การตอบสนองของไฟโบรบลาสท์จากเหงือกของคนต่อการกระตุ้นด้วย โทลล์ไลค์รีเซฟเตอร์ไลแกน

นางสาว พัทนินทร์ มนตรีขจร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาปริทันตศาสตร์ ภาควิชาปริทันตวิทยา
คณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2548
ISBN 974-17-5940-1

THE RESPONSE OF HUMAN GINGIVAL FIBROBLASTS TO TOLL-LIKE RECEPTOR LIGANDS

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Periodontics

Department of Periodontology

Faculty of Dentistry

Chulalongkorn University

Academic Year 2005

ISBN 974-17-5940-1

Thesis Title	The response of human gingival fibroblasts to Toll-like receptor ligands
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Field of study	Periodontics
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พัทนินทร์ มนตรีขจร : การตอบสนองของไฟโบรบลาสท์จากเหงือกของคนต่อการกระตุ้นด้วยโทลล์ไลค์รีเซฟเตอร์ไลแกน (THE RESPONSE OF HUMAN GINGIVAL FIBROBLASTS TO TOLL-LIKE RECEPTOR LIGANDS). อ. ที่ปรึกษา: ผศ.ดร. รังสินี มหานนท์, อ.ที่ปรึกษาร่วม : ดร.สาธิต พิชญางกูร, 59 หน้า. ISBN 974-17-5940-1.

โรคปริทันต์อักเสบ คือโรคติดเชื้อแบคทีเรียชนิดหนึ่ง ซึ่งมีลักษณะการอักเสบเรื้อรังของเหงือก อันนำไปสู่การสูญเสียของอวัยวะรองรับฟัน การศึกษาเมื่อไม่นานมานี้พบว่า เซลล์ไฟโบรบลาสท์จากเหงือกของคนมีการแสดงออกของโทลล์ไลค์รีเซฟเตอร์ที่ 2, 4 และ 9 โทลล์ไลค์รีเซฟเตอร์เป็นแพทเทิน รีคอกนิชั่น รีเซฟเตอร์ (pattern recognition receptors) ที่สำคัญในการกระตุ้นระบบภูมิคุ้มกันที่มีมาแต่กำเนิดของร่างกายโดยการรับรู้ส่วนที่เรียกว่า คอนเซิร์ฟ พาโทเจน-แอดโซซิเอท โมเลกูลลา แพทเทิร์น (conserved pathogen-associated molecular patterns (PAMPs)) จึงตั้งสมมติฐานว่าการรับรู้ถึงสาเหตุการก่อโรคของเซลล์ไฟโบรบลาสท์ด้วยโทลล์ไลค์รีเซฟเตอร์จะนำไปสู่การตอบสนองของระบบภูมิคุ้มกันที่มีมาแต่กำเนิด ในการศึกษานี้ได้ศึกษาถึงบทบาทของเซลล์ไฟโบรบลาสท์เกี่ยวกับระบบภูมิคุ้มกันที่มีมาแต่กำเนิดของอวัยวะปริทันต์ จึงได้ทำการตรวจหาการแสดงออกของเมสเซนเจอร์อาร์เอนเอ (mRNA) ของโทลล์ไลค์รีเซฟเตอร์ ที่ 1-10 ด้วยวิธีรีเวอร์สทรานส์คริปเตส-โพลิเมอร์เรสเชนรีแอกชั่น (reverse transcriptase-polymerase chain reaction) จากเหงือกของคนซึ่งมีอวัยวะปริทันต์สุขภาพดี นอกจากนี้ได้ทำการกระตุ้นเซลล์ไฟโบรบลาสท์ด้วยโทลล์ไลค์รีเซฟเตอร์ไลแกนแบบเดี่ยวคือ ไลแกนของโทลล์ไลค์รีเซฟเตอร์ที่ 2, 3, 4, 5, 7, 8 และ 9 ดังนี้ 1). TLR2 ligand (*Porphyromonas gingivalis* LPS); 2). TLR3 ligand (Polyinosine-polycytidylic acid); 3). TLR4 ligand (*Escherichia coli* LPS); 4). TLR5 ligand (*Salmonella thyphimurium* flagellin); 5). TLR7 ligand (Loxoribine); 6). TLR8 ligand (ssPolyU), และ 7). TLR9 ligand (CpG2006) และกระตุ้นด้วยไลแกนร่วมกัน 2 ชนิด ทำการตรวจวัดระดับความสามารถในการกระตุ้นการผลิตอินเตอร์ลิวคิน-8 ด้วยวิธีอีไลซ่า ผลการศึกษาพบว่าเซลล์ไฟโบรบลาสท์มีการแสดงออกของ เมสเซนเจอร์อาร์เอนเอของโทลล์ไลค์รีเซฟเตอร์ที่ 1, 2, 3, 4, 5, 6 และ 9 แต่ไม่พบการแสดงแสดงออกของเมสเซนเจอร์อาร์เอนเอของโทลล์ไลค์รีเซฟเตอร์ที่ 7, 8 และ 10 ไลแกนของโทลล์ไลค์รีเซฟเตอร์ที่ 2, 3, 4 และ 5 สามารถกระตุ้นให้เซลล์ไฟโบรบลาสท์ผลิตอินเตอร์ลิวคิน-8 ได้ แต่ไลแกนของโทลล์ไลค์รีเซฟเตอร์ที่ 9 ไม่สามารถกระตุ้นได้ ข้อมูลนี้บ่งชี้ถึงการไม่สามารถทำหน้าที่หรือการไม่สามารถจับกับไลแกนของโทลล์ไลค์รีเซฟเตอร์ที่ 9 ได้ เมื่อกระตุ้นด้วยไลแกนของโทลล์ไลค์รีเซฟเตอร์ร่วมกัน 2 ชนิด มีผลในการกระตุ้นการผลิตอินเตอร์ลิวคิน-8 เป็นจำนวนเพิ่มมากขึ้นเล็กน้อยหรือไม่เปลี่ยนแปลง เมื่อเทียบกับการกระตุ้นด้วยไลแกนแบบเดี่ยว ที่น่าสนใจคือเมื่อกระตุ้นด้วยไลแกนของโทลล์ไลค์รีเซฟเตอร์ที่ 3 ร่วมกับ 9 พบการกดการผลิตอินเตอร์ลิวคิน-8 ลงอย่างมากเมื่อเทียบกับการกระตุ้นด้วยไลแกนของโทลล์ไลค์รีเซฟเตอร์ที่ 3 เพียงอย่างเดียว ซึ่งยังต้องมีการศึกษาเพื่อหากลไกที่แน่ชัดต่อไป สรุปว่างานวิจัยครั้งนี้ได้แสดงถึงความสำคัญของเซลล์ไฟโบรบลาสท์ ในแง่ของการมีบทบาทในระบบภูมิคุ้มกันที่มีมาแต่กำเนิดของอวัยวะปริทันต์

ภาควิชา .....ปริทันตวิทยา..... ลายมือชื่อนิสิต.....พัทนินทร์ มนตรีขจร.....

สาขาวิชา.....ปริทันตศาสตร์..... ลายมือชื่ออาจารย์ที่ปรึกษา.....

ปีการศึกษา.....2548..... ลายมือชื่ออาจารย์ที่ปรึกษาร่วม.....

4676115832 : MAJOR PERIODONTICS

KEY WORD: HUMAN GINGIVAL FIBROBLASTS / TOLL-LIKE RCEPTORS / PERIODONTITIS / IMMUNOPATHOGENESIS / INNATE IMMUNITY / IL-8

PATTANIN MONTREEKACHON: THE RESPONSE OF HUMAN GINGIVAL FIBROBLASTS T0 TOLL-LIKE RECEPTOR LIGANDS. THESIS ADVISOR: ASST. PROF. RANGSINI MAHANONDA, Ph.D, THESIS CO-ADVISOR : SATHIT PICHYANGKUL, Ph.D, 59 pp. ISBN 974-17-5940-1.

Periodontitis is a bacterial infection characterized by chronic gingival inflammation, which leads to the loss of tooth-supporting tissues. Recent data demonstrated that human gingival fibroblasts (HGFs) expressed Toll-like receptors (TLRs) 2, 4 and 9. Toll-like receptors (TLRs) are important pattern recognition molecules that trigger innate immune responses via the recognition of conserved pathogen-associated molecular patterns (PAMPs). We hypothesize that pathogen recognition by gingival fibroblast TLRs may contribute to the development of innate immune response. In this study, we investigated the role of HGFs in the innate immunity of periodontium. The mRNA expression of TLRs (TLRs1-10) in HGFs from healthy gingiva was assessed using reverse transcriptase-polymerase chain reaction (RT-PCR). In addition, the responses of HGFs to different TLR ligands were determined. HGFs were stimulated with a single TLR ligand 1). TLR2 ligand (*Porphyromonas gingivalis* LPS); 2). TLR3 ligand (Polyinosine-polycytidylic acid); 3). TLR4 ligand (*Escherichia coli* LPS); 4). TLR5 ligand (*Salmonella thyphimurium* flagellin); 5). TLR7 ligand (Loxoribine); 6). TLR8 ligand (ssPolyU), and 7). TLR9 ligand (CpG2006) or the combination of TLR ligands. After 24 hour incubation, culture supernatants were collected and analyzed for IL-8 production using enzyme-linked immunosorbent assay. RT-PCR results revealed that HGFs expressed mRNA of TLRs1, 2, 3, 4, 5, 6, and 9 but did not express mRNA of TLRs 7, 8 and 10. We found that HGFs produced IL-8 in response to TLR 2, 3, 4, 5 ligands, but not TLR9 ligand. The data may indicate non-functional TLR9 or inability to bind/up-take CpG2006 by HGFs. When HGFs were stimulated with the combination of TLR ligands, the IL-8 production was minimally enhanced or remained relatively unchanged as compared with those from single ligand-stimulated HGFs. It is interesting to note that CpG2006 greatly inhibited IL-8 production induced by TLR3 ligand, but not by other TLR ligands. Further investigation for underlying mechanism of this inhibitory effect is needed. In conclusions, our results suggest that HGFs play an important role in innate immunity of the periodontium.

Department .....Periodontology..... Student's signature.....

Field of study.....Periodontics..... Advisor's signature.....

Academic year....2005..... Co-advisor's signature.....

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude and appreciation to my advisor, Assistant Professor Dr. Rangsini Mahanonda, for her guidance, encouragement, supervision, suggestion and kindness throughout the course of my Master degree program. I am extremely indebted to my co-advisor, Dr. Sathit Pichyangkul, Department of Immunology and Medical Component, AFRIMS, for providing the laboratory facilities and his grateful guidance, supervision, valuable technical advice and correction of this thesis. I wish to thank my thesis committee members; Associated Professor Naulchavee Hongprasong, Assistant Professor Dr. Chakrit Hirunpetcharat and Dr. Kitti Torrungruang for their suggestions and kindness in being committee members.

Sincere appreciation is expressed to Mr. Noppadol Sa-Ard-Iam for his assistance in setting the experiments and preparing this manuscript. I also would like to thank Mr. Manop Pachantabut and Ms. Pimprapa Rerkyen for kind advice and technical assistance.

I would like to acknowledge research grant from the Graduate School, Chulalongkorn University for the partial financial support for this study. My sincere appreciation is also extended to the staff of Periodontology Department, Faculty of Dentistry, Chulalongkorn University for keeping the gingival biopsy and for their kindness, guidance and encouragement. Finally, I would like most sincerely to thank my father, my mother, my brothers and my friends for their love, caring, understanding and encouragement.

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
CD	cluster of differentiation
CpG ODN	cytidine-phosphate-guanosine oligonucleotide
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
GAPDH	glyceraldehyde-3–phosphate dehydrogenase
HGFs	human gingival fibroblasts
IFN	interferon
IL	interleukin
LPS	lipopolysaccharide
MyD88	myeloid differentiation primary-response protein 88
MMPs	matrix metalloproteinases
NAP-1	neutrophil-activating peptide-1
P. gingivalis	*Porphyromonas gingivalis*
P.g. LPS	*Porphyromonas gingivalis* lipopolysaccharide
P. intermedia	*Prevotella intermedia*
PAMPs	pathogen–associated molecular patterns
PBMC	peripheral blood mononuclear cells
PGE	prostaglandin
Poly I:C	polyinosine-polycytidylic acid
PRRs	pattern recognition-receptors
RT-PCR	reverse transcriptase–polymerase chain reaction
SEM	standard error of mean
ssPolyU	single strand poly-uridine
TLRs	toll–like receptors
T. forsythia	*Tannerella forsythia*
TNF	tumor necrosis factor

CHAPTER I

INTRODUCTION

1.1 Background of Present Study

The host defense response to pathogens depends on the immune system. Studies of the host defense system in fruit flies, *Drosophila*, provide the first clue as to the mechanism of innate immune recognition. *Toll* gene products were first discovered in 1985 as critical for the embryonic development of dorsal-ventral polarity (Anderson et al., 1985a; 1985b). Subsequently, homologues of *Drosophila* Toll, the so-called Toll-like receptors (TLRs), were identified in mammals (Medzhitov et al., 1997). To date 10 TLRs in humans and 12 TLRs in mice have been described (Beutler et al., 2004). It is now clear that TLRs function as key pattern recognition-receptors (PRRs) of the innate immune system (Janeway and Medzhitov, 2002). They recognize and distinguish highly conserved structures present in large groups of microorganisms. The structures are referred to as pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are bacterial lipopolysaccharide (LPS), peptidoglycan, lipoproteins, bacterial DNA, and double-stranded RNA. In the innate immune system, TLRs sense invasion by microorganisms and trigger immune responses to clear such pathogens. Upon interaction with a pathogen, TLRs transmit this information through intracellular signaling pathways, resulting in activation of innate immune cells. The TLR-mediated innate immune response is also critical for the development

and direction of the adaptive immune system. Today, TLR signaling has become a central topic in immunology.

Periodontal disease is a chronic bacterial infection that affects the gingiva and bone supporting the teeth. Bacterial plaque stimulates the host inflammatory response leading to tissue damage. Since gingival tissue is consistently exposed to bacterial PAMPs, TLR sensing and signaling in periodontal tissue could indeed play important role in the innate immune response. Little is known about the role of TLRs in periodontal health and disease. Human gingival fibroblasts (HGFs) are the major constituents of periodontal connective tissue and maintain gingival tissue integrity. It becomes clear that they also play an important role in the non-immune innate cells. They secrete inflammatory cytokines such as interleukin (IL)-1, IL-6 and IL-8 in response to bacteria and their products (Takada et al., 1991; Tamura et al., 1992; Yamazaki et al., 1992). These cytokines are known to associate with chronic periodontal tissue inflammation and tissue destruction. Recent studies demonstrated that HGFs constitutively expressed TLR2, TLR4 and TLR9 (Tabeta et al., 2000; Wang et al., 2000; Nonnenmacher et al., 2003). The presence of these TLRs would indicate the ability for HGFs to respond to their ligands such as LPS, lipoprotein, and bacterial DNA. The binding of TLRs and their specific ligands on HGFs leads to TLR signaling and subsequently gene expression of inflammatory mediators/cytokines which is critical for the immune regulation in periodontal environment.

1.2 Objective

So far, only TLR2, TLR4, TLR9 expression on HGFs have been reported, therefore, in this study we were investigated HGFs with regards to

1.2.1 a panel of TLR expression (TLRs 1,2,3,4,5,6,7,8,9,10).

1.2.2 the ability to produce IL-8 in response to TLR ligands.

1.3 Hypothesis

Human gingival fibroblasts express a variety of TLRs and produce IL-8 upon TLR ligation.

1.4 Field of Research

To investigate the TLR expression of human gingival fibroblasts and ability to produce IL-8 in response to TLR ligands.

1.5 Criteria Inclusions

1.5.1 HGFs were grown from gingival biopsies which were obtained from healthy adult subjects.

1.5.2 Peripheral blood mononuclear cells (PBMC) were obtained from healthy adult subjects who had no taken any antibiotics or anti-inflammatory drugs within the past 3 months prior to blood donation.

1.5.3 Subjects who have clinically healthy periodontium with probing depth less than 4 mm. were included.

1.5.4 Analyses of TLR expression (TLRs1-10) were determined by reverse transcriptase-polymerase chain reaction (RT-PCR).

1.5.5 The responses of HGFs to different TLR ligands (IL-8 production) were measured by enzyme-linked immunosorbent assay (ELISA).

1.6 Limitation of Research

This study cannot investigate many HGFs samples due to limitation of grant support.

1.7 Application and Expectation of Research

An important advance in our understanding of early events in microbial recognition and the subsequent development of immune response has been the identification of TLRs as key PRRs of the innate immune system. Periodontal disease, a chronic inflammation of the periodontium, provides a unique opportunity to investigate the host-microbial interactions. Gingival tissue is continually exposed to at least 500 species of both commensal and pathogenic bacteria (Paster et al., 2001). To date, research data indicate that the cells of periodontium express different types of TLRs. But how the periodontal cells including gingival epithelium and gingival fibroblasts orchestrate their response via TLR signaling is indeed a very interesting issue. In this proposal, we aim to evaluate HGFs with regards to their TLR expression and function as assessed by the ability to induce IL-8 production in response to a variety of TLR ligands. We anticipate that the results from this line of investigation would provide a better understanding in the immune response and the immunopathogenesis of periodontal disease. With the proper

knowledge of the TLR signaling in periodontal tissue should provide new insight into the role of the immune system in maintaining health and combating disease in the periodontium.

CHAPTER II

LITERATURE REVIEW

2.1 Toll-Like Receptors

2.1.1 Introduction

Innate immunity represents the first line of immunologic defense. The main distinction between the innate and the adaptive immune systems lies in the receptors used for the immune recognition. The antigen receptors of T and B cells are generated somatically, whereas the innate PRRs are encoded in the germ line (Akira et al., 2001). It has long been our perception that innate immunity mediates non-specific immune responses, via ingestion and digestion of microorganisms and foreign substances by macrophages and neutrophils. However, innate immunity is now recognized to show remarkable specificity by means of discriminating between the host and pathogens, through a sophisticated TLR-based system.

TLRs are predominantly expressed on cells of the innate immune system, including neutrophils, monocytes/macrophages, and antigen presenting cells (Langerhans and dendritic cells). Subsets of these cells express different TLRs, allowing them to induce a wide variety of immune responses to specific pathogens. Neutrophils, the predominant innate immune cells in blood, express TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, TLR8, TLR9, and TLR10, but not TLR3 (Hayashi et al., 2003). Being the first innate immune cells to migrate to the site of infection, neutrophils utilize relevant TLRs

to recognize and respond to different types of microbial challenge. Like neutrophils, macrophages/monocytes are also considered as a first line of defense against microbial pathogens. They play a key role in host defense by recognizing, engulfing and killing microorganisms. Macrophages/monocytes express TLR1, TLR2, TLR4, TLR5, TLR6, TLR7, and TLR8 (Iwasaki and Medzhitov, 2004).

2.1.2 Toll-like receptor ligands and signaling

Each of the 10 known human TLRs is believed to respond to a distinctive aspect of microbial infection. As specificity for broad categories of PAMPs is provided by a relatively limited diversity of TLRs, in some cases combinations of TLRs are required for recognition of certain PAMPs (Ozinsky et al., 2000; Hajjar et al., 2001). For example, TLR2 forms heterophilic dimers with TLR1 and TLR6 to recognize diacyl and triacyl lipopeptides, respectively (Takeda and Akira, 2005). Many other human TLRs and their ligands are known as shown in table 1. For example TLR2 recognizes peptidoglycan, TLR3 recognizes viral double-stranded RNA, TLR4 recognizes LPS, TLR5 recognizes flagellin, TLR7 recognizes viral single-stranded RNA, and TLR9 recognizes bacterial DNA. It should be noted that cell surface TLRs (TLR1, TLR2, TLR4, TLR5, and TLR6) seem to recognize microbial products whereas intracellular TLRs (TLR3, TLR7, TLR8, and TLR9) recognize nucleic acids. Much research is underway, to identify additional ligands recognized by TLRs, and to identify accessory molecules that may aid in recognition of PAMPs by TLRs. Recognition of microbial components by TLRs initiates signal transduction pathways, which triggers expression of genes. These gene products control innate immune responses and further instruct the development of antigen-specific acquired immunity. Current information

suggests that TLR signaling pathways are divergent, although Myeloid differentiation primary-response protein 88 (MyD88), a key adaptor molecule, is used by most TLRs and this signaling pathway is called MyD88-dependent. Stimulation of TLR3 and some TLR4 utilize different adaptor molecules and the signaling pathway is called MyD88-independent pathways (Takeda and Akira 2005).

Table 1: Human Toll-like receptors and their ligands

Receptor	Ligand	References
TLR1	Triacyl lipopeptides	Takeuchi et al.,2002
TLR2	Lipoprotein/lipopeptides	Aliprantis et al., 1999
	Peptidoglycan/Lipoteichoic acid	Schwandner et al., 1999
	Porphyromonas gingivalis lipopolysaccharide	Hirschfeld et al., 2001
	Porphyromonas gingivalis fimbriae	Asai et al., 2001
	Mycobacterial lipoarabinomannan	Means et al., 1999
	Zymosan	Underhill et al., 1999
TLR3	Double-stranded RNA	Alexopoulou et al., 2001
	Polyinosine-polycytidylic acid	Alexopoulou et al., 2001
TLR4	*Escherichia coli* Lipopolysaccharide	Tapping et al., 2000
	Porphyromonas gingivalis LPS	Darveau et al., 2004
TLR5	Flagellin	Gewirtz et al., 2004
TLR6	Peptidoglycan/Lipoteichoic acid	Schwandner et al., 1999
	Diacyl lipopeptides	Takeuchi et al.,2002
	Zymosan	Ozinsky et al., 2000
TLR7	Imidazoquinoline	Hemmi et al., 2002
TLR8	Single-stranded RNA	Heil et al., 2004
	Imidazoquinoline	Jurk et al., 2002
TLR9	Bacterial DNA	Bauer et al., 2001
	CpG Oligonucleotides	Hemmi et al.,2000
TLR10	N.D.	

N.D. = not determined

2.2 Periodontal Disease

The periodontium, a tooth supporting structure, consists of gingiva, cementum, periodontal ligament and alveolar bone. Histological examination of healthy periodontal tissue reveals the presence of a very low numbers of immune cells such as macrophages, Langerhans cells, tissue dendritic cells, and migratory neutrophils in gingival crevice and the epithelial cell layer. In addition to immune cells, cells of the periodontium express TLRs. Gingival epithelium constitutively expressed a repertoire of TLRs: TLR2, TLR3, TLR4, TLR5, TLR6, TLR9 (Kusumoto et al., 2004) and HGFs constitutively expressed TLR2, TLR4, and TLR9 (Tabeta et al., 2000; Wang et al., 2000; Nonnenmacher et al., 2003). Since the gingiva is consistently exposed to bacterial PAMPs, TLR sensing and signaling in periodontal tissue could indeed play important role in the innate immune response and maintain periodontal health.

Periodontal disease is a chronic bacterial infection. Dental plaque biofilms have been well recognized as etiologic agents. The disease initiation and progression result from host response to plaque bacteria. In healthy periodontal tissue, low amounts of Gram-positive aerobes and facultative anaerobes, such as *Streptococcus* species and *Actinomyces* species are found supragingivally (Moore and Moore, 1994). More accumulation of plaque leads to gingival inflammation (or gingivitis) with increased cellular infiltrates. T cells are the dominant cell type in gingivitis lesions. In contrast, in the more advanced form of periodontal disease, periodontitis, cellular infiltrates including numerous T and B cells are observed together with high levels of inflammatory mediators such as IL-1β, tumor necrosis factor (TNF)-α, prostaglandin E_2 (PGE_2), and interferon (IFN)-γ in tissues and gingival

crevicular fluid (Page et al.,1997). B cells and plasma cells are the dominant cell type in periodontitis lesions, and numerous Gram-negative anaerobes are found in subgingival biofilms (Seymour, 1991). The differences in microbial compositions and quantities between health/gingivitis and periodontitis may influence the local inflammatory response. Key periodontal pathogens, *Porphyromonas gingivalis* (*P.g.*), *Actinobacillus actinomycetemcomitans* (*A.a.)* and *Tanerella forsythia* (*T.f.*), which are frequently detected in deep periodontal pockets, are well recognized for their virulence as etiologic agents in human periodontitis (Consensus report, 1996). Chronic TLR stimulation in periodontal tissues by bacterial PAMPs can lead to excessive production of pro-inflammatory mediators, resulting in tissue destruction.

2.3 Cytokine: Interleukin-8

Cytokines are known to be major participants in acute and chronic inflammation regardless of its location, and there is strong evidence for participation of these mediators in periodontitis. They are produced by activated resident gingival cells and infiltrating immune cells. In periodontitis lesion, high levels of inflammatory mediators/cytokines such as IL-1β, TNF-α, IFN-γ, IL-6, IL-10, IL-13, IL-4, IL-8, matrix metalloproteinases (MMPs) and PGE_2 have been detected (Kornman et al., 1997; Okada and Murakami, 1998).

IL-8 is a chemoattractant cytokine produced by a variety of tissue and blood cells, it is formerly known as neutrophil-activating peptide-1 (NAP-1). It has distinct target specificity for the neutrophil, with weaker effect on other blood cells (Bickel, 1993; Baggiolini, 1994). IL-8 attracts neutrophils by inducing neutrophil extravasation at the site of inflammation. It then activates

the cells to undergo the metabolic burst and to degranulate on arrival at the site of the challenge (Kornman et al., 1997). This cytokine has been thought to play a significant role in various forms of periodontitis (Takashiba, 1992; Bickel, 1993; Fitzgerald, 1995; Gainet, 1998).

IL-8 concentration was shown to be increased in gingival crevicular fluid from patients with periodontitis (Tsai et al., 1995), and remained elevated in patients who did not respond to treatment (Chung, 1997). High levels of IL-8 in plasma were detected in patients with various forms of periodontitis and the presence of mRNA for IL-8 was observed in gingival neutrophils (Gainet et al., 1998). In inflamed gingival tissues, it was observed that IL-8 was produced in epithelial cells, macrophages and fibroblasts (Tonetti et al., 1993). As mentioned earlier, HGFs IL-8 could be induced by stimulation with bacterial LPS or other cytokines (Takashiba et al., 1992; Takigawa et al., 1994; Sakuta et al., 1998; Steffen et al., 2000). The decisive role of IL-8 in periodontal disease is not clear. It is possible that at an early phase of periodontal inflammation, IL-8 may be required to attract neutrophils and leukocytes to eliminate the infection. On the other hand, at the chronic stage of periodontal inflammation, excessive IL-8 may be unwanted but inevitably present due to continual activation by etiologic bacterial plaque and the local cellular/cytokine network in the lesion. Therefore, additional work is required to determine the significance of this cytokine in periodontal disease.

2.4 Gingival Fibroblasts

Fibroblasts are the dominant resident cells, which inhabit the periodontal tissues. As such, they are crucial for maintaining the connective tissues, which support and anchor the tooth. In the past they had been considered to function as the simple supporting framework for other cell types. Now it is firmly established that fibroblasts are anything but simple. Rather, they have been found to be a dynamic cell type involved in many local tissue functions and in host defense (Phipps et al., 1997).

2.4.1 Response of human gingival fibroblasts to stimuli

Gingival fibroblasts are also function as a regulator of the cytokine network in periodontal tissues, because they produce several kinds of cytokines when stimulated by inflammatory cytokines or bacterial cell components (Takada, 1991). Fibroblasts not only respond to extrinsic environmental signals, but are also able to receive and process signals from other host cells by direct contact and through cell-cell receptors and ligands (Fries, 1995).

Gingival fibroblasts could secrete various soluble mediators of inflammation including PGE_2, IL-1, IL-6 and IL-8 in response to extrinsic environmental factors such as plaque bacterial pathogens and their products and cytokines. These fibroblast-derived mediators are thought to play important role in inflammatory response in local periodontal lesions. Many plaque bacterial products e.g. LPS derived from *P. gingivalis*, *A. actinomycetemcomitans* and *Prevotella intermedia (P.i.)* were shown to

enhance IL-6 and IL-8 production from gingival fibroblasts (Ohmori, 1987; Takada et al., 1991; Tamura et al., 1992; Dongari-Bagtzoglou and Ebersole, 1996a, 1996b; Imatani, 2001). Fibroblasts are considered to be major sources of these IL-6 and IL-8 cytokines which are secreted in high amount both constitutively (Bartold and Haynes, 1991; Dongari-Bagtzoglou and Ebersole, 1998) and in response to bacteria, IL-1, TNF-α (Takashiba et al., 1992) and IFN-γ (Takashiba et al., 1992; Sakuta et al., 1998; Daghigh et al., 2002).

2.4.2 Toll-like receptors on human gingival fibroblasts

Human gingival fibroblasts produce various inflammatory cytokines such as IL-1, IL-6 and IL-8 upon stimulation by bacteria and their components (Takada et al., 1991; Tamura et al., 1992; Yamazaki et al., 1992). HGFs constitutively express mRNA of TLR2, TLR4, and TLR9 (Tabeta et al., 2000; Wang et al., 2000; Nonnenmacher et al., 2003) and other TLR-related molecules, e.g., CD14 (a co-receptor for LPS) and MyD88 (Hiraoka et al., 1998; Tabeta et al., 2000; Hatakeyama et al., 2003). A recent study, using DNA microarray analysis, demonstrated that expressed levels of TLR2, TLR4 and CD14 in the HGFs were higher in periodontitis than in healthy individuals (Wang et al., 2003). Interestingly, upon *in vitro* stimulation with *P.gingivalis* LPS, levels of TLR2, TLR4, CD14, and MD-2 (a co-receptor for TLR4) expression in HGFs increased (Tabeta et al., 2000; Wang et al., 2000; Wang and Ohura, 2002). The data suggest that *P. gingivalis* LPS may responsible for the observed up-regulation of TLR2, TLR4 and CD14 in periodontitis. To date, it is not clear at this point how response of immune and non-immune cells are appropriately tailored by individual TLRs to the advantage of the host.

CHAPTER III

MATERIAL AND METHODS

3.1 Medium

Dulbecco's modified Eagle's medium (DMEM) supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml) and fungizone (2.5 μg/ml) and 10% heat-inactivated fetal calf serum (FCS) (Gibco Laboratory, Grand Island, NY) were used throughout the study. ELISA kits for human IL-8 were obtained from R&D system Inc. (Minneapolis, MA, USA). RPMI 1640 medium supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml) and 10% heat inactivated fetal calf serum (FCS) (Gibco Laboratory, Grand Island, NY) were used. Histopaque 1.077 was obtained from Sigma (St. Louis, MO).

3.2 Toll-Like Receptor Ligands

We were used specific ultrapure TLR ligands for TLRs 2,3,4,5,7,8 and 9 obtained from InvivoGen (San Diego, CA, USA) as shown in table 2.

Table 2: Toll-like receptors and Toll-like receptor-specific ligands used in stimulated experiments

Receptor	Ligand
TLR2	Ultrapure LPS from *Porphyromonas gingivalis*
TLR3	Polyinosine-polycytidylic acid (Poly I:C)(Synthetic analog of double stranded RNA)
TLR4	Purified LPS from *Escherichia coli* K12 strain
TLR5	Purified flagellin from *Salmonella thyphimurium*
TLR7	Loxoribine (Guanosine analog)
TLR8	Single-stranded polyU oligonucleotide complexed with LyoVec™
TLR9	CpG oligonucleotide (CpG ODN) 2006

3.3 Fibroblast Preparation and Cell Culture

3.3.1 Gingival tissue samples

Gingival tissue samples were collected from subjects who have clinically healthy periodontium with probing depth less than 4 mm. The biopsies of gingiva were obtained at the time of crown lengthening procedure for prosthetic reasons from Periodontal Clinic, Faculty of Dentistry, Chulalongkorn University. Informed consent was obtained prior to inclusion in the study. The protocol was approved by the ethics committee of Faculty of Medicine, Chulalongkorn University. The excised tissues were immediately placed in sterile tube containing culture medium, keep on ice and transferred to the laboratory within a few hours for explantation.

3.3.2 Fibroblast cell culture

The method to obtain fibroblasts from the gingival tissues was that described by Murakami et al. (1999). Briefly, the biopsies were washed twice with culture medium to remove blood clots and adherent erythrocytes. Then, the biopsies were minced with scissors into fragment of 1-3 mm^2 and placed in 35 mm. tissue culture dishes. These tissue explants were cultured with culture medium supplemented with 10% heat-inactivated FCS at 37^{o}C in humidified atmosphere of 5% CO_2 in air. Culture medium was changed twice a week. After a confluent monolayer of cells was reached, HGFs were trypsinized, washed twice and then sub-cultured to new tissue culture flasks. The HGFs at passage (P) 4-8 were used throughout the study.

3.3.3 Peripheral blood mononuclear cell isolation

Heparinized peripheral blood (20 ml) was obtained from healthy adult volunteers. PBMC were prepared as previously described by Boyum (1968). Briefly, heparinized peripheral blood was layered on Ficoll-Hypaque (Histopaque), and centrifuged for 30 minutes at 700 x g at 25 ^{o}C. PBMC were collected and washed twice with PBS and then resuspended in RPMI 1640 medium with 10% heat inactivated autologous serum. Cells were counted in haemocytometer and cell viability was assessed by trypan blue exclusion.

3.3.4 mRNA expression of Toll-like receptors on human gingival fibroblasts

HGFs $1x10^6$ cells were trypsinized and washed twice. Total RNA were separated by using RNeasy Mini kit from Qiagen (Chatworth, CA, USA),

according to the manufacturer's instructions. The RNA samples were further purified by successive treatment with DNase I (Qiagen, Chatworth, CA, USA). 4 μl of total RNA were reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR, according to the manufacturer's instructions (Promega, Madison, WI, USA). TLR primer sequences shown in table 3 were used. PCR amplification were performed using Taq DNA polymerase (Qiagen, Chatworth, CA, USA) by the Mastercycler gradient (Eppendorf, Germany) for 35 cycles of 94°C for 30 seconds, 55°C (TLRs1-2, 4-8, 10) or 50°C (TLR3) or 65°C (TLR9) for 30 seconds, 72°C for 1 min, and then a final extension of 72°C for 2 min. Forward and reverse primer pairs selected for human TLRs 1-10 were purchased from Proligo (Singapore). The PCR products were separated on a 1.2 % agarose gel containing ethidium bromide and visualized by UV illumination. Glyceraldehyde - 3 - phosphate dehydrogenase (GAPDH) was used as an internal standard. As a negative control, a PCR reaction was performed without an RT sample. As a positive control, PBMC were used. The methods of RT-PCR for measuring mRNA expression of TLR on PBMC followed the same procedure as those of HGFS.

Table 3: Toll-like receptors primer sequence

Product	Forward primer	Reverse primer	Amplicon size (bp)
TLR1*	CGTAAAACTGGAAGCTTTGCAAGA	CCTTGGGCCATTCCAAATAAGTCC	885
TLR2*	GGCCAGCAAATTACCTGTGTG	CCAGGTAGGTCTTGGTGTTCA	614
TLR3*	ATTGGGTCTGGGAACATTTCTCTTC	GTGAGATTTAAACATTCCTCTTCGC	319
TLR4*	CTGCAATGGATCAAGGACCA	TCCCACTCCAGGTAAGTGTT	622
TLR5#	CCTCATGACCATCCTCACAGTCAC	GGCTTCAAGGCACCAGCCATCTC	355
TLR6$	ACTGACCTTCCTGGATGTGG	TGGCACACCATCCTGAGATA	404
TLR7$	ACAAGATGCCTTCCAGTTGC	ACATCTGTGGCCAGGTAAGG	207
TLR8*	CAGAATAGCAGGCGTAACACATCA	AATGTCACAGGTGCATTCAAAGGG	636
TLR9#	GCGAGATGAGGATGCCCTGCCCTACG	TTCGGCCGTGGGTCCCTGGCAGAAG	510
TLR10$	GGCCAGAAACTGTGGTCAAT	AACTTCCTGGCAGCTCTGAA	287

(* primer sequence by Saikh 2003, # primer sequence by Schaefer 2004, $ nucleotide sequences were determined from PubMed (National Center for Biomedical Information), and the primers were custom designed using primer 3 software.)

3.3.5 Toll-like receptor ligation and cytokine production

HGFs ($1.5x10^5$ cells/ml) seeded in 96-well plates (Corning Inc. Corning, NY, USA) were stimulated with various TLR ligands. After 24 hr., the supernatants were harvested to measure the expression of IL-8. Production of IL-8 in supernatants of TLR ligands stimulated HGFs were measured by commercially available ELISA kit (R&D system Inc.). The assay was performed according to the manufacturer's protocol, cytokine concentrations were calculated by comparison with a standard curve. The detection limit of ELISA assay for IL-8 is 3.5 pg/ml.

3.4 Statistic Analysis

For statistical analysis, we used the Mann – Whitney test for compare the IL-8 production from control wells and TLR ligand stimulation. Results were expressed as mean ± standard error of mean (SEM). Differences with a *P* values of 0.05 or less were considered significant.

CHAPTER IV

RESULT

4.1 mRNA Expression of Toll-Like Receptors on Human Gingival Fibroblasts.

TLRs have been found on many cell types and known to play a central role in pathogen recognition of the innate immunity. Some members of TLR family i.e.TLR2, TLR4, and TLR9 were previously shown to express in cultured human gingival fibroblasts by RT-PCR (Tabeta et al., 2000; Wang et al., 2000; Nonnenmacher et al., 2003). In this study we first established 4 HGF lines prepared from healthy periodontium. A panel of mRNA expression of TLRs (TLRs1-10) on HGFs (P 4-8) was analyzed by RT-PCR. A representative HGF line (HGF5 P5) in figure 1 expressed mRNA of TLRs 1, 2, 3, 4, 5, 6 and 9 but not TLRs 7, 8, and 10. The results of TLR mRNA expression in HGFs from the other three experiments revealed a very similar pattern, although the density of the bands specific for each TLR product was slightly different among individual subjects (Appendix: figure 8, 9 and 10). The levels of TLR3 and/or TLR4 and /or TLR5 mRNA expression seem to be pronounced while those of TLR2 and TLR9 mRNA expression seem to be low, as compared with GAPDH, the reference house keeping gene. Human PBMC, as a positive control, markedly expressed TLR 1-10 mRNA (figure 2). RT-PCR analysis of GAPDH expression confirmed the quality of all RNA preparations used for RT-PCR. No band was detected in the non-RT sample by PCR (figure 3).

HGF5 P5

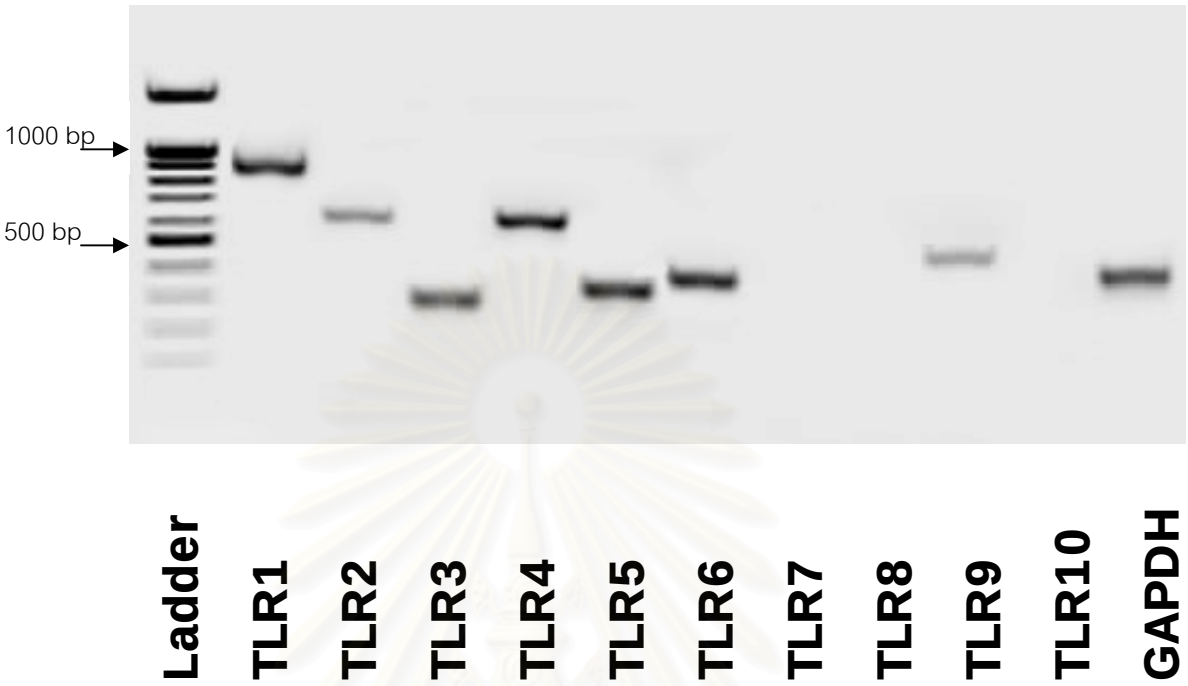

Figure 1. TLR mRNA expression (TLRs 1-10) of HGF5 (1x 10^6 cells) at passage 5 was analyzed by RT-PCR (normalized by GAPDH) as detailed in Materials and Methods. The GAPDH gene was assayed as a positive control. Size marker (Ladder) is the far left lane. Ethidium bromide-stained PCR products were photographed, and then the images were digitalized and analyzed. Data were representative of four independent experiments. PCR was performed once for each assay.

PBMC

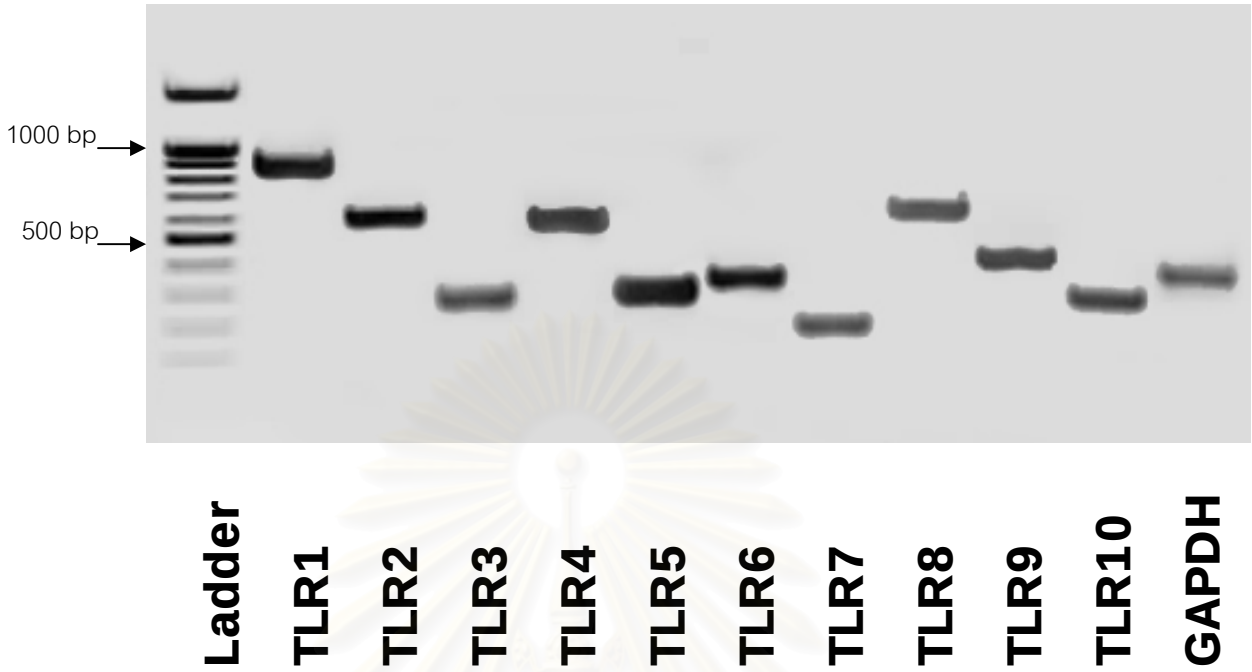

Figure 2. TLR mRNA expression (TLRs 1-10) of PBMC. Human PBMC were used as positive sources of TLRs 1-10 mRNA expression to confirm the specificity of the primers and PCR. The GAPDH gene was assayed as a positive control. Size marker (Ladder) is the far left lane. Ethidium bromide-stained PCR products were photographed, and then the images were digitalized and analyzed. PCR was performed once for each assay.

Negative control

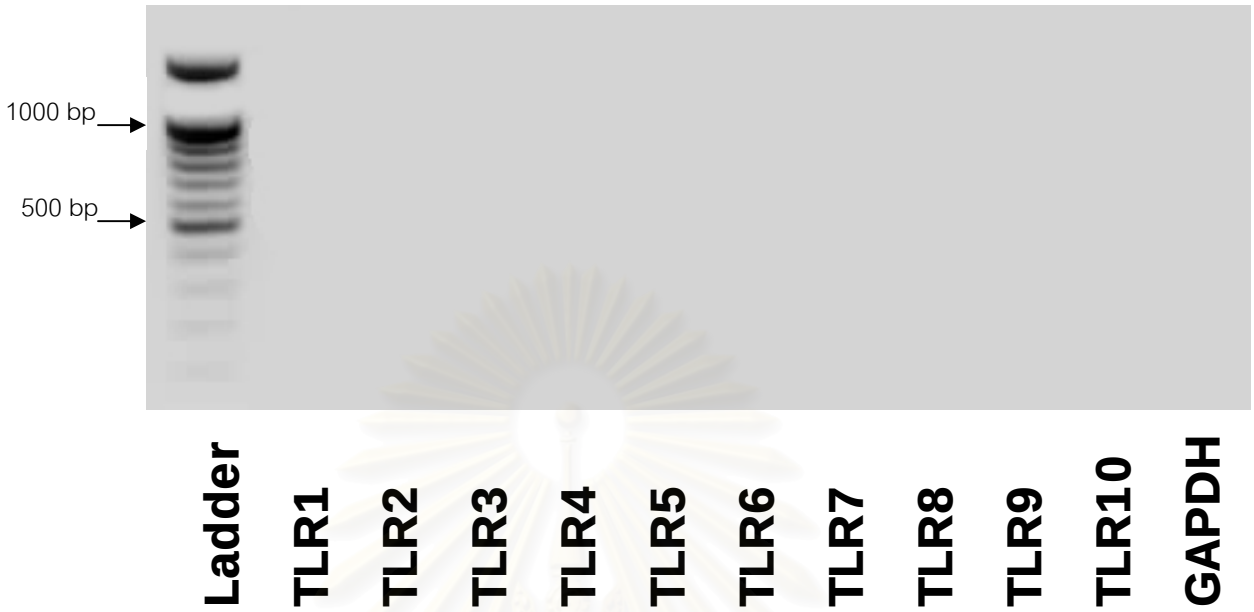

Figure 3. TLR mRNA expression (TLRs 1-10) of PCR products from non-RT samples were examined as a negative control. Size marker (Ladder) is the far left lane. Ethidium bromide-stained PCR products were photographed, and then the images were digitalized and analyzed. PCR was performed once for each assay.

4.2 Toll-Like Receptor Ligation and Interleukin-8 Production.

In order to characterize the functional relevance of TLRs in HGFs, IL-8 production was determined after TLR ligation on HGFs in 4 separate experiments. HGFs were stimulated without and with various TLRs ligands (LPS from *P. gingivalis* – TLR2 ligand; Polyinosine-polycytidylic acid – TLR3 ligand; LPS from *Escherichia coli* – TLR4 ligand; flagellin from *Salmonella thyphimurium* – TLR5 ligand; Loxoribine – TLR7 ligand; Single-stranded polyU oligonucleotide – TLR8 ligand; CpG oligonucleotide 2006 – TLR9 ligand) at a predetermined concentration for 24 hr. Subsequently, the induction of the proinflammatory chemokine IL-8 was quantified by IL-8 ELISA. As shown in a representative HGF line in figure 4, *P. gingivalis* LPS, Poly I:C, *E.Coli* LPS, and flagellin induced strong IL-8 production, whereas stimulation of Loxoribine, ssPolyU, and CpG2006 resulted in no induction of IL-8 production. The IL-8 levels in *P. gingivalis* LPS, Poly I:C, *E.Coli* LPS, and flagellin-stimulated HGF were significantly higher than those in control HGF (unstimulated) ($P < 0.05$) as shown in figure 5. The IL-8-production coincided with their mRNA expression in HGFs of 6 experiments (Appendix: figure 11-15). On the contrary, no IL-8 production was observed in HGF-stimulated with CpG2006 even though the cells express TLR9.

HGF4.1 P6

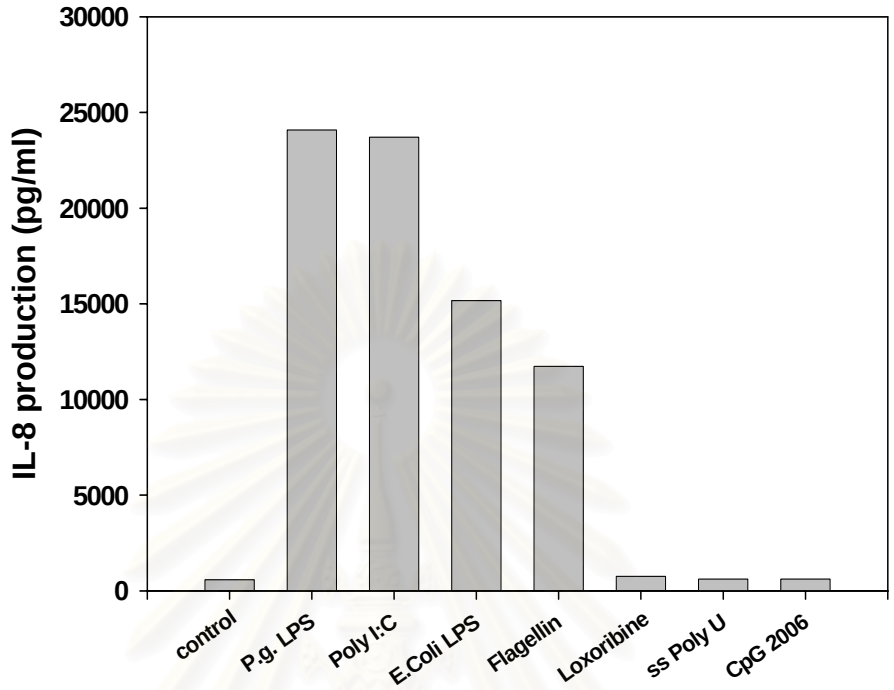

Figure 4. Stimulatory effect of various TLR ligands on IL-8 production by human gingival fibroblasts (HGF4.1 P6). HGFs (1.5×10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml; TLR 5 ligand – flagellin 5 μg/ml; TLR 7 ligand – Loxoribine 100 μM; TLR 8 ligand – ssPolyU 5 μg/ml and TLR 9 ligand - CpG2006 10 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA. Data were representative of four separate experiments.

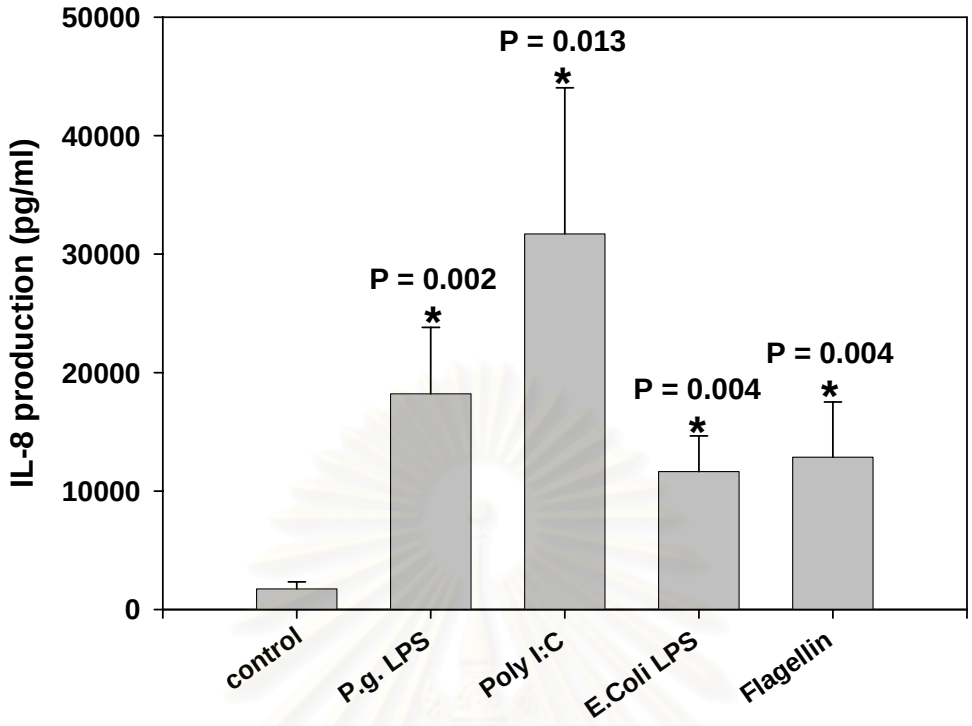

Figure 5. Stimulatory effect of various ligands on IL-8 production by human gingival fibroblasts from six separate experiments. HGFs (1.5×10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml; TLR 5 ligand – flagellin 5 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA. Results are expressed as mean ± standard error of mean. * , significantly different between the group with and without the TLR ligands using Mann Whitney U test ($P < 0.05$).

4.3 The Combination Effect of Toll-Like Receptor Ligands on Interleukin-8 Production by Human Gingival Fibroblasts.

Gingival tissues are consistently exposed to many PAMPs from dental plaque. Simulataneous stimulation of different PAMPs may have different effects upon gingival cells. We next investigated the combination effects of TLR ligands (*P.gingivalis* LPS + Poly I:C; *P.gingivalis* LPS + *E.Coli* LPS; *P.gingivalis* LPS + flagellin; *P.gingivalis* LPS + CpG2006; Poly I:C + *E.Coli* LPS; Poly I:C + flagellin; Poly I:C + CpG2006; *E.Coli* LPS + flagellin; *E.Coli* LPS + CpG2006 and flagellin + CpG2006) on HGFs. Culture supernatants from the HGFs stimulated with single and combination of TLR ligand(s) were collected after 24 hr. incubation. IL-8 production by HGFs was measured by ELISA. We found that nearly all TLR ligand combinations resulted in minimally enhanced IL-8 production when compared with single ligand stimulation (Table 4). The combination of *P.gingivalis* LPS and Poly I:C tend to induce high HGF IL-8 as compared with other combinations (Figure 6, Appendix: figure 17-19). Surprisingly, CpG2006 significantly suppressed IL-8 production when combined with Poly I:C ($P = 0.028$), but not with other TLR ligands as shown in figure 7.

HGF4.1 P6

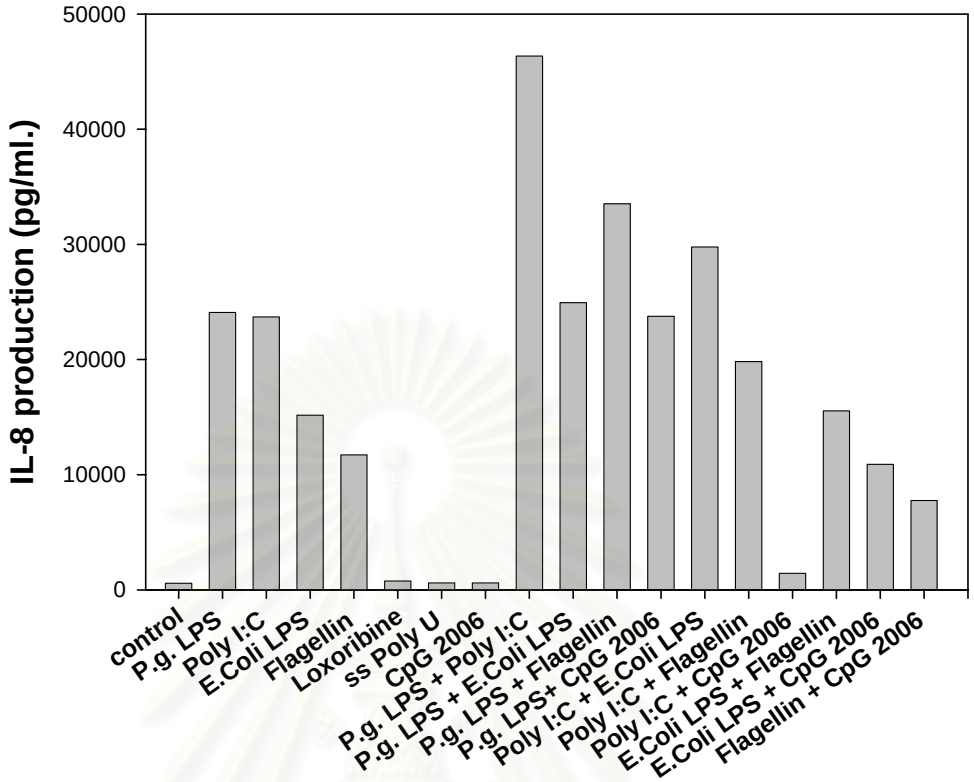

Figure 6. Stimulatory effect of combination TLR ligands on IL-8 production by human gingival fibroblasts (HGF4.1 P6). HGFs (1.5×10^5 cells/ml) were stimulated with single or combination of TLR ligand(s) (control - medium only; *P.gingivalis* LPS 50 μg/ml + Poly I:C 100 μg/ml; *P.gingivalis* LPS + *E.Coli* LPS 10 μg/ml; *P.gingivalis* LPS + flagellin 5 μg/ml; *P.gingivalis* LPS + CpG2006 10 μg/ml; Poly I:C + *E.Coli* LPS; Poly I:C + flagellin; Poly I:C + CpG2006; *E.Coli* LPS + flagellin; *E.Coli* LPS + CpG2006 and flagellin + CpG2006). The culture supernatants were harvested 24 hr. later and then IL-8 production was measured by ELISA. Data were representative of four separate experiments.

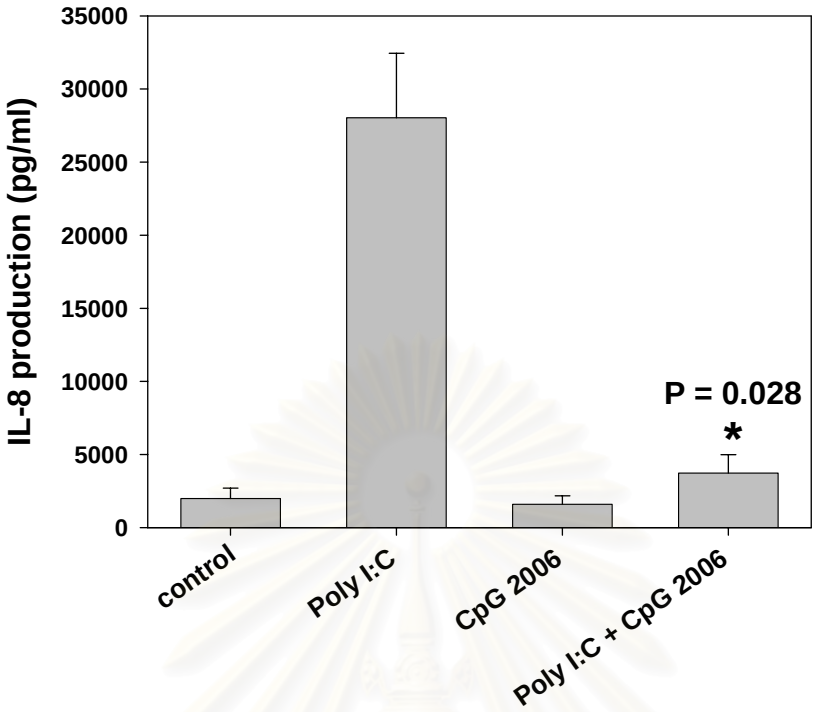

Figure 7. Inhibitory effect of CpG 2006 on Poly I:C-stimulated HGF. HGFs 1.5 x 10^5 cells/ml were stimulated with single TLR ligand (Poly I:C or CpG2006), combined TLR ligands (Poly I:C + CpG2006) or not stimulated. After 24 hr. Incubation, supernatants were harvested and IL-8 production was measured by ELISA. Results are expressed as mean ± standard error of mean. * , significantly different between the group with and without the TLR ligands using Mann Whitney U test ($P < 0.05$). Data from control wells and Poly I:C stimulated HGFs was from 8 experiments, data from CpG stimulated HGFs and combined TLR ligand stimulation was from 7 experiments.

Table 4. Summative data of IL-8 production from HGFs when stimulated with single TLR ligand or combined TLR ligands. Data from control wells and single ligand stimulation for *P.gingivalis* LPS, Poly I:C, *E.Coli* LPS and Flagellin were carried out by 6 experiments and for Loxoribine, ssPolyU, CpG2006 and combined TLR ligands stimulation was carried out by 4 experiments.

HGFs stimulated with TLR ligands	IL-8 production (pg/ml) Mean ± SEM
Control	1,748 ± 785
1 .*P.g.* LPS 50 μg/ml. (TLR2)	18,192 ± 5,617
2. Poly I:C 100 μg/ml.(TLR3)	31,703 ± 11,369
3. *E.Coli* LPS 10 μg/ml. (TLR4)	11,633 ± 3,023
4. flagellin 5 μg/ml. (TLR5)	12,844 ± 4,660
5. Loxoribine 100 μg/ml. (TLR7)	1,549 ± 801
6. ss Poly U 5 μg/ml. (TLR8)	1,279 ± 677
7. CpG 2006 10 μg/ml. (TLR9)	1,301 ± 681
8. *P.g.* LPS 50 μg/ml.+ Poly I:C 100 μg/ml.(TLR2+3)	37,652 ± 16,998
9. *P.g.* LPS 50 μg/ml.+ *E.Coli* LPS 10 μg/ml.(TLR2+4)	20,616 ± 9,053
10. *P.g.* LPS 50 μg/ml.+ flagellin 5 μg/ml.(TLR2+5)	26,223 ± 11,567
11. *P.g.* LPS 50 μg/ml.+ CpG 2006 10 μg/ml.(TLR2+9)	21,024 ± 10,348
12. Poly I:C 100 μg/ml.+ *E.Coli* LPS 10 μg/ml.(TLR3+4)	20,926 ± 9,388
13. Poly I:C 100 μg/ml.+ flagellin 5 μg/ml.(TLR3+5)	19,359 ± 8,574
14. Poly I:C 100 μg/ml.+ CpG 2006 10 μg/ml.(TLR3+9)	2,657 ± 1,557 *
15. *E.Coli* LPS 10 μg/ml.+ flagellin 5 μg/ml.(TLR4+5)	14,199 ±6,464
16. *E.Coli* LPS 10 μg/ml.+ CpG 2006 10 μg/ml.(TLR4+9)	10,162 ± 5,056
17. Flagellin 5 μg/ml.+ CpG 2006 10 μg/ml.(TLR5+9)	9,155 ±4,500

***** Significantly suppressed IL-8 production when compare to Poly I:C activation.

CHAPTER V

DISCUSSION AND CONCLUSION

Human gingival fibroblasts are the dominant cells, which support and anchor the tooth. Recent evidence demonstrates that HGFs also play crucial role in local immunity of the local periodontal tissues. In response to bacterial products, HGFs produce various inflammatory mediators. Previous studies reported TLRs 2, 4 and 9 mRNA expression on HGFs (Tabeta et al., 2000; Wang et al., 2000; Nonnenmacher et al., 2003). In this study, we investigated a panel of TLRs expression on HGFs analyzed by RT-PCR. We demonstrated the expression of mRNA of TLRs 1, 2, 3, 4, 5, 6 and 9 but not TLRs 7, 8 and 10 in all HGF lines (n = 4). Our results support the current study of nasal mucosal fibroblasts which demonstrated the mRNA expression of TLRs 1, 2, 3, 4, 5, 6 and 9, but not TLRs 7, 8 and 10 (Takahashi et al., 2006). By using immunohistochemical staining, expression of TLR2 and TLR4 was observed in inflamed periodontal tissues. Most TLR2 and TLR4-positive cells detected were morphologically macrophages (Mori et al., 2003). It's not surprisingly, since HGFs expressed very low surface TLRs 2 and 4 as monitored by flowcytometric analysis (our own observations).

To date, there has been no study to evaluate the response of HGFs to a variety of TLR ligand stimulation. Therefore, we next measured the ability of highly purified TLR ligands to activate HGFs *in vitro*. We found that TLR ligands for TLRs 2, 3, 4 and 5 (*P.gingivalis* LPS, Poly I:C, *E.Coli* LPS and flagellin respectively) were able to stimulate IL-8 production but TLR ligands for TLRs 7, 8 and 9 (Loxoribine, ssPolyU and CpG2006) were unable to induce

IL-8 production. The ability to induce IL-8 production coincided with the expression of gingival fibroblast TLRs. Surprisingly, CpG2006 was unable to induce IL-8 production. *E. coli* DNA, which recognizes by TLR9, also did not induce IL-8. Our data may indicate non-functional TLR9 or inability of HGFs to bind/up-take CpG2006. Recent data showed that DNA preparation from periodontopathic bacteria activated HGFs to produce IL-6 and IL-8 (Takeshita et al., 1999; Nonnenmacher, et al., 2003). This discrepancy could be due to the LPS contamination in periodontopathic bacteria DNA preparation, since highly purified ligands were used in our study.

Subgingival plaque consist of many PAMPs that recognized by TLRs. For examples, *P. gingivalis* LPS, *Bacteroides fragilis* LPS (Erridge et al., 2004) and *Capnocytophaga ochracea* LPS bind TLR2 (Yoshimura et al., 2002). In contrast, *A. actinomycetemcomitans* and *Fusobacterium nucleatum* LPS bind to TLR4 (Mochizuki et al., 2004; Yoshimura et al., 2002). The expression of many TLRs on HGFs may provide the opportunity in recognition of various bacterial PAMPs in the periodontal lesions and thus suggesting the critical role of HGFs in innate immune response.

We further evaluated that simultaneous stimulation of different TLR ligands may result in an augmented HGF-IL-8 response. HGFs were stimulated with single and combinations of several TLR ligands (*P.gingivalis* LPS + Poly I:C, *P.gingivalis* LPS + *E.Coli* LPS, *P.gingivalis* LPS + flagellin, *P.gingivalis* LPS + CpG2006, Poly I:C + *E.Coli* LPS, Poly I:C + flagellin, Poly I:C + CpG2006, *E.Coli* LPS + flagellin, *E.Coli* LPS + CpG2006 and flagellin + CpG2006). Generally those combinations minimally enhanced HGF IL-8 production as compared to those stimulated with single TLR ligand.

Interestingly, IL-8 production from Poly I:C activation was markedly suppressed when combined with CpG2006 (*P* value < 0.05). Data from our laboratory also indicated that not only CpG2006 but also other DNAs such as non-CpGDNA, microbial DNA and calf thymus DNA could inhibit IL-8 production. At present, it is not clear about the mechanism underlying the inhibitory effect. Further studies are required.

In conclusion, this is the first study to fully evaluate the mRNA expression of TLRs 1-10 on HGFs as well as their responses to a variety of TLR ligands and their combinations. The expression of TLRs on HGFs (TLRs 1, 2, 3, 4, 5, 6 and 9) and their IL-8 response to TLR ligation indicate the role of HGFs in innate immunity in the local periodontal tissue. TLR triggering by TLR ligand stimulation on HGFs is indeed a very interesting issue. It is known that many types of pro-inflammatory cytokines are frequently found in high levels in inflamed periodontal lesions. The cytokine, especially IFN-γ is known for its synergistic effect with TLR4 ligand (LPS). For example, IFN-γ-primed HGFs significantly enhance IL-8 production when stimulated with *Salmonella enterica* LPS (Tamai R. et al., 2002). Further study into the effect of the combinations between cytokines such as IFN-γ, TNF-α and IL-1β and TLR ligand (local PAMPs) on HGFs should be investigated.

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APPENDICES

HGF1 P5

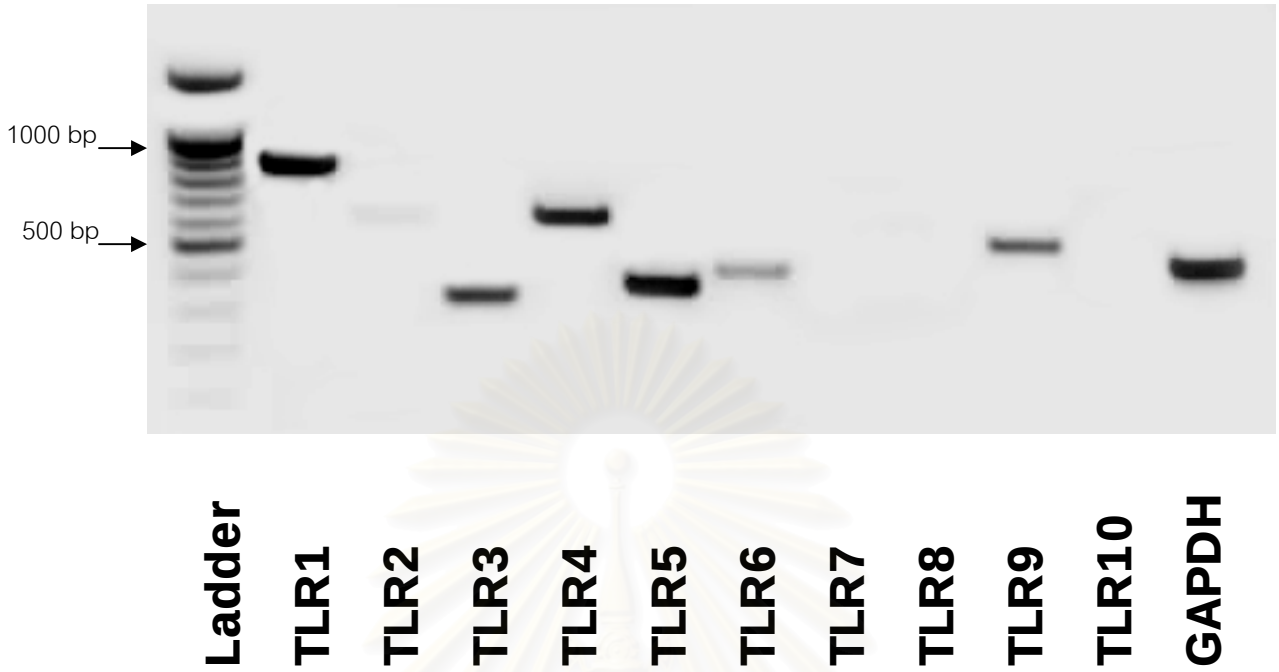

Figure 8. TLR mRNA expression of HGF1 (1x 10^6 cells) at passage 5 was analyzed by RT-PCR (normalized by GAPDH) as detailed in Materials and Methods. Size marker (Ladder) is the far left lane. Ethidium bromide-stained PCR products were photographed, and then the images were digitalized and analyzed. PCR was performed once for each assay.

HGF3 P5

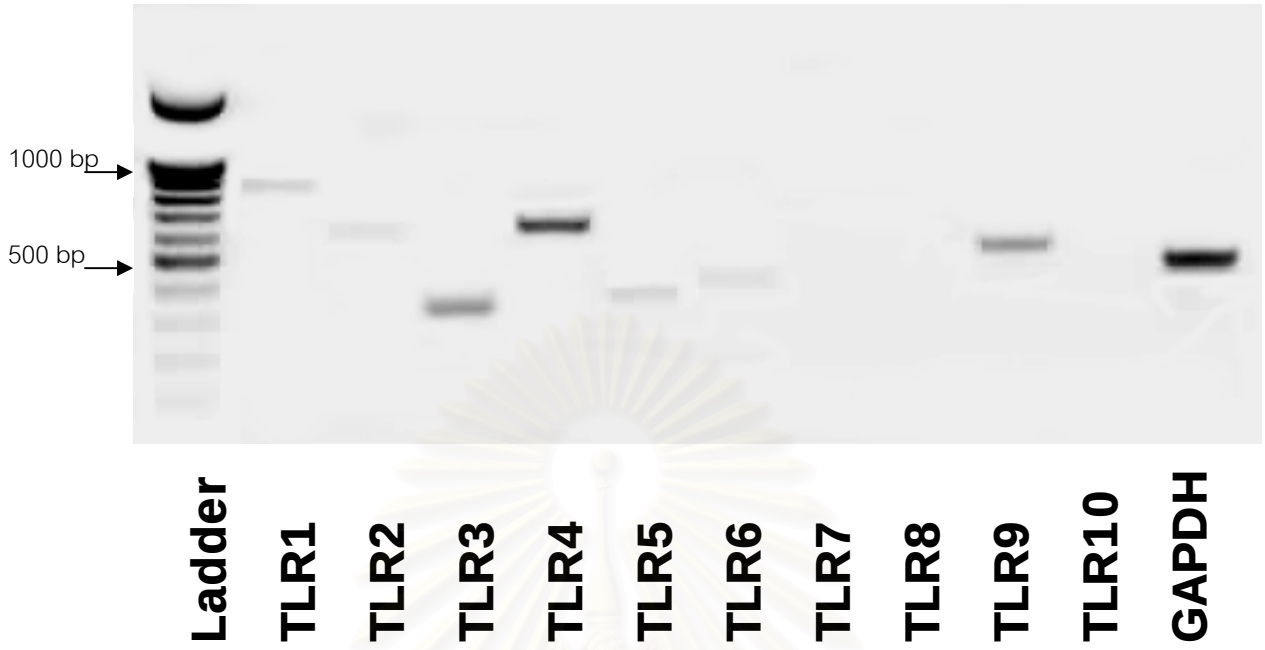

Figure 9. TLR mRNA expression of HGF3 (1x 10^6 cells) at passage 5 was analyzed by RT-PCR (normalized by GAPDH) as detailed in Materials and Methods. Size marker (Ladder) is the far left lane. Ethidium bromide-stained PCR products were photographed, and then the images were digitalized and analyzed. PCR was performed once for each assay.

HGF4 P5

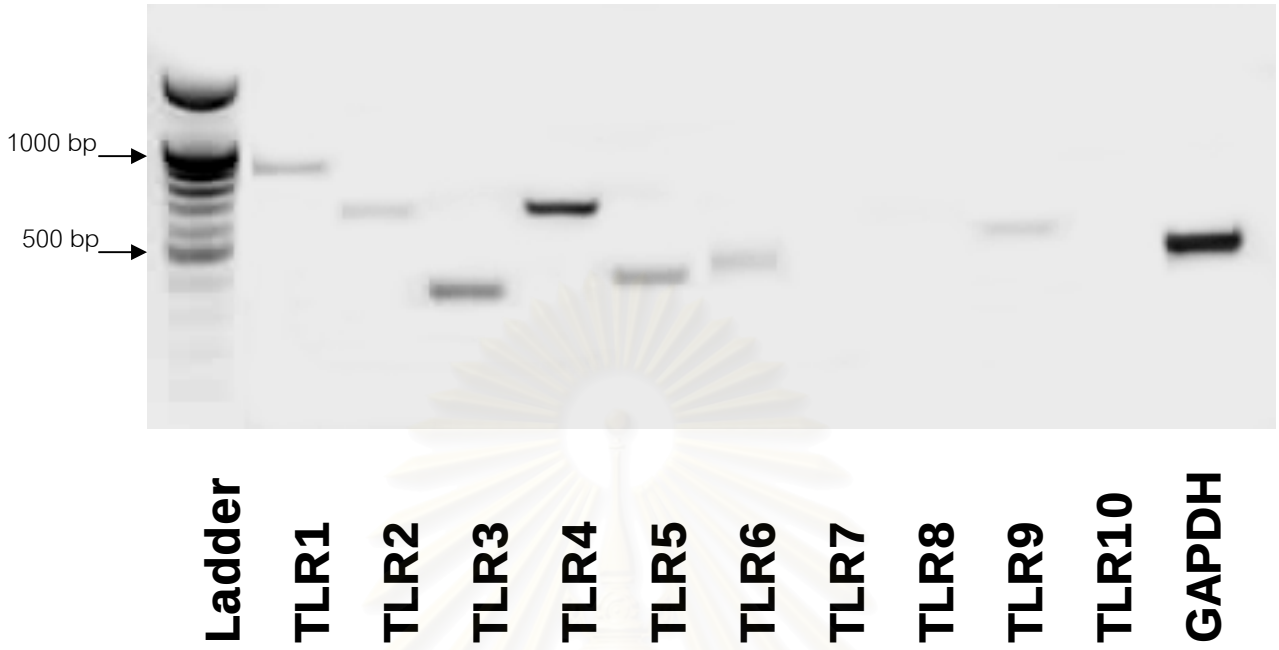

Figure 10. TLR mRNA expression of HGF4 (1x 10^6 cells) at passage 5 was analyzed by RT-PCR (normalized by GAPDH) as detailed in Materials and Methods. Size marker (Ladder) is the far left lane. Ethidium bromide-stained PCR products were photographed, and then the images were digitalized and analyzed. PCR was performed once for each assay.

HGF3 P4

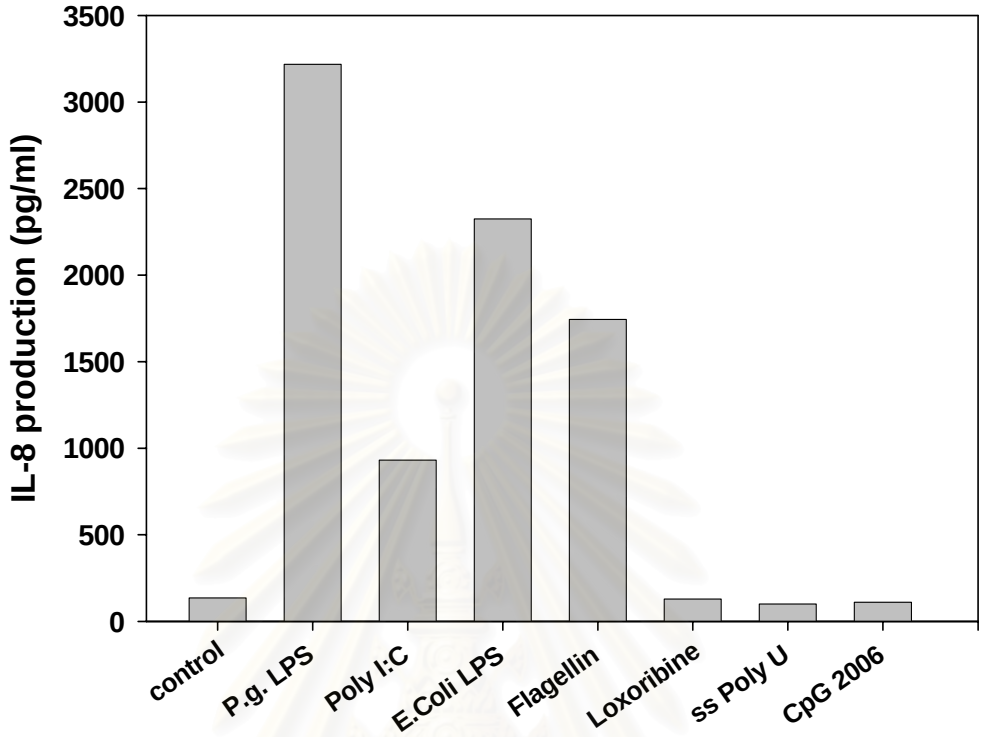

Figure 11. Stimulatory effect of various TLR ligands on IL-8 production by human gingival fibroblasts (HGF3 P4). HGFs (1.5×10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml; TLR 5 ligand – flagellin 5 μg/ml; TLR 7 ligand – Loxoribine 100 μM; TLR 8 ligand – ssPolyU 5 μg/ml and TLR 9 ligand - CpG2006 10 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA.

HGF4.2 P6

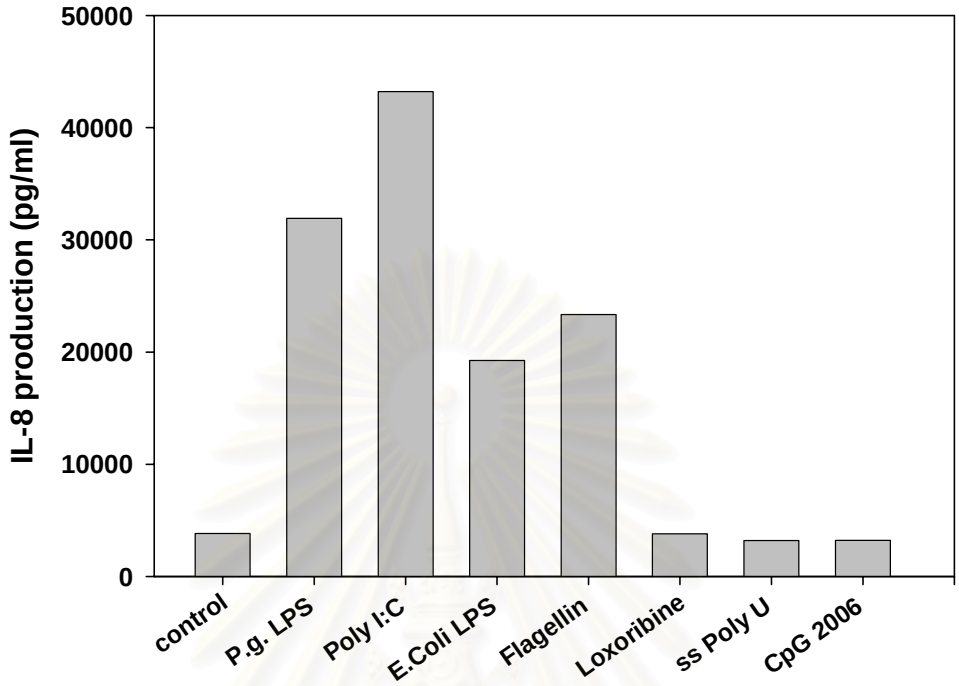

Figure 12. Stimulatory effect of various TLR ligands on IL-8 production by human gingival fibroblasts (HGF4.2 P6). HGFs (1.5 x 10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml; TLR 5 ligand – flagellin 5 μg/ml; TLR 7 ligand – Loxoribine 100 μM; TLR 8 ligand – ssPolyU 5 μg/ml and TLR 9 ligand - CpG2006 10 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA.

HGF5 P7

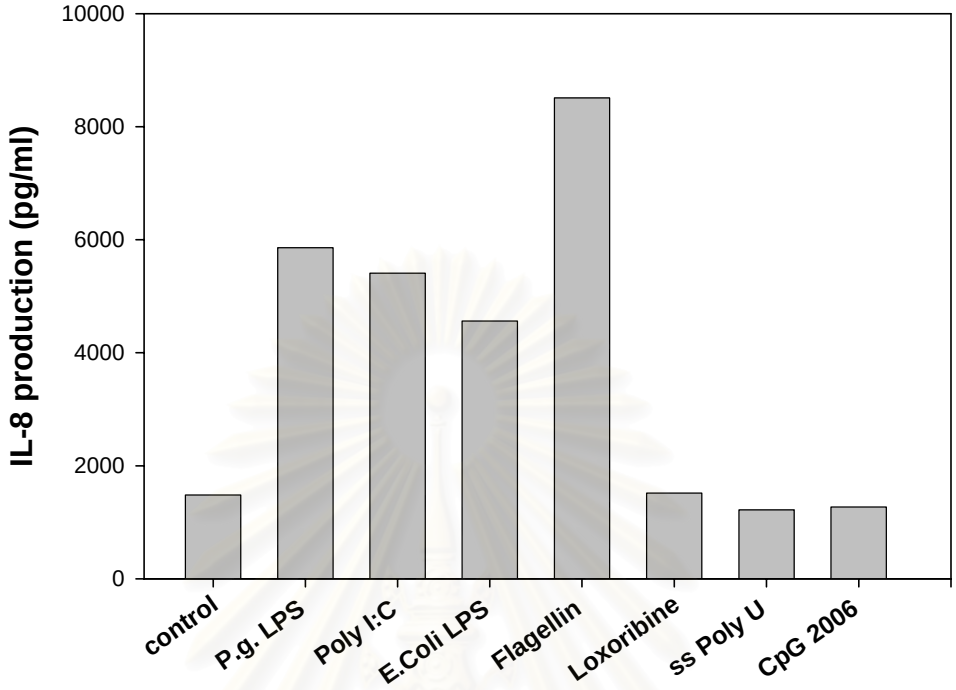

Figure 13. Stimulatory effect of various TLR ligands on IL-8 production by human gingival fibroblasts (HGF5 P7). HGFs (1.5 x 10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml; TLR 5 ligand – flagellin 5 μg/ml; TLR 7 ligand – Loxoribine 100 μM; TLR 8 ligand – ssPolyU 5 μg/ml and TLR 9 ligand - CpG2006 10 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA.

HGF7 P6

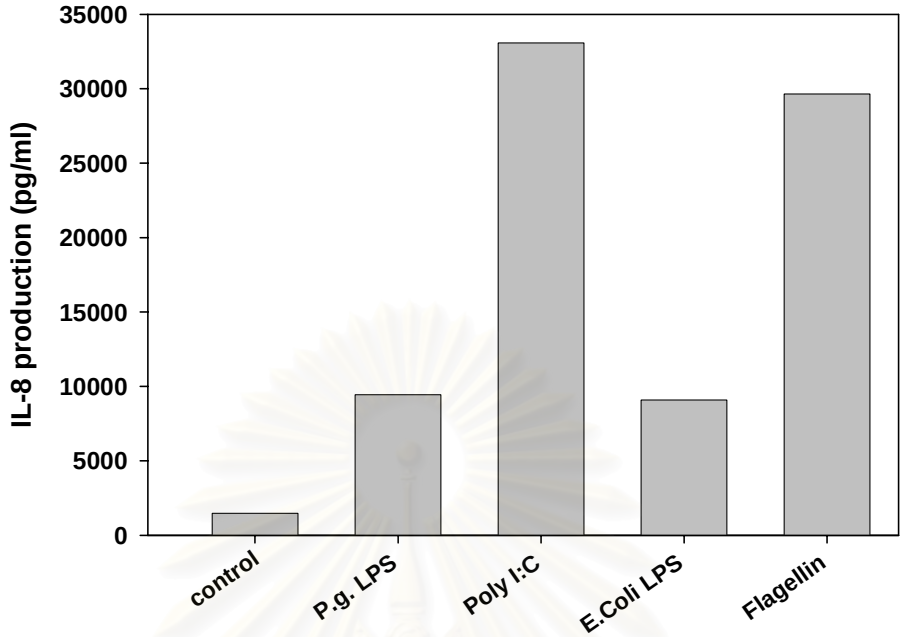

Figure 14. Stimulatory effect of various TLR ligands on IL-8 production by human gingival fibroblasts (HGF7 P6). HGFs (1.5 x 10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml and TLR 5 ligand – flagellin 5 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA.

HGF8 P5

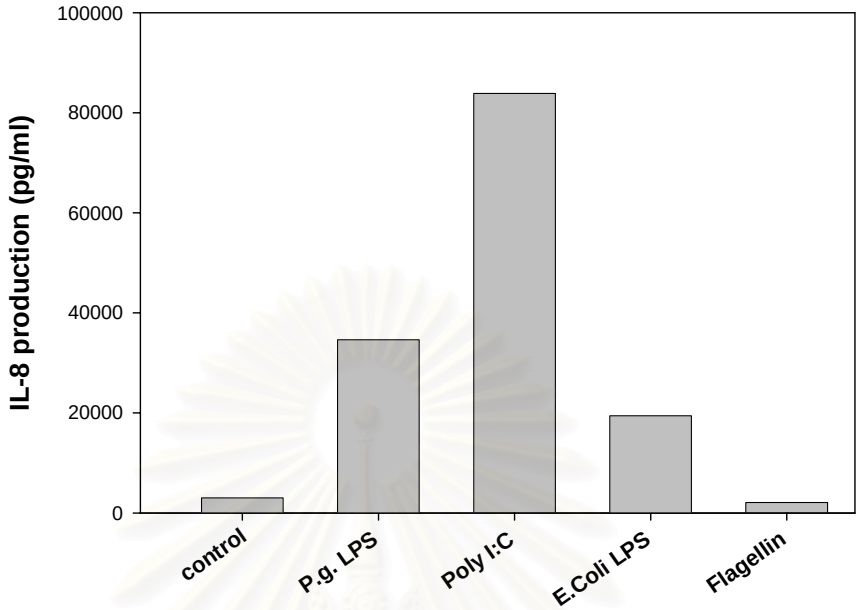

Figure 15. Stimulatory effect of various TLR ligands on IL-8 production by human gingival fibroblasts (HGF8 P5). HGFs (1.5 x 10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml and TLR 5 ligand – flagellin 5 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA.

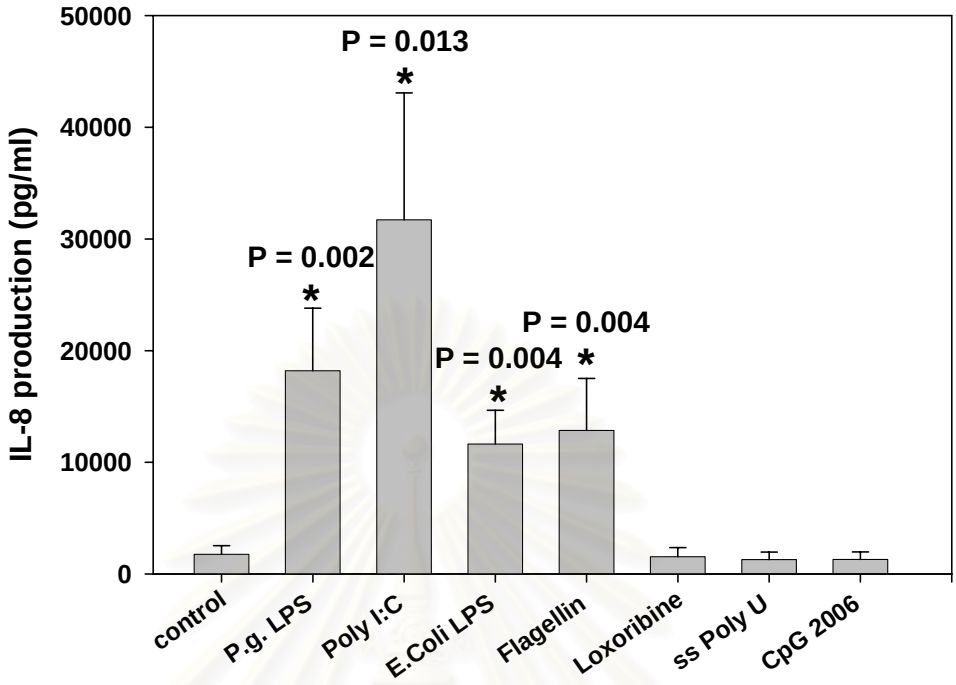

Figure 16. Stimulatory effect of various ligands on IL-8 production by human gingival fibroblasts. HGFs (1.5 x 10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml; TLR 5 ligand – flagellin 5 μg/ml; TLR 7 ligand – Loxoribine 100 μM; TLR 8 ligand – ssPolyU 5 μg/ml and TLR 9 ligand - CpG2006 10 μg/ml). The culture supernatants were harvested 24 hr. and IL-8 production was measured by ELISA. Results are expressed as mean ± standard error of mean. * , significantly different between the group with and without the TLR ligands using Mann Whitney U test ($P < 0.05$). Data from control wells and single ligand stimulated HGF (*P.gingivalis* LPS, Poly I:C, *E.Coli* LPS and flagellin) was from 6 experiments, data from Loxoribine, ssPolyU and CpG2006 stimulated HGF was from 4 experiments.

HGF3 P4

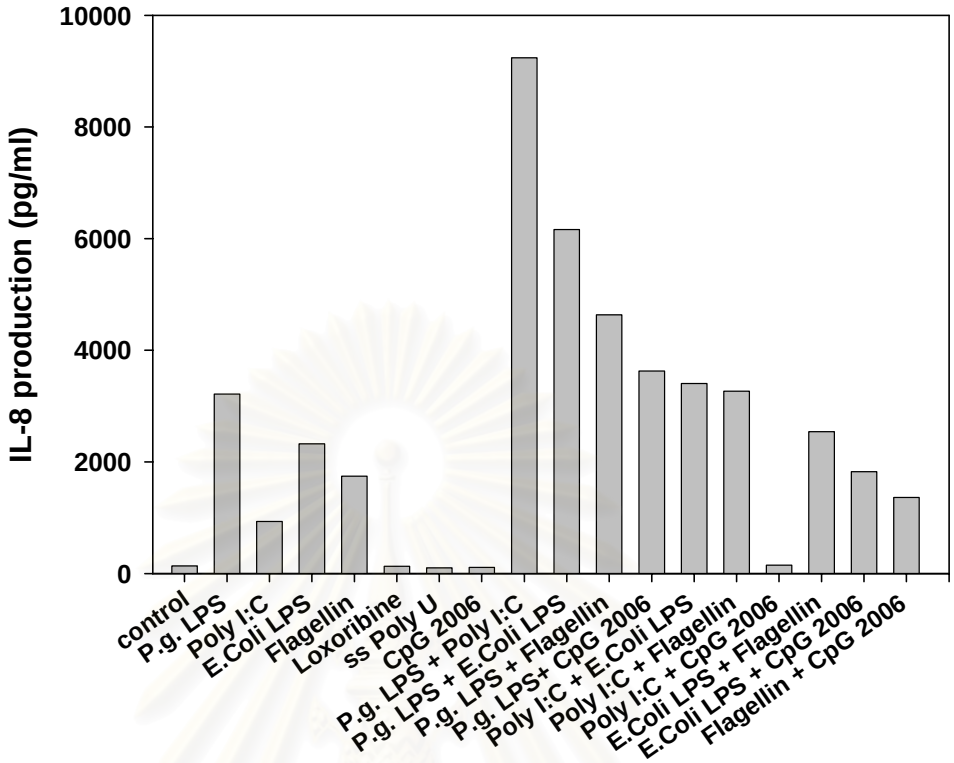

Figure 17. Stimulatory effect of combination TLR ligands on IL-8 production by human gingival fibroblasts (HGF3 P4). HGFs (1.5 x 10^5 cells/ml) were stimulated with single or combination TLR ligand(s) (control - medium only; *P.gingivalis* LPS 50 μg/ml + Poly I:C 100 μg/ml; *P.gingivalis* LPS + *E.Coli* LPS 10 μg/ml; *P.gingivalis* LPS + flagellin 5 μg/ml; *P.gingivalis* LPS + CpG2006 10 μg/ml; Poly I:C + *E.Coli* LPS; Poly I:C + flagellin; Poly I:C + CpG2006; *E.Coli* LPS + flagellin; *E.Coli* LPS + CpG2006 and flagellin + CpG2006). The culture supernatants were harvested 24 hr. later and then IL-8 production was measured by ELISA.

HGF4.2 P6

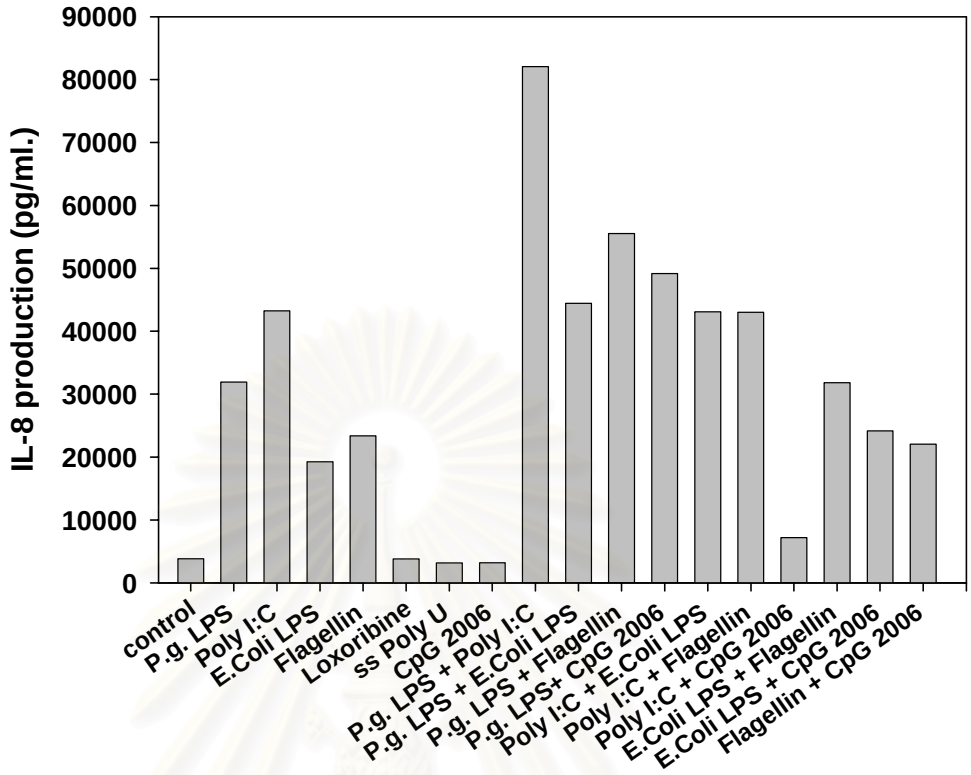

Figure 18. Stimulatory effect of combination TLR ligands on IL-8 production by human gingival fibroblasts (HGF4.2 P6). HGFs (1.5 x 10^5 cells/ml) were stimulated with single or combination TLR ligand(s) (control - medium only; *P.gingivalis* LPS 50 μg/ml + Poly I:C 100 μg/ml; *P.gingivalis* LPS + *E.Coli* LPS 10 μg/ml; *P.gingivalis* LPS + flagellin 5 μg/ml; *P.gingivalis* LPS + CpG2006 10 μg/ml; Poly I:C + *E.Coli* LPS; Poly I:C + flagellin; Poly I:C + CpG2006; *E.Coli* LPS + flagellin; *E.Coli* LPS + CpG2006 and flagellin + CpG2006). The culture supernatants were harvested 24 hr. later and then IL-8 production was measured by ELISA.

HGF5 P7

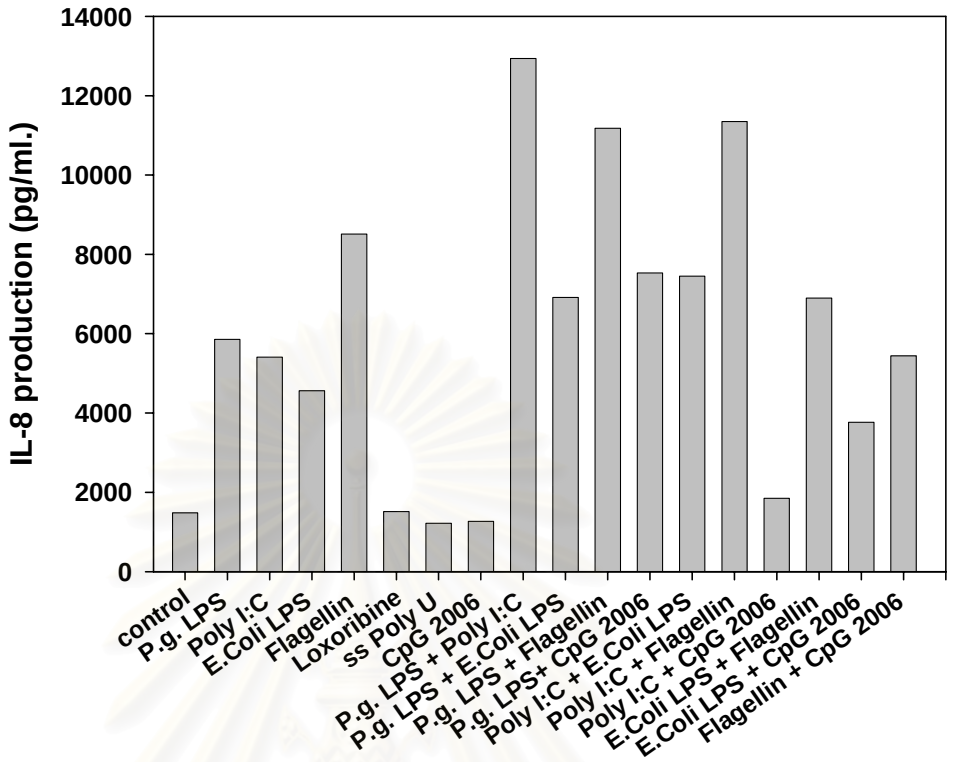

Figure 19. Stimulatory effect of combination TLR ligands on IL-8 production by human gingival fibroblasts (HGF5 P7). HGFs (1.5 x 10^5 cells/ml) were stimulated with single or combination TLR ligand(s) (control - medium only; *P.gingivalis* LPS 50 μg/ml + Poly I:C 100 μg/ml; *P.gingivalis* LPS + *E.Coli* LPS 10 μg/ml; *P.gingivalis* LPS + flagellin 5 μg/ml; *P.gingivalis* LPS + CpG2006 10 μg/ml; Poly I:C + *E.Coli* LPS; Poly I:C + flagellin; Poly I:C + CpG2006; *E.Coli* LPS + flagellin; *E.Coli* LPS + CpG2006 and flagellin + CpG2006). The culture supernatants were harvested 24 hr. later and then IL-8 production was measured by ELISA.

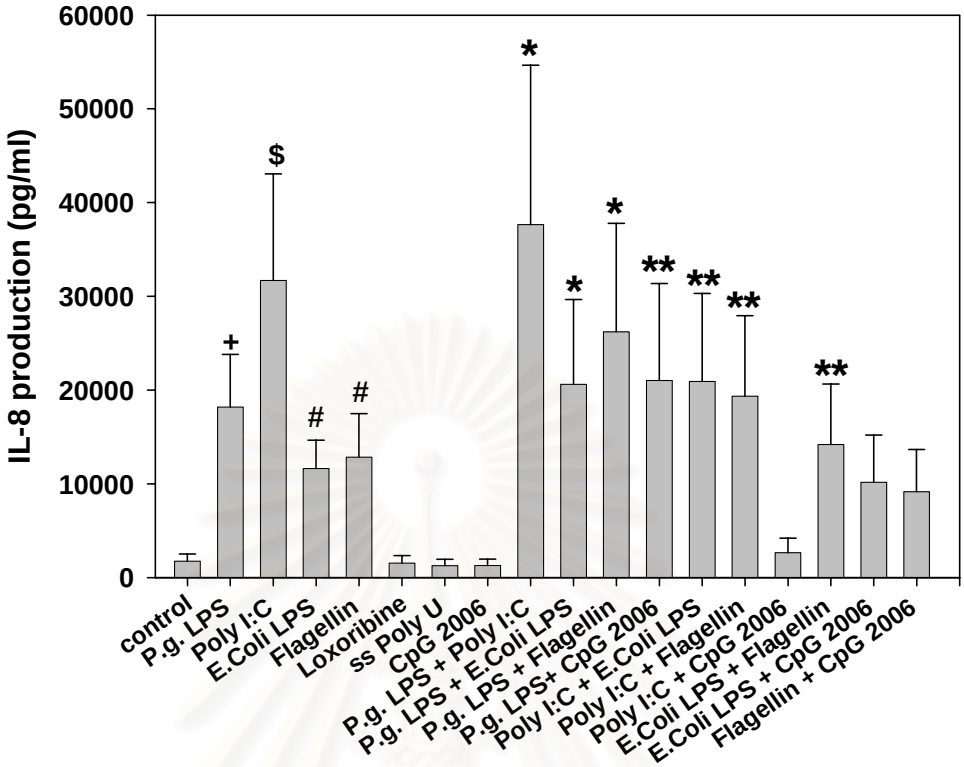

Figure 20. Stimulatory effect of single and combination TLR ligand(s) on IL-8 production by HGFs. HGFs (1.5 x 10^5 cells/ml) were stimulated or not stimulated with various TLR ligands (control - medium only; TLR 2 ligand - *P.gingivalis* LPS 50 μg/ml; TLR 3 ligand - Poly I:C 100 μg/ml; TLR 4 ligand - *E.Coli* LPS 10 μg/ml; TLR 5 ligand – flagellin 5 μg/ml; TLR 7 ligand – Loxoribine 100 μM; TLR 8 ligand – ssPolyU 5 μg/ml; TLR 9 ligand - CpG2006 10 μg/ml; *P.gingivalis* LPS + Poly I:C; *P.gingivalis* LPS + *E.Coli* LPS; *P.gingivalis* LPS + flagellin; *P.gingivalis* LPS + CpG2006; Poly I:C + *E.Coli* LPS; Poly I:C + flagellin; Poly I:C + CpG2006; *E.Coli* LPS + flagellin; *E.Coli* LPS + CpG2006 and flagellin + CpG2006). The culture supernatants were harvested 24 hr. later and then IL-8 production was measured by ELISA. Results are expressed as mean ± standard error of mean. Significantly different between the group with and without the TLR ligands using Mann Whitney U test ($P < 0.05$). ($^{+}$ P = 0.002, $^{\#}$ P = 0.004, $^{\$}$ P = 0.013, * $P = 0.014$, ** $P = 0.029$). Data from control wells and single stimulated HGF (*P.gingivalis* LPS, Poly I:C, *E.Coli* LPS and flagellin) was from six experiments, data from Loxoribine, ssPolyU and CpG2006 stimulated HGF and combined TLR ligands stimulation was from four experiments.

Table 5: The effect of TLR ligands on IL-8 production by human gingival fibroblasts (n = 4: HGF3 P4, HGF4.1 P6, HGF4.2 P6 and HGF5 P7)

TLR ligands	IL-8 production (pg/ml)			
	HGF3 P4	HGF4.1 P6	HGF4.2 P6	HGF5 P7
Control	135.1	578.0	3,829.1	1,480.0
P.g. LPS 50 μg/ml. (TLR2)	3,217.5	24,086.0	31,922.0	5,858.0
Poly I:C 100 μg/ml.(TLR3)	931.7	23,706.0	43,222.0	5,408.0
E.Coli LPS 10 μg/ml. (TLR4)	2,324.0	15,163.3	19,250.0	4,562.0
Flagellin 5 μg/ml. (TLR5)	1,743.8	11,726.8	23,349.0	8,510.0
Loxoribine 100 μg/ml.(TLR7)	128.1	754.5	3,795.8	1,516.0
ss Poly U 5 μg/ml. (TLR8)	100.0	605.5	3,192.3	1,220.0
CpG 2006 10 μg/ml. (TLR9)	110.4	609.2	3,215.6	1,270.0
P.g. LPS 50 μg/ml.+ Poly I:C 100 μg/ml.	9,239.4	46,350.0	82,078.0	12,940.0
P.g. LPS 50 μg/ml.+ *E.Coli* LPS 10 μg/ml.	6,163.7	24,934.0	44,452.0	6,913.0
P.g. LPS 50 μg/ml.+ Flagellin 5 μg/ml.	4,634.0	33,535.0	55,542.0	11,180.0
P.g. LPS 50 μg/ml.+ CpG 2006 10 μg/ml.	3,626.4	23,755.0	49,181.0	7,534.0
Poly I:C 100 μg/ml.+ *E.Coli* LPS 10 μg/ml.	3,404.3	29,765.0	43,081.0	7,453.0
Poly I:C 100 μg/ml.+ Flagellin 5 μg/ml.	3,266.9	19,822.4	42,998.0	11,350.0
Poly I:C 100 μg/ml.+ CpG 2006 10 μg/ml.	151.8	1,429.5	7,201.4	1,847.0
E.Coli LPS 10 μg/ml.+ Flagellin 5 μg/ml.	2,541.2	15,541.0	31,816.0	6,897.0
E.Coli LPS 10 μg/ml.+ CpG 2006 10 μg/ml.	1,823.9	10,900.4	24,156.0	3,769.0
Flagellin 5 μg/ml.+ CpG 2006 10 μg/ml	1,361.4	7,760.6	22,059.0	5,438.0

BIOGRAPHY

Miss Pattanin Montreekachon was born on 19 th of September 1976 in Lampang province. She graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Chiangmai University in 1999, and became a staff member at Kasetwisai Hospital, Roiet province. In 2001, she became a staff member of the Faculty of Dentistry, Chiangmai University, Chiangmai. She studied in a Master degree program in Periodontology at Graduate School, Chulalongkorn University in 2003.