Human antimicrobial peptides in ocular surface defense

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Abstract

Sight depends on the passage of light through the transparent cornea and being focused on the fovea. Its exposed position renders it vulnerable to microbial infection. The cornea has developed a wide array of defense mechanisms against infection, of which endogenous antimicrobial peptides (AMPs) are key. AMPs are essentially small molecular weight cationic peptides with a wide range of activity against virus, bacteria, fungi and parasites. Some proteins such as RNases and S100As are also included in this group. Several AMPs act synergistically allowing low expression of multiple AMPs to act efficiently. AMPs also have a range of non-microbicidal functions and serve as signaling molecules, immunomodulators; show anti-tumour activity, and influence vascularization and wound healing. Different toll-like receptors (TLR) have been implicated in the preferential induction of specific AMPs. A range of bacteria, including mycobacteria tuberculosis, viruses including herpes virus, fungi and parasites including acanthamoeba, that cause ocular infections have been shown to induce specific AMPs via TLR activation. Non-TLR mediated induction of AMP expression can occur and several molecules such as L-isoleucine, sodium butyrate, vitamin D3, phenylbutyrate, vasoactive intestinal peptide, and etinostat have been identified in this regard. Given the rising microbe resistance to antibiotics, the slow rate of development of new antibiotics and the limited access to effective antibiotics by patients living in the developing world, an ideal solution would be to find AMPs that are effective singly or in combination with each other or other antimicrobial proteins to reduce, if possible eliminate reliance on antibiotics alone.

Table of Contents

Abbreviations:

HBD – Human beta-defensins

mBD – Murine beta-defensins

HD – Human alpha-defensins

LL-37 – Human cathelicidin

RNases – Ribonucleases

LEAP - Liver-expressed antimicrobial peptide

NF-κB – Nuclear factor kappa B

MAPK – Mitogen-activated protein kinases

IL – Interleukin

IFN – Interferon

OS – Ocular surface

LPS – Lipopolysaccharide

CD14 – Cluster of differentiation 14

MD2 – Myeloid differentiation 2

TLR – Toll-like receptor

AMPs – Antimicrobial peptides

CXCR – C-X-C motif chemokine receptor

CCR – C-C chemokine receptor

HSV1 – Herpes simplex virus-1

HSK – Herpetic stromal keratitis

138   **Keywords**:

139

140   Antimicrobial peptides

141   Host defense peptides

142   Eye

143   Ocular surface

144   Corneal epithelium

145   Bacteria

146   Virus

147   Fungi

148   Protozoa

149   Keratitis

150   Conjunctivitis

151   Toll-like receptors

152

153

1. Introduction

Sight is arguably the most important of the special senses across species, especially so in humans. The cornea, the clear window in the front of the eye, is the critical part of the ocular surface. The ocular surface is an anatomical and functional unit. It is made of the tear film, the conjunctival, limbal and corneal epithelium, the lacrimal, mucous and meibomian glands and the lids and blink reflex. The transparency of the cornea and its optical properties allows for two thirds of the focusing of light rays to occur at the air-cornea interface, *en route* to the focal point on the retina (Cursiefen, 2007). The tear film provides the optical polish to the cornea. The tear film is composed of a basal layer of mucin derived from conjunctival goblet cells and the underlying corneal and conjunctival epithelium (Gipson, 2016). Mucin makes the hydrophobic epithelial surface into a hydrophilic surface that holds the aqueous component of the tear film derived from the lacrimal gland. This in turn is covered by a thin layer of lipid secreted by the meibomian glands located in the lids and discharging their content on to the ocular surface through tiny orifices along the lid margins (Gipson, 2007; Yanez-Soto et al., 2014).

To fulfill its function, the cornea is naturally and constantly exposed to the environment, which poses risks of contamination with environmental pathogens and irritants. This is compounded by the lack of an established vascular or lymphatic system in the cornea, which is integral to its transparency (Cursiefen, 2007). The battle with microbes and irritants is not won by just getting rid of the offending agent but also requires maintenance of transparency while doing so. A healed cornea, which is opaque, is functionally compromised and leads to visual impairment or blindness. It is not surprising therefore that the ocular surface has developed an array of innate and adaptive protective and defense mechanisms to gain a survival advantage. The physical lid blink reflex; the relative lower temperature of the ocular surface due to tear fluid evaporation making the environment less conducive to bacterial proliferation; the OS cellular defense including neutrophils, macrophages and antigen presenting Langerhans cells (Hamrah and Dana, 2007) and the humoral constituents of the tear fluid including secretory immunoglobulins, lactoferrin, lysozyme, lipocalin and a host of cationic proteins, the antimicrobial peptides make for a formidable defense armamentarium (McDermott, 2013).

185 Epidemiology of corneal diseases is complex and determined by varied environmental

186 influences in different geographical regions (Shah et al., 2011). The prevalence of

187 corneal blindness in developed and industrialized countries is not the same as in

188 developing countries, reflecting the diverse standards of eye care and socio-economic

189 conditions. For instance, incidence of viral keratitis in America is declining and in Africa it

190 is increasing with corneal ulceration being the major cause of monocular blindness

191 (WHO, 2010). According to the World Health Organization (WHO) 2010 report, it was

192 estimated that about 4.9 million of the world's population is blind from corneal diseases

193 with 98% cases existing in developing countries (WHO, 2010). In 2013, the global

194 initiative, "Universal Eye Health: A global action plan 2014-2019", was endorsed by the

195 WHO and the International Agency for the Prevention of Blindness (IAPB) with the aim

196 to reduce the avoidable causes of visual impairment by improving preventive care and

197 initiation of public health programs (WHO, 2013).

198 The emergence of microbial resistance to conventional antibiotics has added another

199 dimension to the challenge in the management of infections (O'Neill, 2016). A report

200 published by the WHO in 2015 "Global action plan on antimicrobial resistance" highlights

201 the increase in incidence of antimicrobial resistance in large parts of the world (WHO,

202 2015). It specifically draws attention for the need to develop newer antimicrobial agents

203 and diagnostic tools to combat antimicrobial resistance. Moreover, the treatment options

204 for viral, fungal and parasitic infections are limited and only provide palliative care to the

205 patients (Upadhyay et al., 2015), where even 'successful treatment' of the infection is

206 associated with visual impairment. There is an urgent need to develop alternative

207 antimicrobial drugs. The identification and exploitation of endogenous host defense

208 proteins (Antimicrobial peptides [AMPs]) as potential agents is a promising avenue in

209 this regard.

210 An antibiotic is classically defined as a substance produced by a living microorganism

211 (prokaryotic) that specifically inhibits or kills another microorganism. AMPs are the

212 eukaryotic analogues of antibiotics. Nowadays synthetics antibiotics, structured on

213 natural antibiotics are commonplace. For example, POL7080, a peptidomimetic is in

214 Phase-I clinical trials for its potent activity against *Pseudomonas aeruginosa.* It is

215 developed based on the scaffold structure of a porcine leukocytes-derived antimicrobial
216 peptide, protegrin-1 (Brown et al., 2014).

217 AMPs are also known as Host Defence Peptides (HDPs). They are naturally occurring
218 molecules that are conserved across the plant and animal kingdom (Broekaert et al.,
219 1995; Brogden et al., 2003; Hancock and Diamond, 2000). AMPs are by and large small,
220 cationic and amphipathic peptides ranging from 12 to 50 amino acids in length (Hancock
221 and Diamond, 2000). They have a net positive charge of +2 to +9 due to low acidic
222 residues (glutamate or aspartate), high cationic residues (arginine or lysine and/or
223 histidine) and around 30-50% of hydrophobic residues. The cationic charge of the
224 peptide provides an ideal motif that binds with the anionic surface of the bacterial
225 cytoplasmic membrane (Shafee et al., 2016). Some larger molecules such as RNases
226 and S100A proteins are also included in the group. There are other molecules with
227 definitive bactericidal activity, notably the Peptidoglycan recognition proteins, which are
228 also found at the ocular surface (Ghosh et al., 2009; Gowda et al., 2015; Hua et al.,
229 2015; Ma et al., 2010) but these have a different structure, mechanism of action and
230 expression from the currently known mammalian AMPs (Royet and Dziarski, 2007).
231 AMPs possess broad-spectrum activity against a variety of microbes such as bacteria,
232 viruses, fungi and parasites (Selsted and Ouellette, 2005). Furthermore, they also
233 participate in immune-modulation, proliferation, wound healing and chemotaxis
234 (Hemshekhar et al., 2016; Mangoni et al., 2016). The antimicrobial potential of AMPs
235 may vary at different sites for a particular organism and between different species. In
236 mammals, they can be systemically expressed or concentrated in a specific tissue, such
237 as skin and mucosal epithelia (Haynes et al., 1999; van Wetering et al., 1999; Zanetti,
238 2004). On ocular mucosa, constitutive production and secretion of AMPs from
239 epithelium in to the tear fluid reflects the constant microbial threat to this site (McDermott,
240 2004). It is now evident that any imbalance in AMPs expression bears a casual
241 relationship with human disease conditions. Because of their diverse functions, AMPs
242 are now being clinically tested to combat antibiotic-resistant superbugs, human
243 immunodeficiency viral infection and cancer (Gordon et al., 2005).

244 In this paper, based on a review of the literature and our own published and unpublished
245 work we discuss our current understanding of AMP function in OS defense. We

246 summarize how AMPs are modulated during OS disease and health and provide insight
247 on the mechanisms associated with their production at the OS epithelium.

2. Antimicrobial peptides

2.1. History

250 Lysozyme was the first protein to be identified as an antimicrobial agent by Alexander
251 Fleming in 1922. The discovery of penicillin in 1928 heralded the era of 'Golden Age of
252 Antibiotics' and overshadowed any existing interest in natural AMPs. Since the 1940s,
253 inappropriate and often widespread usage of antibiotics has led to emergence of multi-
254 drug resistant pathogens. This issue was further compounded by the lack of substantial
255 antibiotic development attributed to the increased cost burden for healthcare companies
256 to develop them. In the 1960s, discovery of lactoferrin from milk reignited the interest in
257 AMP research and ushered the interest in identifying AMPs in different species. In the
258 1980s, cecropin and magainin were identified as potential AMPs from silk moth and frog,
259 respectively. AMPs have since been studied extensively resulting in the identification of
260 several other sub-classes of AMPs. In 1997, cecropin-A and melittin were the first to be
261 tested for their antimicrobial efficacy in a *Pseudomonas aeruginosa* keratitis model in
262 rabbits (Nos-Barbera et al., 1997). In humans, our group was the first to report the
263 presence of defensins on the ocular surface (Haynes et al., 1998, 1999) and intra-ocular
264 tissue (Haynes et al., 2000). To date, there are more than 2500 AMPs identified in six
265 life kingdoms and they can be found in the Antimicrobial peptide database (APD) (refer
266 http://aps.unmc.edu/AP/main.php). APD provides details on AMPs discovery, source,
267 nomenclature, classification and prediction tools. A recent review by Wang and co-
268 workers provides a complete update on APD as a tool for AMP research (Wang et al.,
269 2016).

270 Natural AMPs are classified on the basis of their secondary structure into four groups:
271 alpha (α)-helical, beta (β)-stranded, loop and extended peptides (contains both α and β).
272 Loop and extended AMPs (*e.g.* θ-defensins) are mainly from non-human sources or
273 represent synthetic peptides (Powers and Hancock, 2003).

2.2. Microbicidal activity of AMPs

Numerous mechanisms of microbial killing are reported for AMPs and these are constantly growing with increase in knowledge of structure-activity relationship of natural AMPs (Huang et al., 2010). Most mechanistic studies available are limited by the use of 'membrane' as a model system. However, few AMPs (*e.g.* dermaseptin and temporin) were studied with whole microbes utilizing fluorescence labeling chemistry (Andre et al., 2015; Pouny et al., 1992). Generally, AMPs are known for their propensity to kill a variety of microbes. They exhibit high cationic charge related to hydrophobic amino acid residues, facilitating their selective binding to the negatively charged surface of pathogens. Based on their electrochemical affinity, AMPs are classified into two categories: membrane disruptive and non-membrane disruptive peptides (Powers and Hancock, 2003).

The membrane disruptive peptides, such as LL-37 and magainin (belong to α-helical group), utilize one of the three well-known killing mechanisms: *carpet model*, *barrel-stave model* and *micellar aggregate model* (Bahar and Ren, 2013; Huang et al., 2010; Powers and Hancock, 2003). In the *carpet model*, the peptides align parallel to the microbial membrane inducing membrane instability and disruption. In the *barrel-stave model*, the peptides align perpendicular to the membrane and form transmembrane pores by virtue of a high electrical gradient leading to leakage of cytoplasmic components. In the *micellar aggregate model*, peptides form micelle-like aggregates and disrupt membrane integrity leading to osmotic cell lysis. In both barrel-stave and micellar aggregate models, AMPs induce pores across the microbial cytoplasmic membrane and create transmembrane channels, leading to membrane depolarization, leakage of internal organelles and cell death. For some peptides, bacterial killing by pore-formation was noted within a few minutes of interaction with AMPs (Friedrich et al., 1999).

Non-membrane disruptive peptides exhibit antimicrobial activity by acting on intracellular targets rather than acting on the membrane surface. These peptides translocate into bacterial cytoplasm and perturb nucleic acids (*e.g.* buforin II and magainin 2) and cellular protein synthesis (*e.g.* pyrrhocoricin, apidaecin and drosocin) (Powers and Hancock, 2003). Pyrrhocoricin, an insect AMP, binds heat shock protein (DnaK) and

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induces bacterial killing by inhibiting protein folding (Kragol et al., 2001). In contrast to membrane disruptive peptides, intracellular acting AMPs exert the action within hours as was demonstrated for phyrrhocoricin (Kragol et al., 2001).

In contradiction to known mechanisms of AMPs, it is also proposed that penetration of a peptide into bacterial cytoplasm does not always induce bacterial killing. For instance, cecropin-A, a lytic peptide, only induces transcriptional changes in *Escherichia coli* but does not affect its survival (Hong et al., 2003). From the expanding body of knowledge on AMP-microbe interaction, it is evident that the study of the response of microbe to AMPs is as important as the mechanisms by which AMPs attack microbes. Both mechanisms are crucial and could enhance our understanding by which bacteria may resist or overcome AMPs to cause infection.

AMPs also possess lytic activities against enveloped viruses (Ganz et al., 1988; Sinha et al., 2003) and some non-enveloped viruses such as adenovirus (Bastian and Schafer, 2001; Gropp et al., 1999) and papillomavirus (Buck et al., 2006). However, unlike antibiotics that generally do not act against fungi, and unlike antifungals that are not antibacterial, AMPs have potential to act across the microbial spectrum. They also have been shown to act against multidrug resistant *Pseudomonas aeruginosa* or methicillin-resistant *Staphylococcus aureus* (MRSA) (Steinberg et al., 1997).

Another important facet of their action is synergy between a number of AMPs, such that a reduced expression of one AMP is balanced by an increase in others (Nagaoka et al., 2000). LL-37 and HBD-2 act synergistically against *S. aureus* (Ong et al., 2002) and the activity of HBD-3 is boosted by Lysozyme (Maisetta et al., 2003). Such synergy enhances not only the killing properties of AMPs but also the production of the chemokine IL-8 (by the action in concert of HBD-1 to-4 and LL-37), which has been shown to induce microbial killing (Niyonsaba et al., 2005). AMPs even synergize with antibiotics and enhance their microbicidal activity (Cirioni et al., 2008; Midorikawa et al., 2003; Scott et al., 1999).

Some AMPs are also shown to inhibit pathogens by limiting metal availability and the process is termed nutritional immunity. Hepcidin or liver-expressed antimicrobial peptide (LEAP-1) and S100 proteins such as psoriasin (S100A7) and calprotectin (S100A8/9)

are known to play an essential role in nutritional immunity (Verga Falzacappa and Muckenthaler, 2005; Zackular et al., 2015). LEAP1/hepcidin has a broad spectrum of activity against bacteria and fungi (Garcia et al., 2001). It is a very important iron hormone (Ganz, 2011), expression of which is increased in inflammation (Lemaitre et al., 1996) and excretion of which has been shown to decrease with resolution of sepsis (Nemeth et al., 2003). LEAP1/hepcidin reduces iron absorption from the gut and increases its retention in macrophages; it was proposed that these strategies would limit the iron available to microbes for growth (Ganz, 2011). Psoriasin was discovered in psoriatic skin with strong microbicidal properties (Madsen et al., 1991). Its antimicrobial activity is primarily shown to be dependent on its ability to limit zinc (Zn^{2+}) to pathogens (Glaser et al., 2005). On the other hand, calprotectin-mediated limitation of manganese (Mn^{2+}) was shown to increase the bacterial susceptibility to neutrophil killing due to weakening of the bacterial Mn^{2+} dependent defense system (Damo et al., 2013; Wheeler et al., 2016). These studies demonstrated that at the host-pathogen interface, sequestration of essential metal ions by these AMPs induces starvation and weakens their defence system against host immunity.

2.3. Non-microbicidal activity of AMPs

AMPs were originally regarded as molecules that kill microbial pathogens, but recent studies have highlighted their multifunctional potential (Elsbach, 2003). At certain sites such as airway surface (Cole et al., 1999) and gut lumen (as opposed to intestinal crypts) (Elphick et al., 2008), concentration of AMPs is too low to exhibit microbicidal activity. It is implied that they act as signalling molecules at these sites. AMPs also stimulate the release of cytokines such as IFN-α, IFN-γ, IL-2 and IL-13, but not TNF-α or IL-6 in some instances (Vallespi et al., 2000) and this has been shown to correlate with increased survival in mice following lethal doses of *P. aeruginosa* (Vallespi et al., 2003). Anti-inflammatory activity is further enhanced by inhibition of immuno-suppressive adrenal steroid hormones by competitive binding to their receptors (Solomon et al., 1991; Zhu and Solomon, 1992). LL-37 has been shown to induce pro-IL-1β processing and mature IL-1β release from LPS-treated human monocytes *via* a purinergic receptor (P2X7 receptor) (Elssner et al., 2004). LL-37 in synergy with IL-1β was shown to

364   enhance human neutrophil activity by increasing production of cytokines (IL-6, IL-8 and

365   IL-10), chemokine (CCL2) as well as synthesis and release of HNP1-3 (Zheng et al.,

366   2007). Subsequently, it was demonstrated that the LL-37 mediates its anti-inflammatory

367   effect on neutrophils *via* binding to CXCR2 (Zhang et al., 2009b).

368   Besides being early effectors of the innate immune system, AMPs act as a link between

369   the innate and the adaptive immune systems. Alpha defensins induce chemotaxis of

370   monocytes (Territo et al., 1989) and T-cells (Yang et al., 2000) and enhance antibody

371   production from B-cells (van Wetering et al., 1999) whereas β-defensins are potent

372   ligands for the chemokine receptor CCR6 on immature dendritic cells (iDC) and T-cells

373   (Yang et al., 2000). Human Beta Defensin 4 (HBD4) also exhibits chemoattractant

374   properties toward monocytes (Rodriguez-Martinez et al., 2005). LL-37, like β-defensins,

375   is a receptor ligand; it uses formyl peptide receptor-like 1 (FPRL-1) to orchestrate

376   activities in neutrophils, monocytes and T cells (Anderson, 2000). Adaptive immunity

377   comes into play with the uptake of antigens by iDC, which subsequently mature and

378   traffic to secondary lymphoid tissue to present those antigens to naive T-cells

379   (Banchereau and Steinman, 1998). Thus, by the recruitment of iDC, defensins start the

380   process, then chaperone it onward by the induction of TNF-α and IL-1β production by

381   monocytes (Chaly et al., 2000), a process important for DC maturation. Bovine

382   lactoferrin was shown to enhance both natural-killer (NK) cell activity by increasing IL-18

383   and IFN-γ production (Kuhara et al., 2006) and the function of intraepithelial

384   lymphocytes in small intestine by increasing IL-10 secretion (Takakura et al., 2006).

385   AMPs are able to curtail immune responses as effectively as they stimulate them in

386   order to protect the host from destructive or even lethal inflammation, which occurs

387   when LL-37 neutralizes LPS and lipotechoic acid (Larrick et al., 1995; Mookherjee et al.,

388   2006). The pig cathelicidin, PR-39, though potent against both gram-positive and gram-

389   negative organisms, has anti-inflammatory action when required (Hoffmeyer et al., 2000;

390   Sayama et al., 2005). Moderate inflammation is beneficial to the host but an excessive

391   response can even lead to death. Neutralisation of such a response is effected by

392   inhibition of LPS binding and suppression of LPS-induced TNFα production (Yan and

393   Hancock, 2001).

There are other less well-known roles of AMPs, which are important nonetheless. They have been shown to have anti-tumour activity (Lichtenstein et al., 1986), inhibit fibrinolysis and stimulate fibroblasts and keratinocytes (McDermott et al., 2006; Panyutich et al., 1995; Sayama et al., 2005). They play a role in smooth muscle contraction (Nassar et al., 2002) and LL-37 and α-defensins are implicated in angiogenesis (Koczulla et al., 2003) (Chavakis et al., 2004). The role of LL-37 in wound healing may have important therapeutic implications; LL-37 is found in abundance in skin wounds and anti-LL-37 antibody inhibits re-epithelialization of skin wounds (Heilborn et al., 2003).

3. Antimicrobial peptides during ocular surface infection

The ocular surface, specifically the cornea, has to balance the contradiction of maintaining an immune privileged status and yet warding off a wide-ranging spectrum of microbes. It is not surprising therefore that the ocular surface epithelium produces a multitude of AMPs. Key AMPs at the ocular surface include defensins and cathelicidin. However, other families of AMPs such as RNases, S100As and LEAPs have also been identified but their specific role at this site remains to be fully elucidated.

3.1. Bacterial infection

Bacterial infections of the ocular surface can range from mild conjunctivitis to severe corneal ulcers. Bacterial keratitis is the most frequent cause of corneal disease worldwide and its risk is mainly associated with contact-lens use and trauma (Shin et al., 2016; Willcox, 2007). *Pseudomonas aeruginosa*, an opportunistic gram-negative bacteria, is a frequently isolated bacterium from the extended contact-lens wearers (Fleiszig and Evans, 2010) and it accounts for 70% of bacterial keratitis in the United States (Schein et al., 1989). Chronic hypoxia was initially thought to be the risk factor related to *P. aeruginosa* biofilm formation between the contact lens and corneal epithelial surface (Zaidi et al., 2004). Although lens technology and storage solutions have improved considerably in recent years, the incidence of bacterial keratitis related to contact-lens use is rising (Dart et al., 2008; Radford et al., 2009). Lack of appropriate experimental models has further compounded the challenges to test available therapies against contact lens-related bacterial keratitis (Fleiszig and Evans, 2010).

Genetic models of bacterial keratitis have provided sizeable evidence on the role of innate and adaptive immunity in host response against infection (Gerke and Magliocco, 1971; Hazlett, 2004; Marquart, 2011). There are a number of factors that have been identified when using specific background strains of mice for studying the pathogenesis of *Pseudomonas aeruginosa* keratitis. It has been shown that IL-12 triggered IFN-γ production contributes to corneal destruction in C57BL/6 background of mouse while IL-18-driven IFN-γ induces more bacterial killing but less destruction to host tissue in Balb/C mice (Hazlett, 2005).

The importance of AMPs against bacterial infections was first demonstrated by the development of cathelicidin knockout mice (*Cnlp$^{-/-}$*) (Nizet et al., 2001). Deficiency of cathelicidin enhanced susceptibility to a wide-variety of bacteria including *Escherichia coli* (Chromek et al., 2006), *Neisseria meningitidis* (Bergman et al., 2006; Merres et al., 2014) and *Klebsiella pneumoniae* (Kovach et al., 2012). Importance of cathelicidin in corneal and retinal defense against bacteria has also been demonstrated. Cathelicidin deficiency increased the susceptibility of mice to *P. aeruginosa* keratitis (Huang et al., 2007) and *S. aureus* endophthalmitis (Talreja et al., 2015). Silencing of murine β-defensins (mBDs) with small interfering RNA (siRNA) demonstrated the key role of mBD-2 and mBD-3 (homolog of HBD-2) but not mBD-1 and mBD-4 (homologs of HBD-1 and HBD-3) in ocular defense against *P. aeruginosa* (Wu et al., 2009a; Wu et al., 2009b). Moreover, induction of murine cathelicidin and defensins during *P. aeruginosa* keratitis (Berger et al., 2013) and *E. coli* infection of urinary tract (Lin et al., 2015) has been shown to be dependent on the hypoxia-inducible factor (HIF)-1α transcription factor.

We have previously profiled a spectrum of AMPs in corneal and conjunctival epithelial specimen collected by impression cytology (IC) from patients with bacterial or viral infections. These are compared to AMP expression in corneal epithelial cells that were sourced from healthy adult eyes, cadaver corneal disc and cultured limbal explants. We showed positive expression of 7 out of 21 AMPs investigated. HBD1 and -2, LEAP1 and -2 and LL-37 were shown to be present in all samples tested while HBD3 was only induced during disease conditions (McIntosh et al., 2005). In 2008, we discovered a novel defensin gene, HBD9, which was constitutively expressed on corneal and

conjunctival epithelium of healthy eyes but during infectious keratitis, HBD9 mRNA was expressed at a very low level (Abedin et al., 2008). We also characterized its protein expression in healthy cadaver sections of OS tissue using polyclonal antibody generated against the HBD9 synthetic peptide. A punctate pattern of HBD9 protein in healthy corneal and limbal epithelium was observed. In stromal keratocytes, conjunctival epithelial and globlet cells and cadaver tonsil tissue sections - HBD9 was ubiquitously present (Mohammed et al., 2010). Since HBD3 was induced and HBD9 was decreased during infective keratitis, we further carried out a detailed ex-vivo study wherein we investigated the expression of HBD3 and HBD9 mRNA in IC samples collected from patients with active infection (gram-negative and gram-positive bacterial keratitis (BK)) and after healing. HBD3 mRNA was increased 10-fold during gram-positive BK and 4-fold during gram-negative BK. It then returned to normal control levels after healing. On the other hand, HBD9 mRNA was decreased 5-fold during both gram-negative and gram-positive BK. Notably, it returned to its normal levels after healing of gram-negative BK but not gram-positive BK (Otri et al., 2012). It could be hypothesized that different bacteria utilize specific mechanisms to dampen the host innate immunity. Non-recovery of HBD9 mRNA to its normal levels post-healing of gram-positive BK indicates that these bacteria may have affected the transcriptional regulatory mechanisms of HBD9. Further studies are underway in our laboratory to understand the mechanism of HBD9 mRNA down regulation during bacterial infections. We extended our search for newer AMPs and also studied the expression of ribonuclease-7 (RNase-7), originally found in skin keratinocytes (Harder and Schroder, 2002), in OS epithelial cells at mRNA and protein level. Unlike HBD9, we noted an increased expression of RNase-7 mRNA in infective keratitis samples (**Figure 1**). RNase-7 was mainly localized to the apical layer of OS epithelium with minimal staining noted in stromal keratocytes (Mohammed et al., 2011b). More recently, we further investigated the mRNA expression of AMPs in corneal epithelial cells treated with *P. aeruginosa* and *S. aureus* (from patient ocular isolates). In response to *P. aeruginosa* infection, 5 of the 8 AMPs increased while HBD9 and LEAP-1 consistently showed a reduced expression pattern. During *S. aureus* infection, all AMPs showed a trend towards increased expression. LL-37 and RNase-7 were notably the

485 most responsive against *P. aeruginosa* and HBD2 was highly induced in response to *S.*

486 *aureus* (Dua et al., 2014).

487 Toll-like receptors (TLRs) are evolutionarily conserved pathogen-recognition receptors

488 (PRRs) that are known to recognize and respond to a variety of microbial stimuli, known

489 as pathogen-associated molecular patterns (PAMPs) (Kawai and Akira, 2009). The 'Toll'

490 was first discovered in *Drosophila melanogaster* as a homologue of the pleiotropic

491 interleukin-1 receptor type–I (IL-1R1) (Anderson et al., 1985). The only known function

492 of Toll was then linked to the development of *D. melanogaster* embryo (Hashimoto et al.,

493 1991; Hashimoto et al., 1988). In the late 1990s, the pioneering work from Beutler's

494 group demonstrated that LPS unresponsiveness in C3H/HeJ and C57BL/10ScCr mice

495 was due to a specific mutation in Lps^d allele (that encodes for TLR4) (Poltorak et al.,

496 1998). This led to the discovery that LPS is specifically recognized by TLR4 (Hoshino et

497 al., 1999) and implicated an unequivocal role of TLRs in the immune system. Further

498 work from laboratories of Akira and Medzhitov, who generated multiple TLR and adaptor

499 molecules gene knockout mice, has provided the basis for discovery of specific PAMPs

500 that are recognized by each TLR (Barton et al., 2006; Horng et al., 2002; Kagan et al.,

501 2008; Yamamoto et al., 2003; Yamamoto et al., 2002). In 2011, the Nobel Prize in

502 Medicine and Physiology was conjointly awarded to Bruce A. Beutler and Jules A.

503 Hoffmann for the work related to the TLRs (Nobel-Prize, 2011). To date, it has been

504 shown that the human genome encodes 10 TLRs and mouse genome encodes 13

505 TLRs. TLRs are localized both on cell surface and in endosomal compartments (Kawai

506 and Akira, 2009). In most cell types, TLR2/1, TLR2/6, TLR4 and TLR5 were found to be

507 present on cell surface, whereas TLR3, TLR7, TLR8 and TLR9 localized to endosomes

508 (Kawai and Akira, 2009). When PAMPs bind to the TLR ectodomain they initiate the

509 activation of myeloid-differentiation protein 88 (MyD88)-dependent or MyD88–

510 independent pathways (Barton and Medzhitov, 2003). Although the general scheme for

511 the activation of TLRs pathway is well known, it is only in the last decade that the

512 associated molecular mechanisms have begun to be understood (O'Neill et al., 2013).

513 On OS epithelium, TLRs are essential for first line defense against invading pathogens.

514 In humans and mice, TLR2, TLR4, TLR5 and TLR9 have been widely shown to play an

515 important role in *P. aeruginosa* and *S. aureus* induced corneal inflammation (Chang et

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al., 2006; Huang et al., 2005; Johnson et al., 2005; Pearlman et al., 2008; Sun et al., 2006b; Sun et al., 2010). Protein and mRNA levels of TLR-1 to -6 and -9 were shown to be constitutively present in human conjunctival, limbal and corneal epithelial cells, while those of TLR7, -8 and -10 were shown to be absent (Li et al., 2007). Previously, we surveyed the profile of TLRs 1 to 10 mRNA expression on OS epithelium collected from patients with infective keratitis. In bacterial keratitis specimen, TLR2 and TLR8 mRNA were moderately increased while the level of TLR1, TLR3, TLR5, TLR6, TLR7 and TLR10 remained unchanged. Notably, TLR4 and TLR9 mRNA were down regulated during bacterial infection (Mohammed et al., 2011a). In the same year, McDermott's group also reported a baseline expression profile of TLRs 1-10 in various OS cell types (Redfern et al., 2011). This study has corroborated to our findings on TLR4 and TLR8 mRNA expression in healthy corneal epithelium (Mohammed et al., 2011a). However, the reports on TLR2 and TLR4 expression and localization on OS epithelium has been conflicting. It was demonstrated that LPS treatment of corneal epithelial cells activates TLR4 signaling and modulates cytokine production (Song et al., 2001) while peptidoglycan (PGN) treatment was shown to increase cytokines and HBD-2 secretion in a TLR2 dependent manner (Kumar et al., 2004). In contrast, two studies later reported that both TLR2 and TLR4 are unresponsive to their specific PAMPs on corneal, limbal and conjunctival epithelium (Li et al., 2007; Ueta et al., 2004). Blais and co-workers demonstrated that human tears secrete soluble lipopolysaccharide binding protein (sLBP) and CD14 and suggested these may regulate the LPS responsiveness on corneal epithelium (Blais et al., 2005). Using structural studies, it was confirmed that LPS recognition and response on a cell-surface is mainly mediated *via* a complex of LPS binding protein (LBP)/CD14 and TLR4/MD-2 (myeloid differentiation protein-2) (**Figure 2**) (Park and Lee, 2013; Park et al., 2009). A subsequent study demonstrated that the LPS unresponsiveness on corneal epithelium was related to the lack of MD-2 expression (Lang et al., 2011; Zhang et al., 2009a). Utilizing MD-2 deficient mice, it was shown that interferon-gamma (IFN-γ) produced during *P. aeruginosa* keratitis could induce the MD-2 cell–surface expression and trigger TLR4 responses to LPS on corneal epithelium (Roy et al., 2011). Furthermore, exogenous supply of MD-2 to the human corneal epithelial cell culture was shown to induced cell-surface expression of TLR4 and

547 CD14 and restored the LPS responsiveness (Lang et al., 2011). Moreover, enhanced
548 TLR4 surface expression during disease condition was further confirmed in an
549 experimental dry eye disease model (Lee et al., 2012). It is notable that this regional
550 specialization of TLR expression is not unique to OS epithelium. For example, low levels
551 of TLR4 and MD-2 have also been demonstrated in the intestinal epithelium, which does
552 not normally respond to LPS derived from the commensal microbes of the gut (Abreu et
553 al., 2001). Similarly, it was also reported that the intestinal epithelium is unresponsive to
554 a TLR2 ligand, PGN (Melmed et al., 2003). TLR5 that recognizes flagellin from gram-
555 negative bacteria was shown to be only localized to basal and wing cell layers of corneal
556 epithelium. It was proposed that TLR5 might only respond when the apical squamous
557 layer is breached (Zhang et al., 2003). The variable expression pattern of TLRs at the
558 OS is suggestive of the presence of an immunosilent milieu to contain the excessive
559 inflammatory responses triggered by aberrant TLR stimulation. This is a useful strategy
560 resulting in efficient microbial kill without undue inflammation. With inflammation, the
561 risk of corneal scarring is high.

562 TLRs were shown to be the major inducers of AMP expression in response to bacterial
563 infection on various cell types including corneal epithelium (**Figure 2**). *P. aeruginosa*
564 derived-LPS treatment of corneal and conjunctival epithelial cells (McNamara et al.,
565 1999), gingival keratinocytes (Mathews et al., 1999), tracheobronchial epithelial cells
566 (Becker et al., 2000) and intestinal epithelial cells (Vora et al., 2004) enhanced HBD-2
567 levels. These studies suggested that LPS-mediated TLR4 activation plays an important
568 role in HBD2 induction. TLR2 activation by *S. aureus* or PGN also enhanced HBD-2 but
569 not HBD-1 and HBD-3 levels in corneal (Kumar et al., 2006) and intestinal epithelial cells
570 (Vora et al., 2004). Further studies demonstrated that *S. aureus* lipopeptide (SaLP) but
571 not *S. aureus* protein A (SpA) increases HBD-2 and LL-37 expression in corneal
572 epithelial cells *via* TLR2 activated NF-κB and mitogen-activated protein kinase (MAPK)
573 pathways (Kumar et al., 2007a; Li et al., 2008). As mentioned before, we have
574 previously demonstrated that HBD9 mRNA, which expresses constitutively in normal
575 control epithelial cells, was found to be down regulated in infective keratitis (Abedin et
576 al., 2008; Otri et al., 2012). This intriguing observation led us to further test the effect of
577 potential inducers of HBD9. Of the tested targets, TLR2 was found to be a major inducer

of HBD9 at shorter durations of culture stimulation (Mohammed et al., 2010). In a subsequent study, we further characterized the signaling mechanisms involved in TLR2-induced HBD9 expression. Both NF-κB and MAPK pathways were shown to play an essential role in TLR2 mediated HBD9 induction (**Figure 2**) (Dua et al., 2014). We have further demonstrated that dexamethasone (clinically used for its anti-inflammatory properties) was able to mitigate the TLR2-induced HBD9 expression. MAPK phosphatase-1 (MKP-1; negative regulator of MAPK signaling pathway) was implicated in this inhibitory effect of dexamethasone as tested by RNAi silencing method (**Figure 2**) (Dua et al., 2014). Our results were consistent with previous studies demonstrating an inhibitory effect of dexamethasone on IL-1β (McDermott et al., 2003) and TLR2 (Winder et al., 2009) induced HBD2 expression in corneal and airway epithelium respectively. Activation of TLR5 with purified flagellin from *P. aeruginosa* was shown to induce proinflammatory cytokines and HBD2 and LL-37 expression in corneal epithelial cells (Zhang et al., 2003). It was further demonstrated that pre-exposure of corneal epithelial cells to low-dose flagellin could mitigate pro-inflammatory responses and induced antimicrobial defense against *P. aeruginosa* infection (Kumar et al., 2007b). This phenomenon of endotoxin tolerance was conceived in the 1940s and currently it is being applied as an adjuvant therapy to attenuate the immunopathology associated with septic shock (Albrecht et al., 2008; Cohen, 2002; Nomura et al., 2000). Previous studies have demonstrated that RNase-7 was induced in response to bacterial infections and inflammatory cytokines in a variety of cell types (Koczera et al., 2016; Koten et al., 2009; Reithmayer et al., 2009; Spencer et al., 2014). We have recently showed that IL-1β displays a rapid but transient effect on RNase-7 in CECs (**Figure 2**). Notably, we reported that IL-1β induced RNase-7 expression is dependent on transforming growth factor β activated kinase-1 (TAK-1)-activated MAPKs but not NF-κB signaling (Mohammed et al., 2011b). This intriguing signaling mechanism is not unique to RNase-7 and this dichotomy also exists with other AMPs. In gingival epithelial cells, HBD-2 expression was also shown to be dependent on MAPKs but not NF-κB in response to *Fusarium nucleatum* (Krisanaprakornkit et al., 2002). More commonly, both MAPKs and NF-κB are known to regulate *P. aeruginosa* induced HBD-2 and HBD-3 in skin keratinocytes (Wehkamp et al., 2006) and IL-1β induced HBD-2 in CECs (McDermott et

609 al., 2003). We have also shown that RNase-7 was induced in CECs infected with *P.*
610 *aeruginosa* and *S. aureus* (Dua et al., 2014). We further elucidated the role of TLRs in
611 regulation of RNase-7 mRNA in corneal epithelial cells. As shown in **figure 3**, in
612 response to activators of TLR2 (Pam3CSK4; 1 µg/mL), TLR3 (Poly I:C; 10 µg/mL) and
613 TLR5 (Flagellin; 100 µg/mL) we noted a modest increase in RNase-7 at 1 hour. Notably,
614 at the 24-hour time point there was a 2-fold reduction compared to untreated controls.
615 However, treatment with PAMPs for other TLRs did not effect RNase-7 expression.

616 *Corynebacterium pseudodiphtheriticum* is an opportunistic commensal organism
617 commonly found in skin and upper respiratory tract (Izurieta et al., 1997). Although *CP* is
618 an uncommon cause of OS infection, a first case of such infection was reported in an
619 immunocompromised patient in Australia (Li and Lal, 2000). Subsequent case studies
620 have also reported other strains of *Corynebacterium spp.* as causative of OS infection
621 (Giammanco et al., 2002; Ruoff et al., 2010; Todokoro et al., 2015). Recently, it was
622 reported that 5% of total bacterial keratitis cases at a single center in India are
623 diagnosed as *Corynebacterium* keratitis and a number of reported cases has been rising
624 in the last decade (Ramesh et al., 2010). A recent study has reported an increased
625 expression of TLR1 to -4, cytokines and antimicrobial peptides such as HBD1, S100A8
626 and S100A9 in corneal scrapings from *Corynebacterium* keratitis patients. These were
627 further substantiated in corneal epithelial cell cultures treated with *Corynebacterium spp.*
628 (Roy et al., 2015).

629 *Mycobacterium tuberculosis* primarily infects lungs and it remains the most common
630 cause of infection related high mortality worldwide (Dirlikov et al., 2015). Extra-
631 pulmonary infections are commonly reported in more than 50% of patients with
632 tuberculosis (Gupta et al., 2007). Both intraocular and ocular surface infections due to
633 *M. tuberculosis* are uncommon but increased incidence of multi-drug resistant strains of
634 *Mycobacterium spp.* has amplified the challenges for its treatment (Gupta et al., 2007).
635 AMPs have been recently shown to possess strong anti-mycobacterial properties and it
636 has been suggested that they could play an important role in tuberculosis control (Dong
637 et al., 2016; Fu, 2003; Ganz, 2002). Recent studies have shown that the human corneal
638 fibroblasts (Castaneda-Sanchez et al., 2013) and human macrovascular endothelial
639 cells (Garcia-Perez et al., 2011) increased production of HBD-1 to 3 in response to

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640   infection with *M. tuberculosis* and non-tuberculous strains such as *M. abscessus and M.*
641   *smegmatis*. Similarly, elevated levels of HBD-2 and HBD-3 but not LL-37 were also
642   been reported in skin specimen collected from patients with cutaneous tuberculosis
643   (Zhao et al., 2016b).   *M. tuberculosis* infection of macrophages was shown to
644   specifically induce HBD4 (Liu et al., 2009). RNase-3 and RNase-7 were shown to
645   display a potent microbicidal activity against *M. vaccae* (Pulido et al., 2013).
646   Furthermore, an amino acid, L-isoleucine was specifically shown to induce expression of
647   defensins *in-vivo* in an experimental animal model of pulmonary tuberculosis (Fehlbaum
648   et al., 2000; Rivas-Santiago et al., 2011). This suggests that the administration of
649   recombinant/synthetic AMPs or the pharmacological induction of AMPs could be
650   beneficial in combating sight-threatening ocular infections. This potential needs to be
651   explored further in in-vitro and in-vivo studies.

3.2. Viral infection

653   Defensins have been shown to display antiviral properties against both enveloped and
654   non-enveloped viruses. Antiviral property of α-defensins is chiefly dependent on the
655   stability of their 3D-structure, i.e., disulphide bonds. It was shown that α-defensins in
656   linear form demonstrated reduced antiviral activity against HSV-1, influenza virus,
657   adenoviruses and HIV-1 (Demirkhanyan et al., 2012; Rapista et al., 2011; Salvatore et
658   al., 2007; Smith et al., 2010). However, β-defensins showed similar antiviral activity in
659   either forms (Nigro et al., 2015; Scudiero et al., 2010). The fundamental differences in
660   antiviral activity of both defensin groups needs to be clarified. It could be suggested from
661   these studies that α-defensins exhibit their antiviral property due to their amphipathic
662   structure or their intrinsic ability to oligomerize rather than their net positive charge. It
663   was also reported that the antiviral activity of HBD-2 and HBD-3 against HIV (Quinones-
664   Mateu et al., 2003) and human α-defensin 5 (HD-5) binding to adenovirus (Gounder et
665   al., 2012) is salt-dependent. Defensins are known to exhibit antiviral property by direct
666   neutralization of enveloped viruses through lipid bilayer interaction or their affinity
667   towards envelope glycoproteins (e.g. HIV-1 and HSV). Human neutrophil peptide-2
668   (HNP-2), an α-defensin, interferes with gp120 glycoprotein interaction with CD4 receptor
669   on T-cells and prevents the initial phase of HIV infection (Furci et al., 2007). HBD3 and
670   HD5 but not HBD1 and HBD2 were shown to inhibit both HSV-1 and HSV-2 entry into

671   host cells by binding gB glycoprotein of HSV (Hazrati et al., 2006). Similarly, HBD2 was

672   shown to exhibit antiviral activity against enveloped respiratory syncytial virus (RSV) by

673   damaging its lipid bilayer (Kota et al., 2008). This activity of defensins is favored by its

674   interaction with positively charged phospholipids hence viruses with inert bilayers

675   (Lorizate and Krausslich, 2011) might show tolerance to defensins. A mechanism

676   involving protein-protein interaction for neutralization of non-enveloped viruses (e.g.

677   Adenoviruses and human papillomavirus) was attributed to HD5 (Buck et al., 2006; Flatt

678   et al., 2013; Tenge et al., 2014). Such variations account for the differential susceptibility

679   of viruses to defensins. The antiviral mechanism of defensins is well described and

680   readers are directed to the following review paper for more information (Wilson et al.,

681   2013).

682   Clinical manifestations of OS viral infections vary greatly depending on the disease-

683   causing viruses (Dua, 2000; Otri et al., 2013). The changes induced by viruses on OS

684   range from benign, self-limiting conjunctivitis to vision threatening ulceration and

685   vascularization. Herpes simplex virus-1 (HSV-1) and adenoviruses (A to G serotypes)

686   are the most common causes of OS viral infections (Dua, 2000; Mukherjee et al., 2015).

687   Ocular manifestations due to other viruses such as West Nile virus (Blitvich et al., 2016),

688   Zika virus (Miner et al., 2016), Varicella-Zoster virus (Khalafallah et al., 2013; Liesegang,

689   1999) and Human Immunodeficiency virus-1 (HIV-1) (Baranwal et al., 2015; Biswas and

690   Sudharshan, 2008) have also been reported.

691   HSV-1 infections are highly prevalent and a majority of the world's population carries

692   virus as a latent load in the trigeminal ganglion (Dua, 2000; Maroui et al., 2016). New

693   cases of ocular HSV-1 infection have been estimated annually at 11.8 per 100,000 in

694   the USA (Young et al., 2010). In Europe, France has a high incidence of HSV-1 cases

695   estimated at 31.5 per 100,000 (Labetoulle et al., 2005). Thus, HSV-1 ocular infections

696   represent a significant burden globally. Primary HSV-1 ocular infections are rare and

697   can be restricted to blepharoconjunctivitis (inflammation of conjunctiva and eyelids) with

698   or without keratitis. When cornea is involved, this is typically referred to as 'Infectious

699   Epithelial Keratitis (IEK)' (Rowe et al., 2013). Clinical symptoms of IEK include pain,

700   photophobia, blurred vision, excessive tearing and redness. In IEK, the corneal lesion

701   starts as punctate vesicular eruptions and often progresses to non-linear geographic

lesions (Green and Pavan-Langston, 2006). More commonly, HSV-1 ocular infections can result from reactivation of latent virus that was originally established in the trigeminal ganglion following non-ocular infection (Maroui et al., 2016; Toma et al., 2008). Recurrent HSV-1 infection can present with dendritic ulcers, which represent active viral replication, or immune mediated stromal keratitis. 'Herpes Stromal Keratitis (HSK)' can occur with or without epithelial involvement and can be further sub-divided into two categories: necrotizing and non-necrotizing (Rowe et al., 2013). The former is a severe vision threatening infection and often managed surgically, whereas non-necrotizing HSK causes stromal inflammation without epithelial defect and is frequently referred to as immune keratitis or disciform keratitis (Knickelbein et al., 2009; Rowe et al., 2013). Studies employing mouse model have provided substantial evidence of the role of macrophages (Cheng et al., 2000), dendritic cells (Jiang et al., 2015), natural killer cells (Carr et al., 2008) and neutrophils (Tumpey et al., 1996) in HSV-1 OS disease pathogenesis. All of these cell types are known to contribute in HSV-1 clearance from the cornea. HSV-1 infection of corneal epithelial cell cultures has been reported to show expression of cytokines and interferons *via* activation of TLR7 (Li et al., 2006) and TLR9 (Takeda et al., 2011). HSV-1 recognition by immune cells is mainly mediated *via* TLR3, TLR7 and TLR9 (Hochrein et al., 2004; Krug et al., 2004; Sarangi et al., 2007; Taube et al., 2015). Activation of these TLRs was shown to induce production of type-I IFNs, proinflammatory cytokines and chemokines, resulting in further recruitment of immune cells (Yang et al., 2005; Zhang et al., 2007). T-cells, in particular, CD4^{+} subsets have been shown to be the principal mediators of HSK immunopathology (Gangappa et al., 1999; Lepisto et al., 2006). Whilst CD8^{+} T-cells are involved in immune surveillance of HSV-1 infected neurons in trigeminal ganglia and prevent virus reactivation from latency (Liu et al., 2000; St Leger et al., 2011).

Adenoviruses are mainly responsible for 75% of cases of conjunctivitis worldwide (Jhanji et al., 2015). Adenoviral ocular infections are presented as epidemic keratoconjunctivitis (EKC; involves both cornea and conjunctiva) whereas isolated adenoviral conjunctivitis without corneal involvement is also reported (Jhanji et al., 2015). The National Surveillance Centre reported about 1 million cases per year of EKC in Japan alone (Aoki and Tagawa, 2002; Kaneko et al., 2011). Other forms of adenoviral infections termed as

733 pharyngoconjunctival fever (PCF) also involves the conjunctiva. Subepithelial multifocal
734 cellular infiltrates and formation of pseudomembranes are two common complications
735 associated with EKC (Chintakuntlawar and Chodosh, 2010). The former may cause
736 visual impairment if it involves the visual axis with persistent subepithelial opacity.
737 However, lack of effective antiviral agents has made the treatment of adenoviral
738 infections difficult. Clinically it is often managed by topical steroids to reduce associated
739 inflammation (Viswalingam, 1993). Adenoviruses are in majority sensed by TLRs such
740 as TLR2, -4, -7, -8 and -9 (Blasius and Beutler, 2010; Fejer et al., 2011; Huang and
741 Yang, 2009). Interaction of DC with adenoviruses activates TLR9-mediated type I IFN
742 responses (Fejer et al., 2008), whereas TLR2, 4 and 9 on macrophages elicits IL-12,
743 MCP-1 and RANTES production (Nociari et al., 2009; Yamaguchi et al., 2007).
744 However, recent studies have reported that the double-stranded DNA (dsDNA) of
745 adenoviruses could also induce type-I IFN and proinflammatory cytokines without
746 activating TLR-dependent pathways (Hendrickx et al., 2014; Ishii et al., 2006; Nociari et
747 al., 2007). Animal models of adenovirus keratitis have provided considerable evidence
748 of neutrophils, macrophages and dendritic cells in disease pathogenesis
749 (Chintakuntlawar et al., 2007; Hamrah and Dana, 2007; Ramke et al., 2016). Corneal
750 fibroblasts from human and mouse were also shown to contribute towards
751 immunopathology by increase production of IL-8, IL-6, IP-10 and MCP-1 in response to
752 adenovirus infection (Chodosh, 2006; Natarajan et al., 2003; Rajaiya et al., 2008; Xiao
753 and Chodosh, 2005).

754 Although as mentioned above that defensins possess potent antiviral activity, it was not
755 known whether OS infection due to adenovirus or HSV has any effect on AMP
756 production. Our group was the first to demonstrate the low levels of HBD-9 during viral
757 keratoconjunctivitis (Abedin et al., 2008). This interesting result led us to further test the
758 expression of known AMPs and TLRs during OS diseased conditions including viral
759 keratoconjunctivitis. Of 6 AMPs tested, we demonstrated an increased expression of LL-
760 37 and LEAP-1 (also known as hepcidin) (Mohammed et al., 2011a). In similar samples,
761 we also showed elevated levels of TLR2, TLR7, TLR8 and TLR10 mRNA (Mohammed
762 et al., 2011a). As there is no known ligand for TLR10 available and it is likely that TLR10
763 could play an essential role during viral infections, at least on OS; It is imperative to

764 further validate the role of TLR10 during viral infections using gene-knockout mice or
765 siRNA-mediated TLR10 knockdown. A recent study has confirmed the anti-inflammatory
766 role of TLR10 demonstrating direct inhibition of TLR2 responses and increase IL-1R
767 antagonist production (Oosting et al., 2014). Similarly, TLR10 knockdown in a monocytic
768 cell line (THP-1) was shown to attenuate the proinflammatory cytokines that are induced
769 in response to activation of TLR2, TLR4 and TLR5 (Le and Kim, 2016). In HSK diseased
770 corneas, increased mRNA expression of TLR4, 7, 8 and 9 was also reported, whereas
771 TLR7 mRNA was only shown to be elevated in non-active HSK cornea (Jin et al.,
772 2007a). Together, these studies indicate an unequivocal role of TLRs in HSV-1 disease
773 pathogenesis. A recent study showed that LEAP-1 was reduced in response to hepatitis
774 C virus (HCV) infection of primary hepatocytes (Liu et al., 2012). In contrast, IL-6 was
775 shown to induce LEAP-1 expression both in-vitro in human hepatocytes (Wrighting and
776 Andrews, 2006) and in-vivo in mouse liver (Pietrangelo et al., 2007). HSV-1 infection of
777 corneal epithelial cells and corneal fibroblast was also shown to induce IL-6 levels in a
778 TLR-dependent manner (Hayashi et al., 2006). From these studies, it could be
779 postulated that elevated levels of LEAP-1 in viral keratoconjunctivitis specimen could be
780 mediated *via* IL-6. However, this needs to be further validated in a separate study and
781 potentially tested in an animal model of viral keratoconjunctivitis. We have also studied
782 and elucidated   RNase-7 mRNA expression in viral keratoconjunctivitis specimen. We
783 noted a modest increased in RNase-7 mRNA levels in viral keratoconjunctivitis samples
784 (**Figure 1**). A recent study has also demonstrated an induced expression of RNase-7 in
785 keratinocytes infected with dengue virus (Surasombatpattana et al., 2011). RNase-1, -2,
786 -3 and -5 are shown to exhibit strong antiviral activity against RSV and HIV-1 (Bedoya et
787 al., 2006; Domachowske et al., 1998a; Domachowske et al., 1998b; Koczera et al.,
788 2016). However, the antiviral activity of RNase-7 against HSV needs to be investigated.

789 ### 3.3.Fungal infection

790 Fungi are opportunistic pathogens and are recognized as the commonest cause of
791 ocular morbidity in sub-tropical countries (Shah et al., 2011). In such regions, fungal
792 keratitis may constitute up to 50% of all cases of ulcerative keratitis (Saad-Hussein et
793 al., 2011). It may occur secondary to trauma, any vegetative injury and contact-lens
794 wear (Klotz et al., 2000). Fungal keratitis is also common in immuno-compromised

795   patients and in those suffering from chronic dry eye (Klotz et al., 2000). More recently,

796   fungal keratitis cases in temperate regions such as UK have risen and its risk is mainly

797   associated with contact-lens use and ocular surface trauma. A recent retrospective

798   study has reported an upward trend of FK incidence from 4.5 cases per year (between

799   1994 to 2006) to 14 cases per year (between 2007 to 2014) at a single centre in the UK

800   (Ong et al., 2016). More than 70 species of fungi are known to cause ocular mycoses.

801   *Candida, Aspergillus and Fusarium* species are common causative organisms (Said et

802   al., 2011). Clinically, fungal keratitis presents as dry and raised satellite lesions with a

803   feathery border, stromal infiltration, endothelial plaque and hypopyon. However, mixed

804   bacterial and fungal infections and severe fungal infections that resemble *Pseudomonas*

805   keratitis are difficult to diagnose (Said et al., 2011). Management of FK is challenging

806   due to several factors such as delayed diagnosis, limited availability of broad-spectrum

807   antifungal agents, and poor corneal penetration of available antifungals (Said et al.,

808   2011). Therefore, it is warranted that alternative antifungal treatment modalities for sight-

809   threatening fungal infections are identified.

810   AMPs offer promise due to their distinct antifungal properties. Both native and synthetic

811   peptides of AMPs have been developed and studied extensively for their antifungal

812   properties. The α-defensins have been shown to exhibit potent fungicidal activity against

813   *Aspergillus fumigatus* (Levitz et al., 1986)*, Cryptococcus neoformans* (Alcouloumre et

814   al., 1993) *and Candida albicans* (Edgerton et al., 2000). The candidacidal activity of

815   HNP-1 and histatin-5 (found in human saliva) was attributed to their non-lytic release of

816   mitochondrial ATP and metal chelation (Edgerton et al., 2000; Puri and Edgerton, 2014).

817   HBD-1, -2 & -3 and LL-37 have also been shown to display antifungal effects against

818   *Candida spp.* (Durnas et al., 2016; Joly et al., 2004; Krishnakumari et al., 2009; Vylkova

819   et al., 2007b). Both β-defensins and cathelicidin have been shown to induce yeast cell

820   death by affecting membrane permeabilisation (den Hertog et al., 2005; Durnas et al.,

821   2016; Krishnakumari et al., 2009). HBD3 and LL-37 are also known to bind to the *C.*

822   *albicans* cell wall component, β-1, 3-exogluconase Xog1p, thereby reducing its infectivity

823   (Chang et al., 2012). Recent studies have reported the potent candidacidal activity of

824   RNase-3 and RNase-7 at a low micromolar concentration (Harder and Schroder, 2002;

825   Koczera et al., 2016; Salazar et al., 2016). They have been shown to exhibit a dual

mechanism of action against *C. albicans, namely* membrane destabilization and perturbation of cellular RNA (Salazar et al., 2016). A recent study has demonstrated the fungicidal role of psoriasin in protection of psoriasis lesions against fungal infection (Hein et al., 2015). Notably, a reduced form of psoriasin (a linear peptide) showed activity against *A. fumigatus* but not *C. albicans*. The mechanism of fungicidal action was imparted *via* zinc chelation and induction of fungal apoptosis (Hein et al., 2015). Similar to psoriasin (S100A7), calprotectin (S100A8/A9 dimer) has also been shown to exhibit antimicrobial activity against a range of pathogens (Clohessy and Golden, 1995; Damo et al., 2013; Zackular et al., 2015). A recent study has demonstrated the essential role of calprotectin-derived from neutrophils in *Aspergillus* keratitis using S100A9 knockout mice (Clark et al., 2016). It was also shown that recombinant calprotectin inhibited hyphal but not conidial growth of *A. fumigatus* (Clark et al., 2016).

Similar to gram positive bacteria (Frick et al., 2003; Omardien et al., 2016; Peschel and Sahl, 2006), fungi have also developed numerous escape mechanisms against AMPs (Swidergall and Ernst, 2014). In particular, *C. albicans* has been shown to evade histatin-5 effect *via* three mechanisms: influx/efflux pumps, activation of stress-response pathways and secretion of proteases (Swidergall and Ernst, 2014). Firstly, *C. albicans* allows intracellular uptake of histatin-5 by influx transporters Dur3/Dur31 (Kumar et al., 2011). Once inside, histatin-5 induces ATP efflux, which triggers the formation of reactive oxygen species (ROS) and further activation of the HOG (high osmolarity glycerol) stress-response pathway (Vylkova et al., 2007a). Ultimately, it leads to extrusion of histatin-5 *via* efflux transporter, Flu1 (Li et al., 2013) and subsequent inactivation by the secreted aspartate proteases (SAP 9/10) when outside the cell (Puri and Edgerton, 2014; Swidergall and Ernst, 2014). *C. albicans* has also been shown to evade other AMPs such as LL-37, HNP-1, histadin-5 and HBD1 *via* secreted glycosylated exodomain protease, Msb2 (Puri et al., 2015; Swidergall et al., 2013; Szafranski-Schneider et al., 2012).

TLR2, 4 and 9 are known to recognize and become activated in response to the fungal PAMPs such as mannans, β-glucan, zymosan and fungal DNA (Romani, 2011; Smeekens et al., 2013). Other innate immune receptors such as nucleotide oligomerization domain 2 (NOD2), galectin 3, complement receptor 3 (CR3) and dectin-

29

857　1 have also been shown to play an essential role in eliciting innate immune response
858　against fungi (Netea et al., 2008; Xu et al., 2012; Zhao et al., 2016a; Zhu et al., 2015).
859　Recent studies have indicated that a polymorphism in TLR4 is linked to pulmonary
860　aspergillosis (Bochud et al., 2008) and systemic candidiasis (Van der Graaf et al., 2006),
861　whereas a point mutation in TLR9 increases the risk to allergic bronchopulmonary
862　aspergillosis (Smeekens et al., 2013). Single nucleotide polymorphism in TLR1
863　(Plantinga et al., 2012), TLR2 (Woehrle et al., 2008) and TLR3 (Nahum et al., 2011)
864　genes have also been reported to increase the risk of candidiasis but these results have
865　not yet been confirmed in larger cohorts.

866　At the OS, TLR2 and TLR4 have also been implicated in host response against
867　*Fusarium solani* (Jin et al., 2008; Jin et al., 2007b), *A. fumigatus* (Guo and Wu, 2009; Jie
868　et al., 2009; Zhao and Wu, 2008) and *C. albicans* (Yuan and Wilhelmus, 2010) infection.
869　TLR5 has not being directly implicated in fungal keratitis, but its exogenous activation
870　with flagellin has been shown to be protective against *C. albicans* infection. Notably, this
871　was mediated through murine cathelicidin and neutrophils (Gao et al., 2011) and in
872　another study through CXCL10 producing CXCR3 positive natural killer (NK) cells (Liu et
873　al., 2014). In a *C. albicans* keratitis model, Yuan and co-workers have demonstrated an
874　increased level of cathelicidin and reduced levels of mBD1 and -2 mRNA (Yuan et al.,
875　2010). Similarly, reduced level of mBD1 has also been reported during oral candidiasis
876　(Tomalka et al., 2015). Polymorphism in human DEFB1 gene (HBD1) has been
877　implicated in susceptibility to oral candidiasis (Jurevic et al., 2003). A recent study has
878　correlated the role of mBD3 to increased susceptibility of CCAAT/Enhancer binding
879　protein-β (C/EBPβ) transcription factor knockout mice to systemic candidiasis (Simpson-
880　Abelson et al., 2015). Similarly, genetic deficiency of cathelicidin has been shown to
881　increase the disease severity of *C. albicans* keratitis (Gao et al., 2011). Using human
882　CECs, increased levels of LL-37, HBD2 and HBD3 have also been reported in response
883　to heat-killed *C. albicans* (Hua et al., 2014). Collectively, these studies indicate an
884　important role of AMPs in host immunity against *C. albicans* infection. During *F. solani*
885　corneal infection, increased expression of murine cathelicidin, murine β-defensin 3
886　(mBD3) and mBD4 have been reported (Kolar et al., 2013). Using siRNA knockdown

887 and genetic knockout mice, this study has also established that mBD-3 and -4 and
888 cathelicidin play an important role in defense against *F. solani* (Kolar et al., 2013).

889 3.4. Protozoan infection

890 Acanthamoeba is a free-living protozoan that is capable of causing vision debilitating
891 corneal infection. The infective form is the motile trophozite, and a dormant form exists
892 as a cyst. Acanthamoeba trophozoites feed on bacteria, fungi and other organism to
893 replicate and propagate. Trophozoites revert to a cyst form during adverse changes of
894 pH, temperature, nutrient supply and desiccation. Risk of acanthamoeba keratitis (AK) is
895 related to contact-lens wear and corneal injury (Otri et al., 2013). A recent genetic study
896 has reported 19 genotypes (T1 to T19) of acanthamoeba (Corsaro et al., 2015) and T4
897 genotype has been frequently isolated from AK patients (Derda et al., 2015). In the
898 developed world, poor hygiene and improper storage of contact-lenses has been
899 identified as risk factors for contact-lens related AK (Cope et al., 2016; Radford et al.,
900 2002). Clinically, AK is associated with severe pain, blurred vision, watery eyes,
901 photophobia, hypopyon, diffuse inflammation and in 50% of patients ring-like corneal
902 infiltrates are also present (Sun et al., 2006a; Tu et al., 2008). Diagnosis and treatment
903 of AK is challenging and if not treated early, could lead to vision loss (Dua et al., 2009).
904 During the early stage, AK is often confused with herpes keratitis and when the patient
905 presents at an advanced stage it is mistaken for fungal keratitis (Alkharashi et al., 2015).
906 To compound matters, AK also presents as mixed infection due to its symbiotic
907 relationship with bacteria and fungi (Iovieno et al., 2010). Currently, there are no
908 licensed drugs available for treatment of AK. Most often it is aggressively managed by
909 combination of antibiotics, antifungals and topical biguanides (Azuara-Blanco et al.,
910 1997; Dart et al., 2009; Otri et al., 2013). Penetrating keratoplasty is required when
911 medical treatment is unsuccessful or when AK has advanced to the formation of
912 extensive corneal abscess or perforation (Alkharashi et al., 2015; Azuara-Blanco et al.,
913 1997).

914 Both innate and adaptive immune systems have been shown to play a pivotal role in
915 host immunity against acanthamoeba. Macrophages and neutrophils are key players in
916 AK and are shown to kill both trophozoites and cysts (Hurt et al., 2003b; Stewart et al.,

917 1992). Immunoglobulin A (IgA) induced immune responses have been shown to be
918 protective against acanthamoeba. IgA has been shown to specifically facilitate
919 complement activation and neutrophil-mediated killing of acanthamoeba (Stewart et al.,
920 1992; Stewart et al., 1994) but there are contrasting reports on the lytic activity of
921 complement against acanthamoeba (Pumidonming et al., 2011; Toney and Marciano-
922 Cabral, 1998). Mannose-induced cytolytic proteins, acanthamoeba plasminogen
923 activator (aPA) (Alizadeh et al., 2007; Hurt et al., 2003a; Tripathi et al., 2014; Yang et al.,
924 1997) and sIgA (Said et al., 2004) have also been implicated in AK disease
925 pathogenesis. Acanthamoeba was shown to establish corneal infection *via* a major
926 virulence protein, mannose-binding protein (MBP), which mediates adhesion to the
927 corneal surface (Garate et al., 2006b; Panjwani, 2010). Oral immunization of animals
928 with MBP but not aPA prior to infection was shown to reduce the AK disease severity
929 (Alizadeh et al., 2007; Garate et al., 2006a; Hurt et al., 2003a). More recently, the role of
930 TLRs as innate immune sensors of *Acanthamoeba spp.* has been demonstrated. Using
931 *in-vitro* and *in-vivo* AK models, TLR4 has been shown to play an essential role in OS
932 immunity against *Acanthamoeba spp.* (Alizadeh et al., 2014; Pan and Wu, 2012; Ren et
933 al., 2010; Ren and Wu, 2011)*.* Similarly, an increased expression of TLR2 and TLR4
934 was also reported in *Acanthamoeba* T4 strain infected murine lungs (Derda et al., 2016)
935 and brain (Wojtkowiak-Giera et al., 2016). Mattana and co-workers have recently
936 demonstrated that acanthamoeba are capable of countering host inflammatory
937 responses by inducing IL-10 production from effector cells (Mattana et al., 2016).

938 AMPs derived from animal sources such as magainins, gomesin and trialysin have been
939 shown to be effective against trophozoites and cysts of *Acanthamoeba spp.* (Ondarza,
940 2007; Sacramento et al., 2009; Schuster and Jacob, 1992). However, acanthamoeba
941 trophozoites have been shown to secrete proteases (Ondarza, 2007) that impair the
942 host responses (Na et al., 2002) including proteolysis of certain AMPs (Sacramento et
943 al., 2009). A recent study has demonstrated that activation of protease-activated
944 receptor-2 (PAR2) on corneal epithelial cells in response to trophozoite protease (aPA)
945 elicits IL-8 production (Tripathi et al., 2014). It was suggested that inhibition of PAR2
946 would alleviate the inflammation associated with AK.

947   We have previously demonstrated that HBD9 mRNA was found to be low in AK patient
948   specimen (Abedin et al., 2008). In subsequent study, we also profiled the expression of
949   other AMPs such as HBD1 to 3, LL-37 and LEAP1 & 2 in samples obtained from AK
950   patients. Unlike HBD9, we did not observe any significant changes in levels of tested
951   AMPs (Mohammed et al., 2011a). We have also investigated the expression of RNase-7
952   in AK specimen (n=3) and as shown in **figure 1,** we noted 3-fold increase in RNase-7
953   levels. The unusual trend of AMPs in AK specimen led us to further characterize the
954   AMPs expression in *in-vitro* CEC cultures exposed to live *A. castellanii* trophozoites (Otri
955   et al., 2010). HBD3 was increased 10 fold, whereas HBD2, LEAP1 & 2 and RNase-7
956   were increased up to 4 fold. LL-37 was slightly increased and HBD1 showed a reduced
957   pattern for all time-points of treatment. HBD9 showed a trend of decreased expression
958   for first 6 hours and modestly increased to 1.5 times at 9 hours (Otri et al., 2010). Unlike
959   in samples taken from AK patients, in-vitro infection study showed variable AMPs
960   expression suggesting that the trophozoite-induced endogenous factors present in the
961   milieu of diseased cornea would have dampened the AMPs production n-vivo. Although
962   we were first to provide the profile of known human AMPs in response to acanthamoeba
963   infection, it is still unknown whether these possess amoebicidal activity. Studies to
964   improve our understanding of the role of AMPs against acanthamoeba such as to
965   establish the role of TLRs or PARs in induction/reduction of AMPs in response to
966   acanthamoeba infection and to elucidate the amoebicidal activity of human AMPs in
967   combination with other potent AMPs or protease inhibitors are warranted

968   Trypanosoma infections are commonly endemic in Central and South America and
969   globally affect about 25 million people (Soares-Silva et al., 2016; Tanowitz et al., 1992).
970   *Trypanosoma cruzi* is responsible for causing Chagas' disease. It occurs when an
971   infected reduviid bug (*Triatoma infestans*) bites humans. The parasite is transmitted to
972   the ocular surface either by systemic dissemination of trypomastigotes or by direct
973   inoculation when the insect bites near the orbit (Klotz et al., 2000). *Trypanosoma cruzi*
974   derived glycosylphosphatidylinositol (GPI) anchor protein is specifically recognized by
975   TLR2 on APCs leading to activation of adaptive immunity against the parasite (Gil-
976   Jaramillo et al., 2016; Tarleton, 2007). In addition, TLR4 and TLR9 have also been
977   implicated in recognition of *T. cruzi* (Bafica et al., 2006; Kayama et al., 2009; Koga et al.,

978   2006). Mice deficient in UNC93B1, an essential protein that is required for TLR3, TLR7

979   and TLR9 activation, are highly susceptible to infection with *T. cruzi* (Caetano et al.,

980   2011; Fukui et al., 2009). These studies indicate the critical role of TLRs in host

981   resistance to *T. cruzi* infections. Human α-defensins such as HD-1 (Madison et al.,

982   2007) and HD-5 (Tanaka et al., 2010), equine cathelicidin (eCATH1) (Cauchard et al.,

983   2016) and bovine AMP (BMAP18) (Haines et al., 2009) were shown to exhibit strong

984   anti-protozoan activity against *Trypanosoma spp*. Furthermore, a recent study has

985   demonstrated an increased expression of HD1 from colonic epithelial cells in response

986   to *T. cruzi* infection (Johnson et al., 2013). The anti-parasitic activity of AMPs against

987   *Trypanosoma brucei* (McGwire et al., 2003) *Cryptosporidium parvum* (Zaalouk et al.,

988   2004) *Toxoplasma gondii* (Morampudi et al., 2011), *Leishmania major* (Dabirian et al.,

989   2013) and microsporidia species (Leitch and Ceballos, 2009) is well known. However,

990   we did not find any reports on the effect of AMPs on *T. cruzi.* The effect of *T. cruzi*

991   infection on OS epithelial cells and AMP production also remains to be elucidated. One

992   reason for lack of AMPs study would be that trypanosoma ocular infections are

993   uncommon in the developed world and are only endemic in Latin America (Requena-

994   Mendez et al., 2016; Tanowitz et al., 1992).

4. Exogenous induction of AMPs for OS disease treatment

996   Rapid emergence of antibiotic-resistant pathogens and steady decline in development of

997   newer antibiotic agents has contributed towards the rise in number of deaths due to

998   infectious diseases globally (Mortality and Causes of Death, 2015, 2016; Spellberg et al.,

999   2008). Alternative approaches such as direct application of AMPs singly or in

1000   combination and exogenous induction of host AMPs to treat infectious diseases need to

1001   be pursued. As mentioned above, AMPs are produced constitutively at the mucosal

1002   surfaces or modulated in response to microbial infection. Numerous studies have

1003   demonstrated the potency of AMPs against a range of microbes. AMPs have also been

1004   shown to enhance the efficacy of conventional antibiotics against *P. aeruginosa* biofilms

1005   (Dosler and Karaaslan, 2014) and MRSA biofilms (Mataraci and Dosler, 2012). TLR

1006   activation in response to microbial infection induces AMP expression that enables

1007   clearance of pathogens (Ganz, 2003; Stolzenberg et al., 1997; Zasloff, 2002) and

1008   accelerates wound healing (Mangoni et al., 2016). Exogenous activation of TLR5 with

1009 flagellin has been shown to protective against *P. aeruginosa* (Kumar et al., 2007b) and
1010 *C. albicans* keratitis (Gao et al., 2011; Liu et al., 2014) and also enhanced wound
1011 healing (Gao et al., 2010) by inducing LL-37, HBD2 and CXCL10 levels.

1012 Exogenous induction of AMPs during disease conditions could be beneficial to host
1013 tissue. This might reduce the unwanted inflammation from proinflammatory cytokines
1014 that are produced particularly in response to TLR activation. One of the first non-TLR
1015 exogenous inducer of AMPs identified was L-isoleucine, an essential amino acid (Figure
1016 4), which has been shown to induce β-defensins in bovine kidney epithelial cells
1017 (Fehlbaum et al., 2000). L-isoleucine induced mBD3 & -4 has been shown to be
1018 protective in a late-stage multi-drug resistant tuberculosis mouse model (Rivas-Santiago
1019 et al., 2011). L-isoleucine was shown to be non-toxic and also increased HBD2 in lung
1020 epithelial cells (Rivas-Santiago et al., 2011). Another exogenous inducer is sodium
1021 butyrate (BA), a short chain fatty acid, which was shown to induce LL-37 expression and
1022 provided protection against shigellosis (Raqib et al., 2006; Schauber et al., 2003).
1023 Similarly, phenylbutyrate (PBA) alone and in synergy with vitamin D3 was also shown to
1024 induce LL-37 expression in a variety of human cell lines (Figure 4) (Steinmann et al.,
1025 2009). Jiang and co-workers have demonstrated that hexanoate, heptanoate and
1026 valerate are more potent than butyrate in promoting LL-37 expression (Jiang et al.,
1027 2013). However, the molecular mechanisms associated with LL-37 induction are unclear
1028 and possibly it could be a result of *de novo* protein synthesis through unknown factors.
1029 Entinostat, a histone deacetylase inhibitor was shown to induce LL-37 and HBD1 but not
1030 HBD2 in HT-29 cells (Miraglia et al., 2016; Ottosson et al., 2016). It was also
1031 demonstrated that STAT3 (signal transducer and activator of transcription-3) and HIF-1α
1032 (hypoxia-inducible factor-1α) are essential for LL-37 induction by Entinostat (Figure 4)
1033 (Miraglia et al., 2016). Fan and co-workers have recently demonstrated that commensal
1034 anaerobic bacteria of gut induce LL-37 *via* activation of HIF-1α and provide protection
1035 against *C. albicans* colonization (Fan et al., 2015). Furthermore, HIF-1 targeting drugs
1036 are in clinical trials for anemia and other infectious diseases (Bhandari and Nizet, 2014).

1037 Vasoactive intestinal peptide (VIP) is an essential neuropeptide that plays a bidirectional
1038 role in communication between immune and neuronal systems (Delgado et al., 2004). It
1039 is secreted by neurons and activates immune cells and modulates both innate and

1040   adaptive immunity (Delgado et al., 2004). During *P. aeruginosa* keratitis, VIP is elevated

1041   and promotes wound healing by reducing proinflammatory cytokines (Szliter et al., 2007).

1042   This protective effect of VIP was attributed to the enhanced production of growth factors,

1043   which in turn are shown to regulate cytokine and mBD2 & -3 production (**Figure 4**)

1044   (Jiang et al., 2011).   VIP has also been shown to protect against *P. aeruginosa* by

1045   modulating the adhesion molecules (Berger et al., 2010) and TLRs expression (Jiang et

1046   al., 2012).

1047   Vitamin D3 plays an important role in calcium homeostasis (Holick et al., 1972; Raisz et

1048   al., 1972). It is mainly obtained from dietary source or from the action of UV-B light on

1049   skin (Wallis and Zumla, 2016). Vitamin D3 was also shown to modulate the immune

1050   system (Lin and White, 2004) and induce the expression of TLR4 co-receptor CD14

1051   (Hmama et al., 1999). It was demonstrated that vitamin D3 could induce LL-37 and

1052   HBD2 both directly and in synergy with TLR4 in a variety of cell types **(Figure 4)**

1053   (Gombart et al., 2005; Wang et al., 2004). Vitamin D3 was also shown to provide

1054   protection against *P. aeruginosa* (Wang et al., 2004) and *M. tuberculosis* (Liu et al.,

1055   2006) through induced secretion of AMPs. TLR2-induced LL-37 and HBD-2 expression

1056   in monocytes was shown to be dependent on IL-1β and vitamin-D receptor (VDR)

1057   pathway (Liu et al., 2009). In human keratinocytes, LL-37 induction by vitamin D was

1058   shown to be dependent on retinoid X receptor α (RXRα) rather than VDR pathway

1059   (Svensson et al., 2016b). Th-1 and Th-2 cytokines on the other hand were shown to

1060   have differential effect on TLR2-mediated vitamin D metabolism and subsequently on

1061   AMP secretion from human monocytes (Edfeldt et al., 2010). Significant levels of active

1062   vitamin D3 metabolite, 1α-hydroxylase enzyme and VDR expression have been

1063   demonstrated in OS epithelium (Yin et al., 2011) and various intraocular epithelial cells

1064   (Alsalem et al., 2014). It was also reported that exogenous application of vitamin D3

1065   could enhance the barrier function of OS and intraocular epithelium (Alsalem et al.,

1066   2014; Yin et al., 2011). Subsequent studies have demonstrated that deficiency of VDR

1067   could modulate the function of epithelial junction proteins (Lu and Watsky, 2014) and

1068   reduce the wound healing ability of corneal epithelium (Elizondo et al., 2014).

1069   Immunomodulatory and antimicrobial function of vitamin D3 too has been demonstrated

1070   (Reins et al., 2015; Reins et al., 2016; Svensson et al., 2016a; Svensson et al., 2016b).

1071 Vitamin D3 in synergy with TLR3 and other viral RNA sensors was shown to induce LL-
1072 37 (Figure 4) and reduce proinflammatory cytokines and MMP9 in corneal epithelial cells
1073 (Reins et al., 2015; Reins et al., 2016).

5. Future directions

1075 Microbial keratitis poses several challenges. It is the commonest cause of corneal
1076 blindness in the world; the efficacy of existing antibiotics is rapidly declining due to
1077 evolving microbial resistance; the pace of development of new antibiotics is very slow
1078 and those that are available are too expensive and inaccessible to the vast majority of
1079 the world's population who need them most. Often keratitis is polymicrobial with
1080 bacterial infections combined with fungal, parasitic or both. Licensed antibiotics for the
1081 latter two are few and far between. By virtue of their wide range of activity against
1082 viruses, bacteria, fungi and parasites; their low propensity to induce microbial resistance
1083 and the limited ability of microbes to counter their effect, AMPs have the potential to
1084 provide answers to most, if not all, of the challenges posed.

1085 A lot more however needs to be done before this potential can be realized. AMPs that
1086 are specific to different groups of organisms have to be clearly defined and the ideal
1087 concentrations of these for maximum efficacy will need to be worked out. The tendency
1088 of AMPs to induce an inflammatory response could be countered by exploiting the
1089 synergistic action of multiple AMPs in low concentrations. This will need to be
1090 systematically worked out. Strategies to promote endogenous secretion of AMPs need
1091 to be explored as this approach holds promise. As is often the case, progress in one
1092 direction uncovers other challenges. The non-microbicidal effects of AMPs could be a
1093 friend or foe. Suppression of inflammation with microbial killing can be an advantage but
1094 other consequences could lead to undesirable side effects. It is likely that in the coming
1095 years answers to these questions will be found as research in the field progresses. Our
1096 group is investigating the synergy between AMPs to determine the optimal
1097 concentrations and combinations that are effective against pathogenic isolates from
1098 clinically infected patients.

1099

1100   Use of AMPs in combination with antibiotics also holds promise in reducing the dose
1101   and strength of the antibiotic required to treat the infection. This has considerable
1102   implications for reducing toxicity of antibiotics, which at present can itself cause damage
1103   to the ocular surface and cause more inflammation and scarring despite combating the
1104   infection.

1105   Synthetic peptides with antimicrobial effects have been developed (Brown et al., 2014;
1106   Silva et al., 2016). This is one direction in which research is very likely to accelerate.
1107   The ability to tailor-make peptides will enable blending of different synthetic peptides in
1108   one dispensation, to combat polymicrobial corneal infections. This approach will also
1109   open the door to utilize and adapt AMP structures from a wide range of species, for
1110   example protegrins (Brown et al., 2014) and clavanins (Silva et al., 2016) to treat human
1111   infections.

1112   With regard to the burden of blindness secondary to cornea infections, another serious
1113   limitation is the paucity, in relation to demand, of donor human corneas to perform vision
1114   restoring corneal transplant surgery. With improving ability to employ AMPs as
1115   alternatives to antibiotics with improved killing efficacy and immunomodulatory
1116   properties, earlier control of infection thereby limiting scarring, can have a huge impact
1117   on eye banks and the need to develop engineered corneas, which is providing a
1118   challenge of its own.

1119

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2349 **Legends for Figures**

2350 **Figure 1: RNase-7 gene expression in healthy control and disease groups.** Quantitative

2351 mRNA levels of RNase-7 in impression cytology specimens collected from healthy control

2352 subjects and patients with bacterial, viral and acanthamoeba (n=3 for each). One-way ANOVA

2353 was applied for statistical analysis of mRNA levels between control and disease groups. Data

2354 are presented as means ± standard error of mean (SEM).

2355

2356 **Figure 2: Mechanisms involved in AMPs production in corneal epithelial cells in response**

2357 **to bacterial infection.** Pathogen-associated molecular pattern (PAMPs) are recognized by toll-

2358 like receptors (TLRs) and trigger multiple intracellular signalling pathways resulting in production

2359 of antimicrobial peptides (AMPs). TLR2/1 and TLR2/6 are shown to recognize diacylated (DAL)

2360 and triacylated (TAL) lipopeptides respectively. Lipopolysaccharide (LPS) on bacterial surface is

2361 recognized by LPS binding protein (LBP) and presented to CD14 (a glycosylphosphatidylinositol

2362 (GPI)-anchored protein; also present in a soluble form in tear fluid). CD14 facilitates the transfer

2363 of LPS to myeloid differentiation-2 (MD-2)/TLR4 complex and modulates LPS recognition. In

2364 naïve OS cells, TLR4 is present intracellularly but upon infection or inflammation it is transported

2365 to the cell surface (Lang et al., 2011; Lee et al., 2012). Flagellin (Flag), a flagellar protein of

2366 gram-negative bacteria is recognized by TLR5 on cell-surface. TLR9 present on endosomes

2367 recognizes CpG containing bacterial DNA, however, its role in production of AMPs and

2368 associated signalling mechanisms in corneal epithelial cells is unknown. Endogenous IL-1β

2369 released in response to bacterial infection is recognized by interleukin-1 receptor (IL-1R) on cell

2370 surface. Upon ligand binding, Toll/IL-1-receptor (TIR) domain of both TLR and IL-1R triggers

2371 recruitment of the adaptor molecule myeloid differentiation primary response protein 88 (MyD88).

2372 TLR4 activates both MyD88 and TIR-domain-containing adaptor protein inducing interferon-β

2373 (TRIF). Both MyD88 and TRIF initiate phosphorylation and ubiquitylation of several other

70

2374   molecules (not shown) leading to activation of transforming growth-factor-β activated kinase-1

2375   (TAK1). In the cytosol, TAK-1 triggers activation of mitogen-activated protein kinases (MAPKs)

2376   and nuclear-factor-κ-B (NF-κB) pathways. This allows nuclear translocation of NF-κB and

2377   activator protein 1 (AP-1; complex of Jun and Fos protein) transcription factors and modulates

2378   expression of target AMPs. Dexamethasone (Dex), an anti-inflammatory steroid binds to

2379   glucocorticoid receptors (GR) in cytosol and leads to production of MAPK phosphatase 1

2380   (MKP1), which in turn inhibits MAPKs and reduces production of AMPs downstream of TLRs.
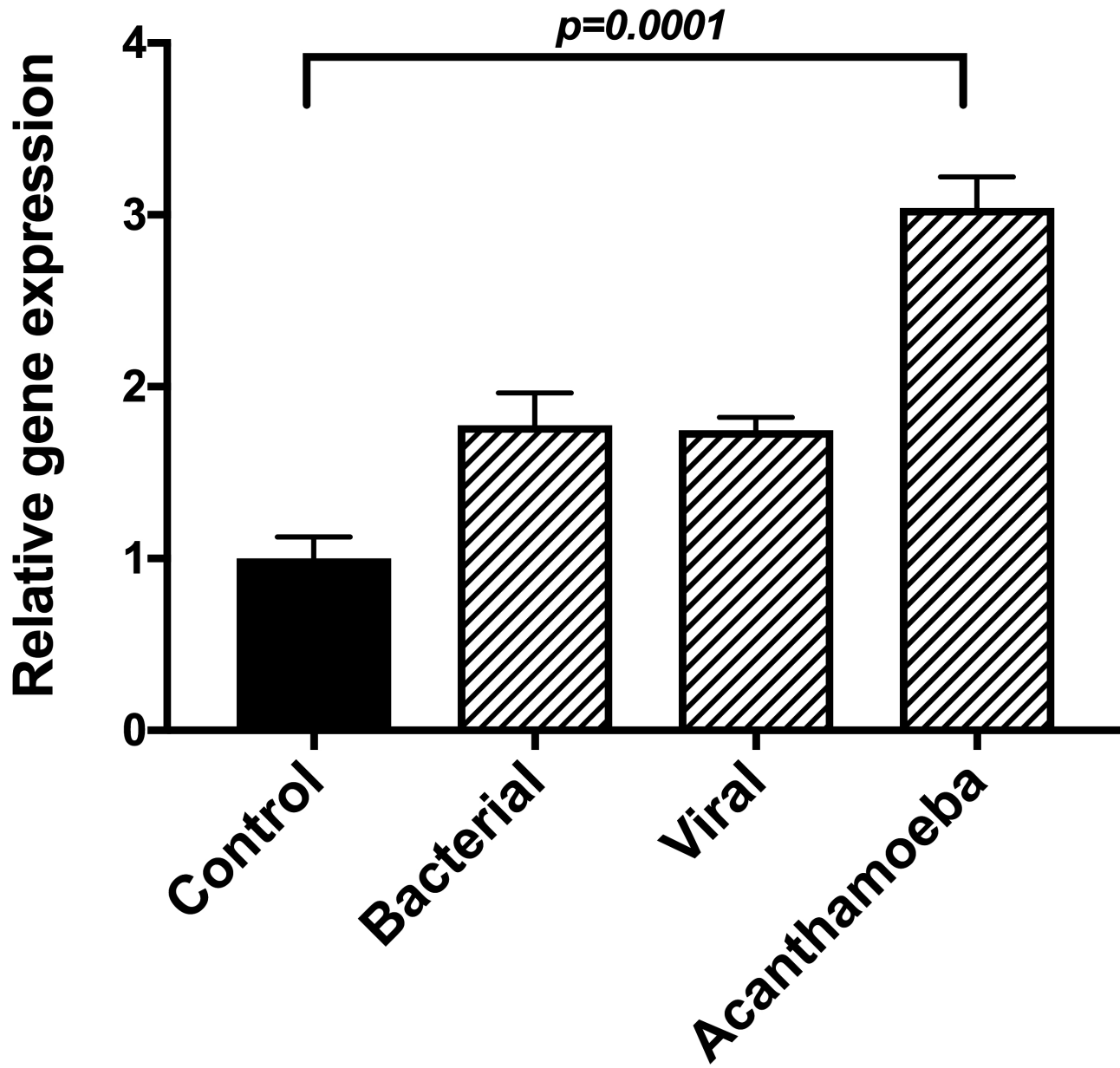
2381

2382   **Figure 3: Role of TLRs in RNase-7 expression in human corneal epithelial cells.** The cells

2383   were incubated with following TLR ligands: Pam3CSK4, Poly I:C, LPS, Flagellin, R848 and CpG

2384   dinucleotide for indicated time-points (in hours). Cell lysate was prepared for RNase-7 analysis

2385   by quantitative polymerase chain reaction (qPCR) using taqman probes on Mx3005p qPCR

2386   machine (Agilent technologies). RNase-7 mRNA expression was normalized to the

2387   housekeeping gene (18s rRNA) and relative gene expression was calculated by delta-delta Ct

2388   method. Data represents means ± standard error of mean (SEM) of triplicate samples repeated

2389   twice. Statistical analysis was performed with One-way ANOVA and Bonferroni posthoc test on

2390   GraphPad prism v7.0a. * denotes *p<0.05* and ** denotes *p<0.001.*
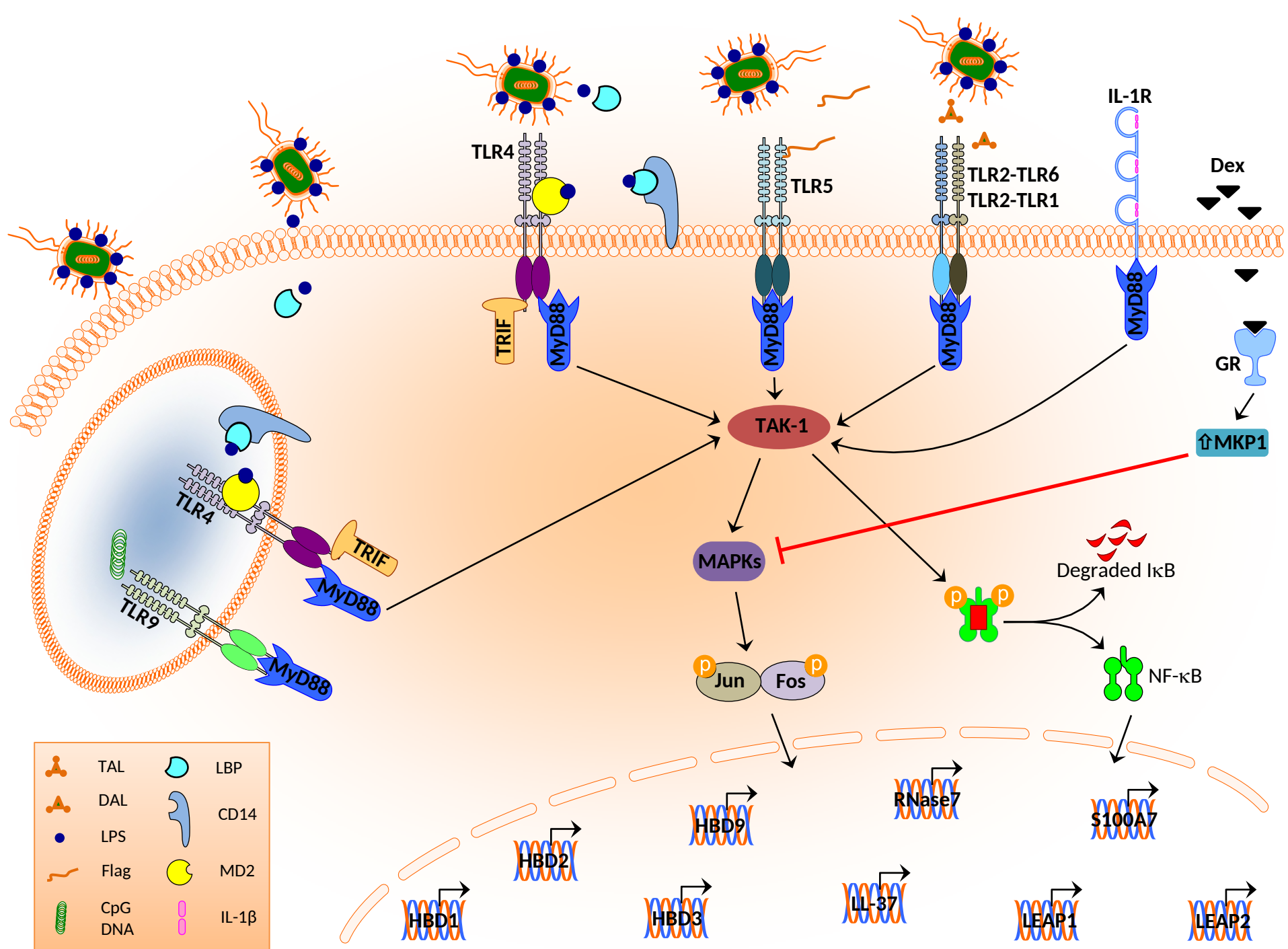
2391

2392   **Figure 4: Schematic diagram representing the exogenous induction of AMPs.** Vasoactive

2393   intestinal peptide (VIP) and L-isoleucine (L-iso) were shown to induce the expression of murine

2394   β-defensins and protect against *P. aeruginosa* keratitis and *M. tuberculosis* lung infection.

2395   Butyrate, phenyl butyrate and Entinostat are shown to induce LL-37 in a variety of cell types.

2396   Entinostat was shown to modulate LL-37 expression *via* STAT3-HIF1α pathway. Of all

2397   exogenous inducers of AMPs, Vitamin D3 has been studied the most and was shown to induce

2398   (via vitamin-D receptor (VDR) and retinoid X receptor-α (RXRα) both LL-37 and HBD2 in

2399   synergy with TLR2, TLR3 and TLR4; RIG1/MDA5 and butyrate.

TAL

DAL

LPS

Flag

CpG
DNA

LBP

CD14

MD2

IL-1β

TLR4

TLR5

TLR2-TLR6
TLR2-TLR1

IL-1R

Dex

TRIF

MyD88

MyD88

MyD88

MyD88

GR

⇑MKP1

TLR4

TRIF

TLR9

MyD88

MyD88

TAK-1

MAPKs

Degraded IκB

p p

NF-κB

Jun

Fos

p

p

HBD9

RNase7

S100A7

HBD2

LL-37

LEAP1

LEAP2

HBD1

HBD3

Pam3CSK4/TLR2

Flagellin/TLR5

Poly I:C/TLR3

R848/TLR7/8

LPS/TLR4

CpG DNA/TLR9