilm, lower chance of development of resistance, and low cytotoxicity to host. In the following sections, we have provided detailed description of AMPs and their protective role in preventing corneal keratitis.

ANTIMICROBIAL PEPTIDES

Antimicrobial peptides are a diverse group of small proteins that acts as the first line of defense and mounts an attack against invading pathogens. They are found to be evolutionarily conserved in the genome and synthesized by all forms of life (Hancock, 2000). In nature, AMPs are produced either by ribosomal translation of mRNA or by non-ribosomal peptide synthesis (Hancock and Chapple, 1999). In mammals, they are mainly found within granules of neutrophils; however, epithelial cells in the cornea, skin, or other mucosal sites also secrete AMPs. Often, several AMPs with different modes of action are encoded in a cluster of the genome and are co-expressed resulting in the accumulation of several AMPs at a specific site (Lai and Gallo, 2009). Due to their distinguished mode of actions, they are often effective against MDR isolates. Several AMPs are often produced in a catalytically inactive form and needs proteolytic cleavage to become active (Bals, 2000). AMPs are commonly classified based on their secondary structures as α-helix, β-sheets, and random coil that lack a secondary structure. Antimicrobial peptides approved by the Food and Drug Administration (FDA) for clinical trials are listed in **Table 1**.

Antimicrobial Peptides in Eye

The cornea and ocular surface are protected from trauma and infection by physical and molecular defenses. The first effective defense involves eyelid closure and blinking, which protects the cornea from physical trauma and removes microbes from the ocular surface. In addition, ocular surface mucins and the tear film restrict pathogen interaction with the corneal epithelium (Mantelli and Argueso, 2008). Corneal epithelium is multilayered in nature and acts as the first line of defense during pathogen attack. Cornea expresses several AMPs (**Figure 2**) that have microbicidal properties or aids in wound healing, either constitutively or in response to microbial antigens (Mc et al., 1999; Mcdermott, 2009; Sharma et al., 2018; Sharma et al., 2019). β-defensins are produced by the ocular surface and α defensins are supplied into the ocular surface fluids by resident or passing neutrophils and lacrimal ductular epithelia secretion (Kumar et al., 2006). Defensins are 3–4 kDa cationic AMPs that are ubiquitous in nature and its structure contains six cysteine residues paired by disulfide bonds (Ganz and Lehrer, 1994). β-defensin-1 to -4, liver expressed antimicrobial peptide (LEAP)-1 and -2, and LL-37/cathelicidin are often found in ocular surface epithelia (Paulsen et al., 2001). LEAP-1 is a cationic peptide made up of twenty five amino acids and contains four disulfide bonds (Krause et al., 2000). LL-37 is another cationic peptide consisting of 37 amino acids and is the only member of human cathelicidin family (Durr et al., 2006). Human β-defensin (hBD)-1 is constitutively expressed in corneal and conjunctival epithelial cells whereas, in contrast, hBD-2 is rarely expressed in normal tissues. The corneal epithelial cells also constitutively express hBD-3, which some have found to be upregulated in response to cytokines (Mcdermott et al., 2003). hBD-4 is often found to be expressed *in vitro*, but rarely *in vivo*. hBD-9 is expressed by corneal and limbal epithelia and corneal stroma at modest levels, whereas conjunctival epithelium shows the presence of high levels of hBD-9 protein (Mohammed et al., 2010). LL-37 is expressed by both corneal and conjunctival epithelial cells (Gordon et al., 2005) and along with β-defensins contribute to the ability to resist pathogen attack by these cells (Augustin et al., 2011). S-100 class of proteins or calgranulins comprises a group of calcium-binding peptides that usually function as dimers (Fano et al., 1995). Constitutive expression of S100A7 has been reported in cornea, conjunctiva, nasolacrimal ducts, and lacrimal glands. S100A8 and S100A9 were also found to be expressed by human corneal stromal cells (Wilkinson et al., 2016). Ocular fluid also contains human neutrophil peptides (hNP-1 and -2), hBD-1, hBD-2, hBD-3, lactoferrin, lysozyme, and transferrin during normal conditions (Silva et al., 2013). Lactoferrin, a member of transferrin family, is an iron-binding protein having different isoforms such as lactoferrin α, β, and γ (Anderson et al., 1990)

TABLE 1 | Antimicrobial peptides in clinical trial.

AMP	Source	Target	Phase	References
hLF1-11	Lactoferricin derivative	MRSA, *K. pneumoniae*, *L. monocytogenes*, fungal infections	I/II	Morici et al. (2016)
PAC113	Histatin-5 analogue	Oral candidiasis	II completed	Mohammad et al. (2015)
POL7080	Protegrin analogue	*P. aeruginosa*, *K. pneumoniae*	III	Butler et al. (2013)
LTX-109 (Lytixar)	Synthetic peptide	Gram-positive MRSA skin infections	IIa completed	Mohammad et al. (2015)
LL-37	Human cathelicidin	Leg ulcer	IIb	Boparai and Sharma (2020)
Novexatin (NP213)	Cyclic cationic peptide	Fungal nail infection	IIb	Javia et al. (2018)
D2A21	Synthetic peptide	Burn wound infections	III	Ballweber et al. (2002)
SGX942	Synthetic peptide	Oral mucositis	III	Butler et al. (2017)
PXL01	Lactoferrin analogue	Postsurgical adhesions	III	Wiig et al. (2011)
OP-145	LL-37 derivative	Chronic middle ear infection	Phase II completed	Ming and Huang (2017)
Mycoprex	Extracted from insects	Fungal infections	III	Hancock (2000)
Murepavadin (POL7080)	Amino acid substitution of protegrin I	Nosocomial pneumonia and ventilator-associated bacterial pneumonia (VABP)	III	Butler et al. (2017)
OP-145	Synthetic 24-mer peptide derived from LL-37	Antibacterial, Chronic bacterial middle ear infection	II completed	Ming and Huang (2017)
Histatin	Naturally occurring peptide	Oral candidiasis	Phase II/III	
MX-226	Cationic Peptide	Dermatology related infections	Phase III b	Felício et al. (2017), Boparai and Sharma (2020)
HB-107	Cecropin B derivative	Wound healing	Preclinical	Mangoni et al. (2016)
PL-5	Alpha helical synthetic peptide	Treatment of skin infections	Phase II	Feng et al. (2015)
Daptomycin	*Streptomyces roseosporus*	Gram-positive bacteria	In market	Cortes-Penfield et al. (2018)

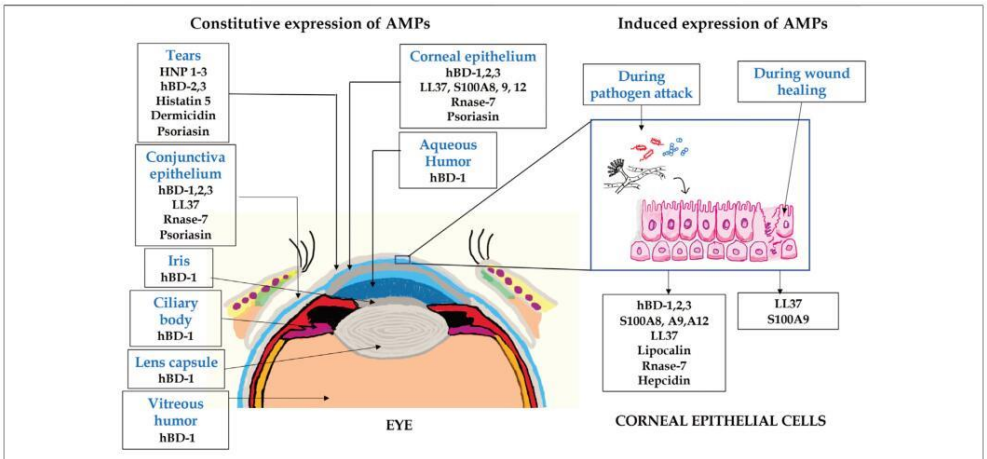

FIGURE 2 | A schematic representation of AMPs expressed in eye. The expression of several AMPs in the anterior part of the eye including tears, conjunctiva, iris, lens, aqueous humor, and corneal epithelium. The corneal epithelial cells express several AMPs constitutively or in response to pathogen attack and wound healing.

and plays an important role in human innate immune system (Baveye et al., 1999). Transferrin is another iron-binding glycoprotein that is responsible for the transport of iron in biological fluids (De Jong et al., 1990). Lysozyme, a 15 kDa protein, present abundantly in tears hydrolyses bacterial cell wall (Mcdermott, 2013). The tear film also contains lactoferrin, β-defensins, calprotectin, and lipocalin (Dartt, 2011; Rusciano et al., 2018). Though their role in tears is not

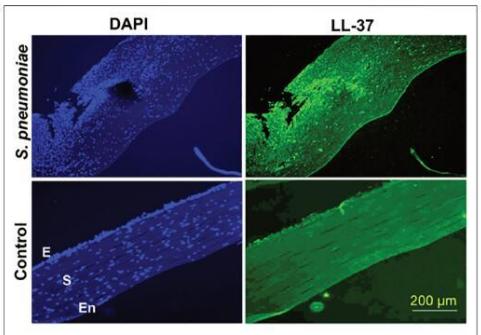

FIGURE 3 | LL-37 expression in corneal tissues obtained from *S. pneumoniae* keratitis patients. Corneal sections of keratitis patients obtained during corneal transplantation and control cadaveric corneas were stained with anti-LL-37 antibody, followed by Alexafluor 488 secondary antibody and imaged under a fluorescent microscope using 10X objective. The sections were counterstained with DAPI. E denotes epithelium, S-stroma, and En-endothelium. This figure is reprinted from reference Sharma et al. (2019).

fully known, it is likely that cation (Fe^{2+} and Zn^{2+}) sequestration in the ocular surface inhibits fungal germination and growth, which requires these essential metals. These constitutively expressed AMPs have a major role to play in the prevention of infections and aids in host defense. Murine β-defensin 3 (mBD-3, an orthologue of hBD-2) contributes to the clearance of *P. aeruginosa in vivo* and additionally limits *P. aeruginosa* adhesion to the corneal epithelial surface, thus suggesting that AMPs are also involved in maintaining normal resistance to infection in the healthy tissues (Augustin et al., 2011). hBD-2 is found more frequently in infected and inflamed ocular surface tissues. Bacterial products such as lipopolysaccharide (LPS) and pro-inflammatory cytokines such as interleukin (IL)-1β have been shown to upregulate hBD-2 expression in corneal and conjunctival epithelial cells *in vitro* through mitogen-activated protein (MAP) kinase and NF-κB pathways. Differential expression of several AMPs was identified in patients with *P. aeruginosa* and *S. pneumoniae* corneal infections (Sharma et al., 2018; Sharma et al., 2019). Increased expression of LL-37 was also detected in the corneal tissues obtained from patients with *S. pneumoniae* keratitis (**Figure 3**) (Sharma et al., 2019). hBD-9 was found to be downregulated in bacterial keratitis caused by both Gram-positive and Gram-negative bacteria (Mohammed et al., 2010). hBD-3 and RNAse7 were found to be significantly increased in corneal epithelial cells in response to *A. castellanii* (Otri et al., 2010). A significant increase in the expression of S100A8, S100A9, and hBD-1 was observed both *in vitro* and in corneal ulcers of patients during the *Corynebacterium pseudodiphtheriticum* infection (Roy et al., 2015). Mohammed et al. (2020) observed increased expression of hBD-1, 2, and 9 in patients with fungal keratitis. However, we have found reduced expression of β-defensins in patients with *A. flavus* corneal infections (Mallela et al., 2021).

Natural Peptides

Peptides are broadly classified into two classes based on their origin, natural peptides, and synthetic peptides. Natural peptides are secreted by a large number of different species such as bacteria, virus, amphibians, mammals, insects, and plants. These peptides can act as natural ligands either as agonists or antagonists and are highly selective and well tolerated. Bacterial infection causes upregulation of several host defense peptides that helps to combat the attack of pathogens. hBD-2 was frequently reported in infected/inflamed ocular surface tissues. Increased expression of defensins was also observed in a murine model of corneal infections, and mBD-3 increased resistance to *Pseudomonas* corneal infections and modulate Toll-like receptor (TLR) signaling in the infected corneas (Wu et al., 2009). Ocular commensal like *C. mastidis* has been shown to induce IL-17 expression that elicits the release of AMPs into tears and protect against *P. aeruginosa* infections (St Leger et al., 2017). The topical application of OH-CATH30, an AMP identified in king cobra is efficacious against drug-resistant *P. aeruginosa* keratitis (Li et al., 2014). LL-37 showed potent antibacterial activity against both laboratory strain and ocular clinical isolates of *S. pneumoniae* (Sharma et al., 2019). It also showed antibacterial activity against *S. aureus* and *S. epidermidis*. Cathelicidin deficit mice also showed increased susceptibility to *P. aeruginosa* keratitis (Huang et al., 2007). S100A8/A9 was found to induce reactive oxygen species (ROS) and aided in macrophage-mediated bacterial killing during *P. aeruginosa* keratitis (Deng et al., 2013). Lipocalin present in tear was reported to inhibit both bacterial and fungal growth (Fluckinger et al., 2004) and also suppressed ocular inflammation in LPS-induced uveitis (Tang et al., 2018). The antimicrobial peptide Css54, isolated from the venom of *Centruroides suffuses*, was found to exhibit antimicrobial activity against bacteria such as *Listeria monocytogenes*, *Streptococcus suis*, *Campylobacter jejuni,* and *Salmonella typhimurium* that cause zoonotic diseases (Park et al., 2020). Using a high-throughput technique of the phage display method, 12-mer peptide phage display library was screened to identify peptides effective against infectious keratitis. Both Pc-C and Pc-E peptides inhibit the adhesion of *A. fumigatus* to human corneal epithelial cells (HCEC) and decreased fungus-mediated corneal disruptions in a mice model of keratitis (Zhao et al., 2012). Endogenous expression of mBD3, mBD4, and CRAMP was found to play an important role in *F. solani* keratitis (Kolar et al., 2013). Increased expression of LL-37, hBD-2, and hBD-3 in corneal epithelial cells was also noted in response to heat-killed *C. albicans* (Hua et al., 2014). Cullor et al. (1990) found defensins, NP-1, and NP-5, effective against *C. albicans*' ocular isolates found in humans and horses. A study by Sengupta et al. (2012) reported the antifungal activity of lactoferricin B against fungal pathogens and its effect on eradication of the biofilms from contact lenses. Lactoferricin B helps to decrease the dosage of antifungal agents by 8 folds against biofilms formed by *A. fumigatus, F. solani*, or *C. albicans*. Another study reported that α defensins are known to show strong antifungal activity against *A. fumigatus*, *C. neoformans,* and *C. albicans* (Delliere et al., 2020). Histatin -5 is a histidine-rich peptide which is

produced by salivary glands in humans and higher primates (Du et al., 2017). Both hNP-1 and histatin-5 were found in human saliva exhibiting candidacidal activity due to their non-lytic release of mitochondrial ATP and chelation of the metal ions (Helmerhorst et al., 2001). hBD-1, -2, -3, histatin-5, and LL-37 were found to show fungicidal activity against *Candida* spp. *via* secreted glycosylated exodomain protease, Msb2 (Swidergall et al., 2013). Human cathelicidin and β-defensins permeabilize the cell membrane of the yeast that leads to the death of the fungi (van Der Weerden et al., 2010). HBD-3 and LL-37 bind to the β-1, 3-exogluconase of the cell wall of *C. albicans*, thereby reducing its infectivity toward the host (Mohammed I. et al., 2017). Histatin-5 kills the fungus by different mechanisms, it activates the stress response pathways and secretion of the proteases. Histatin-5 enter into the fungus *via* Dur3/Dur 31 transporters, induce ATP efflux and release of ROS, and finally activate the high osmolarity glycerol stress response pathway, followed by extrusion of histatin-5 *via* efflux transporter, Flu1 (Edgerton et al., 2000). RNase-3 and RNase-7 were known to show fungicidal activity at lower micromolar concentration against *C. albicans* by perturbing the cellular RNA and disturbing the stability of the cell membrane (Berman, 2012). A reduced form of psoriasin (S100A7), a linear peptide, showed killing activity against *A. fumigatus* by chelation of zinc metals and leading to fungal apoptosis (Mohammed I. et al., 2017). Calprotectin (S100A8/A9) exhibited antifungal activity, blocked hyphal growth, and limited the growth of *Aspergillus* spp. by inhibiting transport of fungal zinc and manganese in a mouse model of keratitis (Clark et al., 2016).

Synthetic Peptides

Synthetic or semi synthetic peptides are generally designed to upregulate the pharmacological properties and to overcome the limitations of naturally derived AMPs including host toxicity, degradation by proteases, and loss of antimicrobial activity in presence of physiological salt concentration (Mohamed et al., 2016). The short synthetic peptides have high electivity and reduced cost of production making them ideal for translational clinical application. Several peptides designed and synthesized have been tested against various bacteria and fungus both *in vitro* and *in vivo*. EC1-17KV, synthetic peptides based on the antimicrobial peptide EeCentrocin 1 from *Echinus esculentus*, was efficient in killing MDR *P. aeruginosa* and exhibited multiple modes of action, including direct membrane disruption and inhibitory effects on cell adhesion and biofilm formation (Ma et al., 2020). Another novel short α-helical hybrid peptide inspired by natural α-helical AMPs, PA-13, showed remarkable broad-spectrum antibacterial activity, especially against *P. aeruginosa* with no toxicity to mammalian cells. It displayed rapid binding and penetration activity which resulted in membrane permeabilization and also exhibited an anti-inflammatory response by neutralizing LPS (Klubthawee et al., 2020). Pam-3, a palmitoleic acid–modified octapeptide fragment designed from hBD-1, was found to be effective *in vitro* against multidrug-resistant ESKAPE pathogens (six nosocomial pathogens, *Enterococcus faecium*, *S. aureus*, *K. pneumoniae*, *A. baumannii*, *P. aeruginosa*, and *Enterobacter species* that exhibit multidrug resistance and virulence). Pam-3 is also highly effective against *A. baumannii* resistant to the last-resort antibiotics, colistin and tigecycline. Additionally, Pam-3 was able to eradicate established biofilms formed by *S. aureus* and *P. aeruginosa in vitro* (Koeninger et al., 2021). SAAP-148, a synthetic LL-37 inspired peptide, is highly effective against MDR Gram-positive and Gram-negative ESKAPE pathogens, as well as isolates of *E. cloacae*, *E. coli*, and *K. pneumoniae* resistant to the last-resort antibiotic colistin. SAAP-148 was also able to kill *Clostridium difficile* under anaerobic conditions and successful in preventing the formation and eradication of established biofilms by *S. aureus* and *A. baumannii* (De Breij et al., 2018). A synthetic peptide, TC19, derived from the human thrombocidin-1–derived peptide L3, showed efficient and rapid killing in human plasma of MDR strains of several ESKAPE bacterial species. FK16, a cathelicidin-derived shorter peptide, was found to show increased killing ability toward *P. aeruginosa*, by itself or in synergy with vancomycin without having any toxicity to the host (Mohammed et al., 2019). A hybrid peptide of cecropin A and mellitin (derived from venom of European honey bee) was found to be effective against *P. aeruginosa* in a rabbit model of keratitis (Nos-Barbera et al., 1997). Cationic antimicrobial protein (CAP37)–derived peptide analogues showed significant antifungal activity against multifarious *Candida* species from patients with vulvovaginitis. Bactericidal permeability-increasing (BPI) protein, a cationic protein derivative has broad spectrum activity against many fungi, including *Candida spp*., *C. neoformans*, and *A. fumigatus*. Shorter synthetic peptides (XMP.284, XMP.366, and XMP.39 1) which are derivatives of BPI protein domain III analogs act against *Candida spp*. When these peptides were used in combination with flucanozole, the minimum inhibitory concentration (MIC) of the antibiotic reduced up to 8-fold. The peptide XMP 391 was effective against murine-disseminated aspergillosis and enhanced the effectiveness of amphotericin B. The peptides ToAP2 (derived from venom of scorpion *Tityus obscurus*) and NDNP-5.7 also showed antifungal properties against *C. albicans* infection in which ToAP2 exhibited a broad spectrum of activity. These peptides show fungicidal nature by permeabilizing the cell membrane and invoked changes in cell morphology such as cell disruption 24-h posttreatment (Do Nascimento Dias et al., 2020). P10, an α-helical amphipathic AMP, exhibited an enhanced antimicrobial spectrum against. *C. albicans* and *A. niger* (Kang et al., 2017). Lipopeptides, which were secreted by the *P. syringae*, was particularly active against several filamentous fungi and yeasts, including *Candida, Cryptococcus,* and *Aspergillus* strains (De Luca and Walsh, 2000). Cecropins, an AMP derived from insects, exhibited antifungal properties against *Aspergillus* and *Fusarium spp*. (Andra et al., 2001). This peptide targets the cell membrane by disrupting it and creates ionic imbalance and intracellular redox states in *C. albicans*. The upregulation of expression of CRAMP, a mouse analogue of human LL-37, leads to a decrease in the gastrointestinal colonization of *C. albicans* (Niyonsaba and Ogawa, 2005). The peptide proteagrin-1 from porcine was particularly active against a broad range of fungi, including several *Candida spp*.

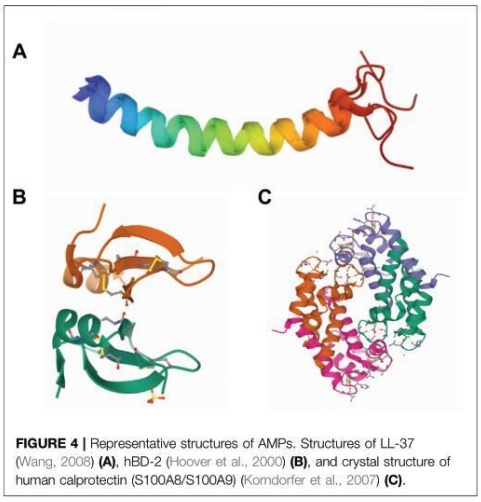

FIGURE 4 | Representative structures of AMPs. Structures of LL-37 (Wang, 2008) **(A)**, hBD-2 (Hoover et al., 2000) **(B)**, and crystal structure of human calprotectin (S100A8/S100A9) (Korndorfer et al., 2007) **(C)**.

(including drug-resistant strains) and *Cryptococcus neoformans* (Buda De Cesare et al., 2020). The same study also reported other peptides like SMAP-29 (cathelicidin in ovine), BMAP-27, and BMAP-28 (two bovine α-helical) to be effective as proteagrin-1 against *C. tropicalis*, *C. glabrata*, and *C. parapsilosis* (Buda De Cesare et al., 2020).

Mode of Action of Antimicrobial Peptides

In general, AMPs are cationic in nature with a net charge of +2 to +11 and an amphipathic structure that aids in insertion in bacterial cell membrane either as alpha-helices or beta-sheets (Luong et al., 2020). Representative structural images of few AMPs are given in **Figure 4**. Cationic AMPs selectively bind to the negatively charged surface of the bacterial membrane (Matsumoto et al., 2006; Strahl and Errington, 2017) that differ from mammalian cell membranes consisting of neutral phospholipids (Van Meer et al., 2008). In case of fungi, AMPs inhibit or kill the pathogenic fungi by inhibiting the spore germination or mycelial growth or by causing the structural deformity in the spores/hyphae by creating pores, twists, and making them swollen and broken (Li et al., 2021). Most of the AMPs show a direct action against pathogens by creating pores on their membrane, hence causing leakage and cell death. The models for the creation of pores on the cell membrane by AMPs that have been hypothesized are as follows:

a. Barrel-stave model, where AMPs form bundles and insert in the membrane like a cylinder creating pores, hydrophobic region of peptide interacting with lipid part, and ultimately causing leakage (Bocchinfuso et al., 2009; Zeth and Sancho-Vaello, 2017). For example, dermicidin and GA IV glycotriazole peptides (Junior et al., 2017).
b. Toroidal model, where AMPs arrange and implant themselves vertically in the membrane to make pores and forces the lipids to form micelles and hence disrupts the membrane (Matsuzaki et al., 1995). For example, magainin 2.
c. Carpet-like model, where AMPs form aggregates and arrange parallelly on the membrane like a carpet and intercalate into the membrane, with hydrophobic sites facing the membrane and disrupts the arrangement of the lipid bilayer (Oren and Shai, 1998). For example, LL37 and PMAP-23.

Apart from these classical models, AMPs like R9F2 and (KFF) 3K have recently been shown to cause cell wall thinning, loss of cytoplasm structure, formation of mesosome-derived multimembrane structures, and "decorated fibers" derived from DNA chains in *S. aureus* (Grigor'eva et al., 2020) instead of pore formation. EP-2, an antifungal peptide isolated from *B subtilis*, can swell and distort the mycelium of the fungi thereby inhibiting the growth of fungi (Wang et al., 2016). As fungal cells are mainly composed of polysaccharides, β-glucans, chitin, and mannan, AMPs inhibit the synthesis of these saccharides (Li et al., 2021). Anidulafungin, a synthetic lipopeptide, inhibits the synthesis of 1,3 β-D glucan synthesis in the cell walls of *Candida* and *Aspergillus* (Martin Mazuelos and Rodriguez-Tudela, 2008). Apart from cell membrane disruption, some AMPs like Bac 7, PR-39, and HNP-1 (Cardoso et al., 2019) are also involved in the inhibition of protein biosynthesis or nucleic acid biosynthesis (Hale and Hancock, 2007); some like histatin-5 causes protease activity, others like pyrrhocoricin and drosocin (Nielsen et al., 2021) causes disruption of DnaK activity. Indolicidin, aurein, magainin II, cecropin A, and LL-37 were also found to enhance the essential lipid transportation causing rapid scrambling of the lipid composition leading to an increase in ion transport and activation of lethal signaling processes (Paredes-Gamero et al., 2012). Apart from their direct microbicidal activity, AMPs also help in the chemotaxis of host neutrophils, mast cells, and monocytes, phagocytosis, angiogenesis, and wound healing. AMPs can also trigger mast cell degranulation and lead to increased blood vessel permeability. Hence, AMPs can target a pathogen directly or indirectly, ultimately leading to clearance and healing. It has also been shown that some AMPs at lower concentrations activate apoptosis and hence lead to regulated cell death (Lee and Lee, 2014).

OTHER COMPOUNDS

During corneal infections, pro-inflammatory cytokines like IL-1β, IL-6, and tumor necrosis factor (TNF)-α are released that triggers different signaling pathways leading to collagen degradation in the stroma as well as necrotic death of keratocytes. These inflammatory damages result in the thinning of corneal stroma followed by scar formation (Yan et al., 2017; Crupi et al., 2019). Studies have reported that the topical antioxidants or corticosteroids or lipid treatment reduced the inflammatory reaction that occurs during acute corneal inflammation and protects the ocular damage (Alio et al., 1995; Lim et al., 2015; Ni et al., 2016a). Here, we will be

discussing about the antioxidants, small molecules, lipids, and corticosteroids that were used to protect the eye from fungal, bacterial or bacterial toxin–induced inflammatory damage.

Antioxidants

The generation of ROS by corneal epithelial cells (CEC) often protects corneas from microbial infections by killing the microbes; however, excess production of ROS induces corneal inflammation and results in irreversible corneal opacification and loss of vision (Ruban et al., 2018). Antioxidants reduce the free radical by donating their electrons and decrease the inflammatory damage of CEC. Various studies have reported the usage of antioxidants to protect the cornea from the microbial-induced inflammatory damage. Resveratrol, a naturally occurring phytoalexin produced in the fruits and leaves of edible plants is known to possess antioxidant and anti-inflammatory activity (Iyori et al., 2008; Speciale et al., 2011). Resveratrol downregulates pro-inflammatory cytokines in cells by suppressing the activation of NF-κB. Marino et al. (2013) found that resveratrol protected the cornea from *S. aureus*–induced inflammatory damage by downregulating the expression of TLR2 and IL-8 in an *ex vivo* model of infection. Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl), a membrane permeable molecule known to exhibit antioxidant activity, reduces the reactive oxygen and nitrogen species and enhances the catalytic activity of catalase (Batinić-Haberle et al., 2010; Wilcox, 2010). Crupi et al. (2019) used this potent antioxidant molecule to explore its protective effect against LPS-induced corneal cell damage and found that tempol protected the corneal cells from inflammatory damage by enhancing the antioxidant activity of SOD and lowering the prostaglandin E2 (PGE2) levels, cytokines, and COX-2 expression. Asiatic acid (a triterpenoid) was found to protect HCEC from the LPS-induced inflammatory damage by inhibiting p-Akt activation, downregulating inflammatory cytokines, and upregulating antioxidant activity (Chen et al., 2017). Quercetin is a natural flavonoid known for antioxidant activity along with anti-inflammatory, antitumor, and cardiovascular protective activity (Li et al., 2016). Previous studies have reported that quercetin exerts antifungal activity against wide variety of fungal strains (Rocha et al., 2019) and also reported the suppression of LPS-induced retinal inflammation in mice (Ho et al., 2020). Yin et al. (2021) reported protective effect of potential quercetin against *A. fumigatus*–induced corneal keratitis. *In vitro* studies showed that quercetin treatment significantly reduced the growth of *A. fumigatus* without affecting the viability of HCEC, whereas in *in vivo* studies, it reduced the fungal load in the cornea with minimal recruitment and infiltration of neutrophils to the corneal stroma (Yin et al., 2021). Baicalein, a flavonoid isolated from *Scutellarin baicalins,* is also known to have an antioxidant activity along with antifungal and anti-inflammatory applications (Zhu et al., 2021). Zhu et al. (2021) have reported the use of baicalein to protect the cornea from *A. fumigatus* infection in a murine model of keratitis. Treatment of infected mice cornea with baicalein significantly reduced fungal growth, biofilm formation, adhesion, and suppressing inflammatory responses *via* downregulating the thymic stromal lymphopoietin (TSLP)/TSLP receptor (TSLPR) pathway (Zhu et al., 2021). Epigallocatechin gallate (EGCG) is a polyphenol obtained from the green tea is known to have good antioxidant and anti-inflammatory activity (Ruban et al., 2018). Topical administration of voriconazole along with EGCG showed the inhibition of *F. solani*–induced keratitis by reducing the fungal growth and inflammatory responses. Systemic supplementation of vitamin C (ascorbic acid) showed reduced corneal opacity caused by infectious keratitis (Cho et al., 2014). Vincamine, an alkaloid with strong antioxidant activity, protected HCEC against LPS-induced oxidative and inflammatory damage by scavenging ROS and reducing the expression of IL-1β, IL-6, IL-8, TNF-α, and the transforming growth factor (TGF)-β (Wu et al., 2018). Antioxidants having anti-inflammatory activity have advantages in protecting the cornea from the microbial toxin–induced inflammatory damage during corneal keratitis.

Small Molecules

To fight the antibiotic resistance that is increasing rapidly on a global scale, the virulence factors of the pathogens are often targeted and explored as alternative therapeutic agents. High-throughput screening are being widely used to identify various compounds that could be developed as small molecule tools to study the pathogen further or as a treatment for preventing infections. Dajcs et al. have reported the inhibitory role of lysostaphin, a zinc metalloproteinase obtained from *S. simulans*, against *S. aureus*–induced corneal keratitis. *In vivo* studies (rabbit models) displayed that lysostaphin treatment significantly killed more methicillin resistant *S. aureus* (MRSA) or methicillin sensitive *S. aureus* on the corneal surface and the corneal tissues than in vancomycin-treated or untreated eyes (Dajcs et al., 2000). Further, the authors also evaluated possible allergic reactions toward lysostaphin and found no adverse reactions (Dajcs et al., 2002). Small molecule like 2-Imino-5-arylidene thiazolidinone was found to inhibit type II secretory system of *P. aeruginosa* and type 3 secretory system (T3SS) in *Yersinia spp*. (Felise et al., 2008). Recently Sharma et al. (2020) have shown that INP0341, a salicyledene acylhydrazide that blocks T3SS, effectively inhibits *Pseudomonas* infection in a murine model of keratitis. The salicyledene acylhydrazides block the T3SS in several pathogens, including *Y. pseudotuberculosis* (Nordfelth et al., 2005; Bailey et al., 2007), *C. trachomatis* (Slepenkin et al., 2011), *S. enterica*, and *P. aeruginosa*. Several small-molecule compounds that inhibit *Pseudomonas* elastase have been reported by various groups (Burns et al., 1990; Cathcart et al., 2011; Konstantinovic et al., 2020). The compound 2-mercaptoacetyl-L-phenylalanyl-L-leucine, effectively inhibited elastase activity in rabbit corneas and reduced corneal melting in a rabbit model of *P. aeruginosa* keratitis (Spierer and Kessler, 1984). Targocil, a teichoic acid biosynthesis inhibitor, significantly reduced intracellular growth of *S. aureus* in HCEC and further aided in reducing the adherence of *S. aureus* to corneal cells (Suzuki et al., 2011). Very recently, pseudolipasin A, an inhibitor of ExoU of *P. aeruginosa*, induced scratch healing and reduced cell death of human corneal epithelial cells during *P. aeruginosa* infection (Foulkes et al., 2021). Glycyrrhizin, a glycoconjugate triterpene isolated from *G. glabra*, significantly reduced the adherence of *P.*

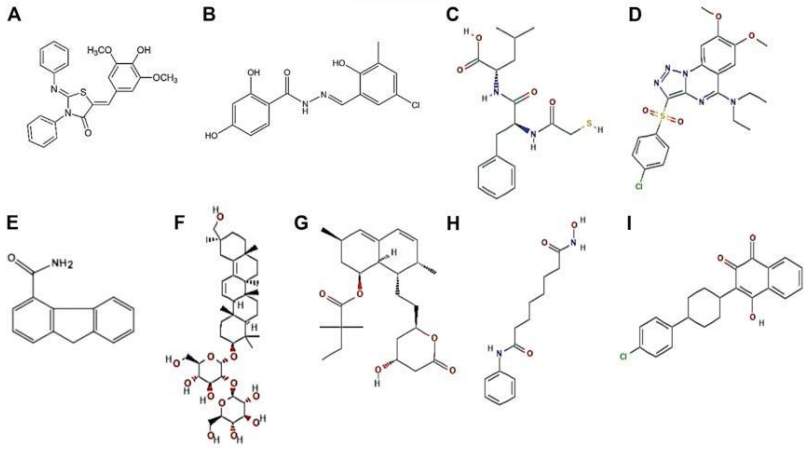

FIGURE 5 | Representative structures of small molecules. 2-Imino-5-Arylidene thiazolidinone (Felise et al., 2008) **(A)**, INP0341 (Nordfelth et al., 2005) **(B)**, 2-mercaptoacetyl-L-phenylalanyl-L-leucine (Kessler et al., 1982) **(C)**, Targocil (Farha et al., 2015) **(D)**, Pseudolipasin A (Lee et al., 2007) **(E)**, Glycyrrhizin (Ji et al., 2016) **(F)**, Simvastatin (Jensen et al., 2016) **(G)**, Suberoylanilide hydroxamic acid (Choi and Pflum, 2012) **(H)**, and Atovaquone (Nixon et al., 2013) **(I)**.

aeruginosa to corneal epithelial cells and inhibited the growth of bacteria in a mouse model of *Pseudomonas* corneal infections, either alone or in combination with antibiotic (Hazlett et al., 2019). Topical application of simvastatin that blocks HMG-CoA reductase required for siderophore biosynthesis, significantly reduced fungal burden during *A. fumigatus* corneal infections by restricting fungal iron acquisition *in vivo* (Leal et al., 2013). Very recently suberoylanilide hydroxamic acid, a histone deacetylase inhibitor, significantly reduced inflammation and expression of pro-inflammatory cytokines in a murine model of *F. solani* keratitis (Li et al., 2019). Atovaquone, a hydroxynapthaquinone, acts as an antifungal agent by disrupting mitochondrial functions and intracellular zinc storage in *Aspergillus* spp. and *Fusarium* spp. Reduced hyphal growth was observed in murine eyes infected with Fusarium and treated with atovaquone compared to infected eyes (Clark et al., 2018). The represented structures of the small molecules are presented in **Figure 5**.

Lipids

The omega-3 fatty acids like docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) exhibits anti-inflammatory activity by inhibiting the oxidation of arachidonic acid (AA), which prevents the production of pro-inflammatory molecules like leukotriene B_4 and prostaglandin E_2 (Lim et al., 2015). Resolvins, an endogenous lipid mediator derived from EPA (resolvin E, RvE) and DHA (resolvin D, RvD) exhibits anti-inflammatory activity by reducing the production of pro-inflammatory cytokines. Among E-series resolvins, RvE1 possess various biological activities, including antioxidant and anti-inflammatory properties, and also they clear neutrophils from the site of inflammation by recruiting non-inflammatory monocytes and macrophages. This potential molecules was used by Lee et al. (2015) to evaluate its protective effect from bacterial toxin(LPS, antibiotic-killed *S. aureus* and *P. aeruginosa*)–induced corneal inflammatory damage. RvE1 treatment significantly reduced the production of chemokines and pro-inflammatory cytokines in HCEC, human neutrophils (IL-6, IL-8, and CXCL1), murine cornea (CXCL1), and macrophages (CXCL1, TNF-α, and IL-1β). Additionally, RvE1 treatment also reduced the neutrophil infiltrations into the corneal stroma and minimized corneal thickness and haze. RvD1 prevented the corneal inflammatory damage by reducing corneal infiltrates, edema, and production of inflammatory cytokines (IL-6 and CXCL1) along with inhibition of neutrophil recruitment (Lee et al., 2016).

Corticosteroids

Corticosteroids having anti-inflammatory activity are known to suppress inflammatory pathways in a wide variety of cells by downregulating the inflammatory genes (Tallab and Stone, 2016). Corticosteroids were used for its anti-inflammatory property to treat bacterial keratitis in combination with antibiotics, in addition they also reduce scarring, stromal melts, and neovascularization (Austin et al., 2017; Ni et al., 2016b). However, the use of topical corticosteroids has always been a topic of debate. There are several studies that report use of corticosteroids to be helpful in reducing severity of corneal stromal melt and neovascularization and improve patient compliance by alleviating pain and discomfort. It has also been shown to reduce neutrophil chemotaxis and thus lower cytokine burden. On the contrary, use of corticosteroid in bacterial keratitis has been argued to delay epithelial healing

and exacerbation of infection (Gritz et al., 1992). Several animal models were developed to explore the protective effect of corticosteroids from microbial keratitis (Bohigian and Foster, 1977; Badenoch et al., 1985; Gritz et al., 1992). Usage of corticosteroids alone for treating microbial keratitis enhanced the microbial count and extended infection period that leads to the corneal stroma thinning and perforation (Badenoch et al., 1985; Hazlett et al., 2016), whereas, in combination with antibiotics they neither have an effect on antimicrobial activity nor on the final outcome of corneal anatomy (Hindman et al., 2009). Animal studies have also displayed that topical administration of corticosteroids after corneal injury decreased corneal wound healing strength (Sugar and Chandler, 1974). Previous clinical trials have reported the use of corticosteroids in treating corneal keratitis and steroids for corneal ulcers trial (SCUT). A clinical study performed by Carmichael et al. (1990) reported that patients treated with antibiotics only (fortified cefazolin 32 g/L and gentamicin 14 g/L) and antibiotics plus steroids (fortified cefazolin 32 g/L, gentamicin 14 g/L, and 0.1% dexamethasone) showed no statistically significant difference in the outcome in terms of best corrected vision and the ulcer healing rate. A randomized clinical study was carried out by Blair et al. (2011) to investigate the protective effect of antibiotics (gatifloxacin) and antibiotics plus corticosteroids (gatifloxacin plus 0.1% dexamethasone) on the bacterial corneal ulcers. The mean residual ulcers measured by clinician at 10 weeks showed a smaller change in the ulcers size of antibiotics plus corticosteroids–treated patient than the only the antibiotic-treated patients group, though the difference was not significant. A preliminary clinical trial was carried out by Srinivasan et al. (2009) to evaluate the effect of adjunctive topical corticosteroids on the outcome in bacterial keratitis. Patients treated with corticosteroid (prednisolone sodium phosphate 1%) after moxifloxacin (0.5%) showed significant delay in re-epithelialization than control patients (corticosteroids-untreated patients).

ANTIMICROBIAL PEPTIDES AND ALTERNATIVE THERAPEUTICS IN OCULAR WOUND HEALING

Corneal wound healing consists of complex sequences of events involving the regeneration of epithelial layers, migration of epithelial cells, fibroblasts for wound closure, and regeneration of tissues in a regulated manner so that the transparency of the cornea is not compromised. Corneal epithelial cells aid in the process by replenishing themselves and mediating cell signaling and crosstalk with stromal cells (Di Girolamo, 2015). The wound healing is facilitated by several growth factors, cytokines, and endogenous AMPs expressed by CEC. Several AMPs that are upregulated in patients with bacterial or fungal corneal infections, like β-defensins, LL-37, S100A8, S100A9, and Reg3γ (Sharma et al., 2018; Sharma et al., 2019), have been shown to also possess wound-healing properties. hBD-2 mRNA was found to be upregulated in the epithelium of wounded corneas and aided in regeneration of corneal epithelium (Mcdermott et al., 2001). hBD-2 also induced expression of several cytokines and enhanced rapid intracellular Ca^{2+} influx that helped in proliferation of keratinocytes in a skin wound model (Mi et al., 2018). Histatin-5 was found to promote cell migration and accelerate wound closure in murine model of corneal epithelial injury (Shah et al., 2020). Increased expression of LL-37 was noted in regenerating corneal epithelium and induced migration of corneal epithelial cells (Huang et al., 2006). Other reports have also shown that LL-37 induced wound healing in the porcine corneal wound model by activation of EGFR signaling *via* PI3K/Akt and ERK signaling pathways (Yin and Yu, 2010). Cationic antimicrobial protein, CAP37, interacts with TLR4 and promotes corneal re-epithelization in mice (Kasus-Jacobi et al., 2020). Very recently, a dipeptide, isolated from human placental extract has been found to accelerate corneal wound healing both *in vitro* and *in vivo* (Nagata et al., 2015). RNase 5 has been shown to facilitate corneal wound healing in rabbit by activation of the PI3K/Akt pathway (Kim et al., 2016). Apart from AMPs, small molecules like Y-27632, a Rho-associated protein kinase inhibitor, has also been shown to induce proliferation of limbal stem cells and improve wound healing in *in vivo* mouse model (Sun et al., 2015) by modulation of cell-cell adhesion. Several lectins have also been shown to promote regeneration of corneal epithelium. KM+, a lectin obtained from *Artocarpus integrifolia*, promotes neutrophil migration and restoration of corneal epithelium in a rabbit corneal wound model (Chahud et al., 2009). Galectin 3 and galectin 7 have also shown to accelerate wound healing in a corneal alkali burn model (Cao et al., 2003). Acacia honey has been reported to accelerate corneal wound healing in rabbit corneal fibroblasts *in vitro* (Abd Ghafar et al., 2016). Another traditional herbal medicine, *Centella asiatica*, helped in proliferation and migration of rabbit corneal epithelial cells (Ruszymah et al., 2012). These data show promises that AMPs or their derivatives and mimetics can be potentially be useful to promote wound healing following corneal damages due to accidental injuries, or various surgeries.

OCULAR DRUG DELIVERY SYSTEMS TO TREAT KERATITIS

Treatment of cornea appears to be easily accessible; however, various physicochemical barriers hinder the drug penetration into this tissue. In addition to this, other factors, like excess tear secretions, blinking reflex, and nasolacrimal drainage system, significantly reduces drug retention time in the ocular surface; only 1–7% of the available drug reaches the corneal stroma (Hewitt et al., 2020). Therefore, a high dosage of the drugs are required for treating the infections, and nasolacrimal regions exposed to those drugs lead to adverse side effects (Phan et al., 2014; Djebli et al., 2017). In order to minimize high usage of drugs and reduce their side effects, the drugs need to be delivered to the site of infection. Drug delivery systems play a critical role in delivering the drugs to the infected sites. In this section, we will discuss the different types of drug delivery systems for delivering therapeutics to treat corneal infections.

Contact Lens

Contact lenses (CLs) are transparent, concave-shaped devices directly placed on the ocular surface for correcting the refractive errors. Additionally, they are used as bandage for protecting the corneal epithelial layer, helping in healing, and sealing wound leaks (Al-Qahtani et al., 2014; Grinninger et al., 2015; Mohammadpour et al., 2015; Ubani-Ukoma et al., 2019). CLs are explored widely as drug carrier systems to enhance the residence time of the therapeutic molecules on the ocular surface. Various materials have been employed in fabricating CLs without effecting their oxygen permeability and light transmittance (Dubald et al., 2018; Garg et al., 2019; Wei et al., 2020). One of the major challenges in developing drug delivery systems is to optimize the relevant drug release kinetics to inhibit the microbes (Hui et al., 2014). Tan et al. (2016) reported the fabrication of drug loaded intra-ocular lens to enhance the bioavailability of the drugs. Levofloxacin solubilized in tetrahydrofuran during fabrication of intra-ocular lens showed sustained release of the drug compared to levofloxacin solubilized in dicholoromethane. The studies carried out using commercially available CLs displayed prolonged drug release (Karlgard et al., 2000). Hui et al. reported silicone hydrogel CLs using a molecular imprinting technique to optimize the release kinetics of ciprofloxacin. However, incorporation of ciprofloxacin during fabrication of CLs unfortunately decreased light transmission. Acrylic acid–modified CLs displayed significantly longer time for drug release than unmodified controls. *In vivo* studies showed that ciprofloxacin released by the modified CLs significantly inhibited *P. aeruginosa* infection than unmodified CLs (Hui et al., 2014). Kakisu et al. (2013) reported the fabrication of soft CLs for assessing their ability to uptake and release antibiotics for a better drug delivery system for treating keratitis. The prolonged release of drugs from the soft CLs was achieved by layer-by-layer (LbL) coating of polyelectrolytes (combinations of sodium alginate, chitosan, sodium hyaluronate, and poly-lysine hydrobromide) with drugs (Silva et al., 2018). LbL coating controlled the release of diclofenac sodium salt from CLs and enhanced antibacterial activity without any adverse effects. Molecular imprinting and LbL coating did not affect the properties of soft CLs, and drugs released from the lenses inhibited the growth of *S. aureus* and *S. epidermis* (Silva et al., 2021). Ciolino et al. developed econzole impregnated poly-lactic-co-glycolic acid (PLGA) and poly-HEMA contact lenses for treating fungal keratitis. Econazole released from the contact lens efficiently inhibited the growth of *C. albicans* and displayed antifungal activity for up to three weeks. Huang et al. (2016) reported delivery of voriconazole using a hybrid CLs developed with quaternized chitosan, silver nanoparticles, and graphene oxide (HTCC/Ag/GO) for treating microbial keratitis. CLs are also used for coating with antimicrobial molecules and peptides and is one of the effective strategies for preventing or treating the microbial keratitis. Various studies have reported the coating of contact lenses with silver nanoparticles to treat microbial keratitis and showed inhibition of bacterial (Bazzaz et al., 2014; Shayani Rad et al., 2016) and fungal growth (Huang et al., 2016; Liu et al., 2018). However, the use of a high concentration of silver nanoparticles induced cytotoxicity to the mitochondrial activity, which became unsuitable for ophthalmic application (Bin Sahadan et al., 2019). Bin Sahadan et al. (2019) have reported the coating of contact lenses with phomopsidione nanoparticles, a ketone derivative, which excellently inhibited the growth of keratitis caused by Gram-negative bacteria. Melimine, a synthetic peptide derived from protamine and melittin, increased the hydrophilicity of the contact lenses and inhibited the growth of various microbes cultured on it (Dutta et al., 2013; Dutta et al., 2016a). It also inhibited *P. aeruginosa*–induced microbial keratitis in rabbit models (Dutta et al., 2016b). In a human trials, use of melimine-coated contact lenses increased mean corneal (depth, extent, and type) staining than control eyes (Dutta et al., 2014). Mel4, a peptide derived from melamine, also exhibited high antimicrobial activity toward *S. aureus* and *P. aeruginosa* as a contact lens coating material (Dutta et al., 2016a) and prevented the bacterial adhesion to contact lenses and displayed no sign of cytotoxicity toward rabbit models. During human trials, no sign of ocular difference was observed between test eyes (with Mel4-coated contact lenses) and the control eyes (Dutta et al., 2017).

In situ Forming Hydrogels

Hydrogels are three-dimensional polymeric networks having high water-holding capacity, which could be made from natural or synthetic hydrophilic polymer (Torres-Luna et al., 2020). They are widely used for tissue engineering and drug delivery applications as they retain the bioactive molecules within the crosslinked matrices (Lynch et al., 2020). High viscosity of the hydrogels also enhances the residence time of the bioactive molecules at ocular surface by preventing their washout. Delivery of the bioactive molecules to a local site in a controlled manner make the hydrogels a suitable drug delivery system for ocular applications. Hydrogels are usually characterized as performed gels (achieved by crosslinking the polymers) or *in situ* gels (Sharma and Taniguchi, 2017; Kurniawansyah et al., 2018). Formation of gels at the site of interest by changing the pH or temperature or ion (electrolyte) composition after reacting with surround environment make the *in situ* gels advantageous for enhancing the bioavailability of drugs. Various studies have reported the use of *in situ* hydrogels to deliver antimicrobial agents for treating corneal infection. Shastri et al. (2010) have reported the formulation of thermoreversible mucoadhesive gels using poloxamer, xanthan gum, and sodium alginate to deliver moxifloxacin hydrochloride (MXF) for corneal application. *In vitro* studies showed that the *in situ* gels were transparent, had satisfactory adhesion to the sheep's cornea, and sustained release of MXF (Shastri et al., 2010). In another study, MXF-loaded *in situ* hydrogels were formulated using sodium alginate and hydroxy propyl methyl cellulose (HPMC) for ocular application. *In vitro* studies showed the MXF released from the *in situ* gels inhibited bacterial growth of *E. coli* and *S. aureus*, whereas *in vivo* studies displayed that the gels were transparent and non-irritating with no ocular damage (Mandal et al., 2012). El-Laithy et al. (2011) reported the preparation of *in situ* gels based on Gelrite to deliver MXF for treating bacterial keratitis. MXF-loaded gels delivered higher

amount of drug into ocular region in comparison to Vigamox® commercial eye drops without causing ocular irritation and efficiently reduced bacterial keratitis (El-Laithy et al., 2011). Nair et al. (2021) also reported the development of *in situ gel* formulation by optimizing the concentration of polymers like gellan gum, sodium alginate, and HPMC for enhancing bioavailability and efficiency of MXF. Selected formulation significantly improved the bioavailability of MXF to the ocular region when compared to commercially available eye (Nair et al., 2021). Mohammed et al. (2017a) reported formulation of MXF or gentamicin-loaded chitosan/β-glycerolphosphate *in situ* gels for treating bacterial keratitis. Gels displayed sustained cumulative release of antibiotics that inhibited the growth of *S. aureus* and also protected the corneal fibroblast from bacteria-induced death (Mohammed et al., 2017b). Gatifloxacin was also delivered using ion-activated mucoadhesive *in situ* hydrogels (combination of sodium alginate, or gellan, and sodium carboxymethylcellulose) to treat bacteria keratitis in rabbit models (Kesavan et al., 2016). *In vivo* studies showed that ion-activated mucoadhesive hydrogels were non-irritants without causing inflammatory reactions and gatifloxacin released from the gels significantly reduced bacterial infection in comparison with marketed drug solution.

Microneedle Ocular Patch

Delivery of effective therapeutic to internal ocular regions are limited due to ocular barriers like cornea epithelium. Drugs delivered using contact lenses enhance the retention time in the ocular surface; however, penetration of drug into ocular regions is questionable. These problems are addressed using microneedle ocular patch. Microneedles ocular patch haves an array of 50–1,000 μm sized needles, which are fabricated using ceramic, metals, polymer, and silicon (Garg et al., 2019). The microneedle ocular patches containing amphotericin have also been found to be effective in treating fungal keratitis in rabbits (Roy et al., 2019). Microneedle could deliver drugs into the corneal stroma by bypassing tear films and penetrating the corneal epithelium (Lee et al., 2018). Several studies have reported the delivery of bioactive molecules or drugs using microneedle ocular patch for various applications, including keratitis. Bhatnagar et al. reported the fabrication of microneedles using polyvinyl pyrrolidone (PVP) and polyvinyl alcohol (PVA) by micromolding technique to deliver besifloxacin for treating microbial keratitis. Besifloxacin delivered into cornea by dissolution of microneedles showed enhanced antibacterial activity and reduced *S. aureus* growth in the *ex vivo* corneal infection model (Bhatnagar et al., 2018). In another study, Lee et al. developed a detachable hybrid microneedle pen for delivering therapeutics to treat keratitis infections. Polyhexamethylene biguanide released into the cornea from detached poly(lactic-coglycolic) acid (PLGA) microneedle showed effective inhibition of *Acanthamoeba*-induced keratitis in mice models (Lee et al., 2018).

Nanocarriers-Based Drug Delivery

Nanotechnology bridges a barrier between the biology and physical sciences by developing nanostructure using synthetic or natural polymers to address various problems in the field of sciences (Patra et al., 2018). Nanocarriers synthesized from biocompatible and biodegradable natural or synthetic polymers overcome corneal barriers and deliver the drug to deeper ocular regions that reduce drug toxicity and precorneal drug loss (Gote et al., 2019). Various studies have been reported regarding delivery of drugs using different nanocarriers for treating ocular disorders including keratitis. Micelles are good drug carriers that entrap the drug in the hydrophobic core; whereas, hydrophilic shells form a steric barrier and prevents micelles aggregation (Guo et al., 2020). Jaiswal et al. reported the development of micelles to deliver itraconazole for treating fungal keratitis. Itraconazole released from itraconazole-loaded micellar *in situ* gel showed greater antifungal activity than commercial eye drops (Jaiswal et al., 2015). Sayed et al. (2018) enhanced the penetration of itraconazole into the deeper cornea of eyes using β-cyclodextrin consolidated micellar dispersions (CCMD) to treat fungal keratitis *ex vivo* and *in vivo* models. In another study, micelles were synthesized using self-assembled poly(ethylene glycol)-block-poly(glycidyl methacrylate) (PEG-b-PGMA) to deliver natamycin for treating fungal keratitis (Guo et al., 2020). Drug-loaded micelles were nontoxic, and the natamycin released from the micelles suppressed the growth of *C. albicans* in rabbit models. Nanoparticles are colloidal carrier systems made of natural or synthetic polymers and stores the drug in the polymer shell and deliver them to the target site (Garg et al., 2019). Ahsan et al. reported the development of anti-TLR4 antibodies–conjugated gelatin nanoparticles for enhancing the residence time of the drug ketoconazole by anchoring to the cornea. Released drug exhibited the reduction of corneal inflammation and inhibited the growth of *A. flavus* in rat infection models (Ahsan and Rao, 2017). Lecithin/chitosan mucoadhesive nanoparticles used or enhancing the drug retention time in ocular regions enhanced the bioavailability and resident time of amphotericin-B in the corneal region of rabbit eyes. *In vitro* studies displayed that amphotericin-B released from nanoparticles inhibited the growth of *C. albicans* and *A. fumigatus* more effectively than marketed formulations (Chhonker et al., 2015). Shivam et al. (2020) reported the synthesis of MXF-loaded nanoparticles entrapped in *in situ* gel to demonstrate sustained drug release to improve efficacy of drug in treating bacterial keratitis; reduced bacterial load and recovery of corneal epithelium was observed in *in vivo* studies treated with MXF-loaded nanoparticles. Nanostructured lipid nanocarriers (NLCs) are new generation formulations of solid lipid nanoparticles, which are used as an alternative for conventional drug delivery. Üstündağ-Okur et al. (2014) reported development of ofloxacin-loaded NLCs to treat bacterial keratitis. Modification of ofloxacin-loaded NLCs with chitosan enhanced the preocular residence time of the nanocarrier. Ofloxacin delivery by ofloxacin-loaded NLCs-chitosan showed a significantly higher rate of penetration into cornea than commercial solution. Niosome are non-ionic surfactant bilayer vesicles that are formed by the self-assembly of non-ionic surfactants in aqueous environment, also acts as a potential carrier to deliver drug for treating ocular disorders (El-Nabarawi et al., 2019). They also showed natamycin-loaded

niosomes entrapped in ketorolac tromethamine gels enhance the clinical efficacy of drug by improving its penetration of corneal tissue and minimizing inflammation associated with microbial keratitis. Natamycin delivered from the formulation inhibited the *Candida albicans* growth in the rabbit model and KT gels minimized the corneal inflammation caused by fungus (El-Nabarawi et al., 2019). In another study, El-Mofty et al. (2020) reported the development of new formulation by modifying natamycin-loaded nanoparticle niosomal formulation along with KT to improve the safety and efficacy in fungal keratitis. Newly developed F1 (natamycin-loaded nanoparticle niosomes/0.5% KT, 4% carboxymethyl cellulose gel) formulation showed higher viscosity and mucoadhesive property than F2 formulation (natamycin-loaded nanoparticle niosomes/0.5% KT, 2% hydroxypropylmethyl cellulose-E4 gel). F1 formulation also enhances the penetration of drug-loaded niosomal into cornea and increased the bioavailability of the natamycin that resulted in inhibition inflammation and growth of *Aspergillus* spp. .

CONCLUSION

Currently, there is an alarming increase in antibiotic resistance and is considered as a global concern that requires more focused research to develop alternative therapeutic interventions to battle the growing trend. The AMR is increasing among ocular pathogens as well and impacting patient care to a large extent. The studies present in this review highlight the significant role of alternative therapeutics to combat increasing antimicrobial resistance and AMPs are explored widely and considered as the most potent new age therapeutics. The challenge has given us an opportunity to explore new antimicrobial molecules, repurpose existing molecules, explore alternative nonantibiotic therapies, and the drug delivery system that ensure concentration effective against drug-resistant pathogens. AMPs are one of the most potent alternatives that can be considered to fight antibiotic resistance as in addition to direct microbial killing activity, and it also plays an integral role in innate immune system. The small molecules targeting the virulence factors of the pathogens are also emerging as promising alternatives as they are not inhibiting microbial growth and generating selective pressure. These alternative interventions provide hope and feasible option for developing AMPs, small molecules, and natural compound analogues as future generation antimicrobials to combat antibiotic resistance.

AUTHOR CONTRIBUTIONS

SR—conceptualize the work; SR and PKJ—design of the work; PS and BB—figure illustrations; PKJ, PS, BB, and SR—wrote the paper.

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REFERENCES

Abd Ghafar, N., Ker-Woon, C., Hui, C. K., Mohd Yusof, Y. A., and Wan Ngah, W. Z. (2016). Acacia Honey Accelerates *In Vitro* Corneal Ulcer Wound Healing Model. *BMC Complement. Altern. Med.* 16, 259. doi:10.1186/s12906-016-1248-0

Ahsan, S. M., and Rao, C. M. (2017). Condition Responsive Nanoparticles for Managing Infection and Inflammation in Keratitis. *Nanoscale* 9, 9946–9959. doi:10.1039/c7nr00922d

Al-Qahtani, B., Asghar, S., Al-Taweel, H. M., and Jalaluddin, I. (2014). Peripheral Ulcerative Keratitis: Our Challenging Experience. *Saudi J. Ophthalmol.* 28, 234–238. doi:10.1016/j.sjopt.2013.12.006

Alio, J. L., Ayala, M. J., Mulet, M. E., Artola, A., Ruiz, J. M., and Bellot, J. (1995). Antioxidant Therapy in the Treatment of Experimental Acute Corneal Inflammation. *Ophthalmic Res.* 27, 136–143. doi:10.1159/000267648

Anderson, B. F., Baker, H. M., Norris, G. E., Rumball, S. V., and Baker, E. N. (1990). Apolactoferrin Structure Demonstrates Ligand-Induced Conformational Change in Transferrins. *Nature* 344, 784–787. doi:10.1038/344784a0

Andrä, J., Berninghausen, O., and Leippe, M. (2001). Cecropins, Antibacterial Peptides from Insects and Mammals, Are Potently Fungicidal against Candida Albicans. *Med. Microbiol. Immunol.* 189, 169–173. doi:10.1007/s430-001-8025-x

Aoki, T., Kitazawa, K., Deguchi, H., and Sotozono, C. (2021). Current Evidence for Corynebacterium on the Ocular Surface. *Microorganisms* 9. 254. doi:10.3390/microorganisms9020254

Asbell, P. A., Sahm, D. F., Shaw, M., Draghi, D. C., and Brown, N. P. (2008). Increasing Prevalence of Methicillin Resistance in Serious Ocular Infections Caused by *Staphylococcus aureus* in the United States: 2000 to 2005. *J. Cataract Refract Surg.* 34, 814–818. doi:10.1016/j.jcrs.2008.01.016

Augustin, D. K., Heimer, S. R., Tam, C., Li, W. Y., Le Due, J. M., Evans, D. J., et al. (2011). Role of Defensins in Corneal Epithelial Barrier Function against *Pseudomonas aeruginosa* Traversal. *Infect. Immun.* 79, 595–605. doi:10.1128/iai.00854-10

Austin, A., Lietman, T., and Rose-Nussbaumer, J. (2017). Update on the Management of Infectious Keratitis. *Ophthalmology* 124, 1678–1689. doi:10.1016/j.ophtha.2017.05.012

Badenoch, P. R., Hay, G. J., Mcdonald, P. J., and Coster, D. J. (1985). A Rat Model of Bacterial Keratitis. *Arch. Ophthalmol.* 103, 718–722. doi:10.1001/archopht.1985.01050050110028

Bailey, L., Gylfe, A., Sundin, C., Muschiol, S., Elofsson, M., Nordström, P., et al. (2007). Small Molecule Inhibitors of Type III Secretion inYersinia block the Chlamydia Pneumoniaeinfection Cycle. *FEBS Lett.* 581, 587–595. doi:10.1016/j.febslet.2007.01.013

Ballweber, L. M., Jaynes, J. E., Stamm, W. E., and Lampe, M. F. (2002). In Vitro microbicidal Activities of Cecropin Peptides D2A21 and D4E1 and Gel Formulations Containing 0.1 to 2% D2A21 against *Chlamydia trachomatis*. *Antimicrob. Agents Chemother.* 46, 34–41. doi:10.1128/AAC.46.1.34-41.2002

Bals, R. (2000). Epithelial Antimicrobial Peptides in Host Defense against Infection. *Respir. Res.* 1, 141–150. doi:10.1186/rr25

Batinić-Haberle, I., Rebouças, J. S., and Spasojević, I. (2010). Superoxide Dismutase Mimics: Chemistry, Pharmacology, and Therapeutic Potential. *Antioxid. Redox signaling* 13, 877–918. doi:10.1089/ars.2009.2876

Baveye, S., Elass, E., Mazurier, J., Spik, G., and Legrand, D. (1999). Lactoferrin: a Multifunctional Glycoprotein Involved in the Modulation of the Inflammatory Process. *Clin. Chem. Lab. Med.* 37, 281–286. doi:10.1515/cclm.1999.049

Berman, J. (2012). Candida Albicans. *Curr. Biol.* 22, R620–R622. doi:10.1016/j.cub.2012.05.043

Bharathi, M. J., Ramakrishnan, R., Meenakshi, R., Padmavathy, S., Shivakumar, C., and Srinivasan, M. (2007). Microbial Keratitis in South India: Influence of Risk Factors, Climate, and Geographical Variation. *Ophthalmic Epidemiol.* 14, 61–69. doi:10.1080/09286580601001347

Bhatnagar, S., Saju, A., Cheerla, K. D., Gade, S. K., Garg, P., and Venuganti, V. V. K. (2018). Corneal Delivery of Besifloxacin Using Rapidly Dissolving Polymeric Microneedles. *Drug Deliv. Transl. Res.* 8, 473–483. doi:10.1007/s13346-017-0470-8

Bin Sahadan, M. Y., Tong, W. Y., Tan, W. N., Leong, C. R., Bin Misri, M. N., Chan, M., et al. (2019). Phomopsidione Nanoparticles Coated Contact Lenses Reduce Microbial Keratitis Causing Pathogens. *Exp. Eye Res.* 178, 10–14. doi:10.1016/j.exer.2018.09.011

Blair, J., Hodge, W., Al-Ghamdi, S., Balabanian, R., Lowcock, B., Pan, Y. I., et al. (2011). Comparison of Antibiotic-Only and Antibiotic-Steroid Combination Treatment in Corneal Ulcer Patients: Double-Blinded Randomized Clinical Trial. *Can. J. Ophthalmol.* 46, 40–45. doi:10.3129/i10-054

Bocchinfuso, G., Palleschi, A., Orioni, B., Grande, G., Formaggio, F., Toniolo, C., et al. (2009). Different Mechanisms of Action of Antimicrobial Peptides: Insights from Fluorescence Spectroscopy Experiments and Molecular Dynamics Simulations. *J. Pept. Sci.* 15, 550–558. doi:10.1002/psc.1144

Bohigian, G. M., and Foster, C. S. (1977). Treatment of Pseudomonas Keratitis in the Rabbit with Antibiotic-Steroid Combinations. *Invest. Ophthalmol. Vis. Sci.* 16, 553–556.

Boparai, J. K., and Sharma, P. K. (2020). Mini Review on Antimicrobial Peptides, Sources, Mechanism and Recent Applications. *Ppl* 27, 4–16. doi:10.2174/0929866526666190822165812

Bremond-Gignac, D., Chiambaretta, F., and Milazzo, S. (2011). A European Perspective on Topical Ophthalmic Antibiotics: Current and Evolving Options. *Ophthalmol. Eye Dis.* 3, S4866. doi:10.4137/oed.s4866

Buda De Cesare, G., Cristy, S. A., Garsin, D. A., and Lorenz, M. C. (2020). Antimicrobial Peptides: a New Frontier in Antifungal Therapy. *mBio* 11. doi:10.1128/mbio.02123-20

Burns, F. R., Paterson, C. A., Gray, R. D., and Wells, J. T. (1990). Inhibition of *Pseudomonas aeruginosa* Elastase and Pseudomonas Keratitis Using a Thiol-Based Peptide. *Antimicrob. Agents Chemother.* 34, 2065–2069. doi:10.1128/aac.34.11.2065

Butler, M. S., Blaskovich, M. A., and Cooper, M. A. (2017). Antibiotics in the Clinical Pipeline at the End of 2015. *J. Antibiot.* 70, 3–24. doi:10.1038/ja.2016.72

Butler, M. S., Blaskovich, M. A., and Cooper, M. A. (2013). Antibiotics in the Clinical Pipeline in 2013. *J. Antibiot.* 66, 571–591. doi:10.1038/ja.2013.86

Cao, Z., Said, N., Wu, H. K., Kuwabara, I., Liu, F. T., and Panjwani, N. (2003). Galectin-7 as a Potential Mediator of Corneal Epithelial Cell Migration. *Arch. Ophthalmol.* 121, 82–86. doi:10.1001/archopht.121.1.82

Cardoso, M. H., Meneguetti, B. T., Costa, B. O., Buccini, D. F., Oshiro, K. G. N., Preza, S. L. E., et al. (2019). Non-Lytic Antibacterial Peptides that Translocate through Bacterial Membranes to Act on Intracellular Targets. *Int. J. Mol. Sci.* 20, 4877. doi:10.3390/ijms20194877

Carmichael, T. R., Gelfand, Y., and Welsh, N. H. (1990). Topical Steroids in the Treatment of central and Paracentral Corneal Ulcers. *Br. J. Ophthalmol.* 74, 528–531. doi:10.1136/bjo.74.9.528

Cathcart, G. R. A., Quinn, D., Greer, B., Harriott, P., Lynas, J. F., Gilmore, B. F., et al. (2011). Novel Inhibitors of the *Pseudomonas aeruginosa* Virulence Factor LasB: a Potential Therapeutic Approach for the Attenuation of Virulence Mechanisms in Pseudomonal Infection. *Antimicrob. Agents Chemother.* 55, 2670–2678. doi:10.1128/aac.00776-10

Chahud, F., Ramalho, L. N. Z., Ramalho, F. S., Haddad, A., and Roque-Barreira, M. C. (2009). The Lectin KM+ Induces Corneal Epithelial Wound Healing in Rabbits. *Int. J. Exp. Pathol.* 90, 166–173. doi:10.1111/j.1365-2613.2008.00626.x

Chang, H. Y., and Chodosh, J. (2011). Diagnostic and Therapeutic Considerations in Fungal Keratitis. *Int. Ophthalmol. Clin.* 51, 33–42. doi:10.1097/IIO.0b013e31822d64dc

Chen, H., Hua, X. M., Ze, B. C., Wang, B., and Wei, L. (2017). The Anti-inflammatory Effects of Asiatic Acid in Lipopolysaccharide-Stimulated Human Corneal Epithelial Cells. *Int. J. Ophthalmol.* 10, 179–185. doi:10.18240/ijo.2017.02.01

Chhonker, Y. S., Prasad, Y. D., Chandasana, H., Vishvkarma, A., Mitra, K., Shukla, P. K., et al. (2015). Amphotericin-B Entrapped Lecithin/chitosan Nanoparticles for Prolonged Ocular Application. *Int. J. Biol. macromolecules* 72, 1451–1458. doi:10.1016/j.ijbiomac.2014.10.014

Cho, Y.-W., Yoo, W.-S., Kim, S.-J., Chung, I.-Y., Seo, S.-W., and Yoo, J.-M. (2014). Efficacy of Systemic Vitamin C Supplementation in Reducing Corneal Opacity Resulting from Infectious Keratitis. *Medicine (Baltimore)* 93, e125. doi:10.1097/md.0000000000000125

Choi, S. E., and Pflum, M. K. H. (2012). The Structural Requirements of Histone Deacetylase Inhibitors: Suberoylanilide Hydroxamic Acid Analogs Modified at the C6 Position. *Bioorg. Med. Chem. Lett.* 22, 7084–7086. doi:10.1016/j.bmcl.2012.09.093

Chojnacki, M., Philbrick, A., Wucher, B., Reed, J. N., Tomaras, A., Dunman, P. M., et al. (2019). Development of a Broad-Spectrum Antimicrobial Combination for the Treatment of *Staphylococcus aureus* and *Pseudomonas aeruginosa* Corneal Infections. *Antimicrob. Agents Chemother.* 63. e01929. doi:10.1128/aac.01929-18

Clark, H. L., Jhingran, A., Sun, Y., Vareechon, C., De Jesus Carrion, S., Skaar, E. P., et al. (2016). Zinc and Manganese Chelation by Neutrophil S100A8/A9 (Calprotectin) Limits Extracellular Aspergillus fumigatus Hyphal Growth and Corneal Infection. *J. Immunol.* 196, 336–344. doi:10.4049/jimmunol.1502037

Clark, H. L., Minns, M. S., Sun, Y., De Jesus, T., Ghannoum, M. G., and Pearlman, E. (2018). Atovaquone Impairs Growth of Aspergillus and Fusarium Keratitis Isolates by Modulating Mitochondrial Function and Zinc Homeostasis. *Invest. Ophthalmol. Vis. Sci.* 59, 1589–1598. doi:10.1167/iovs.17-22585

Cortes-Penfield, N., Oliver, N. T., Hunter, A., and Rodriguez-Barradas, M. (2018). Daptomycin and Combination Daptomycin-Ceftaroline as Salvage Therapy for Persistent Methicillin-Resistant *Staphylococcus aureus* Bacteremia. *Infect. Dis.* 50, 643–647. doi:10.1080/23744235.2018.1448110

Crupi, R., Impellizzeri, D., Gugliandolo, E., Cordaro, M., Siracusa, R., Britti, D., et al. (2019). Effect of Tempol, a Membrane-Permeable Free Radical Scavenger, on *In Vitro* Model of Eye Inflammation on Rabbit Corneal Cells. *J. Ocul. Pharmacol. Ther.* 35, 571–577. doi:10.1089/jop.2019.0016

Cullor, J. S., Mannis, M. J., Murphy, C. J., Smith, W. L., Selsted, M. E., and Reid, T. W. (1990). *In Vitro* antimicrobial Activity of Defensins against Ocular Pathogens. *Arch. Ophthalmol.* 108, 861–864. doi:10.1001/archopht.1990.01070080105044

Dajcs, J. J., Hume, E. B., Moreau, J. M., Caballero, A. R., Cannon, B. M., and O'Callaghan, R. J. (2000). Lysostaphin Treatment of Methicillin-Resistant *Staphylococcus aureus* Keratitis in the Rabbit. *Invest. Ophthalmol. Vis. Sci.* 41, 1432–1437.

Dajcs, J. J., Thibodeaux, B. A., Girgis, D. O., Shaffer, M. D., Delvisco, S. M., and O'Callaghan, R. J. (2002). Immunity to Lysostaphin and its Therapeutic Value for Ocular MRSA Infections in the Rabbit. *Invest. Ophthalmol. Vis. Sci.* 43, 3712–3716.

Dartt, D. A. (2011). Tear Lipocalin: Structure and Function. *Ocul. Surf.* 9, 126–138. doi:10.1016/s1542-0124(11)70022-2

Das, S., Samantaray, R., Mallick, A., Sahu, S., and Sharma, S. (2019). Types of Organisms and *In-Vitro* Susceptibility of Bacterial Isolates from Patients with Microbial Keratitis: A Trend Analysis of 8 Years. *Indian J. Ophthalmol.* 67, 49–53. doi:10.4103/ijo.ijo_500_18

De Breij, A., Riool, M., Cordfunke, R. A., Malanovic, N., De Boer, L., Koning, R. I., et al. (2018). The Antimicrobial Peptide SAAP-148 Combats Drug-Resistant Bacteria and Biofilms. *Sci. Transl Med.* 10, eaan4044. doi:10.1126/scitranslmed.aan4044

De Jong, G., Van Dijk, J. P., and Van Eijk, H. G. (1990). The Biology of Transferrin. *Clinica Chim. Acta* 190, 1–46. doi:10.1016/0009-8981(90)90278-z

De Luca, A. J., and Walsh, T. J. (2000). Antifungal Peptides: Origin, Activity, and Therapeutic Potential. *Rev. Iberoam Micol* 17, 116–120.

Dellière, S., Sze Wah Wong, S., and Aimanianda, V. (2020). Soluble Mediators in Anti-fungal Immunity. *Curr. Opin. Microbiol.* 58, 24–31. doi:10.1016/j.mib.2020.05.005

Deng, Q., Sun, M., Yang, K., Zhu, M., Chen, K., Yuan, J., et al. (2013). MRP8/14 Enhances Corneal Susceptibility toPseudomonas aeruginosaInfection by Amplifying Inflammatory Responses. *Invest. Ophthalmol. Vis. Sci.* 54, 1227–1234. doi:10.1167/iovs.12-10172

Di Girolamo, N. (2015). Moving Epithelia: Tracking the Fate of Mammalian Limbal Epithelial Stem Cells. *Prog. Retin. Eye Res.* 48, 203–225. doi:10.1016/j.preteyeres.2015.04.002

Djebli, N., Khier, S., Griguer, F., Coutant, A.-L., Tavernier, A., Fabre, G., et al. (2017). Ocular Drug Distribution after Topical Administration: Population Pharmacokinetic Model in Rabbits. *Eur. J. Drug Metab. Pharmacokinet.* 42, 59–68. doi:10.1007/s13318-016-0319-4

Do Nascimento Dias, J., De Souza Silva, C., De Araujo, A. R., Souza, J. M. T., De Holanda Veloso Junior, P. H., Cabral, W. F., et al. (2020). Mechanisms of Actíon of Antimicrobial Peptides ToAP2 and NDBP-5.7 against Candida Albicans Planktonic and Biofilm Cells. *Sci. Rep.* 10, 10327. doi:10.1038/s41598-020-67041-2

Du, H., Puri, S., Mccall, A., Norris, H. L., Russo, T., and Edgerton, M. (2017). Human Salivary Protein Histatin 5 Has Potent Bactericidal Activity against ESKAPE Pathogens. *Front Cel Infect Microbiol* 7, 41. doi:10.3389/fcimb.2017.00041

Dubald, M., Bourgeois, S., Andrieu, V., and Fessi, H. (2018). Ophthalmic Drug Delivery Systems for Antibiotherapy-A Review. *Pharmaceutics* 10, 10. doi:10.3390/pharmaceutics10010010

Dürr, U. H. N., Sudheendra, U. S., and Ramamoorthy, A. (2006). LL-37, the Only Human Member of the Cathelicidin Family of Antimicrobial Peptides. *Biochim. Biophys. Acta (Bba) - Biomembranes* 1758, 1408–1425. doi:10.1016/j.bbamem.2006.03.030

Dutta, D., Cole, N., Kumar, N., and Willcox, M. D. P. (2013). Broad Spectrum Antimicrobial Activity of Melimine Covalently Bound to Contact Lenses. *Invest. Ophthalmol. Vis. Sci.* 54, 175–182. doi:10.1167/iovs.12-10989

Dutta, D., Kumar, N., and D. P. Willcox, M. (2016a). Antimicrobial Activity of Four Cationic Peptides Immobilised to Poly-Hydroxyethylmethacrylate. *Biofouling* 32, 429–438. doi:10.1080/08927014.2015.1129533

Dutta, D., Ozkan, J., and Willcox, M. D. P. (2014). Biocompatibility of Antimicrobial Melimine Lenses. *Optom. Vis. Sci.* 91, 570–581. doi:10.1097/opx.0000000000000232

Dutta, D., Vijay, A. K., Kumar, N., and Willcox, M. D. P. (2016b). Melimine-coated Antimicrobial Contact Lenses Reduce Microbial Keratitis in an Animal Model. *Invest. Ophthalmol. Vis. Sci.* 57, 5616–5624. doi:10.1167/iovs.16-19882

Dutta, D., Zhao, T., Cheah, K. B., Holmlund, L., and Willcox, M. D. P. (2017). Activity of a Melimine Derived Peptide Mel4 against Stenotrophomonas, Delftia, Elizabethkingia, Burkholderia and Biocompatibility as a Contact Lens Coating. *Contact Lens and Anterior Eye* 40, 175–183. doi:10.1016/j.clae.2017.01.002

Edgerton, M., Koshlukova, S. E., Araujo, M. W. B., Patel, R. C., Dong, J., and Bruenn, J. A. (2000). Salivary Histatin 5 and Human Neutrophil Defensin 1 Kill Candida Albicans via Shared Pathways. *Antimicrob. Agents Chemother.* 44, 3310–3316. doi:10.1128/aac.44.12.3310-3316.2000

Egrilmez, S., and Yildirim-Theveny, Ş. (2020). Treatment-Resistant Bacterial Keratitis: Challenges and Solutions. *Clin. Opthamol.* Vol. 14, 287–297. doi:10.2147/opth.s181997

El-Laithy, H. M., Nesseem, D. I., El-Adly, A. A., and Shoukry, M. (2011). Moxifloxacin-Gelrite *In Situ* Ophthalmic Gelling System against Photodynamic Therapy for Treatment of Bacterial Corneal Inflammation. *Arch. Pharm. Res.* 34, 1663–1678. doi:10.1007/s12272-011-1011-5

El-Mofty, H. M., El-Nabarawi, M. A., Abd El Rehem, R. T., Teaima, M. H., Abary, M. Y. S., Salah, M., et al. (2020). Niosomes: Do They Increase the Potency of Topical Natamycin Ketorolac Formula in Treating Aspergillus Keratitis? an Experimental Study. *J. Ocul. Pharmacol. Ther.* 36, 545–554. doi:10.1089/jop.2019.0128

El-Nabarawi, M. A., Abd El Rehem, R. T., Teaima, M., Abary, M., El-Mofty, H. M., Khafagy, M. M., et al. (2019). Natamycin Niosomes as a Promising Ocular Nanosized Delivery System with Ketorolac Tromethamine for Dual Effects for Treatment of candida Rabbit Keratitis; *In Vitro*/*In Vivo* and Histopathological Studies. *Drug Dev. Ind. Pharm.* 45, 922–936. doi:10.1080/03639045.2019.1579827

Fairbrother, R. W. (1956). Mixed Staphylococcal Infections. *The Lancet* 267, 716–719. doi:10.1016/s0140-6736(56)90746-2

Fanò, G., Biocca, S., Fulle, S., Mariggiò, M. A., Belia, S., and Calissano, P. (1995). The S-100: a Protein Family in Search of a Function. *Prog. Neurobiol.* 46, 71–82. doi:10.1016/0301-0082(94)00062-m

Farha, M. A., Czarny, T. L., Myers, C. L., Worrall, L. J., French, S., Conrady, D. G., et al. (2015). Antagonism Screen for Inhibitors of Bacterial Cell wall Biogenesis Uncovers an Inhibitor of Undecaprenyl Diphosphate Synthase. *Proc. Natl. Acad. Sci. USA* 112, 11048–11053. doi:10.1073/pnas.1511751112

Fazly Bazzaz, B. S., Khameneh, B., Jalili-Behabadi, M. M., Malaekeh-Nikouei, B., and Mohajeri, S. A. (2014). Preparation, Characterization and Antimicrobial Study of a Hydrogel (Soft Contact Lens) Material Impregnated with Silver Nanoparticles. *Cont Lens Anterior Eye* 37, 149–152. doi:10.1016/j.clae.2013.09.008

Felise, H. B., Nguyen, H. V., Pfuetzner, R. A., Barry, K. C., Jackson, S. R., Blanc, M.-P., et al. (2008). An Inhibitor of Gram-Negative Bacterial Virulence Protein Secretion. *Cell Host & Microbe* 4, 325–336. doi:10.1016/j.chom.2008.08.001

Felício, M. R., Silva, O. N., Gonçalves, S., Santos, N. C., and Franco, O. L. (2017). Peptides with Dual Antimicrobial and Anticancer Activities. *Front. Chem.* 5, 5. doi:10.3389/fchem.2017.00005

Feng, Q., Huang, Y., Chen, M., Li, G., and Chen, Y. (2015). Functional Synergy of α-helical Antimicrobial Peptides and Traditional Antibiotics against Gram-Negative and Gram-Positive Bacteria In Vitro and In Vivo. *Eur. J. Clin. Microbiol. Infect. Dis.* 34, 197–204. doi:10.1007/s10096-014-2219-3

Fluckinger, M., Haas, H., Merschak, P., Glasgow, B. J., and Redl, B. (2004). Human Tear Lipocalin Exhibits Antimicrobial Activity by Scavenging Microbial Siderophores. *Antimicrob. Agents Chemother.* 48, 3367–3372. doi:10.1128/aac.48.9.3367-3372.2004

Foulkes, D. M., Mclean, K., Zheng, Y., Sarsby, J., Haneef, A. S., Fernig, D. G., et al. (2021). A Pipeline to Evaluate Inhibitors of the *Pseudomonas aeruginosa* Exotoxin U. *Biochem. J.* 478, 647–668. doi:10.1042/bcj20200780

Ganz, T., and Lehrer, R. I. (1994). Defensins. *Curr. Opin. Immunol.* 6, 584–589. doi:10.1016/0952-7915(94)90145-7

Garg, P., Venuganti, V. V. K., Roy, A., and Roy, G. (2019). Novel Drug Delivery Methods for the Treatment of Keratitis: Moving Away from Surgical Intervention. *Expert Opin. Drug Deliv.* 16, 1381–1391. doi:10.1080/17425247.2019.1690451

Gordon, Y. J., Huang, L. C., Romanowski, E. G., Yates, K. A., Proske, R. J., and Mcdermott, A. M. (2005). Human Cathelicidin (LL-37), a Multifunctional Peptide, Is Expressed by Ocular Surface Epithelia and Has Potent Antibacterial and Antiviral Activity. *Curr. Eye Res.* 30, 385–394. doi:10.1080/02713680590934111

Gote, V., Sikder, S., Sicotte, J., and Pal, D. (2019). Ocular Drug Delivery: Present Innovations and Future Challenges. *J. Pharmacol. Exp. Ther.* 370, 602–624. doi:10.1124/jpet.119.256933

Grigor'eva, A., Bardasheva, A., Tupitsyna, A., Amirkhanov, N., Tikunova, N., Pyshnyi, D., et al. (2020). Changes in the Ultrastructure of *Staphylococcus aureus* Treated with Cationic Peptides and Chlorhexidine. *Microorganisms* 8. 1991. doi:10.3390/microorganisms8121991

Grinninger, P., Verbruggen, A. M. J., Kraijer-Huver, I. M. G., Djajadiningrat-Laanen, S. C., Teske, E., and Boevé, M. H. (2015). Use of Bandage Contact Lenses for Treatment of Spontaneous Chronic Corneal Epithelial Defects in Dogs. *J. Small Anim. Pract.* 56, 446–449. doi:10.1111/jsap.12360

Gritz, D. C., Kwitko, S., Trousdale, M. D., Gonzalez, V. H., and Mcdonnell, P. J. (1992). Recurrence of Microbial Keratitis Concomitant with Antiinflammatory Treatment in an Animal Model. *Cornea* 11, 404–408. doi:10.1097/00003226-199209000-00008

Guo, Y., Karimi, F., Fu, Q., G. Qiao, G. G., and Zhang, H. (2020). Reduced Administration Frequency for the Treatment of Fungal Keratitis: a Sustained Natamycin Release from a Micellar Solution. *Expert Opin. Drug Deliv.* 17, 407–421. doi:10.1080/17425247.2020.1719995

Hale, J. D., and Hancock, R. E. (2007). Alternative Mechanisms of Action of Cationic Antimicrobial Peptides on Bacteria. *Expert Rev. Anti-infective Ther.* 5, 951–959. doi:10.1586/14787210.5.6.951

Hancock, R. E. (2000). Cationic Antimicrobial Peptides: towards Clinical Applications. *Expert Opin. Investig. Drugs* 9, 1723–1729. doi:10.1517/13543784.9.8.1723

Hancock, R. E. W., and Chapple, D. S. (1999). Peptide Antibiotics. *Antimicrob. Agents Chemother.* 43, 1317–1323. doi:10.1128/aac.43.6.1317

Hazlett, L. D., Ekanayaka, S. A., Mcclellan, S. A., and Francis, R. (2019). Glycyrrhizin Use for Multi-Drug Resistant *Pseudomonas aeruginosa*: *In Vitro* and *In Vivo* Studies. *Invest. Ophthalmol. Vis. Sci.* 60, 2978–2989. doi:10.1167/iovs.19-27200

Hazlett, L., Suvas, S., Mcclellan, S., and Ekanayaka, S. (2016). Challenges of Corneal Infections. *Expert Rev. Ophthalmol.* 11, 285–297. doi:10.1080/17469899.2016.1203254

Heidari, H., Hadadi, M., Sedigh Ebrahim-Saraie, H., Mirzaei, A., Taji, A., Hosseini, S. R., et al. (2018). Characterization of Virulence Factors, Antimicrobial Resistance Patterns and Biofilm Formation of *Pseudomonas aeruginosa* and Staphylococcus Spp. Strains Isolated from Corneal Infection. *J. Français d'Ophtalmologie* 41, 823–829. doi:10.1016/j.jfo.2018.01.012

Helmerhorst, E. J., Troxler, R. F., and Oppenheim, F. G. (2001). The Human Salivary Peptide Histatin 5 Exerts its Antifungal Activity through the Formation of Reactive Oxygen Species. *Proc. Natl. Acad. Sci.* 98, 14637–14642. doi:10.1073/pnas.141366998

Hewitt, M. G., Morrison, P. W. J., Boostrom, H. M., Morgan, S. R., Fallon, M., Lewis, P. N., et al. (2020). *In Vitro* Topical Delivery of Chlorhexidine to the Cornea: Enhancement Using Drug-Loaded Contact Lenses and β-Cyclodextrin Complexation, and the Importance of Simulating Tear Irrigation. *Mol. Pharmaceutics* 17, 1428–1441. doi:10.1021/acs.molpharmaceut.0c00140

Hindman, H. B., Patel, S. B., and Jun, A. S. (2009). Rationale for Adjunctive Topical Corticosteroids in Bacterial Keratitis. *Arch. Ophthalmol.* 127, 97–102. doi:10.1001/archophthalmol.2008.504

Ho, T.-Y., Lo, H.-Y., Liu, I.-C., Lin, K.-A., Liao, Y.-F., Lo, Y.-C., et al. (2020). The Protective Effect of Quercetin on Retinal Inflammation in Mice: the Involvement of Tumor Necrosis Factor/nuclear Factor-Kb Signaling Pathways. *Food Funct.* 11, 8150–8160. doi:10.1039/d0fo01324b

Hoover, D. M., Rajashankar, K. R., Blumenthal, R., Puri, A., Oppenheim, J. J., Chertov, O., et al. (2000). The Structure of Human β-Defensin-2 Shows Evidence of Higher Order Oligomerization. *J. Biol. Chem.* 275, 32911–32918. doi:10.1074/jbc.m006098200

Hoshi, S., Todokoro, D., and Sasaki, T. (2020). Corynebacterium Species of the Conjunctiva and Nose: Dominant Species and Species-Related Differences of Antibiotic Susceptibility Profiles. *Cornea* 39, 1401–1406. doi:10.1097/ico.0000000000002445

Hua, X., Yuan, X., Tang, X., Li, Z., Pflugfelder, S. C., and Li, D.-Q. (2014). Human Corneal Epithelial Cells Produce Antimicrobial Peptides LL-37 and β-Defensins in Response to Heat-Killed Candida Albicans. *Ophthalmic Res.* 51, 179–186. doi:10.1159/000357977

Huang, J.-F., Zhong, J., Chen, G.-P., Lin, Z.-T., Deng, Y., Liu, Y.-L., et al. (2016). A Hydrogel-Based Hybrid Theranostic Contact Lens for Fungal Keratitis. *ACS nano* 10, 6464–6473. doi:10.1021/acsnano.6b00601

Huang, L. C., Petkova, T. D., Reins, R. Y., Proske, R. J., and Mcdermott, A. M. (2006). Multifunctional Roles of Human Cathelicidin (LL-37) at the Ocular Surface. *Invest. Ophthalmol. Vis. Sci.* 47, 2369–2380. doi:10.1167/iovs.05-1649

Huang, L. C., Reins, R. Y., Gallo, R. L., and Mcdermott, A. M. (2007). Cathelicidin-Deficient (Cnlp−/−) Mice Show Increased Susceptibility toPseudomonas aeruginosaKeratitis. *Invest. Ophthalmol. Vis. Sci.* 48, 4498–4508. doi:10.1167/iovs.07-0274

Hui, A., Willcox, M., and Jones, L. (2014). *In Vitro* and *In Vivo* Evaluation of Novel Ciprofloxacin-Releasing Silicone Hydrogel Contact Lenses. *Invest. Ophthalmol. Vis. Sci.* 55, 4896–4904. doi:10.1167/iovs.14-14855

Iyori, M., Kataoka, H., Shamsul, H. M., Kiura, K., Yasuda, M., Nakata, T., et al. (2008). Resveratrol Modulates Phagocytosis of Bacteria through an NF-κb-dependent Gene Program. *Antimicrob. Agents Chemother.* 52, 121–127. doi:10.1128/aac.00210-07

Jaiswal, M., Kumar, M., and Pathak, K. (2015). Zero Order Delivery of Itraconazole via Polymeric Micelles Incorporated *In Situ* Ocular Gel for the Management of Fungal Keratitis. *Colloids Surf. B: Biointerfaces* 130, 23–30. doi:10.1016/j.colsurfb.2015.03.059

Javia, A., Amrutiya, J., Lalani, R., Patel, V., Bhatt, P., and Misra, A. (2018). Antimicrobial Peptide Delivery: an Emerging Therapeutic for the Treatment of Burn and Wounds. *Ther. Deliv.* 9, 375–386. doi:10.4155/tde-2017-0061

Jensen, M. R., Bajic, G., Zhang, X., Laustsen, A. K., Koldsø, H., Skeby, K. K., et al. (2016). Structural Basis for Simvastatin Competitive Antagonism of Complement Receptor 3. *J. Biol. Chem.* 291, 16963–16976. doi:10.1074/jbc.m116.732222

Ji, S., Li, Z., Song, W., Wang, Y., Liang, W., Li, K., et al. (2016). Bioactive Constituents ofGlycyrrhiza uralensis(Licorice): Discovery of the Effective Components of a Traditional Herbal Medicine. *J. Nat. Prod.* 79, 281–292. doi:10.1021/acs.jnatprod.5b00877

Junior, E. F. C., Guimarães, C. F. R. C., Franco, L. L., Alves, R. J., Kato, K. C., Martins, H. R., et al. (2017). Glycotriazole-peptides Derived from the Peptide HSP1: Synergistic Effect of Triazole and Saccharide Rings on the Antifungal Activity. *Amino Acids* 49, 1389–1400. doi:10.1007/s00726-017-2441-2

Kakisu, K., Matsunaga, T., Kobayakawa, S., Sato, T., and Tochikubo, T. (2013). Development and Efficacy of a Drug-Releasing Soft Contact Lens. *Invest. Ophthalmol. Vis. Sci.* 54, 2551–2561. doi:10.1167/iovs.12-10614

Kang, H.-K., Kim, C., Seo, C. H., and Park, Y. (2017). The Therapeutic Applications of Antimicrobial Peptides (AMPs): a Patent Review. *J. Microbiol.* 55, 1–12. doi:10.1007/s12275-017-6452-1

Karlgard, C., Jones, L., and Moresoli, C. (2000). (Cl-176)Uptake and Release of Acular from Silicone-Hydrogel and Conventional Hydrogel Contact Lens Materials. *Optom. Vis. Sci.* 77, 179. doi:10.1097/00006324-200012001-00296

Kasus-Jacobi, A., Land, C. A., Stock, A. J., Washburn, J. L., and Pereira, H. A. (2020). Antimicrobial Peptides Derived from the Immune Defense Protein CAP37 Inhibit TLR4 Activation by S100A9. *Invest. Ophthalmol. Vis. Sci.* 61, 16. doi:10.1167/iovs.61.4.16

Kesavan, K., Kant, S., and Pandit, J. K. (2016). Therapeutic Effectiveness in the Treatment of Experimental Bacterial Keratitis with Ion-Activated Mucoadhesive Hydrogel. *Ocul. Immunol. Inflamm.* 24, 489–492. doi:10.3109/09273948.2015.1005238

Kessler, E., Israel, M., Landshman, N., Chechick, A., and Blumberg, S. (1982). *In Vitro* inhibition of *Pseudomonas aeruginosa* Elastase by Metal-Chelating Peptide Derivatives. *Infect. Immun.* 38, 716–723. doi:10.1128/iai.38.2.716-723.1982

Kim, K. W., Park, S. H., Lee, S. J., and Kim, J. C. (2016). Ribonuclease 5 Facilitates Corneal Endothelial Wound Healing via Activation of PI3-kinase/Akt Pathway. *Sci. Rep.* 6, 31162. doi:10.1038/srep31162

Klepser, M. (2011). The Value of Amphotericin B in the Treatment of Invasive Fungal Infections. *J. Crit. Care* 26, 225–210. doi:10.1016/j.jcrc.2010.08.005

Klubthawee, N., Adisakwattana, P., Hanpithakpong, W., Somsri, S., and Aunpad, R. (2020). A Novel, Rationally Designed, Hybrid Antimicrobial Peptide, Inspired by Cathelicidin and Aurein, Exhibits Membrane-Active Mechanisms against *Pseudomonas aeruginosa*. *Sci. Rep.* 10, 9117. doi:10.1038/s41598-020-65688-5

Koeninger, L., Osbelt, L., Berscheid, A., Wendler, J., Berger, J., Hipp, K., et al. (2021). Curbing Gastrointestinal Infections by Defensin Fragment Modifications without Harming Commensal Microbiota. *Commun. Biol.* 4, 47. doi:10.1038/s42003-020-01582-0

Kolar, S. S. N., Baidouri, H., Hanlon, S., and Mcdermott, A. M. (2013). Protective Role of Murine β-Defensins 3 and 4 and Cathelin-Related Antimicrobial Peptide in Fusarium Solani Keratitis. *Infect. Immun.* 81, 2669–2677. doi:10.1128/iai.00179-13

Konstantinović, J., Yahiaoui, S., Alhayek, A., Haupenthal, J., Schönauer, E., Andreas, A., et al. (2020). N-Aryl-3-Mercaptosuccinimides as Antivirulence Agents Targeting *Pseudomonas aeruginosa* Elastase and Clostridium Collagenases. *J. Med. Chem.* 63, 8359–8368. doi:10.1021/acs.jmedchem.0c00584

Korndörfer, I. P., Brueckner, F., and Skerra, A. (2007). The Crystal Structure of the Human (S100A8/S100A9)2 Heterotetramer, Calprotectin, Illustrates How Conformational Changes of Interacting α-Helices Can Determine Specific Association of Two EF-Hand Proteins. *J. Mol. Biol.* 370, 887–898. doi:10.1016/j.jmb.2007.04.065

Krause, A., Neitz, S., Mägert, H.-J., Schulz, A., Forssmann, W.-G., Schulz-Knappe, P., et al. (2000). LEAP-1, a Novel Highly Disulfide-Bonded Human Peptide, Exhibits Antimicrobial Activity. *FEBS Lett.* 480, 147–150. doi:10.1016/s0014-5793(00)01920-7

Kumar, A., Zhang, J., and Yu, F.-S. X. (2006). Toll-like Receptor 2-mediated Expression of β-defensin-2 in Human Corneal Epithelial Cells. *Microbes Infect.* 8, 380–389. doi:10.1016/j.micinf.2005.07.006

Kurniawansyah, I., Sopyan, I., Aditya, W., Nuraini, H., Alminda, F., and Nurlatifah, A. (2018). Preformed Gel vs *In Situ* Gel: a Review. *Int. Res. J. Pharm.* 9, 1–5. doi:10.7897/2230-8407.098155

Lai, Y., and Gallo, R. L. (2009). AMPed up Immunity: How Antimicrobial Peptides Have Multiple Roles in Immune Defense. *Trends Immunol.* 30, 131–141. doi:10.1016/j.it.2008.12.003

Leal, S. M., Jr., Roy, S., Vareechon, C., Carrion, S., Clark, H., Lopez-Berges, M. S., et al. (2013). Targeting Iron Acquisition Blocks Infection with the Fungal Pathogens Aspergillus fumigatus and Fusarium Oxysporum. *Plos Pathog.* 9, e1003436. doi:10.1371/journal.ppat.1003436

Leck, A. K., Thomas, P. A., Hagan, M., Kaliamurthy, J., Ackuaku, E., John, M., et al. (2002). Aetiology of Suppurative Corneal Ulcers in Ghana and South India, and Epidemiology of Fungal Keratitis. *Br. J. Ophthalmol.* 86, 1211–1215. doi:10.1136/bjo.86.11.1211

Lee, J.-E., Sun, Y., Gjorstrup, P., and Pearlman, E. (2015). Inhibition of Corneal Inflammation by the Resolvin E1. *Invest. Ophthalmol. Vis. Sci.* 56, 2728–2736. doi:10.1167/iovs.14-15982

Lee, K., Song, H. B., Cho, W., Kim, J. H., Kim, J. H., and Ryu, W. (2018). Intracorneal Injection of a Detachable Hybrid Microneedle for Sustained Drug Delivery. *Acta Biomater.* 80, 48–57. doi:10.1016/j.actbio.2018.09.039

Lee, V. T., Pukatzki, S., Sato, H., Kikawada, E., Kazimirova, A. A., Huang, J., et al. (2007). Pseudolipasin A Is a Specific Inhibitor for Phospholipase A 2 Activity of *Pseudomonas aeruginosa* Cytotoxin ExoU. *Infect. Immun.* 75, 1089–1098. doi:10.1128/iai.01184-06

Lee, W., and Lee, D. G. (2014). Lycopene-induced Hydroxyl Radical Causes Oxidative DNA Damage in *Escherichia coli*. *J. Microbiol. Biotechnol.* 24, 1232–1237. doi:10.4014/jmb.1406.06009

Lee, J. E., Lee, J., Lee, S., Kim, S., and Lee, S. (2016). The Effect of Resovin D1 on Infectious Keratitis of Experimental Animal to Prevent Corneal Scar. *Invest. Ophthalmol Visual Sci.* 60, 4643.

Li, S.-A., Liu, J., Xiang, Y., Wang, Y.-J., Lee, W.-H., and Zhang, Y. (2014). Therapeutic Potential of the Antimicrobial Peptide OH-CATH30 for Antibiotic-Resistant *Pseudomonas aeruginosa* Keratitis. *Antimicrob. Agents Chemother.* 58, 3144–3150. doi:10.1128/aac.00095-14

Li, T., Li, L., Du, F., Sun, L., Shi, J., Long, M., et al. (2021). Activity and Mechanism of Action of Antifungal Peptides from Microorganisms: A Review. *Molecules* 26, 3438. doi:10.3390/molecules26113438

Li, X., Yuan, M., Yin, R., Liu, X., Zhang, Y., Sun, S., et al. (2019). Histone Deacetylase Inhibitor Attenuates Experimental Fungal Keratitis in Mice. *Sci. Rep.* 9, 9859. doi:10.1038/s41598-019-46361-y

Li, Y., Yao, J., Han, C., Yang, J., Chaudhry, M., Wang, S., et al. (2016). Quercetin, Inflammation and Immunity. *Nutrients* 8, 167. doi:10.3390/nu8030167

Lim, A., Wenk, M. R., and Tong, L. (2015). Lipid-based Therapy for Ocular Surface Inflammation and Disease. *Trends Molecular Medicine* 21, 736–748. doi:10.1016/j.molmed.2015.10.001

Lin, A., Rhee, M. K., Akpek, E. K., Amescua, G., Farid, M., Garcia-Ferrer, F. J., et al. (2019). Bacterial Keratitis Preferred Practice PatternBacterial Keratitis Preferred Practice Pattern(R). *Ophthalmology* 126, P1–P55. doi:10.1016/j.ophtha.2018.10.018

Liu, X., Chen, J., Qu, C., Bo, G., Jiang, L., Zhao, H., et al. (2018). A Mussel-Inspired Facile Method to Prepare Multilayer-AgNP-Loaded Contact Lens for Early Treatment of Bacterial and Fungal Keratitis. *ACS Biomater. Sci. Eng.* 4, 1568–1579. doi:10.1021/acsbiomaterials.7b00977

Luong, H. X., Thanh, T. T., and Tran, T. H. (2020). Antimicrobial Peptides - Advances in Development of Therapeutic Applications. *Life Sci.* 260, 118407. doi:10.1016/j.lfs.2020.118407

Lynch, C. R., Kondiah, P. P., Choonara, Y. E., Du Toit, L. C., Ally, N., and Pillay, V. (2020). Hydrogel Biomaterials for Application in Ocular Drug Delivery. *Front. Bioeng. Biotechnol.* 8, 228. doi:10.3389/fbioe.2020.00228

Ma, L., Ye, X., Sun, P., Xu, P., Wang, L., Liu, Z., et al. (2020). Antimicrobial and Antibiofilm Activity of the EeCentrocin 1 Derived Peptide EC1-17KV via Membrane Disruption. *EBioMedicine* 55, 102775. doi:10.1016/j.ebiom.2020.102775

Mallela, L. S., Sharma, P., Rao, T. S. R., and Roy, S. (2021). Recombinant IL-22 Promotes protection in a Murine Model of Aspergillus flavus Keratitis and Mediates Host Immune Responses in Human Corneal Epithelial Cells. *Cell Microbiol* 24, e13367. doi:10.1111/cmi.13367

Mandal, S., Prabhushankar, G., Thimmasetty, M., and Geetha, M. (2012). Formulation and Evaluation of an *In Situ* Gel-Forming Ophthalmic Formulation of Moxifloxacin Hydrochloride. *Int. J. Pharma Investig.* 2, 78–82. doi:10.4103/2230-973x.100042

Mangoni, M. L., Mcdermott, A. M., and Zasloff, M. (2016). Antimicrobial Peptides and Wound Healing: Biological and Therapeutic Considerations. *Exp. Dermatol.* 25, 167–173. doi:10.1111/exd.12929

Mantelli, F., and Argüeso, P. (2008). Functions of Ocular Surface Mucins in Health and Disease. *Curr. Opin. Allergy Clin. Immunol.* 8, 477–483. doi:10.1097/aci.0b013e32830e6b04

Marangon, F. B., Miller, D., Muallem, M. S., Romano, A. C., and Alfonso, E. C. (2004). Ciprofloxacin and Levofloxacin Resistance Among Methicillin-Sensitive *Staphylococcus aureus* Isolates from Keratitis and Conjunctivitis. *Am. J. Ophthalmol.* 137, 453–458. doi:10.1016/j.ajo.2003.10.026

Marino, A., Santoro, G., Spataro, F., Lauriano, E. R., Pergolizzi, S., Cimino, F., et al. (2013). Resveratrol Role inStaphylococcus Aureus-Induced Corneal Inflammation. *Pathog. Dis.* 68, 61–64. doi:10.1111/2049-632x.12046

Martín Mazuelos, E., and Rodríguez-Tudela, J. L. (2008). Actividad *In Vitro* de anidulafungina. Comparación con la actividad de otras equinocandinas. *Enfermedades Infecciosas y Microbiología Clínica* 26 (Suppl. 14), 7–13. doi:10.1016/s0213-005x(08)76587-x

Matsumoto, K., Kusaka, J., Nishibori, A., and Hara, H. (2006). Lipid Domains in Bacterial Membranes. *Mol. Microbiol.* 61, 1110–1117. doi:10.1111/j.1365-2958.2006.05317.x

Matsuzaki, K., Murase, O., Fujii, N., and Miyajima, K. (1995). Translocation of a Channel-Forming Antimicrobial Peptide, Magainin 2, across Lipid Bilayers by Forming a Pore. *Biochemistry* 34, 6521–6526. doi:10.1021/bi00019a033

Mcdermott, A. M. (2013). Antimicrobial Compounds in Tears. *Exp. Eye Res.* 117, 53–61. doi:10.1016/j.exer.2013.07.014

Mcdermott, A. M., Redfern, R. L., and Zhang, B. (2001). Human SS-Defensin 2 Is Up-Regulated during Re-epithelialization of the Cornea. *Curr. Eye Res.* 22, 64–67. doi:10.1076/ceyr.22.1.64.6978

Mcdermott, A. M., Redfern, R. L., Zhang, B., Pei, Y., Huang, L., and Proske, R. J. (2003). Defensin Expression by the Cornea: Multiple Signalling Pathways Mediate IL-1β Stimulation of hBD-2 Expression by Human Corneal Epithelial Cells. *Invest. Ophthalmol. Vis. Sci.* 44, 1859–1865. doi:10.1167/iovs.02-0787

Mcdermott, A. M. (2009). The Role of Antimicrobial Peptides at the Ocular Surface. *Ophthalmic Res.* 41, 60–75. doi:10.1159/000187622

McNAMARA, N. A., Van, R., Tuchin, O. S., and Fleiszig, S. M. (1999). Ocular Surface Epithelia Express mRNA for Human Beta Defensin-2. *Exp. Eye Res.* 69, 483–490. doi:10.1006/exer.1999.0722

Mi, B., Liu, J., Liu, Y., Hu, L., Liu, Y., Panayi, A. C., et al. (2018). The Designer Antimicrobial Peptide A-hBD-2 Facilitates Skin Wound Healing by Stimulating Keratinocyte Migration and Proliferation. *Cell Physiol Biochem* 51, 647–663. doi:10.1159/000495320

Mohamed, M. F., Abdelkhalek, A., and Seleem, M. N. (2016). Evaluation of Short Synthetic Antimicrobial Peptides for Treatment of Drug-Resistant and Intracellular *Staphylococcus aureus*. *Sci. Rep.* 6, 29707. doi:10.1038/srep29707

Mohammadpour, M., Amouzegar, A., Hashemi, H., Jabbarvand, M., Kordbacheh, H., Rahimi, F., et al. (2015). Comparison of Lotrafilcon B and Balafilcon A Silicone Hydrogel Bandage Contact Lenses in Reducing Pain and Discomfort after Photorefractive Keratectomy: A Contralateral Eye Study. *Contact Lens and Anterior Eye* 38, 211–214. doi:10.1016/j.clae.2015.01.014

Mohammed, I., Mohanty, D., Said, D. G., Barik, M. R., Reddy, M. M., Alsaadi, A., et al. (2020). Antimicrobial Peptides in Human Corneal Tissue of Patients with Fungal Keratitis. *Br. J. Ophthalmol.* 105, 1172–1177. doi:10.1136/bjophthalmol-2020-316329

Mohammed, I., Said, D. G., and Dua, H. S. (2017a). Human Antimicrobial Peptides in Ocular Surface Defense. *Prog. Retin. Eye Res.* 61, 1–22. doi:10.1016/j.preteyeres.2017.03.004

Mohammed, I., Said, D. G., Nubile, M., Mastropasqua, L., and Dua, H. S. (2019). Cathelicidin-Derived Synthetic Peptide Improves Therapeutic Potential of Vancomycin against *Pseudomonas aeruginosa*. *Front. Microbiol.* 10, 2190. doi:10.3389/fmicb.2019.02190

Mohammed, I., Suleman, H., Otri, A. M., Kulkarni, B. B., Chen, P., Hopkinson, A., et al. (2010). Localization and Gene Expression of Human β-Defensin 9 at the Human Ocular Surface Epithelium. *Invest. Ophthalmol. Vis. Sci.* 51, 4677–4682. doi:10.1167/iovs.10-5334

Mohammed, S., Chouhan, G., Anuforom, O., Cooke, M., Walsh, A., Morgan-Warren, P., et al. (2017b). Thermosensitive Hydrogel as an *In Situ* Gelling Antimicrobial Ocular Dressing. *Mater. Sci. Eng. C* 78, 203–209. doi:10.1016/j.msec.2017.04.065

Mohammad, H., Thangamani, S., and Seleem, M. (2015). Antimicrobial Peptides and Peptidomimetics - Potent Therapeutic Allies for Staphylococcal Infections. *Cpd* 21, 2073–2088. doi:10.2174/1381612821666150310102702

Morici, P., Fais, R., Rizzato, C., Tavanti, A., and Lupetti, A. (2016). Inhibition of Candida Albicans Biofilm Formation by the Synthetic Lactoferricin Derived Peptide hLF1-11. *PloS one* 11, e0167470. doi:10.1371/journal.pone.0167470

Ming, L., and Huang, J.-A. (2017). The Antibacterial Effects of Antimicrobial Peptides OP-145 against Clinically Isolated Multi-Resistant Strains. *Jpn. J. Infect. Dis.* 70, 601–603. doi:10.7883/yoken.JJID.2017.090

Nagata, M., Nakamura, T., Hata, Y., Yamaguchi, S., Kaku, T., and Kinoshita, S. (2015). JBP485 Promotes Corneal Epithelial Wound Healing. *Sci. Rep.* 5, 14776. doi:10.1038/srep14776

Nair, A. B., Shah, J., Jacob, S., Al-Dhubiab, B. E., Sreeharsha, N., Morsy, M. A., et al. (2021). Experimental Design, Formulation and *In Vivo* Evaluation of a Novel Topical *In Situ* Gel System to Treat Ocular Infections. *PloS one* 16, e0248857. doi:10.1371/journal.pone.0248857

Nami, S., Aghebati-Maleki, A., Morovati, H., and Aghebati-Maleki, L. (2019). Current Antifungal Drugs and Immunotherapeutic Approaches as Promising Strategies to Treatment of Fungal Diseases. *Biomed. Pharmacother.* 110, 857–868. doi:10.1016/j.biopha.2018.12.009

Naranjo, A., Arboleda, A., Martinez, J. D., Durkee, H., Aguilar, M. C., Relhan, N., et al. (2019). Rose Bengal Photodynamic Antimicrobial Therapy for Patients with Progressive Infectious Keratitis: A Pilot Clinical Study. *Am. J. Ophthalmol.* 208, 387–396. doi:10.1016/j.ajo.2019.08.027

Ni, N., Srinivasan, M., Mcleod, S. D., Acharya, N. R., Lietman, T. M., and Rose-Nussbaumer, J. (2016a). Use of Adjunctive Topical Corticosteroids in Bacterial Keratitis. *Curr. Opin. Ophthalmol.* 27, 353–357. doi:10.1097/icu.0000000000000273

Ni, N., Srinivasan, M., Mcleod, S. D., Acharya, N. R., Lietman, T. M., and Rose-Nussbaumer, J. (2016b). Use of Adjunctive Topical Corticosteroids in Bacterial Keratitis. *Curr. Opin. Ophthalmol.* 27, 353–357. doi:10.1097/icu.0000000000000273

Nielsen, J. E., Bjørnestad, V. A., Pipich, V., Jenssen, H., and Lund, R. (2021). Beyond Structural Models for the Mode of Action: How Natural Antimicrobial Peptides Affect Lipid Transport. *J. Colloid Interf. Sci.* 582, 793–802. doi:10.1016/j.jcis.2020.08.094

Nixon, G. L., Moss, D. M., Shone, A. E., Lalloo, D. G., Fisher, N., O'neill, P. M., et al. (2013). Antimalarial Pharmacology and Therapeutics of Atovaquone. *J. Antimicrob. Chemother.* 68, 977–985. doi:10.1093/jac/dks504

Niyonsaba, F., and Ogawa, H. (2005). Protective Roles of the Skin against Infection: Implication of Naturally Occurring Human Antimicrobial Agents β-defensins, Cathelicidin LL-37 and Lysozyme. *J. Dermatol. Sci.* 40, 157–168. doi:10.1016/j.jdermsci.2005.07.009

Nordfelth, R., Kauppi, A. M., Norberg, H. A., Wolf-Watz, H., and Elofsson, M. (2005). Small-molecule Inhibitors Specifically Targeting Type III Secretion. *Infect. Immun.* 73, 3104–3114. doi:10.1128/iai.73.5.3104-3114.2005

Nos-Barbera, S., Portoles, M., Morilla, A., Ubach, J., Andreu, D., and Paterson, C. A. (1997). Effect of Hybrid Peptides of Cecropin A and Melittin in an Experimental Model of Bacterial Keratitis. *Cornea* 16, 101–106. doi:10.1097/00003226-199701000-00017

O'day, D. M., Head, W. S., Robinson, R. D., and Clanton, J. A. (1986). Corneal Penetration of Topical Amphotericin B and Natamycin. *Curr. Eye Res.* 5, 877–882. doi:10.3109/02713688609029240

Oren, Z., and Shai, Y. (1998). Mode of Action of Linear Amphipathic α-helical Antimicrobial Peptides. *Biopolymers* 47, 451–463. doi:10.1002/(sici)1097-0282(1998)47:6<451::aid-bip4>3.0.co;2-f

Otri, A. M., Mohammed, I., Abedin, A., Cao, Z., Hopkinson, A., Panjwani, N., et al. (2010). Antimicrobial Peptides Expression by Ocular Surface Cells in Response to Acanthamoeba Castellanii: an *In Vitro* Study. *Br. J. Ophthalmol.* 94, 1523–1527. doi:10.1136/bjo.2009.178236

Paradiso, P., Serro, A. P., Saramago, B., Colaço, R., and Chauhan, A. (2016). Controlled Release of Antibiotics from Vitamin E-Loaded Silicone-Hydrogel Contact Lenses. *J. Pharm. Sci.* 105, 1164–1172. doi:10.1016/s0022-3549(15)00193-8

Paredes-Gamero, E. J., Martins, M. N. C., Cappabianco, F. A. M., Ide, J. S., and Miranda, A. (2012). Characterization of Dual Effects Induced by Antimicrobial Peptides: Regulated Cell Death or Membrane Disruption. *Biochim. Biophys. Acta (Bba) - Gen. Subjects* 1820, 1062–1072. doi:10.1016/j.bbagen.2012.02.015

Park, J., Oh, J. H., Kang, H. K., Choi, M. C., Seo, C. H., and Park, Y. (2020). Scorpion-Venom-Derived Antimicrobial Peptide Css54 Exerts Potent Antimicrobial Activity by Disrupting Bacterial Membrane of Zoonotic Bacteria. *Antibiotics (Basel)* 9, 831. doi:10.3390/antibiotics9110831

Patra, J. K., Das, G., Fraceto, L. F., Campos, E. V. R., Del Pilar Rodriguez-Torres, M., Acosta-Torres, L. S., et al. (2018). Nano Based Drug Delivery Systems: Recent Developments and Future Prospects. *J. nanobiotechnology* 16, 1–33. doi:10.1186/s12951-018-0392-8

Paulsen, F. P., Pufe, T., Schaudig, U., Held-Feindt, J., Lehmann, J., Schröder, J. M., et al. (2001). Detection of Natural Peptide Antibiotics in Human Nasolacrimal Ducts. *Invest. Ophthalmol. Vis. Sci.* 42, 2157–2163.

Pearce, J. W., Giuliano, E. A., and Moore, C. P. (2009). In Vitrosusceptibility Patterns ofAspergillusandFusariumspecies Isolated from Equine Ulcerative Keratomycosis Cases in the Midwestern and Southern United States with Inclusion of the New Antifungal Agent Voriconazole. *Vet. Ophthalmol.* 12, 318–324. doi:10.1111/j.1463-5224.2009.00721.x

Phan, C.-M., Subbaraman, L., and Jones, L. (2014). Contact Lenses for Antifungal Ocular Drug Delivery: a Review. *Expert Opin. Drug Deliv.* 11, 537–546. doi:10.1517/17425247.2014.882315

Price, M. O., and Price, F. W., Jr. (2016). Corneal Cross-Linking in the Treatment of Corneal Ulcers. *Curr. Opin. Ophthalmol.* 27, 250–255. doi:10.1097/icu.0000000000000248

Rocha, M. F. G., Sales, J. A., Da Rocha, M. G., Galdino, L. M., De Aguiar, L., Pereira-Neto, W. d. A., et al. (2019). Antifungal Effects of the Flavonoids Kaempferol and Quercetin: a Possible Alternative for the Control of Fungal Biofilms. *Biofouling* 35, 320–328. doi:10.1080/08927014.2019.1604948

Roy, G., Galigama, R. D., Thorat, V. S., Mallela, L. S., Roy, S., Garg, P., et al. (2019). Amphotericin B Containing Microneedle Ocular Patch for Effective Treatment of Fungal Keratitis. *Int. J. Pharmaceutics* 572, 118808. doi:10.1016/j.ijpharm.2019.118808

Roy, S., Marla, S., and Praneetha, D. (2015). Recognition of Corynebacterium Pseudodiphtheriticum by Toll-like Receptors and Up-Regulation of Antimicrobial Peptides in Human Corneal Epithelial Cells. *Virulence* 6, 716–721. doi:10.1080/21505594.2015.1066063

Ruban, V. V., Archana, P. T., Sundararajan, M., Geraldine, P., and Thomas, P. A. (2018). Inflammation and Oxidative Stress in Corneal Tissue in Experimental Keratitis Due to Fusarium Solani : Amelioration Following Topical Therapy with Voriconazole and Epigallocatechin Gallate. *Mycoses* 61, 159–171. doi:10.1111/myc.12718

Rusciano, D., Pezzino, S., Olivieri, M., Cristaldi, M., Gagliano, C., Lupo, G., et al. (2018). Age-Related Dry Eye Lactoferrin and Lactobionic Acid. *Ophthalmic Res.* 60, 94–99. doi:10.1159/000489093

Ruszymah, B. H. I., Chowdhury, S. R., Manan, N. A. B. A., Fong, O. S., Adenan, M. I., and Saim, A. B. (2012). Aqueous Extract of *Centella asiatica* Promotes Corneal Epithelium Wound Healing *In Vitro*. *J. Ethnopharmacology* 140, 333–338. doi:10.1016/j.jep.2012.01.023

Sagerfors, S., Ejdervik-Lindblad, B., and Söderquist, B. (2020). Infectious Keratitis: Isolated Microbes and Their Antibiotic Susceptibility Pattern during 2004-2014 in Region Örebro County, Sweden. *Acta Ophthalmol.* 98, 255–260. doi:10.1111/aos.14256

Sayed, S., Elsayed, I., and Ismail, M. M. (2018). Optimization of β-cyclodextrin Consolidated Micellar Dispersion for Promoting the Transcorneal Permeation of a Practically Insoluble Drug. *Int. J. pharmaceutics* 549, 249–260. doi:10.1016/j.ijpharm.2018.08.001

Sengupta, J., Saha, S., Khetan, A., Sarkar, S. K., and Mandal, S. M. (2012). Effects of Lactoferricin B against Keratitis-Associated Fungal Biofilms. *J. Infect. Chemother.* 18, 698–703. doi:10.1007/s10156-012-0398-3

Shah, D., Son, K.-N., Kalmodia, S., Lee, B.-S., Ali, M., Balasubramaniam, A., et al. (2020). Wound Healing Properties of Histatin-5 and Identification of a Functional Domain Required for Histatin-5-Induced Cell Migration. *Mol. Ther. - Methods Clin. Develop.* 17, 709–716. doi:10.1016/j.omtm.2020.03.027

Sharma, A., and Taniguchi, J. (2017). Review: Emerging Strategies for Antimicrobial Drug Delivery to the Ocular Surface: Implications for Infectious Keratitis. *Ocul. Surf.* 15, 670–679. doi:10.1016/j.jtos.2017.06.001

Sharma, P., Elofsson, M., and Roy, S. (2020). Attenuation of *Pseudomonas aeruginosa* Infection by INP0341, a Salicylidene Acylhydrazide, in a Murine Model of Keratitis. *Virulence* 11, 795–804. doi:10.1080/21505594.2020.1776979

Sharma, P., Guha, S., Garg, P., and Roy, S. (2018). Differential Expression of Antimicrobial Peptides in Corneal Infection and Regulation of Antimicrobial Peptides and Reactive Oxygen Species by Type III Secretion System of *Pseudomonas aeruginosa*. *Pathog. Dis.* 76. doi:10.1093/femspd/fty001

Sharma, P., Sharma, N., Mishra, P., Joseph, J., Mishra, D. K., Garg, P., et al. (2019). Differential Expression of Antimicrobial Peptides in Streptococcus Pneumoniae Keratitis and STAT3-dependent Expression of LL-37 by

Streptococcus Pneumoniae in Human Corneal Epithelial Cells. *Pathogens* 8. 31. doi:10.3390/pathogens8010031

Sharma, S. (2011). Antibiotic Resistance in Ocular Bacterial Pathogens. *Indian J. Med. Microbiol.* 29, 218–222. doi:10.4103/0255-0857.83903

Shastri, D. H., Prajapati, S. T., and Patel, L. D. (2010). Design and Development of Thermoreversible Ophthalmic *In Situ* Hydrogel of Moxifloxacin HCl. *Curr. Drug. Deliv.* 7, 238–243. doi:10.2174/156720110791560928

Shayani Rad, M., Khameneh, B., Sabeti, Z., Mohajeri, S. A., and Fazly Bazzaz, B. S. (2016). Antibacterial Activity of Silver Nanoparticle-Loaded Soft Contact Lens Materials: the Effect of Monomer Composition. *Curr. Eye Res.* 41, 1286–1293. doi:10.3109/02713683.2015.1123726

Shivam, U. U., Siddhi, C., Devarshi, G., Umeshkumar, M. U., and Jayvadan, K. P. (2020). Nanoparticles Laden *In Situ* Gel for Sustained Drug Release after Topical Ocular Administration. *J. Drug Deliv. Sci. Technol.* 57, 101736. doi:10.1016/j.jddst.2020.101736

Shrivastava, S., Shrivastava, P., and Ramasamy, J. (2018). Responding to the challenge of Antibiotic Resistance: World Health Organization. *J. Res. Med. Sci.* 23, 21. doi:10.4103/1735-1995.228593

Silva, D., De Sousa, H. C., Gil, M. H., Santos, L. F., Amaral, R. A., Saraiva, J. A., et al. (2021). Imprinted Hydrogels with LbL Coating for Dual Drug Release from Soft Contact Lenses Materials. *Mater. Sci. Eng. C* 120, 111687. doi:10.1016/j.msec.2020.111687

Silva, D., Sousa, H. C. d., Gil, M. H., Santos, L. F., Moutinho, G. M., Serro, A. P., et al. (2018). Antibacterial Layer-By-Layer Coatings to Control Drug Release from Soft Contact Lenses Material. *Int. J. Pharmaceutics* 553, 186–200. doi:10.1016/j.ijpharm.2018.10.041

Silva, N. C., Sarmento, B., and Pintado, M. (2013). The Importance of Antimicrobial Peptides and Their Potential for Therapeutic Use in Ophthalmology. *Int. J. Antimicrob. Agents* 41, 5–10. doi:10.1016/j.ijantimicag.2012.07.020

Slepenkin, A., Chu, H., Elofsson, M., Keyser, P., and Peterson, E. M. (2011). Protection of Mice from a *Chlamydia trachomatis* Vaginal Infection Using a Salicylidene Acylhydrazide, a Potential Microbicide. *J. Infect. Dis.* 204, 1313–1320. doi:10.1093/infdis/jir552

Speciale, A., Chirafisi, J., Saija, A., and Cimino, F. (2011). Nutritional Antioxidants and Adaptive Cell Responses: an Update. *Curr. Mol. Med.* 11, 770–789. doi:10.2174/156652411798062395

Spierer, A., and Kessler, E. (1984). The Effect of 2-Mercaptoacetyl-L-Phenylalanyl-L-Leucine, a Specific Inhibitor of *Pseudomonas aeruginosa* Elastase, on Experimental Pseudomonas Keratitis in Rabbit Eyes. *Curr. Eye Res.* 3, 645–650. doi:10.3109/02713688409003066

Srinivasan, M., Lalitha, P., Mahalakshmi, R., Prajna, N. V., Mascarenhas, J., Chidambaram, J. D., et al. (2009). Corticosteroids for Bacterial Corneal Ulcers. *Br. J. Ophthalmol.* 93, 198–202. doi:10.1136/bjo.2008.147298

St. Leger, A. J., Desai, J. V., Drummond, R. A., Kugadas, A., Almaghrabi, F., Silver, P., et al. (2017). An Ocular Commensal Protects against Corneal Infection by Driving an Interleukin-17 Response from Mucosal γδ T Cells. *Immunity* 47, 148–158. doi:10.1016/j.immuni.2017.06.014

Strahl, H., and Errington, J. (2017). Bacterial Membranes: Structure, Domains, and Function. *Annu. Rev. Microbiol.* 71, 519–538. doi:10.1146/annurev-micro-102215-095630

Subedi, D., Vijay, A. K., and Willcox, M. (2018). Overview of Mechanisms of Antibiotic Resistance in *Pseudomonas aeruginosa*: an Ocular Perspective. *Clin. Exp. Optom.* 101, 162–171. doi:10.1111/cxo.12621

Sugar, J., and Chandler, J. W. (1974). Experimental Corneal Wound Strength. *Arch. Ophthalmol.* 92, 248–249. doi:10.1001/archopht.1974.01010010256018

Sun, C. C., Chiu, H. T., Lin, Y. F., Lee, K. Y., and Pang, J. H. (2015). Y-27632, a ROCK Inhibitor, Promoted Limbal Epithelial Cell Proliferation and Corneal Wound Healing. *PLoS One* 10, e0144571. doi:10.1371/journal.pone.0144571

Suzuki, T., Swoboda, J. G., Campbell, J., Walker, S., and Gilmore, M. S. (2011). In VitroAntimicrobial Activity of Wall Teichoic Acid Biosynthesis Inhibitors againstStaphylococcus aureusIsolates. *Antimicrob. Agents Chemother.* 55, 767–774. doi:10.1128/aac.00879-10

Swidergall, M., Ernst, A. M., and Ernst, J. F. (2013). Candida Albicans Mucin Msb2 Is a Broad-Range Protectant against Antimicrobial Peptides. *Antimicrob. Agents Chemother.* 57, 3917–3922. doi:10.1128/aac.00862-13

Tallab, R. T., and Stone, D. U. (2016). Corticosteroids as a Therapy for Bacterial Keratitis: an Evidence-Based Review of 'who, when and Why'. *Br. J. Ophthalmol.* 100, 731–735. doi:10.1136/bjophthalmol-2015-307955

Tan, D. W., Lim, S. G., Wong, T. T., and Venkatraman, S. S. (2016). Sustained Antibiotic-Eluting Intra-ocular Lenses: A New Approach. *PLoS One* 11, e0163857. doi:10.1371/journal.pone.0163857

Tang, W., Ma, J., Gu, R., Ding, X., Lei, B., Wang, X., et al. (2018). Lipocalin 2 Suppresses Ocular Inflammation by Inhibiting the Activation of NF-Kβ Pathway in Endotoxin-Induced Uveitis. *Cel Physiol Biochem* 46, 375–388. doi:10.1159/000488472

Thomas, P. A. (2003). Fungal Infections of the Cornea. *Eye* 17, 852–862. doi:10.1038/sj.eye.6700557

Thomas, R. K., Melton, R., and Asbell, P. A. (2019). Antibiotic Resistance Among Ocular Pathogens: Current Trends from the ARMOR Surveillance Study (2009–2016). *Clin. Optom.* Vol. 11, 15–26. doi:10.2147/opto.s189115

Torres-Luna, C., Fan, X., Domszy, R., Hu, N., Wang, N. S., and Yang, A. (2020). Hydrogel-based Ocular Drug Delivery Systems for Hydrophobic Drugs. *Eur. J. Pharm. Sci.* 154, 105503. doi:10.1016/j.ejps.2020.105503

Ubani-Ukoma, U., Gibson, D., Schultz, G., Silva, B. O., and Chauhan, A. (2019). Evaluating the Potential of Drug Eluting Contact Lenses for Treatment of Bacterial Keratitis Using an *Ex Vivo* Corneal Model. *Int. J. pharmaceutics* 565, 499–508. doi:10.1016/j.ijpharm.2019.05.031

Urwin, L., Okurowska, K., Crowther, G., Roy, S., Garg, P., Karunakaran, E., et al. (2020). Corneal Infection Models: Tools to Investigate the Role of Biofilms in Bacterial Keratitis. *Cells* 9, 2450. doi:10.3390/cells9112450

Üstündağ-Okur, N., Gökçe, E. H., Bozbıyık, D. I., Eğrilmez, S., Özer, Ö., and Ertan, G. (2014). Preparation and In Vitro–In Vivo Evaluation of Ofloxacin Loaded Ophthalmic Nano Structured Lipid Carriers Modified with Chitosan Oligosaccharide Lactate for the Treatment of Bacterial Keratitis. *Eur. J. Pharm. Sci.* 63, 204–215. doi:10.1016/j.ejps.2014.07.013

Van Der Weerden, N. L., Hancock, R. E. W., and Anderson, M. A. (2010). Permeabilization of Fungal Hyphae by the Plant Defensin NaD1 Occurs through a Cell wall-dependent Process. *J. Biol. Chem.* 285, 37513–37520. doi:10.1074/jbc.m110.134882

Van Meer, G., Voelker, D. R., and Feigenson, G. W. (2008). Membrane Lipids: where They Are and How They Behave. *Nat. Rev. Mol. Cel Biol* 9, 112–124. doi:10.1038/nrm2330

Wang, G. (2008). Structures of Human Host Defense Cathelicidin LL-37 and its Smallest Antimicrobial Peptide KR-12 in Lipid Micelles. *J. Biol. Chem.* 283, 32637–32643. doi:10.1074/jbc.m805533200

Wang, N. N., Yan, X., Gao, X. N., Niu, H. J., Kang, Z. S., and Huang, L. L. (2016). Purification and Characterization of a Potential Antifungal Protein from Bacillus Subtilis E1R-J against Valsa mali. *World J. Microbiol. Biotechnol.* 32, 63. doi:10.1007/s11274-016-2024-5

Wei, Y., Hu, Y., Shen, X., Zhang, X., Guan, J., and Mao, S. (2020). Design of Circular-Ring Film Embedded Contact Lens for Improved Compatibility and Sustained Ocular Drug Delivery. *Eur. J. Pharmaceutics Biopharmaceutics* 157, 28–37. doi:10.1016/j.ejpb.2020.09.010

Whitcher, J. P., Srinivasan, M., and Upadhyay, M. P. (2001). Corneal Blindness: a Global Perspective. *Bull. World Health Organ.* 79, 214–221.

Wilcox, C. S. (2010). Effects of Tempol and Redox-Cycling Nitroxides in Models of Oxidative Stress. *Pharmacol. Ther.* 126, 119–145. doi:10.1016/j.pharmthera.2010.01.003

Wiig, M., Olmarker, K., Håkansson, J., Ekström, L., Nilsson, E., and Mahlapuu, M. (2011). A Lactoferrin-Derived Peptide (PXL01) for the Reduction of Adhesion Formation in Flexor Tendon Surgery: an Experimental Study in Rabbits. *J. Hand Surg. Eur. Vol.* 36, 656–662. doi:10.1177/1753193411410823

Wilkinson, A., Kawaguchi, N., Geczy, C., and Di Girolamo, N. (2016). S100A8 and S100A9 Proteins Are Expressed by Human Corneal Stromal Dendritic Cells. *Br. J. Ophthalmol.* 100, 1304–1308. doi:10.1136/bjophthalmol-2016-308827

Willcox, M. D. P. (2011). Review of Resistance of Ocular Isolates of *Pseudomonas aeruginosa* and Staphylococci from Keratitis to Ciprofloxacin, Gentamicin and Cephalosporins. *Clin. Exp. Optom.* 94, 161–168. doi:10.1111/j.1444-0938.2010.00536.x

Wu, L., Ye, M., and Zhang, J. (2018). Vincamine Prevents Lipopolysaccharide Induced Inflammation and Oxidative Stress via Thioredoxin Reductase Activation in Human Corneal Epithelial Cells. *Am. J. Transl Res.* 10, 2195–2204.

Wu, M., Mcclellan, S. A., Barrett, R. P., Zhang, Y., and Hazlett, L. D. (2009). β-Defensins 2 and 3 Together Promote Resistance toPseudomonas aeruginosaKeratitis. *J. Immunol.* 183, 8054–8060. doi:10.4049/jimmunol.0902140

Yan, H., Wang, Y., Shen, S., Wu, Z., and Wan, P. (2017). Corticosteroids Effects on LPS-Induced Rat Inflammatory Keratocyte Cell Model. *PloS one* 12, e0176639. doi:10.1371/journal.pone.0176639

Yin, J., Peng, X., Lin, J., Zhang, Y., Zhang, J., Gao, H., et al. (2021). Quercetin ameliorates Aspergillus Fumigatus keratitis by Inhibiting Fungal Growth, Toll-like Receptors and Inflammatory Cytokines. *Int. Immunopharmacology* 93, 107435. doi:10.1016/j.intimp.2021.107435

Yin, J., and Yu, F.-S. X. (2010). LL-37 via EGFR Transactivation to Promote High Glucose-Attenuated Epithelial Wound Healing in Organ-Cultured Corneas. *Invest. Ophthalmol. Vis. Sci.* 51, 1891–1897. doi:10.1167/iovs.09-3904

Zeth, K., and Sancho-Vaello, E. (2017). The Human Antimicrobial Peptides Dermcidin and LL-37 Show Novel Distinct Pathways in Membrane Interactions. *Front. Chem.* 5, 86. doi:10.3389/fchem.2017.00086

Zhao, G., Li, S., Zhao, W., He, K., Xi, H., Li, W., et al. (2012). Phage Display against Corneal Epithelial Cells Produced Bioactive Peptides that Inhibit Aspergillus Adhesion to the Corneas. *PLoS One* 7, e33578. doi:10.1371/journal.pone.0033578

Zhu, Y., Peng, X., Zhang, Y., Lin, J., and Zhao, G. (2021). Baicalein Protects against Aspergillus fumigatus Keratitis by Reducing Fungal Load and Inhibiting TSLP-Induced Inflammatory Response. *Invest. Ophthalmol. Vis. Sci.* 62, 26. doi:10.1167/iovs.62.6.26

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PhD THESIS SYNOPSIS

ANTIMICROBIAL PEPTIDE MEDIATED HOST DEFENSE IN BACTERIAL KERATITIS

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A. INTRODUCTION:

Microbial keratitis is a painful, progressive corneal infection causing inflammation, severe corneal opacity, ulcer and visual impairment (Acharya et al., 2019) **(Figure 1).** The World Health Organisation (WHO) has identified 'corneal opacity' as one of the leading causes of visual impairment, preceded by uncorrected refractive error, cataract and glaucoma (*Blindness and Vision Impairment*, n.d.). In another study, 'corneal opacity' was found to be the fifth main cause of blindness in global population (Flaxman et al., 2017). In India, the national programme for control of blindness and visual impairment (NPCB&VI) also lists 'corneal blindness' as one of the main causes of blindness (*Directorate General Of Health Services*, n.d.). Microbial keratitis has been found to be the most common cause of corneal opacity and blindness worldwide (Ung et al., 2019).

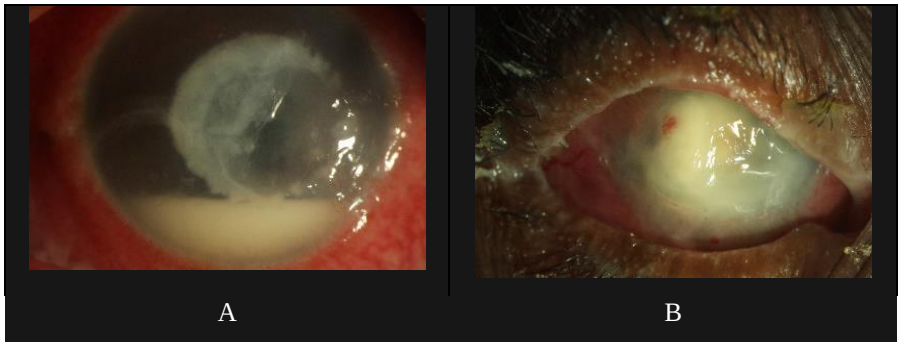

Figure 1: Representative images of bacterial keratitis. **A.** *P. aeruginosa* keratitis; **B.** *S. pneumoniae* keratitis

The common risk factors of microbial keratitis are corneal trauma, prolonged use of contact lenses and ocular surgical procedures (Acharya et al., 2020; Bharathi et al., 2007; Chidambaram et al., 2018; Grünauer-Kloevekorn et al., 2004; Maharana et al., 2018; Palmer & Hyndiuk, 1993). The most common microorganisms causing microbial keratitis are fungus and bacteria (Acharya et al., 2020; Chidambaram et al., 2018; Gopinathan et al., 2009; Ung et al., 2019). In a recent study from North India, 60.6% of microbial keratitis was found to be caused by bacteria (Acharya et al., 2020). In another study conducted at a tertiary centre in west India, 65.1% of culture positive keratitis was found to be of bacterial etiology (Tewari et al., 2012). Bacterial keratitis has been found to be caused by both gram negative and gram positive group of bacteria and *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* are important etiological agents (Bharathi et al., 2007; Gopinathan et al., 2009; Rautaraya et al., 2014; Ung et al., 2019). At present, bacterial keratitis is mostly managed by antibiotic therapy (Acharya et al., 2019). However, there

has been increasing reports of emergence and re-emergence of antibiotic resistance in bacterial pathogens (*WHO Publishes List of Bacteria for Which New Antibiotics Are Urgently Needed*, n.d.). Antibiotic resistance in ocular pathogens has also been identified (Das et al., 2019; Garg et al., 1999; Kaliamurthy et al., 2013; Ting et al., 2021; Willcox, 2011). Therefore, alternative therapeutics for treatment of bacterial keratitis is necessary. One such alternative is antimicrobial peptides (AMPs), either used alone or in conjunction with the currently used antibiotics (Li et al., 2014; Mohammed et al., 2019; Tummanapalli & Willcox, 2021). The intrinsic expression of AMPs can also be positively modulated to achieve enhanced action against the bacteria (Carion et al., 2020; Rodríguez-Carlos et al., 2021). AMPs are short cationic peptides, usually 10-50 amino acids in length, that are naturally produced by living organisms or can be chemically synthesized (Li et al., 2014; Mohammed et al., 2019). They act as the first line of defense against pathogen invasion. AMPs can directly act on bacteria by creating pores on their cell membrane or can target other internal sites of the pathogen. AMPs also show immunomodulatory effect on the host, thereby, indirectly controlling the host response to pathogen **(Figure 2).**

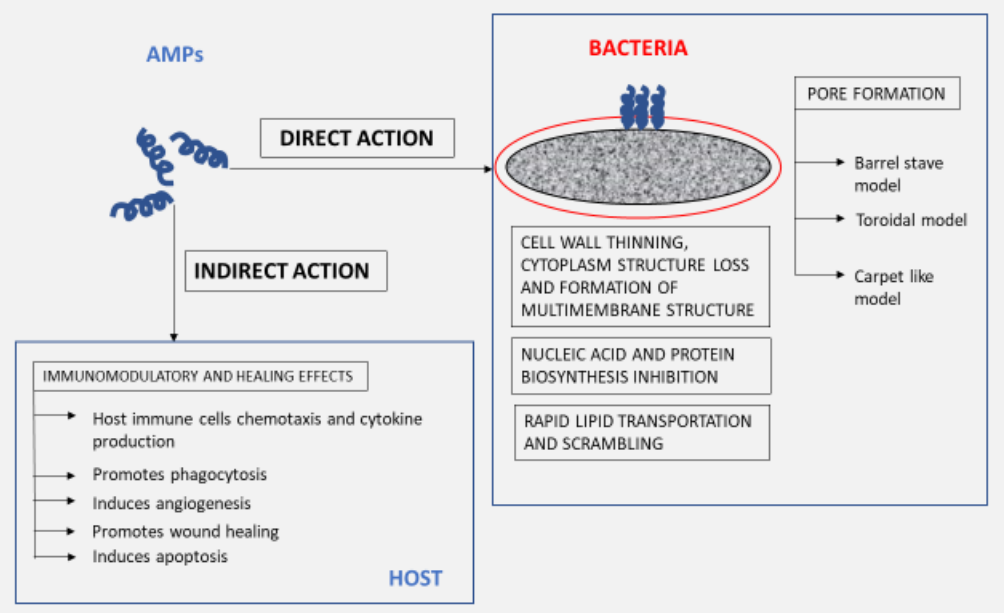

Figure 2. Mechanism of action of AMPs

B. PURPOSE OF THE STUDY:

The World Health Organisation (WHO) in its 2017-18 report has listed out some bacteria in which the emergence of antibiotic resistance needs to be taken care of on a priority basis.

Out of all the listed pathogens, the emergence of antibiotic resistance in *P. aeruginosa* and *S. pneumoniae* has also been pointed out ("WHO | Global Priority List of Antibiotic-Resistant Bacteria to Guide Research, Discovery, and Development of New Antibiotics," 2017). Hence, it becomes an utmost priority to establish an alternative to antibiotic usage in bacterial diseases. Keeping these facts in mind the current study focuses on deciphering the role of AMPs in bacterial keratitis, both gram-negative *Pseudomonas aeruginosa* and gram-positive *Streptococcus pneumoniae*.

C. OBJECTIVES:

1. To determine the expression of antimicrobial peptides during bacterial keratitis in patients.

2. To determine the role of antimicrobial peptides in keratitis caused by gram negative bacteria, *Pseudomonas aeruginosa*.

3. To determine the role of antimicrobial peptides in keratitis caused by gram positive bacteria, *Streptococcus pneumoniae*.

D. STRUCTURE OF THESIS:

The contents of the PhD thesis entitled "Antimicrobial peptide mediated host defense in bacterial keratitis" has been divided into five chapters. **Chapter I** gives an introduction and recent review of literature as a background, aims and objectives. **Chapter II** gives a description of all the materials and methods used for this study. **Chapter III – V** elucidates the background, results, discussion and conclusion covering the objectives 1-3. Chapter III focuses on objective 1, chapter IV focuses on objective 2, and chapter V focuses on objective 3. **Chapter VI** gives a summary of the work done, contributions and limitations of the study along with the future scopes. The **references** are added after it.

Chapter I: Introduction- Review of literature, Aim of the study and objectives

The first chapter gives an introduction about the structure of eye, importance of cornea in vision, loss of vision due to microbial keratitis with specific focus on bacterial keratitis and various therapeutics being used as a treatment. The chapter also introduces the pathogenesis and virulence factors of *Pseudomonas aeruginosa* and *Streptococcus pneumoniae*, the two major bacterial pathogens that are responsible for causing corneal infections. This chapter also has a section about the emerging antimicrobial resistance in microorganisms,

especially antibiotic resistance in bacteria, its mechanism, spread and how it has become difficult to treat infections like bacterial keratitis caused by these antibiotic resistant bacteria. Another section is dedicated to the main highlight of my research, which is antimicrobial peptides (AMPs), and how they have a potential to be used as an alternative therapeutic for treating bacterial keratitis caused by antibiotic resistant bacteria. Also, there is a section explaining the cell signaling pathways relevant to host defense in response to infections and inflammation like TLR, MAPK, NF-κβ, JAK-STAT3, apoptosis, autophagy etc. This chapter also includes the review of literature of the work done in this area so far.

Chapter II: Material and methods

This chapter explains in detail all the material and methods that we have used for our study like bacterial and cell culture for maintenance and experiments, RNA isolation by TRIzol method, cDNA synthesis and quantitative PCR for checking the level of expression of AMPs and signaling markers at mRNA level, immunohistochemistry and immunocytochemistry of corneal sections to check the expression and localisation of markers, western blotting to check the protein expression of various signaling markers, cell viability assay by 3-[4,5-dimethylthiazole-2-yl]-2,5-diphenyltetrazolium bromide (MTT), lactate dehydrogenase (LDH) or propidium iodide (PI) staining, hematoxylin and eosin staining of corneal sections to check the severity of infection and immune cell infiltration. We have also included methods for transient silencing of genes by siRNA, followed by lysing and plating the bacteria to count CFUs.

Chapter III: The expression of antimicrobial peptides (AMPs) during bacterial keratitis in patients.

In this chapter we elucidate the background, results, discussion and conclusion pertaining to the study done to determine the expression of antimicrobial peptides (AMPs) in bacterial keratitis patients' samples. The highlights of this chapter are:

1. Detection of differential expression of AMPs in corneal scrapings of *P. aeruginosa* keratitis patients. Some AMPs like LL-37 are upregulated indicating that they might play a role in infection **(Figure 3).**
2. We found differential expression of AMPs in *S. pneumoniae* keratitis patients' samples and like in case of *P. aeruginosa* keratitis samples some AMPs including LL-37 was found to be upregulated **(Figure 4).**

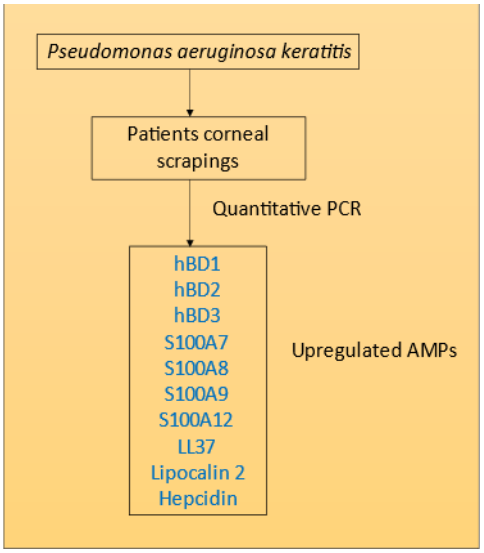

Figure 3: AMPs upregulated in *P. aeruginosa* keratitis patients' samples

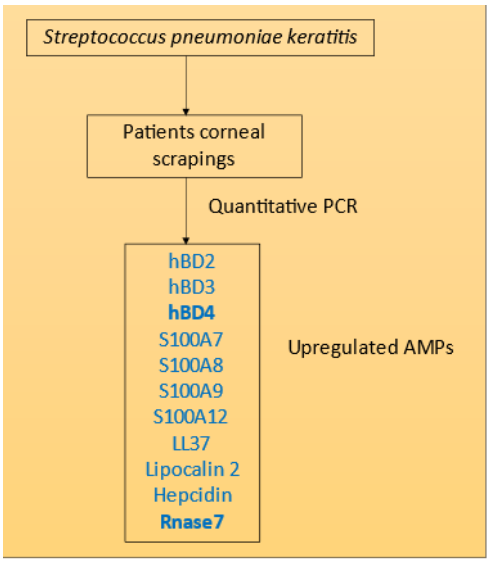

Figure 4: AMPs upregulated in *S. pneumoniae* keratitis patients' samples

Chapter IV: Role of antimicrobial peptides in gram negative *P. aeruginosa*

In this chapter we elucidate the background, results, discussion and conclusion pertaining to the study done to decipher the role of AMPs in gram negative *P. aeruginosa*. The highlights of this chapter are:

1. We found reduced expression of AMPs in human corneal epithelial cells (HCECs) in response to wild-type PAO1 (PA Wt), a laboratory strain of *P. aeruginosa*,

compared to a type-3 secretion system (T3SS) mutant bacteria. Similar result was observed in a monocyte cell line U937 as well, under same infection conditions. The fact that there was no difference in the expression of AMPs in HCECs infected with either wild type or T3SS mutant bacteria for a longer time period (24h) indicates that T3SS mediated suppression of AMPs expression is only active during the initial stages of infection. This confirms a role of T3SS of *P. aeruginosa* in expression of AMPs and host response during the initial stages of infection.

2. Apart from AMPs expression, *P. aeruginosa* was also found to inhibit various signaling pathways in HCECs and U937 like NF-κβ, MAPK (p38 and ERK) in a T3SS dependent manner. Generation of reactive oxygen species in HCECs and U937 was also found to be supressed by *P. aeruginosa* in a T3SS dependent manner.

3. To further corroborate our findings, we used a T3SS inhibitor INP0341, a salicylidene acylhydrazide derivative, in our *in vitro* infection studies on HCECs. INP0341 was found to reduce wild type *P. aeruginosa* mediated cytotoxicity, prevent actin cytoskeleton rearrangement, prevent the suppression of AMPs expression and revive ROS generation in HCECs. In our *in vivo* study on C57BL/6 mice, INP0341 was also found to relatively alleviate the condition in mice eyes when compared to eyes infected with wild type bacteria only. We saw lesser opacity, reduced colony forming units per eye (CFU/eye), lesser cellular infiltration in cornea and increased expression of AMPs in infected murine eyes treated with INP0341 as compared to untreated infected eyes. The eyes infected with T3SS mutant *P. aeruginosa* showed similar results as the eyes infected with wild type bacteria along with INP0341. This indicates that T3SS plays a role in suppressing AMP expression in host and contributes to the severity of keratitis.

Chapter V: Role of antimicrobial peptides in gram positive *S. pneumoniae*

In this chapter we elaborate the background, results, discussion and conclusion to decipher the role of AMPs in gram positive *S. pneumoniae*. The highlights of this chapter are:

1. In *in vitro* model of infection, we saw significant upregulation of some AMPs in HCECs infected by ATCC strain of *S. pneumoniae* (SP ATCC). However, the expression of AMPs in HCECs infected by clinical strain of *S. pneumoniae* (SP CS)

was significantly reduced that was not related to cell death as confirmed by MTT and propidium iodide staining. We also saw significant upregulation of hBD3, S100A8, S100A9 and LL-37 in primary HCECs infected with SP ATCC. There was a significant increase in the expression of S100A9 in primary HCECs infected with clinical strain of *S. pneumoniae*. There was no significant change in expression of AMPs in monocyte derived cell line, U937 when either infected with ATCC strain or clinical strain of *S. pneumoniae*.

2. Further, we focused on LL-37, the only AMP belonging to the cathelicidin family in human, to further study the role of AMPs in *S. pneumoniae* infection. We took tissue sections from patients who underwent therapeutic penetrating keratoplasty, and stained it to detect the presence and localisation of LL-37. We found LL-37 expression in epithelium and stroma of cornea. *In vitro*, we found increased expression of LL-37 mRNA at 4h in HCECs infected with ATCC strain of *S. pneumoniae*. Immunostaining and western blotting further confirmed the *in vitro* expression of LL-37 peptide. Further studies confirmed that this expression was *de novo*. LL-37 also showed potent antimicrobial activity against both the strains of *S. pneumoniae*.

3. The expression of LL-37 in HCECs infected with the ATCC strain of *S. pneumoniae* was found to be mediated by STAT3 **(Figure 5).**

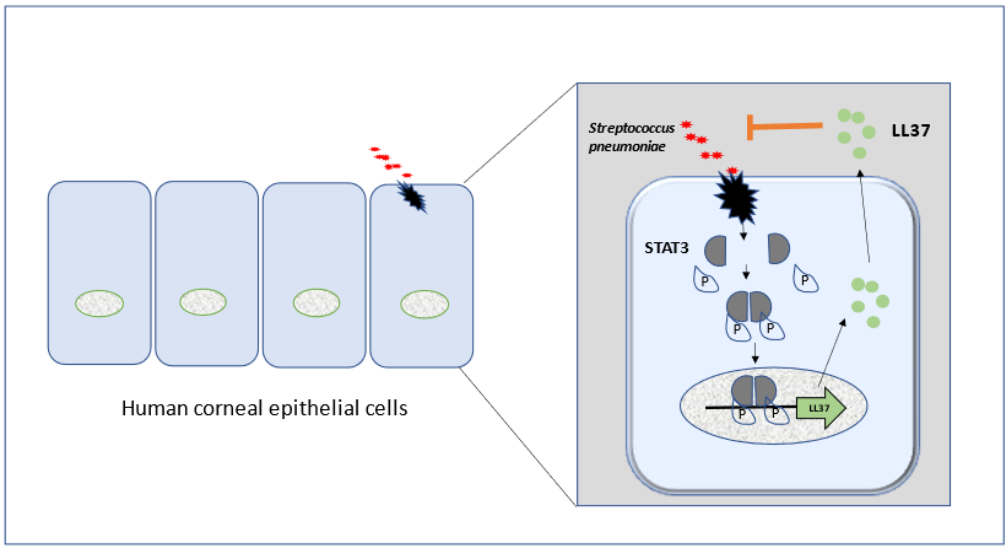

Figure 5: STAT3 mediated expression of LL37 in *S. pneumoniae* infected HCECs

4. We also found that *S. pneumoniae* induces oxidative stress in corneal tissues and *in vitro*, supresses Nrf2 activation and hence antioxidative responses mediated by it. This we speculate, is because of *S. pneumoniae* affinity towards oxidative stress that helps in its pathogenesis. *S. pneumoniae* was also found to inhibit autophagy in HCECs, thereby meddling with one of the arms of innate immunity. In further studies, we found that tert-butylhydroquinone (tBHQ), a known inducer of Nrf2 was able to reduce oxidative stress, activate autophagy and also upregulate AMP expression including LL-37 in infected HCECs. There was also a reduction in bacterial CFUs in infected HCECs upon simultaneous treatment with tBHQ, indicating that LL-37 might be involved in it. We got further confirmation when we saw that tHBQ by itself was not able to inhibit bacterial growth.

Chapter VI: Summary

In this chapter we give the overall gist of our findings. We summarise the role that AMPs play in bacterial infection, with a focus on *P. aeruginosa* and *S. pneumoniae*. We further focus on possible use of T3SS inhibitor as an alternative to antibiotics in treating corneal infections. The representative AMP that we focused on in this study, LL-37, shows potent antibacterial activity against *S. pneumoniae*. AMPs show a promising future in curbing bacterial infections caused by antibiotic-resistant bacteria. AMPs can not only be synthesised and applied externally on the infection sites but can also be upregulated intrinsically by using inhibitors and chemicals that either supress bacterial virulence or enhance AMPs expression.

E: LIST OF PUBLICATIONS:

Research articles from PhD work:

- **Sharma, P**., Guha, S., Garg, P., & Roy, S. (2018). Differential expression of antimicrobial peptides in corneal infection and regulation of antimicrobial peptides and reactive oxygen species by type III secretion system of *Pseudomonas aeruginosa*. *Pathogens and Disease,* 76(1), 10.1093/femspd/fty001.

- **Sharma, P**., Sharma, N., Mishra, P., Joseph, J., Mishra, D. K., Garg, P., & Roy, S. (2019). Differential Expression of Antimicrobial Peptides in *Streptococcus pneumoniae* Keratitis and STAT3-Dependent Expression of LL-37 by

Streptococcus pneumoniae in Human Corneal Epithelial Cells. *Pathogens* (Basel, Switzerland), 8(1),31.

- **Sharma, P**., Elofsson, M., & Roy, S. (2020). Attenuation of *Pseudomonas aeruginosa* infection by INP0341, a salicylidene acylhydrazide, in a murine model of keratitis. *Virulence*, 11(1),795–804.

- Jadi, P. K., **Sharma, P**., Bhogapurapu, B., & Roy, S. (2021). Alternative Therapeutic Interventions: Antimicrobial Peptides and Small Molecules to Treat Microbial Keratitis. Frontiers in Chemistry, 9, 694998. https://doi.org/10.3389/fchem.2021.694998

- **Sharma, P**. & Roy, S. Tert-Butylhydroquinone alleviates oxidative stress, induces autophagy in a Nrf2 dependent manner, and mediates protection against *Streptococcus pneumoniae* by induction of antimicrobial peptides in human corneal epithelial cells. (MANUSCRIPT UNDER REVIEW)

Research articles from collaborations:

- Mallela, L. S., **Sharma, P**., Rao, T., & Roy, S. (2021). Recombinant IL-22 promotes protection in a murine model of *Aspergillus flavus* keratitis and mediates host immune responses in human corneal epithelial cells. Cellular Microbiology, 23(9), e13367. https://doi.org/10.1111/cmi.13367

F. CONFERENCES AND SEMINARS ATTENDED:

- Attended ARVO-IERG, 2016 held at L.V. Prasad Eye Institute, Hyderabad, India.

- Presented a poster entitled “Role of antimicrobial peptides in *Pseudomonas* keratitis” in IMMUNOCON, 2017, held at GITAM UNIVERSITY, Vishakhapatnam, India.

- Presented a poster entitled “Host defense mediated by antimicrobial peptides in *Streptococcus pneumoniae* keratitis” in ARVO-IERG, 2018 held at L.V. Prasad Eye Institute, Hyderabad, India.

- Presented a poster entitled “Role of type III secretion system inhibitor in *Pseudomonas* keratitis” in ARVO-IERG, 2019 held at Sankara Nethralaya, Chennai, India.

- Presented thesis in a 3-minute thesis competition held at LVPEI, Hyderabad, on 28 February, 2019. Won 2nd Prize.

G. REFERENCES:

Acharya, M., Farooqui, J., Gaba, T., Gandhi, A. & Mathur, U. (2020). Delhi infectious keratitis study: Update on clinico-Microbiological profile and outcomes of infectious keratitis. *Journal of Current Ophthalmology*, *32*(3), 249–255. https://doi.org/10.4103/JOCO.JOCO_113_20

Acharya, M., Farooqui, J. H., Jain, S. & Mathur, U. (2019). Pearls and paradigms in Infective Keratitis. *Romanian Journal of Ophthalmology*, *63*(2), 119–127. https://doi.org/10.22336/rjo.2019.18

Bharathi, M. J., Ramakrishnan, R., Meenakshi, R., Padmavathy, S., Shivakumar, C. & Srinivasan, M. (2007). Microbial keratitis in South India: Influence of risk factors, climate, and geographical variation. *Ophthalmic Epidemiology*, *14*(2), 61–69. https://doi.org/10.1080/09286580601001347

Blindness and vision impairment. (n.d.). Retrieved April 22, 2021, from https://www.who.int/news-room/fact-sheets/detail/blindness-and-visual-impairment

Carion, T. W., Ebrahim, A. S., Alluri, S., Ebrahim, T., Parker, T., Burns, J., Sosne, G. & Berger, E. A. (2020). Antimicrobial effects of thymosin beta-4 and ciprofloxacin adjunctive therapy in pseudomonas aeruginosa induced keratitis. *International Journal of Molecular Sciences*, *21*(18), 1–11. https://doi.org/10.3390/ijms21186840

Chidambaram, J. D., Venkatesh Prajna, N., Srikanthi, P., Lanjewar, S., Shah, M., Elakkiya, S., Lalitha, P. & Burton, M. J. (2018). Epidemiology, risk factors, and clinical outcomes in severe microbial keratitis in South India. *Ophthalmic Epidemiology*, *25*(4), 297–305. https://doi.org/10.1080/09286586.2018.1454964

Das, S., Samantaray, R., Mallick, A., Sahu, S. K. & Sharma, S. (2019). Types of organisms and in-vitro susceptibility of bacterial isolates from patients with microbial keratitis: A trend analysis of 8 years. *Indian Journal of Ophthalmology*, *67*(1), 49–53. https://doi.org/10.4103/ijo.IJO_500_18

Directorate General Of Health Services. (n.d.). Retrieved May 17, 2021, from https://dghs.gov.in/content/1354_3_NationalProgrammeforControlofBlindnessVisual.aspx

Flaxman, S. R., Bourne, R. R. A., Resnikoff, S., Ackland, P., Braithwaite, T., Cicinelli, M. V., Das, A., Jonas, J. B., Keeffe, J., Kempen, J., Leasher, J., Limburg, H., Naidoo, K., Pesudovs, K., Silvester, A., Stevens, G. A., Tahhan, N., Wong, T., Taylor, H., … Zheng, Y. (2017). Global causes of blindness and distance vision impairment 1990–2020: a systematic review and meta-analysis. *The Lancet Global Health*, *5*(12), e1221–e1234. https://doi.org/10.1016/S2214-109X(17)30393-5

Garg, P., Sharma, S. & Rao, G. N. (1999). Ciprofloxacin-resistant Pseudomonas keratitis. *Ophthalmology*, *106*(7), 1319–1323. https://doi.org/10.1016/S0161-6420(99)00717-4

Gopinathan, U., Sharma, S., Garg, P. & Rao, G. (2009). Review of epidemiological features, microbiological diagnosis and treatment outcome of microbial keratitis: Experience of over a decade. *Indian Journal of Ophthalmology*, *57*(4), 273–279. https://doi.org/10.4103/0301-4738.53051

Grünauer-Kloevekorn, C., Wilhelm, F., Duncker, G. I. W. & Hammer, T. (2004). Nosokomiale Pseudomonas-Aeruginosa-Assoziierte Keratitis nach Tragen Weicher Kontaktlinsen. *Klinische Monatsblatter Fur Augenheilkunde*, *221*(1), 52–55. https://doi.org/10.1055/s-2003-812639

Kaliamurthy, J., Kalavathy, C. M., Parmar, P., Nelson Jesudasan, C. A. & Thomas, P. A. (2013). Spectrum of bacterial keratitis at a tertiary eye care centre in India. *BioMed Research International*, *2013*, 181564–181564. https://doi.org/10.1155/2013/181564

Li, S. A., Liu, J., Xiang, Y., Wang, Y. J., Lee, W. H. & Zhang, Y. (2014). Therapeutic potential of the antimicrobial peptide oh-cath30 for antibiotic-resistant pseudomonas aeruginosa keratitis. *Antimicrobial Agents and Chemotherapy*, *58*(6), 3144–3150. https://doi.org/10.1128/AAC.00095-14

Maharana, P. K., Sahay, P., Sujeeth, M., Singhal, D., Rathi, A., Titiyal, J. S. & Sharma, N. (2018). Microbial keratitis after accelerated corneal collagen cross-linking in keratoconus. *Cornea*, *37*(2), 162–167. https://doi.org/10.1097/ICO.0000000000001439

Mohammed, I., Said, D. G., Nubile, M., Mastropasqua, L. & Dua, H. S. (2019). Cathelicidin-Derived Synthetic Peptide Improves Therapeutic Potential of Vancomycin Against Pseudomonas aeruginosa. *Frontiers in Microbiology*, *10*, 2190. https://doi.org/10.3389/fmicb.2019.02190

Palmer, M. L. & Hyndiuk, R. A. (1993). Contact lens-related infectious keratitis. In *International Ophthalmology Clinics* (Vol. 33, Issue 1, pp. 23–49). Int Ophthalmol Clin. https://doi.org/10.1097/00004397-199303310-00005

Rautaraya, B., Sharma, S., Ali, M. H., Kar, S., Das, S. & Sahu, S. K. (2014). A 3½-Year Study of Bacterial Keratitis From Odisha, India. *Asia-Pacific Journal of Ophthalmology*, *3*(3), 146–150. https://doi.org/10.1097/apo.0b013e3182a3f301

Rodríguez-Carlos, A., Jacobo-Delgado, Y. M., Santos-Mena, A. O. & Rivas-Santiago, B. (2021). Modulation of cathelicidin and defensins by histone deacetylase inhibitors: A potential treatment for multi-drug resistant infectious diseases. In *Peptides* (Vol. 140). Elsevier Inc. https://doi.org/10.1016/j.peptides.2021.170527

Tewari, A., Sood, N., Vegad, M. M. & Mehta, D. C. (2012). Epidemiological and microbiological profile of infective keratitis in Ahmedabad. *Indian Journal of Ophthalmology*, *60*(4), 267–272. https://doi.org/10.4103/0301-4738.98702

Ting, D. S. J., Ho, C. S., Deshmukh, R., Said, D. G. & Dua, H. S. (2021). Infectious keratitis: an update on epidemiology, causative microorganisms, risk factors, and antimicrobial resistance. In *Eye (Basingstoke)* (Vol. 35, Issue 4, pp. 1084–1101). Springer Nature. https://doi.org/10.1038/s41433-020-01339-3

Tummanapalli, S. S. & Willcox, M. D. D. P. (2021). Antimicrobial resistance of ocular microbes and the role of antimicrobial peptides. *Clinical and Experimental Optometry*, *104*(3), 295–307. https://doi.org/10.1111/cxo.13125

Ung, L., Bispo, P. J. M., Shanbhag, S. S., Gilmore, M. S. & Chodosh, J. (2019). The persistent dilemma of microbial keratitis: Global burden, diagnosis, and antimicrobial resistance. In *Survey of Ophthalmology* (Vol. 64, Issue 3, pp. 255–271). Elsevier USA. https://doi.org/10.1016/j.survophthal.2018.12.003

WHO | Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antibiotics. (2017). *WHO*. http://www.who.int/medicines/publications/global-priority-list-antibiotic-resistant-bacteria/en/

WHO publishes list of bacteria for which new antibiotics are urgently needed. (n.d.). Retrieved July 23, 2020, from https://www.who.int/news-room/detail/27-02-2017-who-publishes-list-of-bacteria-for-which-new-antibiotics-are-urgently-needed

Willcox, M. D. P. (2011). Review of resistance of ocular isolates of Pseudomonas aeruginosa and staphylococci from keratitis to ciprofloxacin, gentamicin and cephalosporins. In *Clinical and Experimental Optometry* (Vol. 94, Issue 2, pp. 161–168). Clin Exp Optom. https://doi.org/10.1111/j.1444-0938.2010.00536.x

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by Prerana Sharma

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