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INVOLVEMENT OF TLR2, MAPKinase and NFκβ PATHWAYS IN REGULATION OF HUMAN BETA DEFENSIN 9 IN HUMAN CORNEAL EPITHELIAL CELLS STIMULATED WITH Pam3CSK4

NURUL HANA ZAINAL BAHARIN

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By

NURUL HANA BINTI HAJI ZAINAL BAHARIN

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NURUL HANA BINTI HAJI ZAINAL BAHARIN

October 2017

Chairman : Associate Professor Nazri Omar, PhD
Faculty : Medicine and Health Sciences

Corneal epithelium was shown to provide immune surveillance against invading pathogens through a variety of pathogen recognition receptors (PRR) such as toll like receptors (TLRs) and nucleotide oligomerisation receptors (NLRs) which are capable of recognizing pathogen associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS) and synthetic triacylated lipoprotein (*Pam3CSK4*), derived from various infection-causing microbes. The elimination of pathogenic microorganisms, including Gram-positive and negative bacteria, fungi and viruses, involves antimicrobial peptides (AMPs). A number of AMPs such as defensins have multiple functions in host defence. Defensins, produced by cells in the course of innate host defence, serve as signals which initiate, mobilise, and amplify adaptive host immune defences. Defensins use multiple cellular receptors, which endow them with the capacity to marshall adaptive host defences against microbial invaders. Human β-defensins (HBDs), one type of defensins family, are an important part of the innate host immune defense at the ocular surface. Unlike other defensins, expression of HBD9, also known as *DEFB109,* at the ocular surface is reduced during microbial infection, but the stimulation of HCECs by Pam3CSK4 was shown to upregulate HBD9 at the initial responds followed by a significant downregulation. The mechanism of infection or inflammation has been linked with alterations in several important cellular signaling pathways such as *TLR2*, *MAPKinase* and *NFκβ* pathways. These pathways are of interest from a therapeutic perspective, because targeting them may help to reverse, delay, or prevent inflammation. The main objectives in this study was to determine the involvement of *TLR2*, *MAPKinase* and *NFκβ* signaling pathways in the expression of *DEFB109* in *Pam3CSK4*-stimulated HCECs. The techniques included cell culture stimulated with *Pam3CSK4*, the exposure of the cells to specific transcription factor inhibitors, qPCR and dot blot analysis. In this study, the evidence to indicate that *TLR2* induces HBD9 mRNA and protein expression in a time- and

dose-dependent manner was presented. *TLR2* siRNA was observed interfering TLR2-mediated induction of *DEFB109* expression. The involvement of *MAPKinase* pathways and *NFκβ* pathways in the expression of *DEFB109* gene also were observed. These pathway-specific molecules can be exploited to modulate the response of HBD9 during microbial infection. The variable expression of different AMPs to specific pathogens would suggest similar but subtly different pathways invoked by the pathogens, probably related to their PAMPs and different TLRs and other receptors they bind to.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

PENGLIBATAN LALUAN ISYARAT *TLR2, MAPKinase* DAN *NFκβ* KE ATAS EKSPRESI DEFB109 DI DALAM SEL EPITELIUM KORNEA TERANSANG OLEH *Pam3CSK4*

Oleh

NURUL HANA BINTI HAJI ZAINAL BAHARIN

Oktober 2017

Pengerusi : Profesor Madya Nazri Omar, PhD
Fakulti : Perubatan dan Sains Kesihatan

Epitelium kornea didapati memberikan pengawasan imun terhadap serangan patogen melalui pelbagai reseptor pengeceman patogen (PRR) seperti reseptor tol (TLR) dan reseptor oligomerisasi nukleotida (NLRs) yang mampu mengenal pasti pola molekul berkaitan patogen (PAMP) seperti lipopolisakarida (LPS) dan lipoprotein triasilat sintetik (*Pam3CSK4*), yang diperoleh daripada pelbagai mikrob penyebab jangkitan. Pemusnaha mikroorganisma patogen termasuk bakteria Gram-positif dan negatif, kulat dan virus, melibatkan peptida antimikrob (AMP). Sejumlah AMP, seperti defensin mempunyai pelbagai fungsi dalam sistem pertahanan. Defensin yang dihasilkan oleh sel berfungsi sebagai isyarat yang mencetuskan, meningkatkan, dan mengukuhkan sistem pertahanan imun perumah. Defensin menggunakan pelbagai reseptor sel, yang membantu dalam sistem pertahanan perumah terhadap jangkitan mikrob. β-defensin manusia (HBDs) adalah salah satu ahli kumpulan defensin yang merupakan komponen penting dalam pertahanan inat di permukaan okular. Tidak seperti defensin lain, ekspresi HBD9 yang juga dekenali sebagai *DEFB109* di permukaan okular berkurang semasa jangkitan mikrob, tetapi rangsangan Pam3CSK4 terhadap HCEC menunjukkan peningkatan ekspresi *DEFB109* pada gerak balas awal diikuti dengan penurunan ekspresi yang signifikan. Mekanisme jangkitan atau keradangan yang berlaku dikaitkan dengan perubahan dalam beberapa laluan isyarat sel penting seperti laluan *TLR2*, MAPKinase dan *NFκβ*. Laluan-laluan ini penting dari perspektif terapeutik, kerana menyasarkan laluan-laluan yang mungkin membantu merencatkan, memulihkan atau mencegah keradangan. Objektif utama dalam kajian ini adalah untuk menentukan penglibatan laluan isyarat *TLR2*, *MAPKinase* dan *NFκβ* dalam ekspresi *DEFB109* di dalam HCEC yang dirangsang oleh *Pam3CSK4.* Teknik-teknik yang terlibat dalam kajian ini termasuk, rangsangan sel kultur oleh Pam3CSK4, pendedahan sel-sel terhadap perencat faktor transkripsi khusus, qPCR dan analisis dot blot. Dalam kajian ini, keterangan menunjukkan bahawa *TLR2* menginduksi mRNA

HBD9 dan ungkapan protein dalam cara yang berkaitan dengan masa dan dos yang diberikan. *TLR2* siRNA dilihat mengganggu induksi *TLR2*-pengantara *DEFB109*. Penglibatan jalur MAPKinase dan laluan NFκβ dalam ekspresi gen *DEFB109* juga diperhatikan. Molekul khusus jalur ini boleh dieksploitasi untuk memodulasi tindak balas HBD9 semasa jangkitan mikrob. Ekspresi bervariasi dari AMP yang berbeza kepada patogen spesifik akan mencadangkan laluan yang serupa tetapi halus yang berbeza dipanggil oleh patogen, mungkin berkaitan dengan PAMP mereka dan TLR yang berlainan dan reseptor lain yang mereka sambungkan.

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All gratification are referred to Allah

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Nazri Omar, MD, PhD
Associate Professor
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Chairman)

Rafidah Md Saleh, MD
Senior Lecturer
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

Nur Shariza Nordin, PhD
Senior Lecturer
Faculty Medicine and Health Sciences
Universiti Putra Malaysia
(Member)

ROBIAH BINTI YUNUS, PhD
Professor and Dean
School of Graduate Studies
Universiti Putra Malaysia

Date:

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Name and Matric No.: Nurul Hana Binti Haji Zainal Baharin, GS42812

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Signature: _____
Name of Chairman of Supervisory Committee: Associate Professor Dr. Nazri Omar

Signature: _____
Name of Member of Supervisory Committee: Dr. Rafidah Md Saleh

Signature: _____
Name of Member of Supervisory Committee: Dr. Nur Shariza Nordin,

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LIST OF ABBREVIATIONS

AA	Antibiotic-Antimycotic
AMPs	Antimicrobial peptides
ATF2	Activating transcription factor 2
BAFF	B cell activating factor
BPE	Bovine Pituitary extract
BSA	Bovine serum albumin
CAP18	Cationic antimicrobial protein of 18 kDA
CAP35	Cationic antimicrobial protein of 35 kDA
CCL20	Chemokine ligand 20
CCR-6	Chemokine receptor 6
cDNA	Complementary DNA
DEFB109	Human beta defensin 9 gene
EGF	Epidermal growth factor
ERK	Extracellular regulated kinase
gDNA	Genomic DNA
HBD9	Human beta defensin 9
HCECs	Human corneal epithelial cell
HEK293	Human embryonic kidney 293
HNP	Herniated nucleus pulposus
IKKα	Inhibitory κB kinase-alpha
IKKβ	Inhibitory κB kinase-beta
IKKγ	Inhibitory κB kinase-gamma

IL-1	Interleukin-1
IL-1β	Interleukin-1 Beta
JIPS	JNK-interacting proteins
JNK	c-Jun N-terminal kinase
LL37	Leucine-leucine 37
LPS	Lipopolysaccharide
LTA	Lipoteicoid acid
MAP2K	Mitogen activated protein kinase kinase
MAP3K	Mitogen activated protein kinase kinase kinase
MAPK	Mitogen activated kinase
MP1	Mouse protamine-1
NADH	Nicotinamide adenine dinucleotide – hydrogen
NAPDH	Nicotinamide adenine dinucleotide phosphate hydrogen
NFκB	Nuclear factor kappa beta
Ng	Nitroglycerin
NLRs	NOD like receptors
p38	p38 mitogen activated kinase
Pam3CSK4	Palmitoyloxy)$_3$-cysteinyl-serine-(lysine)$_4$
PAMPs	Pathogen associated molecular patterns
PA01	Pseudomonas aeruginosa 01
PGN	Peptidoglycan
PRR	Pathogen recognition receptors

RANKL	Receptor Activator of Nuclear Factor Kappa B Ligand
siRNA	Short interference ribonucleic acid
TAK-1	TGFbeta activated kinase 1
TLR2	Toll-like receptor 2
TLRs	Toll-like receptors
TNFSF3	TNF superfamily 3
TNF-α	Tumor necrosis factor – alpha
TX	Texas
WB	Wipeout buffer

CHAPTER 1

INTRODUCTION

1.1 Background

Corneal epithelium is constantly exposed to numerous pathogens and to physical insult from the environment. It is responsible as a first line of defense, providing a barrier against external assaults (Chalovich & Eisenberg, 2005). Frequently, the damage of this highly specialized structure could lead to vision loss (Bashir et al., 2017). The inflammation of the eyes has become among the most severe issues in this world, proven by prevalence that has been stated in the population study in the United States, which dry eye, the symptom of keratitis, becoming among the most frequent eye disorders, ranges from 5% to less than 35% at various ages of the adult population (Kenneth, 2017).

One of the factors that can caused damage to the corneal epithelium is eye infections or inflammation, which it can be occurred when harmful microorganism such as bacteria, fungi and viruses invade any part of the eyeball or surrounding area (Fini & Stramer, 2005). Among the microorganisms, bacteria such as *Staphylococci, Haemophillus, Streptococci* and *Psedomonas* are the most frequently responsible for the inflammation of the eye (Akpek & Gottsch, 2003).

To fight against infections, corneal epithelium has several defense mechanisms such as immunological defense mechanism with features characteristic of the immune system. In the inflammatory response, the innate immune system employs Toll-like receptors (TLRs) to acknowledge and tie-up the pathogen associated molecular patterns (PAMPs). TLRs are the recognition system that utilizes PAMPs through epithelial cells in respond to the exposure of microbial infection (Akira et al., 2001). Like other mucosal epithelial cells, corneal epithelial cells will conscript inflammatory cells in response to pathogenic bacteria and their products (Solomon et al., 2004), by the recognition of the pathogen engage in the host response. This reaction will lead to the expression and the secretion of proinflammatory cytokines (Kumar et al., 2005).

The inflammatory response is characterized by coordinated activation of various signaling pathways that regulate expression of both pro- and anti-inflammatory mediators in tissues. The frequent pathways that were believed to be involved in the inflammatory response were TLRs, *MAPKinase* and NF-κB pathways. Among the TLRs family, *TLR2* was shown to accept a broad spectrum of PAMPs, including lipoteichoic acids (LTA), peptidoglycan (PGN) and bacterial lipoproteins from Gram-positive bacteria cell wall (Takeuchi et al., 2000) which, successively, initiate MAPK- or NF-κB-dependent cascades that peak in a pro-inflammatory response. This reaction requires the secretion of chemokines, cytokines and defensins (Takeuchi et al., 2000).

In inflammatory response, cytokines and chemokines activate the humoral and cellular adaptive immune system. In contrast to the effects of cytokines and chemokines, the action of defensins is double-speared (Esche et al., 2005). Defensins have direct lytic effect on the infective agent and served as the rapid, first line of host defense. Thus, defensin was shown to play an important role in the innate immune systems (Roby & Nardo, 2013). They protect the host against invading organisms such as bacteria (Bals, 2000; Koczulla & Bals, 2007), fungi (Krishnakumari et al., 2009) and viruses (Mateua et al., 2003). In addition to the antimicrobial effect, defensins have also been proven as chemo-attractant (Niyonsaba et al., 2002), anti-cancer (Droin et al., 2009; Okumura et al., 2004) and promote wound healing (Koczulla & Bals, 2007; Steinstraesser et al., 2008)

Defensins are small cationic antimicrobial peptides between 20-50 amino acids with six evolutionary conserved cysteine residues (Ganz & Lehrer, 1995). They can be classified into α, β and θ-defensins. Among of these types of defensins, only α and β-defensin have been isolated in human. To date, more than 30 genes belonging to the β-defensin family have been identified in human (Schutte et al., 2002), especially in ocular surface, the localization of human β-defensin (HBD)-1 to HBD3 was reported in superficial layers of normal and inflamed corneal (Terai et al., 2004). Besides, newer member of β-defensin family, *DEFB109*, also known as HBD9 was first reported on the ocular surface by Dua and co-workers in 2008 (Abedin et al., 2008). They demonstrated that *DEFB109* gene was expressed constitutively but was down regulated in the presence of ocular surface infection and inflammation. This was in agreement with earlier findings of Premratanachai and co-workers who demonstrated down regulation of the *DEFB109* gene in gingival candidiasis (Premratanachai et al., 2004). With this unique characteristic, the expression of HBD9 were found interesting to be investigated.

HBD9 is a relatively newly-found defensin in ocular surface and the pathways involve in the expression of this type of defensin was investigated. The existing data interestingly points towards possibly different or additional roles it plays in the host defence (Dua et al., 2014). This study extended the perimeter of our study regarding HBD9 as a potential source of broad spectrum efficacious therapeutic agent in the future.

Although the more established defensins such as the HBD1-3 were shown to act via the *MAPKinase* pathway in tissue such as the lung and urinary tract, the pathway may varied for HBD9 in the ocular surface environment. In this project, the pathways that were believed to be involved inflammation were unravelled by blocking the mediator molecules in HCECs after treated with *Pam3CSK4* which is believed to express *DEFB109*, while observing the resultant relative mRNA expression. The importance of the involvement of nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) families molecules such as p38, c-Jun N-terminal kinase (JNK) and extracellular signal-regulated kinase (ERK), in infection and inflammation, have made all of these pathways interestingly to be studied (Mohammed et al., 2011). The results improved the current knowledge on the inflammation pathways involves in the expression of HBD9 in immortalized HCECs.

1.2 Problem Statement

Corneal epithelium was proven to provide immune defense, against attacking pathogens through a variety of pathogen recognition receptors (PRR) which are capable of recognizing PAMPs, derived from various infection-causing microbes (Mohammed et al., 2010). It will conscript inflammatory cells in response to pathogenic bacteria and their products (Solomon et al., 2004), by the recognition of the pathogen engage in the host response. This reaction will lead to the expression defensins.

Pam3CSK4 is one of the synthetic PAMPs that is believed to be associated in the HBD9 expression. Stimulation of HCECs with *Pam3CSK4* was shown to up regulated the expression of *DEFB109*, which is also known as HBD9 (Mohammed et al., 2010). *DEFB109* was first reported on the ocular surface by Dua and co-workers in 2008 (Abedin et al., 2008). In this study, they demonstrated that *DEFB109* gene was expressed constitutively but was down regulated in the presence of ocular surface infection and inflammation. With this special characteristic, HBD9 has become one of the defensins that draw attention of many researchers nowadays.

Although there were previous researches that studied the effect of *Pam3CSK4* on the expression of HBD9, the pathway associated in the expression of *DEFB109* have been not fully studied yet. Therefore in this research, various steps of the potential pathways in inflammation and infection of the eye such as *TLR2*, *MAPKinase* and *NFκβ* were tested and the expression of *DEFB109* was analyzed. The new information can be used as a reference in the future investigation. This analysis might be benefit by others scientist or researchers to increase understanding and find a new treatment method for corneal eye diseases. This will drive towards discovery of a broad spectrum, safe but effective resistant-free antibiotic in the future.

1.3 Research Objectives

1.3.1 General Objectives

To determine the signaling pathways involved in the expression of HBD9 at RNA and protein level in *Pam3CSK4*-stimulated culture HCECs.

1.3.2 Specific Objectives

1. To determine the optimal concentration and incubation time of *Pam3CSK4* in stimulating the upregulation of HBD9 in HCECs.
2. To assess the cytotoxicity of *TLR2* siRNA and inhibitors for *MAPKinase* and *NFκβ* pathways in stimulated HCECs.
3. To determine the effects on the expression of HBD9 in *Pam3CSK4*-stimulated HCECs after in treated by *TLR2* siRNA and inhibitors for *MAPKinase* and *NFκβ* pathways.

1.4 Hypotheses

- Ho 1: There is no significant difference between the expressions of *DEFB109* gene in HCECs before and after stimulated with *Pam3CSK4*

- H_1 1: There is a significant difference between the expressions of *DEFB109* gene in HCECs before and after stimulated with *Pam3CSK4*

- Ho 2: There is no significant difference between the expressions of *DEFB109* gene in HCECs following the transfection of *TLR2* siRNA and inhibition of *MAPKinase* and *NF-κβ* signaling pathways.

- H_1 2: There is a significant difference between the expressions of *DEFB109* gene in HCECs following the transfection of *TLR2* siRNA and inhibition of *MAPKinase* and *NF-κβ* signaling pathways.

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