รายงานวิจัยฉบับสมบูรณ์

โครงการวิจัยเรื่อง การศึกษาเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์
ซึ่งเป็นเซลล์สำคัญในระบบอินเนต และบทบาทในการควบคุม
ระบบภูมิคุ้มกันในโรคปริทันต์

โดย

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กรกฎาคม 2552

สัญญาเลขที่ BRG4980006

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สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย (สกว.)

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

กิตติกรรมประกาศ

คณะผู้วิจัยขอขอบคุณ สกว. ที่ได้ให้การสนับสนุนในการทำงานอย่างต่อเนื่อง และคณะผู้วิจัยขอขอบคุณ สถาบันวิจัยวิทยาศาสตร์การแพทย์ทหารที่ให้ความร่วมมือในการทำวิจัย และสุดท้ายนี้ขอขอบคุณคณะทันตแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ที่เล็งเห็นความสำคัญในงานวิจัยและสนับสนุนคณะผู้วิจัยเป็นอย่างดียิ่งมาโดยตลอด

Human gingival fibroblast as a critical innate non-immune cell and its immunoregulatory role in periodontium

ABSTRACT

Gingival fibroblasts, the major cell type in periodontal connective tissues, provide a tissue framework for tooth anchorage. Until recently, they were presumed to be immunologically inert. Currently, however, our observations and other researchers recognize their active role in host defense. By using an in vitro model of primary human gingival fibroblasts (HGFs) derived from healthy gingival biopsies, we demonstrate that these cells express a variety of pattern recognition receptors- Toll like receptors (TLR) which are key sensors to microbial invasion. Our study demonstrates that HGFs express mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9. Triggering with *P. gingivalis* LPS, poly I:C, *E. coli* LPS, and *S. typhimurium* flagellin, ligands specific for TLRs 2, 3, 4, and 5 respectively, led to the expression of chemokine-IL-8 and immune suppressive enzyme-IDO. In contrast, the potent TLR9 ligand CpG ODN 2006 did not induce IL-8 and IDO expression which requires further research. The ability to induce IL-8 and IDO expression in ligand-stimulated HGFs was enhanced when combined with inflammatory cytokine TNF-α and IFN-γ respectively. Finally, that HGFs can enhance IDO expression and down-regulate T cell response, when stimulated with some PAMP-cytokine combinations, resulting in immune suppression. We then further investigated the effect of recently identified IL-17 especially in the combined stimulation with IFN-γ via HGF activation. IL-17, a product of T-helper(Th)17, which has emerged as a crucial regulator of inflammatory responses. Apparently, IL-17 or IFN-γ differentially controls HGF activation, possibly via different intracellular signaling pathways. IL-17 triggers IL-8 production, whereas IFN-γ triggers adhesion molecule-ICAM-1 and co-stimulatory molecule- HLA-DR, as well as IDO. These effects of individual cytokine are potentiated by their combination. Overall our systematical investigation therefore suggests that the critical role of these strategically-placed cells, HGFs, not only in orchestrating the innate immune response, but also in dampening potentially harmful hyperactive inflammation in periodontal tissue.

การศึกษาเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์ ซึ่งเป็นเซลล์สำคัญในระบบอินเนต และบทบาทในการควบคุมระบบภูมิคุ้มกันในโรคปริทันต์

บทคัดย่อ

เซลล์ไฟโบรบลาสต์จากเหงือกเป็นเซลล์ที่พบมากที่สุดในเนื้อเยื่อเกี่ยวพันทางปริทันต์ (periodontal connective tissue) ที่จัดเตรียมโครงร่างของเนื่อเยื่อสำหรับยึดเหนี่ยวฟัน กระทั้งเมื่อไม่นานมานี้ เซลล์เหล่านี้ยังถูกสันนิษฐานว่าไม่มีการตอบสนองทางภูมิคุ้มกัน ในปัจจุบันนี้ ถึงอย่างไรคณะวิจัยของเราและนักวิจัยอื่นๆ ได้ยอมรับถึงบทบาทที่มีประสิทธิภาพในการป้องกันตัวเองของร่างกาย โดยใช้การศึกษาในหลอดทดลองของเซลล์ไฟโบรบลาสต์ที่ได้จากการเพาะเลี้ยงจากเหงือกของมนุษย์ (HGFs) ที่มีสุขภาพเหงือกปกติ คณะผู้วิจัยแสดงให้เห็นว่าเซลล์เหล่านี้มีการแสดงออกของตัวรับ (pattern recogniton receptors) ที่สามารถจดจำโครงสร้างของโมเลกุลที่เรียกว่า โทลล์ไลค์รีเซฟเตอร์ (TLR) ซึ่งเป็นตัวรับที่สำคัญต่อการรุกรานของจุลินทรีย์ งานวิจัยของเราแสดงให้เห็นว่า HGFs มีการแสดงออกของอาร์เอ็นเอนำรหัสของ TLR ที่ 1, 2, 3, 4, 5, 6 และ 9 เมื่อกระตุ้นด้วย *P. gingivalis* LPS, poly I:C, *E. coli* LPS และ *S. typhimurium* flagellin ซึ่งเป็นไลแกนที่มีความจำเพาะต่อ TLR 2, 3, 4 และ 5 ตามลำดับแล้ว นำไปสู่การผลิต Chemokine-IL-8 และเอนไซม์ IDO ที่มีสามารถกดการตอบสนองทางภูมิคุ้มกันได้ ในทางตรงกันข้าม ไลแกนที่มีความจำเพาะต่อ TLR 9 อย่าง CpG ODN 2006 ไม่สามารถกระตุ้นได้ซึ่งต้องการการศึกษาวิจัยต่อไป ความสามารถในการกระตุ้น HGFs ให้ผลิต IL-8 และเอนไซม์ IDO ของไลแกนนั้นถูกยกระดับขึ้นเมื่อกระตุ้นร่วมกับสารสื่อการอักเสบ (inflammatory cytokine) TNF-α และ IFN-γ ตามลำดับ ในที่สุด HGFs นั้นก็สามารถยกระดับการแสดงของเอนไซม์ IDO และลดการควบคุมการตอบสนองของ T cell เมื่อทำการกระตุ้นด้วย PAMP ร่วมกับไซโตไคน์บางชนิดมีผลทำให้เกิดการกดระบบภูมิคุ้มกัน คณะผู้วิจัยยังทำการตรวจสอบถึงผลของ IL-17 ซึ่งเป็นไซโตไคน์ชนิดใหม่ โดยเฉพาะอย่างยิ่งเมื่อกระตุ้น HGFs ร่วมกับ IFN-γ โดย IL-17 นี้เป็นผลผลิตของ T-helper(Th)17 ซึ่งเป็นที่รู้จักว่าเป็นตัวควบคุมที่สำคัญมากในการตอบสนองเกี่ยวกับการอักเสบ เป็นที่ชัดเจนว่า IL-17 และ IFN-γ ควบคุมการกระตุ้น HGF อย่างแตกต่างกัน อาจจะเป็นไปได้ว่าผ่านกลไกภายในที่แตกต่างกัน IL-17 ชักนำให้ผลิต IL-8 แต่ทว่า IFN-γ ชักนำการสร้างโมเลกุลเกียวกับการยึดเกาะ ICAM-1 และ co-stimulatory molecule HLA-DR และเอนไซม์ IDO ผลต่างๆ เหล่านี้ของไซโตไคน์แต่ละตัวจะเสริมกันเมื่อมีการกระตุ้นร่วมกัน รวมทั้งหมดทั้งสิ้นในการตรวจสอบทั้งระบบของคณะผู้วิจัยนี้จึงชี้ให้เห็นถึงบทบาทที่จำเป็นของเซลล์ที่ประจำอยู่ในพื้นที่ของ HGFs ไม่เฉพาะในระบบภูมิคุ้มกันที่มีมาแต่กำเนิด แต่ยังรวมถึงลดขบวนการอักเสบมากเกินไปที่มีแนวโน้มว่าจะเป็นอันตรายในอวัยวะปริทันต์

หน้าสรุปโครงการ (Executive Summary)

1. **ชื่อโครงการ** การศึกษาเซลล์ไฟโบรบลาสต์จากเหงือกมนุษย์ ซึ่งเป็นเซลล์สำคัญในระบบอินเนต และบทบาทในการควบคุมระบบภูมิคุ้มกันในโรคปริทันต์

   Human gingival fibroblast as a critical innate non-immune cell and its immunoregulatory role in periodontium

2. **ชื่อหัวหน้าโครงการ** รังสินี มหานนท์

   Rangsini Mahanonda

ตำแหน่งทางวิชาการ รองศาสตราจารย์

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4. **ระยะเวลาดำเนินงาน** 3 ปี

5. **ปัญหาที่ทำการวิจัยและความสำคัญของปัญหา**

For decades, gingival fibroblasts, the major cell type in periodontal connective tissue, were perceived as bystander cells, providing tissue framework for tooth anchorage. But now it is changing and researchers have begun to recognize their active role in innate immunity as well as immune regulation in local periodontal tissue. Recent data demonstrate that human gingival fibroblasts (HGFs) express Toll-like receptors (TLRs) 2, 4, 9. TLRs are key pathogen recognition receptors for sensing microbial pathogens and their products. They have been recognized as powerful screening tools of the innate immunity. TLR ligation triggers inflammatory innate immune response which is critical for pathogen elimination. It is now known that activation of the innate immunity is also crucial for activating and controlling the adaptive immunity. Recently we observed mRNA expression of indoleamine 2,3-dioxygenase (IDO) enzyme in HGFs when stimulated with periodontopathic bacterial products, *Porphyromonas gingivalis* LPS. IDO is an enzyme that catalyzes tryptophan, an essential amino acid and known to have anti-bacterial activity. Recent studies have now been focused on IDO immune suppressive effect on the adaptive T cell response. The role of gingival fibroblast TLRs in recognition of bacterial pathogens leading to innate immune response and local immunoregulation is indeed an interesting area. Therefore, in the present study, we propose to systematically evaluate the function of HGFs in the innate immune responses and immunoregulation, particularly focusing on TLR expression, production of mediators (as a result of TLR triggering), and the role of fibroblast mediators in periodontal immunoregulation as well as anti-bacterial activity.

6. วัตถุประสงค์

1) To establish HGF cultures from healthy gingival tissues for routine study.
2) To investigate the mRNA expression of TLRs (1 – 10) in HGFs.
3) To evaluate the response of HGFs to a variety of highly purified TLR ligand stimulation (LPS from *P. gingivalis* – TLR2 ligand; Polyinosine-polycytidylic acid (poly I:C) – 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) by measuring

   3.1 IL-8 production (neutrophil recruiting cytokine)

   3.2 IDO production

4) To examine the combination effect of cytokine (interferon gamma (IFN-γ) or tumor necrosis factor alpha (TNF-α)) and TLR ligands on production of IL-8 and IDO
5) To evaluate the role of gingival fibroblast IDO in negative feed back suppression of T cell responses
6) To study the role of TLR ligand stimulated-HGFs in promoting infiltration of dendritic cells, especially plasmacytoid dendritic cells

7. ผลการดำเนินการวิจัย

1. HGF cultures from healthy gingival tissues were established for for routine study.
2. We evaluated the expression of TLRs in HGFs by RT-PCR using a panel of specific primers of TLRs 1–10. We found the mRNA expression of TLRs 1, 2, 3,4, 5, 6, and 9 on HGFs but not TLRs 7, 8, and 10.
3. We characterized the functional relevance of TLRs in HGFs. We found expression of IL-8 and IDO in HGFs after stimulated with highly purified TLR ligand(s). TLR2 ligand (*P.gingivalis* LPS), TLR3 ligand (poly(I:C)), TLR4 ligand (*Escherichia coli* LPS), and TLR5 ligand (*Salmonella typhimurium* flagellin). A potent TLR 9 ligand, CpG oligodeoxynucleotide 2006 had no effect, No significant enhancement on IL-8 or IDO expression was observed when HGFs were stimulated with various combinations of TLR ligands.
4. We investigated the effects on HGF production of IL-8 and IDO by either cytokine or by the combination of cytokine with different TLR ligands. We foundTNF-α enhanced TLR ligand-induced IL-8 production in HGFs, whereas IFN-γ enhanced TLR ligand-induced IDO expression.
5. We evaluated the role of gingival fibroblast IDO in negative feed back suppression of T cell responses. We found production of IDO from HGFs when stimulated with *P. gingivalis* LPS, IFN-γ, or the two in combination inhibited T cell proliferation in MLRs. The observed T cell inhibition could be reversed by addition of either 1-methyl-DL-tryptophan or L-tryptophan.

6. We evaluated the expression of IL-17R mRNA in HGFs by RT-PCR and IL-17R proteins on surface of HGFs by flow cytometry. We found the mRNA expression of IL-17R on HGFs and IL-17R proteins on surface of HGFs.
7. We investigated the effects on HGF production IDO by either cytokine or by the combination of cytokine. We found that only IFN-γ can induce IDO production in HGFs but IL-17 cannot, whereas IL-17 enhanced IFN-γ-induced IDO expression.
8. We investigated the effects on HGF production IDO by the combination of IL-17 with different TLR ligands (highly purified TLR ligand stimulation (LPS from *P. gingivalis* – TLR2 ligand; Polyinosine-polycytidylic acid (Poly I:C) – TLR3 ligand; LPS from *Escherichia coli* – TLR4 ligand; flagellin from *Salmonella thyphimurium* – TLR5 ligand)). We found IL17 enhanced TLR ligand-induced IDO production in HGFs.
9. We investigated the infiltration of plasmacytoid dendritic cells in gingival tissue section from healthy and periodontitis subjects. We found the infiltration of plasmacytoid dendritic cells in periodontitis gingival tissue (7/9) whereas found in healthy gingival tissue (1/8).
10. We investigated the effects on plasmacytoid dendritic cells by the periodontopathic bacterial DNA. We found that different DNAs from plaque bacteria (Pg, Aa, Bf) have different ability to stimulate PDC to produce IFN-alpha.

8. แผนการดำเนินการวิจัยตลอดโครงการ

Year plan	1st year		2nd year		3rd year	
	1-6	7-12	13-18	19-24	25-30	31-36
1. Collect gingival biopsies	←	→				
2. Immunohistochemistry (PDC staining)		←	→			
3. Establish the technique to optimize HGF cultures		←	→			
4. TLR expression		←	→			
5. TLR ligand stimulation			←	→	→	
6. IL-8 measurement				←	→	
7. IDO measurement				←	→	→
8. T cell suppression					←	→
9. PDC culture and activation					←	→
10. Report and publication						↔

9. Publication

1. Mahanonda R and Pichyangkul S (2007) Toll-like receptors and their role in periodontal heakth and disease. *Periodontol 2000* 43:41-55. Impact factor = 3.493 (ที่มา : Journal Citation Reports, 2008)
2. Mahanonda R, Sa-Ard-Iam N, Montreekachon P, Pimkhaokham A, Yongvanichit K, Fukuda MM, Pichyangkul S. (2007). IL-8 and IDO expression by human gingival fibroblasts via TLRs. *J Immunol* 178:1151-1157. Impact factor = 6.000 (ที่มา : Journal Citation Reports, 2008)
3. Mahanonda R, Jitprasertwong P, Sa-Ard-Iam N, Rerkyen P, Charatkulangkun O, Jansisyanont P, Nisapakultorn K, Yongvanichit K, Pichyangkul S. (2008).Effects of IL17 on human gingival fibroblasts. *J Dent Res* 87:267-272. Impact factor = 3.142 (ที่มา : Journal Citation Reports, 2008)

Introduction

Periodontal disease

The periodontium, a tooth supporting structure, consists of gingiva, cementum, periodontal ligament and alveolar bone. Periodontal disease is a chronic bacterial infection of the periodontium which leads to connective tissue and bone destruction. Patients with periodontal disease suffer from bleeding gums, bad breath, gingival recession and in the severe case loose tooth/teeth. The disease has long been implicated as a major cause of adult tooth loss (Papapanou, 1996) and a global health problem (Albandar and Rams, 2002).

Dental plaque biofilms have been well recognized as etiologic agents of periodontal disease. The disease initiation and progression results 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). 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 crevicular fluid and the epithelial cell layer. More accumulation of plaque leads to gingival inflammation (or gingivitis) with increased cellular infiltration of lymphocytes, monocytes and antigen presenting dendritic cells (Page et al., 1997; Jotwani and Cutler, 2003). 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, TNF-α, PGE_2, and 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, *P. gingivalis, Actinobacillus actinomycetemcomitans* and *Tanerella forsythia*, which are frequently detected in deep periodontal pockets, are well recognized for their virulence as etiologic agents in human periodontitis (Anonymous, 1996).

Pathogen induced innate immunity via Toll-like receptors

Gingiva, the outermost component of peridontium, consistently expose to bacteria and their released products/components which are known as pathogen-associated molecular patterns (PAMPs). Examples of PAMPs are bacterial LPS, peptidoglycan, lipoproteins, bacterial DNA, and double-stranded RNA. It is now known that the innate immune response applies a family of pattern-recognition receptors (PRRs) called Toll-like receptors (TLRs) as a tool to recognize PAMPs or sense invasion by microorganisms such as bacteria, viruses, fungi and protozoa. Upon interaction with PAMPs, TLRs transmit this information through intracellular signaling pathways, resulting in activation of innate immune cells and the release of inflammatory mediators in order to clear such pathogens. 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.

To date 10 TLRs in humans and 12 TLRs in mice have been described (Beutler, 2004). For example TLR2 recognizes peptidoglycan, TLR3 recognizes viral double-stranded RNA, TLR4 recognizes LPS, TLR5 recognizes flagellin, TLR8 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.

TLRs are predominantly expressed on cells of the innate immune system, including neutrophils, monocytes/macrophages, and dendritic cells (DCs). 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. In addition to immune cells, recent studies demonstrated that cells of the periodontium express TLRs (Tabeta et al., 2000; Hatakeyama et al., 2003; Kusumoto et al., 2004). Due to continual exposure to oral bacterial PAMPs, TLR sensing and signaling in periodontium could indeed play important role in the innate immune response and maintain periodontal health. On the contrary, chronic TLR stimulation in periodontium by bacterial PAMPs can lead to excessive production of pro-inflammatory mediators, resulting in periodontal tissue destruction.

Cytokines in periodontitis and related cytokines in the present study

Cytokines can be defined as small proteins (8-80 kDa molecular weights) that usually act in autocrine or paracrine manner. They are cell regulators that have a major influence on the production and activation of different effector cells. T cells and macrophages are major source although they are produced by a wide range of cells that play important roles on physiologic and inflammatory responses (Gemmell et al., 1997). They are usually produced transiently, are extremely potent, generally acting at picomolar concentrations and interact with specific receptors at the cell membrane, setting of a cascade that leads to induction, enhancement or inhibition of a number of cytokine-regulated genes in the nucleus (Balkwill and Burke, 1989).

Many cytokines are pleiotrophic, having multiple and overlapping activities on different target cells. Cytokine function may not be identical. The response of a cell to a given cytokine depends on the local concentration, the cell type and other cell regulators to which it is constantly exposed. Cytokine interact in a network: first by inducing each other, second by transmodulating cell surface recepters, and third by synergistic, additive or antagonistic interactions on cell function (Cohen and Cohen, 1996).

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, MMP and PGE_2 have been detected (Kornman et al., 2000; Okada and Murakami, 1998).

Interleukin-8

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 a distinct target specificity for the neutrophil, with weaker effect on other blood cells (Baggiolini et al., 1994; Bickel, 1993). 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., 2000). This cytokine has been thought to play a

significant role in various forms of periodontitis (Bickel, 1993; Fitzgerald and Kreutzer, 1995; Gainet et al., 1998; Takashiba et al., 1992).

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 et al., 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, HGF IL-8 could be induced by stimulation with bacterial LPS or other cytokines (Sakuta et al., 1998; Steffen et al., 2000; Takashiba et al., 1992; Takigawa et al., 1994). 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.

Interferon-γ

IFN-γ has a major role in the regulation of immune response. It has a wide variety of biological activities on immune cells. Its regulatory effect includes the activation of macrophages to enhance their phagocytosis and tumor killing capability as well as activation and growth enhancement of cytolytic T cells and natural killer (NK) cell (O'Garra, 1998). IFN-γ up-regulates Class I MHC antigen expression, and induces Class II MHC and Fcγ receptor expression on macrophages and many other cell types including lymphoid cells, mast cells and fibroblasts so that it may influence the capacity of cells to present antigen (Shimabukuro et al., 1996). IFN-γ also plays a major role in B-cell maturation and immunoglobulin secretion.

High levels of IFN-γ mRNA are detectable in inflammed gingival tissues (Shimabukuro et al., 1996; Takeichi et al., 2000). Lundqvist et al. (1994) reported that not only αβ T cells but also γδ T cells from adult periodontitis patients expressed mRNA for IFN-γ. In addition, IFN-γ could be demonstrated in supernatant of gingival mononuclear cells from rapidly progressive periodontitis patients. Furthermore, IFN-γ was detected in gingival crevicular fluid of periodontitis patients (Salvi et al., 1998). The presence of IFN-γ is likely to prime local

HGF and these primed HGF could subsequently induce further immune reaction. For example, T cell proliferation could be induced *in vitro* by IFN-γ treated HGF, as mentioned.

Interleukin-17

Human IL-17, a 20-30 kDa glycoprotein, is a recently described T cell cytokine which has been speculated to play an essential role in immunopathogenesis of periodontitis. The major source is the activated memory ($CD45RO^{+}CD4^{+}$) T cells (Aarvak et al., 1999; Yao et al., 1995). IL-17 exhibits pleiotrophic biologic activities on various types of cells, such as fibroblasts, endothelial cells, and epithelial cells, mediating a wide range of responses, mostly proinflammatory and hematopoietic (Chabaud et al., 2001; Fossiez et al., 1996; Rouvier et al., 1993; Yao et al., 1995). Many IL-17 studies were done in area of rheumatoid arthritis (Yao et al., 1995) suggesting that IL-17 has the potential to be an important cytokine in the immune system, and associated with disease states. Interestingly, IL-17 was also detected in inflamed gingival tissues, both gingivitis and periodontitis (Oda et al., 2003), particularly, in 4 to 5 mm pockets (Johnson et al., 2004).

It was shown that IL-17 stimulated transcriptional NF-kB activity and IL-6 and IL-8 secretion in mouse fibroblasts, endothelium and epithelial cells and also induced T cell proliferation (Rouvier et al., 1993). Futhermore, IL-17 stimulates stromal cells to secrete cytokines and growth factors (Fossiez et al., 1996). It enhances IL-1 mediated-IL-6 production by rheumatoid arthritis synoviocytes *in vitro* (Chabaud et al., 1998). In combination with IFN-γ, IL-17 showed a synergism in the stimulation of IL-8 secretion and the induction of intercellular adhesion molecule-1 (ICAM-1) and HLA-DR expression by keratinocytes (Albanesi et al., 1999; Teunissen et al., 1998). Moreover, IL-17 stimulates granulopoiesis (Schwarzenberger et al., 1998) and is a strong inducer of neutrophil recruitment through chemokines release (Laan et al., 1999). Apart from its role in inflammatory responses, IL-17 has potential to induce bone destruction. A recent work showed that IL-17 gene transfer strongly up-regulated the synovial receptor activator of nuclear factor-kappa B ligand (RANKL) / osteoprotegerin (OPG) ratio and enhanced the formation of osteoclast-like cells and bone erosion compared with the control groups (Lubberts et al., 2003). RANKL and the decoy receptor OPG has been identified as an important positive and negative regulator of osteclastogenesis and bone erosion (Kong et al., 1999). Periodontitis is a chronic inflammatory disease, which involves alveolar bone destruction. Even though, the presence of IL-17 in gingivitis and periodontitis tissue has been recently demonstrated, the role of this cytokine in periodontitis is still largely unknown.

Gingival fibroblasts

Gingival 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 have been found to be a dynamic cell type involved in many local tissue functions and in host defense (Phipps et al., 1997).

Gingival fibroblasts could secrete various soluble mediators of inflammation including PGE2, 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* 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).

Cytokine production by HGFs is likely to result from TLR triggering. 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 human gingival fibroblasts were higher in periodontitis than in healthy individuals (Wang et al., 2003). We recently demonstrated that HGFs were able to produce indoleamine 2,3-dioxygenase (IDO) enzyme in response to P. gingivalis LPS (Mahanonda et al., 2007). IDO is an enzyme that catalyzes tryptophan, an essential amino acid and known to have anti-bacterial activity. It is produced by macrophages, dendritic cells, epithelial cells, and fibroblasts (Daubener and MacKenzie, 1999; Mellor and Munn, 2003). Recent data shows that IDO is able to inhibit T cell proliferation (Hwu P et al., 2000). The ability of IDO positive dendritic cells to inhibit T cell

expansion suggesting their possible role in promoting immune tolerance. The immunological function of IDO to down-regulate adaptive T cell response has been the area of intense investigation.

The role of gingival fibroblast TLRs in recognition of microbial and their released PAMPs leading to local innate immune response and immunoregulation is an interesting area. Therefore, in the present study, we propose to systematically evaluate the function of HGFs in the innate immune responses and immunoregulation, particularly focusing on TLR expression, production of mediators (as a result of TLR triggering), and the role of fibroblast mediators in periodontal immunoregulation.

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Materials and Methods

Reagents and Abs

Medium for HGF cultures was DMEM which was purchased from Gibco Laboratory (Grand Island, NY). The medium was supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml), fungizone (2.5 μg/ml) and 10% heat-inactivated fetal calf serum. Highly purified TLR ligands, including LPS from *P. gingivalis* (TLR2 ligand); poly I:C (TLR3 ligand); LPS from *Escherichia coli* K12 strain (TLR4 ligand); flagellin from *Salmonella typhimurium* (TLR5 ligand); Loxoribine (Guanosine analog) (TLR7 ligand); Single-stranded polyU oligonucleotide complexed with LyoVec™ (TLR8 ligand) were obtained from InvivoGen (San Diego, CA). CpG ODN 2006 (TLR9 ligand) was obtained from Coley Pharmaceutical Group (Wellesley, MA). IFN-γ, TNF-α and IL-17 were purchased from R&D System Inc. (Minneapolis, MA). Anti-human TLR3 mAb (TLR3.7) was obtained from eBiosciences (San

Diego, CA). Anti-human IL-17R mAb was obtained from R&D System Inc. (Minneapolis, MA). 1-Methyl-DL-tryptophan and L-tryptophan were purchased from Sigma (St. Louis, MO).

Human gingival fibroblasts

Gingival tissue samples were collected from subjects who had clinically healthy periodontium and no history of periodontitis. The gingival biopsies were obtained at the time of crown lengthening procedure for prosthetic reasons from the Periodontal Clinic, Faculty of Dentistry, Chulalongkorn University. Before operation, ethical approval was obtained from the ethics committee of the Faculty of Medicine, Chulalongkorn University, and informed consent was obtained from each subject. Fibroblasts from the gingival tissues were obtained following established procedure (Murakami et al., 1999). Briefly, the excised tissue was immediately washed and then minced with scissors into fragments (1-3 mm^2) and placed in 60 mm. tissue culture dishes. After a confluent monolayer of cells was reached, HGFs were trypsinized, washed twice and then transferred to new tissue culture flasks. The HGF cultures at passage 4-8 were used throughout the study.

Detection of TLRs on HGFs

- mRNA expression of TLRs in HGFs

Total RNA from HGFs was isolated by using RNeasy Mini kit from Qiagen (Chatworth, CA). One μg of DNase I-treated total RNA was reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR (Promega, Madison, WI). TLRs 1-10 and GAPDH were amplified using specific primers purchased from Proligo (Singapore) as described below and the PCR conditions were described as in previous studies(Saikh et al., 2003; Schaefer et al., 2004).

TLR1 (5'-CGTAAAACTGGAAGCTTTGCAAGA-3'/5'- CCTTGGGCCATTCCAAATAAGTCC-3');
TLR2 (5'-GGCCAGCAAATTACCTGTGTG-3'/5'- CCAGGTAGGTCTTGGTGTTCA-3');
TLR3 (5'-ATTGGGTCTGGGAACATTTCTCTTC-3'/5'-GTGAGATTTAAACATTCCTCTTCGC -3');
TLR4 (5'-CTGCAATGGATCAAGGACCA-3'/5'-TCCCACTCCAGGTAAGTGTT-3');
TLR5 (5'-CCTCATGACCATCCTCACAGTCAC-3'/5'-GGCTTCAAGGCACCAGCCATCTC-3');
TLR6 (5'-ACTGACCTTCCTGGATGTGG-3'/5'-TGGCACACCATCCTGAGATA-3');
TLR7 (5'-ACAAGATGCCTTCCAGTTGC-3'/5'-ACATCTGTGGCCAGGTAAGG-3');
TLR8 (5'-CAGAATAGCAGGCGTAACACATCA-3'/5'-AATGTCACAGGTGCATTCAAAGGG-3');

TLR9 (5'-GCGAGATGAGGATGCCCTGCCCTACG-3'/5'-TTCGGCCGTGGGTCCCTGGCAGAAG-3');
TLR10 (5'-GGCCAGAAACTGTGGTCAAT-3'/5'-AACTTCCTGGCAGCTCTGAA-3'); and
GAPDH (5'-TCATCTCTGCCCCCTCTGCTG-3'/5'-GCCTGCTCACCACCTTCTTG-3').

- Flow cytometric analysis of TLR3 expression

The specific localization of TLR3 of HGFs was investigated by flow cytometry. For surface TLR3 staining, HGFs were incubated with PE-conjugated anti-human TLR3 mAb (clone TLR3.7, 1 μg) for 30 min at 4°C. For intracellular staining, cells were pretreated with fixation/permeabilization solution (BD Pharmingen, San Diego, CA) for 20 min at 4 ºC, washed once with PBS and then incubated with the same mAb for 1 h at 4 ºC. Mouse isotype mAbs conjugated with PE was used as control. The stained cells were then analyzed on a FACSCalibur (BD Biosciences, Mountain View, CA).

Detection of IL-17R on HGFs

- mRNA expression of IL-17R

The total RNA of stimulated HGFs was isolated by using RNeasy Mini kit from Qiagen (Chatworth, CA). One μg of DNase I-treated total RNA was reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR (Promega, Madison, WI). IL-17R was amplified using specific primer (5'-CTAAACTGCACGGTCAAGAAT-3'/5'-ATGAACCAGTACACCCAC-3')(Proligo) (Yao *et al*., 1997). The expected size of the PCR product was 833 bp. For semi-quantitative RT-PCR analysis, band intensities on scanned gels were analyzed (GeneTools, Syngene, Cambridge, UK) using specific bands of the housekeeping gene GAPDH as a reference.

- Flow cytometric analysis of IL-17R

The stimulated cells were stained with mAbs anti-IL-17R at 4°C for 30 min, washed in PBS, reconstituted with 1% paraformaldehyde and were analyzed by FACSCalibur (BD Biosciences, Mountain View, CA). Mouse isotype mAbs were used as control. The levels of surface molecule expression were measured by mean fluorescence intensity(MFI).

TLR ligation on HGFs after stimulation with TLR ligand(s) and/or cytokine

HGFs ($1.5x10^5$ cells/ml) in 96-well plates or 24-well plates (Corning Inc. Corning, NY) were treated with either a). Various single TLR ligands: *P. gingivalis* LPS (50 μg/ml), poly I:C (100 μg/ml), *E. coli* LPS (10 μg/ml), *S. typhimurium* flagellin (5 μg/ml); Loxoribine (100 μM), ssPolyU (5 μg/ml), and CpG ODN 2006 (10 μg/ml); b). TLR ligand combination: *P. gingivalis* LPS (50 μg/ml) + poly I:C (100 μg/ml), *P. gingivalis* LPS (50 μg/ml) + *E. coli* LPS (10 μg/ml), *P. gingivalis* LPS (50 μg/ml) + *S. typhimurium* flagellin (5 μg/ml), *P. gingivalis* LPS (50 μg/ml) + CpG ODN 2006 (10 μg/ml), poly I:C (100 μg/ml) + *E. coli* LPS (10 μg/ml), poly I:C (100 μg/ml) + *S. typhimurium* flagellin (5 μg/ml), poly I:C (100 μg/ml) + CpG ODN 2006 (10 μg/ml), *E. coli* LPS (10 μg/ml) + *S. typhimurium* flagellin (5 μg/ml), *E. coli* LPS (10 μg/ml) + CpG ODN 2006 (10 μg/ml), *S. typhimurium* flagellin (5 μg/ml) + CpG ODN 2006 (10 μg/ml); c). Cytokine: IFN-γ (100 U/ml) and TNF-α (50 ng/ml); or d). TLR ligand and cytokine combination: *P. gingivalis* LPS (50 μg/ml) + IFN-γ (5 U/ml), poly I:C (10 μg/ml) + IFN-γ (5 U/ml), *E. coli* LPS (10 μg/ml) + IFN-γ (5 U/ml), *S. typhimurium* flagellin (5 μg/ml) + IFN-γ (5 U/ml), *P. gingivalis* LPS (50 μg/ml) + TNF-α (1 ng/ml), poly I:C (10 μg/ml) + TNF-α (1 ng/ml), *E. coli* LPS (10 μg/ml) + TNF-α (1 ng/ml), and *S. typhimurium* flagellin (5 μg/ml) + TNF-α (1 ng/ml).

After stimulation with TLR ligand(s) and/or cytokine for 12-24 h, the cells and culture supernatants were collected for measurement of IL-8 and IDO expression.

Stimulation of human gingival fibroblasts with IL-17, IFN- γ, or combined cytokines.

HGFs ($1x10^5$ cells/mL) in 48-well plates (Corning Inc. Corning, NY) were stimulated with predetermined concentrations of IL-17 and IFN-γ, separately and in combination. The HGF responses were monitored by IDO. Based on preliminary kinetic experiments, we selected 12 hr incubation for determination of IDO mRNA expression by RT-PCR. The cytokine concentrations for HGF stimulation used in (1). IDO mRNA and activity assay were IL-17 at 5, 50, 500 ng/mL; IFN-γ at 1, 5, 25 U/mL; or combined cytokines at 500 ng/mL IL-17+ 1 U/mL IFN-γ, 500 ng/mL IL-17 + 5 U/mL IFN-γ, 500 ng/mL IL-17 + 25 U/mL IFN-γ . After stimulation with TLR ligand(s) and/or cytokine for 12-72 h, the cells and culture supernatants were collected for measurement of IDO expression.

Stimulation of human gingival fibroblasts with TLR ligand(s) and/or IL-17.

HGFs ($1x10^5$ cells/mL) in 24-well plates (Corning Inc. Corning, NY) were stimulated with predetermined concentrations of IL-17 and highly purified TLR ligands stimulation (LPS from *P. gingivalis* – TLR2 ligand; Polyinosine-polycytidylic acid (Poly I:C) – TLR3 ligand; LPS from *Escherichia coli* – TLR4 ligand; flagellin from *Salmonella thyphimurium* – TLR5 ligand), separately and in combination. The HGF responses were monitored by

IDO. Based on preliminary kinetic experiments, we selected 12 hr incubation for determination of IDO mRNA expression by RT-PCR. The cytokine concentrations for HGF stimulation used in (1). IDO mRNA and activity assay were IL-17 500 ng/mL (2) Various single TLR ligands: *P. gingivalis* LPS (50 μg/ml), poly I:C (100 μg/ml), *E. coli* LPS (10 μg/ml), *S. typhimurium* flagellin (5 μg/ml). (3) TLR ligand and cytokine combination: *P. gingivalis* LPS (50 μg/ml) + IL-17 (500 ng/ml), poly I:C (10 μg/ml) + IL-17 (500 ng/ml), *E. coli* LPS (10 μg/ml) + IL-17 (500 ng/ml), *S. typhimurium* flagellin (5 μg/ml) + IL-17 (500 ng/ml), After stimulation with TLR ligand(s) and/or cytokine for 12-72 h, the cells and culture supernatants were collected for measurement of IDO expression.

Determination of IL-8

The supernatants of HGFs after stimulation with TLR ligand(s) and/or cytokine were harvested and assessed for IL-8 production by ELISA (R&D systems, Minneapolis, MN).

Detection of IDO expression and activity

- mRNA expression of IDO

The kinetics study of IDO mRNA expression (6, 12, and 24 h) was carried out using IFN-γ- or TNF-α-treated HGFs. Twelve hour treated cells showed optimal mRNA expression of IDO. The total RNA of HGFs after 12 h stimulation with TLR ligand(s) and/or cytokine were reverse transcribed and treated with DNase I as previously mentioned. IDO was amplified using specific primer (5'-CTTCCTGGTCTCTCTATTGG-3'/5'-GAAGTTCCTGTGAGCTGGT-3') (Proligo)(von Bubnoff et al., 2002). The expected size of the PCR product was 430 bp. For semi-quantitative RT-PCR analysis, band intensities on scanned gels were analyzed (GeneTools, Syngene, Cambridge, UK) using specific bands of the house keeping gene GAPDH as a reference.

- IDO activity: Kynurenine assay

IDO-dependent catabolism of tryptophan produces kynurenine. Hence, the biological activity of IDO was evaluated by measuring the level of kynurenine in HGF culture supernatants(Braun et al., 2005). One hundred microliters culture supernatants of HGFs after stimulation with TLR ligand(s) and/or cytokine was mixed with 50 μl of 30% tricholroacetic acid, vortexed, and centrifuged at 8000 *g* for 5 min. Then, 75 μl of the supernatant was added to an equal volume of Ehrlich reagent (100 mg p-dimethylbenzaldehyde in 5 ml glacial acetic acid) in a 96

well microtiter plate, and the absorbance was read at OD at 492 nm. A standard curve of defined kynurenine concentration (0-100 μM) permitted analysis of unknowns.

Suppression of T cell response in MLR

To assess whether IDO-expressed HGFs inhibit allogeneic T cell response, MLRs were performed on a layer of HGFs that had been treated for 2 days with either *P. gingivalis* LPS (50 μg/ml), IFN-γ (5 U/ml), or the combination of *P. gingivalis* (50 μg/ml) and IFN-γ (5 U/ml). PBMC were isolated from blood of healthy human donors. Mixed leukocyte cultures were performed by mixing PBMC (each at $4x10^7$ cells/ml in PBS) from 2 healthy donors. A total of $4x10^5$ mixed PBMC in 10 μl of PBS were co-cultured with confluent layer of stimulated HGFs (200 μl / well) in 96-well plates. 3H thymidine (0.5 μCi / 200 μl / well) was added on day 5 and cell cultures were incubated for another 18 h. Cells were harvested onto glass filter paper and radioactivity was measured (Beta plate: Wallac, Turku, Finland). In order to confirm the inhibitory effect of IDO, 1-Methyl-DL-tryptophan (1000 μg/ml) or L-tryptophan (1000 μM) was added during the co-culture of the stimulated HGFs with mixed PBMC.

Statistical analysis

Statistical comparisons among treatment conditions with respect to production of IL-8 and IDO, and to inhibition of T cell response were analyzed using SigmaStat (Jandel Scientific, San Rafael, CA). The parametric Student's *t* test was used for normally distributed data, and the non-parametric Mann-Whitney rank-sum test was used for non-normally distributed data. A value of $p \leq 0.05$ was considered statistically significant.

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Results

mRNA expression of TLRs on HGFs

TLRs have been found on many cell types, and are known to play a central role in pathogen recognition in the innate immune system. To evaluate the expression of TLRs in HGFs, total RNA from HGFs was analyzed by RT-PCR using a panel of specific primers of TLRs 1-10. We found the mRNA expression of TLRs 1, 2, 3, 4, 5, 6, and 9 on HGFs except TLRs 7, 8, and 10 (Fig. 1*A*). The results were reproducible in all 4 HGF lines. Human PBMC were used as a positive control and shown to express all mRNA of TLRs 1-10 (Fig. 1*B*).

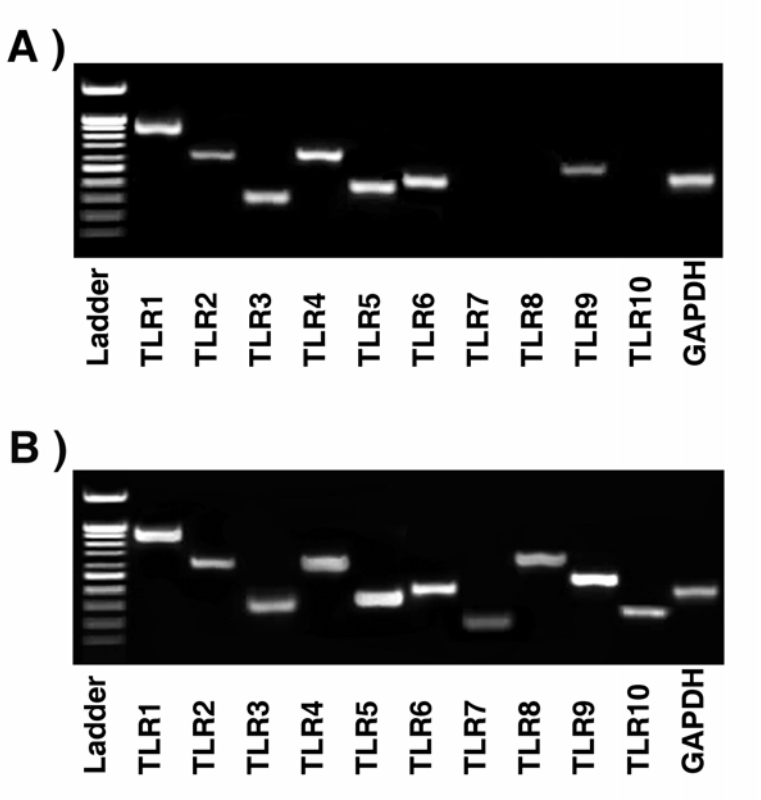

Figure 1. TLR expression in HGFs. TLRs1-10 mRNA expression was measured in cultured HGFs by RT-PCR (*A*). PBMC mRNA was used as positive control (*B*). GAPDH mRNA was used as an internal control. Data are representative of 4 separate HGF lines and PBMC.

TLR3 is generally recognized as an intracellular receptor, but a recent study showed a surface TLR3 on human skin and lung fibroblasts (Matsumoto et al., 2002). Thus, we analyzed the specific location of TLR3 expression in HGFs. Using the same mAb specific to TLR3 (clone TLR 3.7), we demonstrated the detection of intracellular TLR3 on HGFs but not on the cell surface (Fig. 2).

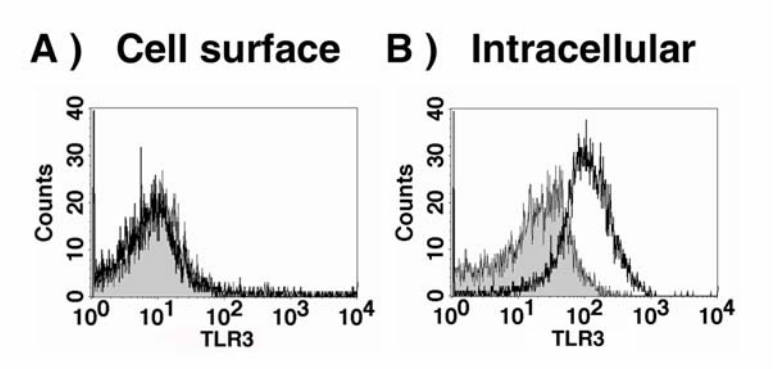

Figure 2. Flow cytometric analysis of cell surface and intracellular TLR3 expression in HGFs. HGFs were stained for cell surface (*A*) or intracellular TLR3 (*B*) with monoclonal Ab against human TLR3 (clone TLR3.7) (open histograms). Shaded histograms represent cells stained with isotype-matched control Abs.

TLR ligands stimulate expression of IL-8 and IDO.

In order to characterize the functional relevance of TLRs in HGFs, expression of IL-8 and IDO was determined after stimulation with highly purified TLR ligand(s). IL-8 production coincided with mRNA expression of TLRs i.e. TLRs 2, 3, 4, and 5 (Fig. 3*A*). On the contrary, no IL-8 production was observed in HGFs stimulated with CpG ODN 2006, even though the cells expressed TLR9 mRNA (Fig. 3*A*).

Skin fibroblasts are known to express IDO when treated with IFN-γ (Holmes., 1998; Sarkhosh et al., 2004). In this study, the IDO expression of HGFs after TLR ligation was also evaluated. Similar to IL-8 production, *P. gingivalis* LPS, poly I:C, *E. coli* LPS, *S. typhimurium* flagellin , respective ligands for TLRs 2, 3, 4, and 5, induced IDO mRNA expression. IFN-γ- and TNF-α-treated HGFs were used as positive controls (Fig. 3*B*)

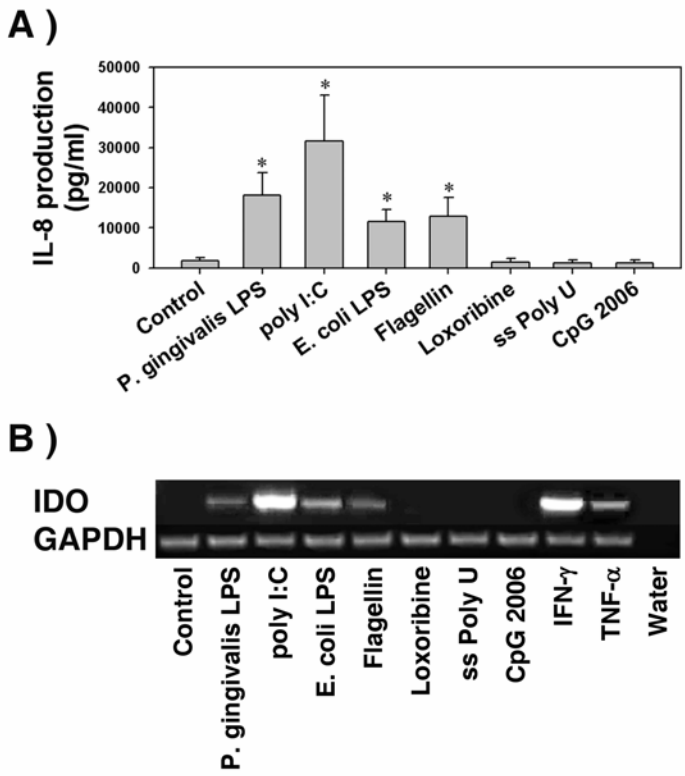

Figure 3. Expression of IL-8 and IDO in HGFs after stimulation with various TLR ligands. HGFs were cultured in 96-well plates or 24-well plates, and stimulated with the following ligands: *P. gingivalis* LPS (TLR2 ligand), poly I:C (TLR3 ligand); *E. coli* LPS (TLR4 ligand); *S. typhimurium* (TLR5 ligand); Loxoribine (TLR7 ligand); polyU (TLR8 ligand); CpG ODN 2006 (TLR9 ligand). Culture medium was used as a control. For assessment of IL-8 production, culture supernatants of stimulated HGFs were harvested after 24 h and then assayed by ELISA. Data shown are mean ± SEM of 4 separate experiments (*, $p < 0.05$, compared with un-stimulated control) (*A*). To measure IDO expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. IFN-γ- and TNF-α-stimulated HGFs were used as positive controls. GAPDH mRNA was used as an internal control. Data are representative of 4 separate experiments (*B*).

Combination of TLR ligands stimulate expression of IL-8 and IDO.

TLR ligand combinations (*P. gingivalis* LPS + poly I:C; *P. gingivalis* LPS + *E. coli* LPS; *P. gingivalis* LPS + *S. typhimurium* flagellin; *P. gingivalis* LPS + CpG ODN 2006; poly I:C + *E. coli* LPS; poly I:C + *S. typhimurium* flagellin; poly I:C + CpG ODN 2006; *E. coli* LPS + *S. typhimurium* flagellin; *E. coli* LPS + CpG ODN 2006; *S.*

typhimurium flagellin + CpG ODN 2006) did not lead to a significant enhancement of IL-8 production (Figure 4*A*) or IDO expression (Fig. 4*B*), as compared to the sum of individual ligands. Surprisingly, CpG ODN 2006 specifically inhibited poly I:C-induced IL-8 production ($p < 0.05$) and poly I:C-induced IDO expression ($p < 0.05$) (Fig. 4, *A* and *B*).

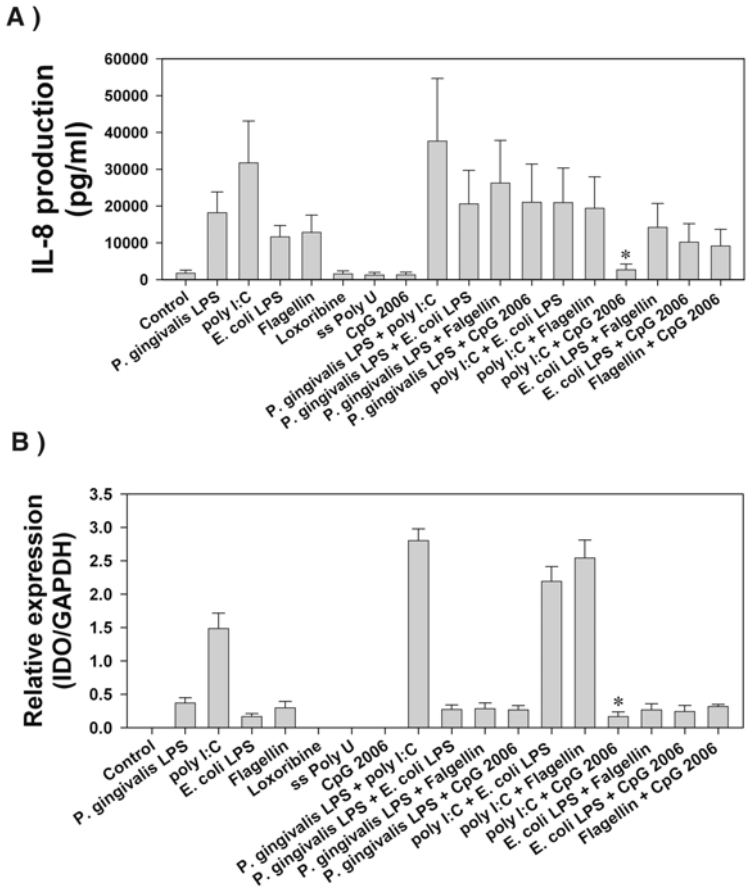

Figure 4. Expression of IL-8 and IDO in HGFs after stimulation with TLR ligand combination. HGFs were cultured in a 96-well plates or 24-well plates and stimulated with the following ligand combinations: *P. gingivalis* LPS + poly I:C; *P. gingivalis* LPS + *E. coli* LPS; *P. gingivalis* LPS + *S. typhimurium* flagellin; *P. gingivalis* LPS + CpG ODN 2006; poly I:C + *E. coli* LPS; poly I:C + *S. typhimurium* flagellin; poly I:C + CpG ODN 2006; *E. coli* LPS + *S. typhimurium* flagellin; *E. coli* LPS + CpG ODN 2006; *S. typhimurium* flagellin + CpG ODN 2006. Culture medium was used as a control. Culture supernatants of stimulated HGFs were harvested after 24 h and IL-8 production was determined by ELISA. Data shown are the mean ± SEM of 4 separate experiments (*, $p < 0.05$, compared with poly I:C treatment) (*A*). For semiquantitative analysis of IDO mRNA expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. IFN-γ- and TNF-α-stimulated HGFs

were used as positive controls. Data shown are the mean ratio ± SEM of IDO:GAPDH from 4 separate experiments (*, $p < 0.05$, compared with poly I:C treatment) (*B*).

Combination of TLR ligand and cytokine stimulates expression of IL-8 and IDO

Cytokines, IFN-γ and TNF-α have been consistently detected in periodontitis lesions (Wang et al., 2003; Shimabukuro et al., 1996). We next investigated the effects on HGF production of IL-8 and IDO by either cytokine or by the combination of cytokine with different TLR ligands, specifically *P. gingivalis* LPS, poly I:C, *E. coli* LPS, or *S. typhimurium* flagellin. Fig. 5*A* demonstrates that unlike IFN-γ, TNF-α when combined with *P. gingivalis* LPS, *E. coli* LPS, or *S. typhimurium* flagellin significantly induced more IL-8 from HGFs than the additive ($p < 0.05$). Interestingly, the results of IDO mRNA expression were quite different. IFN-γ, but not TNF-α when combined with *P. gingivalis* LPS, *E. coli* LPS, or *S. typhimurium* flagellin significantly induced IDO mRNA expression greater than the sum of individual stimulators ($p < 0.05$) (Fig. 5*B*).

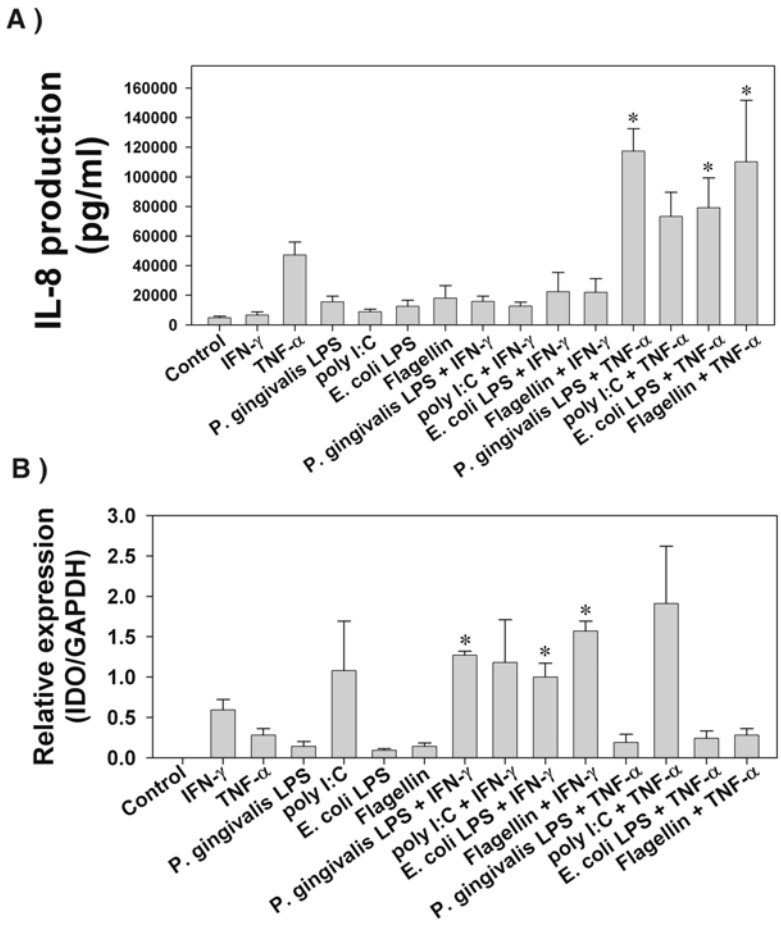

Figure 5. Expression of IL-8 and IDO in HGFs after stimulation with TLR ligand and cytokine combination. HGFs were cultured in a 96-well plates or 24-well plates and stimulated with the following ligand and cytokine combination: *P. gingivalis* LPS + IFN-γ; poly I:C + IFN-γ; *E. coli* LPS + IFN-γ; *S. typhimurium* flagellin + IFN-γ; : *P. gingivalis* LPS + TNF-α; poly I:C + TNF-α; *E. coli* LPS + TNF-α; *S. typhimurium* flagellin + TNF-α. Culture medium was used as a control. Culture supernatants of stimulated HGFs were harvested after 24 h and IL-8 production was determined by ELISA. Data shown are the mean ± SEM of 4 separate experiments (*, $p < 0.05$, compared to the sum of two individual stimulators) (*A*). For semiquantitative analysis of IDO expression, stimulated HGFs were harvested after 12 hr and mRNA expression of IDO was analyzed by RT-PCR. Data shown are the mean ratio ± SEM of IDO:GAPDH from 4 separate experiments (*, $p < 0.05$, compared to the sum of two individual stimulators) (*B*).

P. gingivalis LPS or/and IFN-γ treated HGFs induce suppression of T response via IDO

According to the enhancement of IDO mRNA expression on HGFs after stimulation with the combination of *P. gingivalis* LPS and IFN-γ , we next assessed the biological activity of IDO by measuring kynurenine concentration in those cultured supernatants. Fig. 6*A* demonstrates that the kynurenine could be detected within 24 h-culture supernatants of stimulated HGFs. The levels of kynurenine continued to increase up to 72 h in cultures.

We further evaluated whether HGFs, which were stimulated with *P. gingivalis* LPS, IFN-γ, or the two in combination could inhibit T cell response in MLRs. Co-cultures of mixed PBMC with 2 days *P. gingivalis* - or IFN-γ-stimulated HGF showed 10 - 32 % inhibition of T cell proliferative response (*, $p < 0.05$) as compared to un-stimulated HGFs. However, the combination of *P. gingivalis* and IFN-γ-stimulated cells markedly inhibited T cell proliferation by 62% (**, $p < 0.001$) (Fig. 6*B*). The response of T cells was restored when 1-Methyl-DL-tryptophan or L-tryptophan was added to the cultures (Fig. 6*B*).

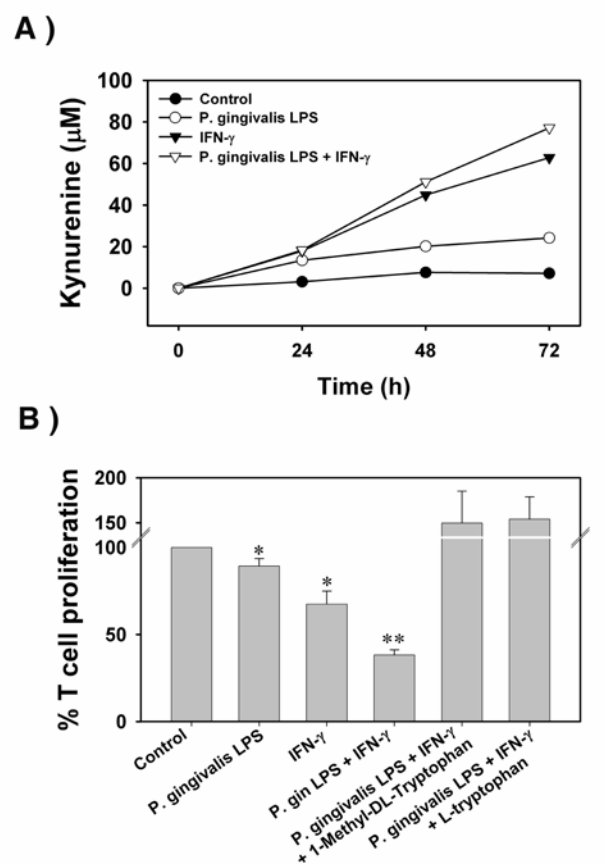

Figure 6. Kinetics of IDO activity and suppression of T cell proliferation in MLRs. HGFs were stimulated with either IFN-γ , *P. gingivalis* LPS or the combination of IFN-γ and *P. gingivalis* LPS. Culture supernatants were harvested at different time points (0, 24, 48, and 72 h) and the concentration of kynurenine was determined (*A*). Suppression of T cell proliferation in MLR was assessed by co-culturing mixed PBMC from 2 donors with *P. gingivalis* LPS, IFN-γ, or the combination of *P. gingivalis* LPS and IFN-γ-stimulated HGFs. 1-Methyl-DL-tryptophan or L-tryptophan was added to the stimulated HGF cultures at the same time as mixed PBMC. After 6 days of incubation, T cell proliferative response was determined by tritiated thymidine uptake. T cell proliferation was calculated as a percentage of control. Data shown are mean ± SEM from 4 separate experiments (*, $p < 0.05$; **, $p < 0.001$, compared with un-stimulated control) (*B*).

The expression of IL-17R on HGFs

Periodontal disease is characterized by high levels of cytokines, cellular infiltration and tissue destruction. Recent studies demonstrate the presence of IL-17, a product of Th17, in periodontal lesions, and suggest a possible role of this cytokine in disease severity (Oda *et al*., 2003; Johnson *et al*., 2004; Takahashi *et al*., 2005; Vernal *et al*., 2005). From our observation, HGFs expressed IL-17 receptor (Fig 7.)

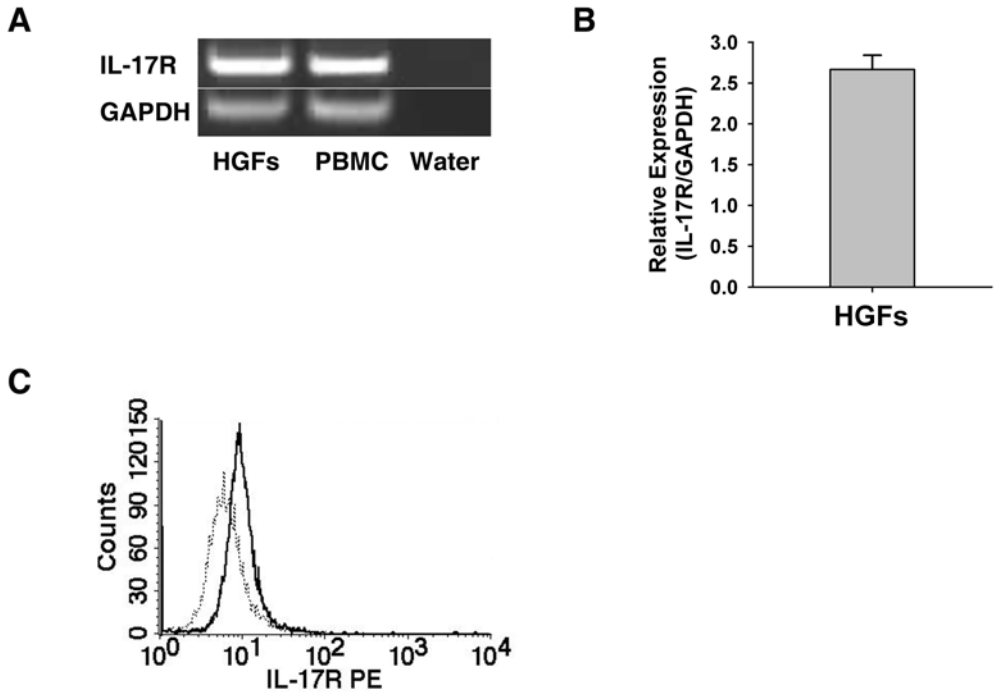

Figure 7. IL-17 receptor expression in HGFs. mRNA expression of IL-17 receptor was analysed by RT-PCR. Peripheral blood mononuclear cells (PBMC) were used as positive controls. GAPDH mRNA was used as an internal control. Data are representative of 4 separate experiments .(A1). Semi-quantitative RT-PCR of IL-17 receptor expression. Mean relative expression of IL-17R/GAPDH ± SEM from 4 separate experiments (A2). Flow cytometric analysis of cell surface IL-17 receptor expression in HGFs. HGFs were stained for cell surface IL-17 receptor with mAb against human IL-17 receptor (clone 133617). Dotted line are represent cells stained with isotype-matched control mAbs and solid line represent cells stained with IL-17 receptor mAbs. Data are representative of 4 separate experiments (A3)

Effects of IL-17 and IFN-γ on IDO expression by HGFs

We previously showed that HGFs expressed IDO when treated with IFN-γ (Mahanonda *et al.*, 2007). In this study, we investigated IDO mRNA expression by HGFs upon stimulation with IL-17 by RT-PCR. Unlike IFN-γ, IL-17 did not induce IDO mRNA expression at any concentration tested (5, 50, 500 ng/mL) (Fig.8A). The combination effect of IL-17 (500 ng/mL) and IFN-γ (1, 5, 25 U/mL) was then investigated. The combined cytokine

stimulation led to a significant enhancement of IDO mRNA expression ($P < 0.05$, as compared to the sum of two individual cytokine stimulation)(Fig. 8B). The biological activity of IDO was also assessed by measuring kynurenine concentration in cultured supernatants. Fig. 8C demonstrates the significant increase in kynurenine levels in the combined treatment cultures ($P < 0.05$, as compared to the sum of effects of the individual treatments), thus exhibiting consistency with the mRNA expression results.

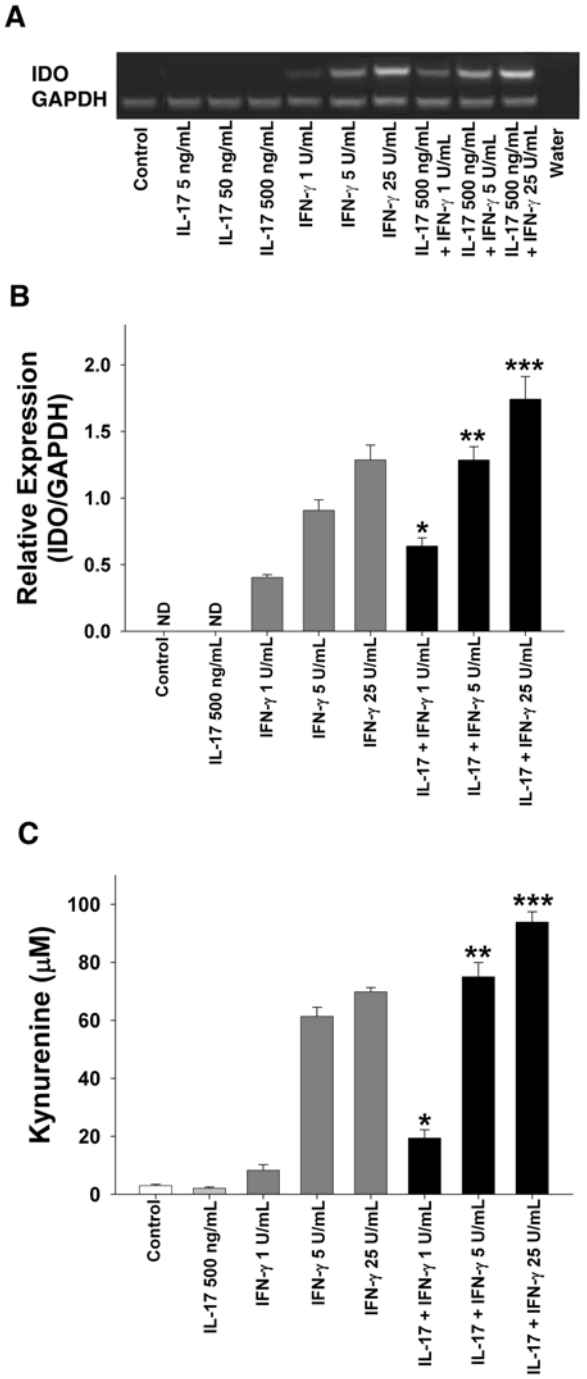

Figure 8. Expression of IDO in HGFs after stimulation with IL-17, IFN-γ or combined cytokines. HGF cultures were stimulated with indicated concentrations of IL-17, IFN-γ, and combined cytokines. Culture medium was used as a control. Stimulated HGFs were harvested and mRNA expression of IDO was analyzed by RT-PCR. GAPDH mRNA was used as an internal control. Data are representative of 4 separate experiments (A). Mean relative expression

of IDO:GAPDH ± SEM from 4 separate experiments was shown (*, $P < 0.05$, compared to the sum of IL-17 and IFN-γ (1 U/mL) stimulation; **, $P < 0.05$, compared to the sum of IL-17 and IFN-γ (5 U/mL) stimulation ***, $P < 0.05$, compared to the sum of IL-17 and IFN-γ (25 U/mL) stimulation) (B). To assess IDO activity, the concentrations of kynurenine in culture supernatants were determined. Data shown are mean concentrations of kynurenine ± SEM from 4 separate experiments (*, $P < 0.05$, compared to the sum of IL-17 and IFN-γ (1 U/mL) stimulation; **, $P < 0.05$, compared to the sum of IL-17 and IFN-γ (5 U/mL) stimulation ***, $P < 0.05$, compared to the sum of IL-17 and IFN-γ (25 U/mL) stimulation) (C).

Effect of Combination of TLR ligand and cytokine stimulates expression and IDO

We next investigated the effects on HGF production of IDO by IL-17 or by the combination of IL-17 with different TLR ligands, specifically *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, or *S. typhimurium* flagellin. Fig. 9 demonstrates that, when combined IL-17 with *P. gingivalis* LPS, *E. coli* LPS, or *S. typhimurium* flagellin, IL-17 does not seem to modulate TLR ligand-induced IDO mRNA expression as compared with single TLR ligand stimulator.

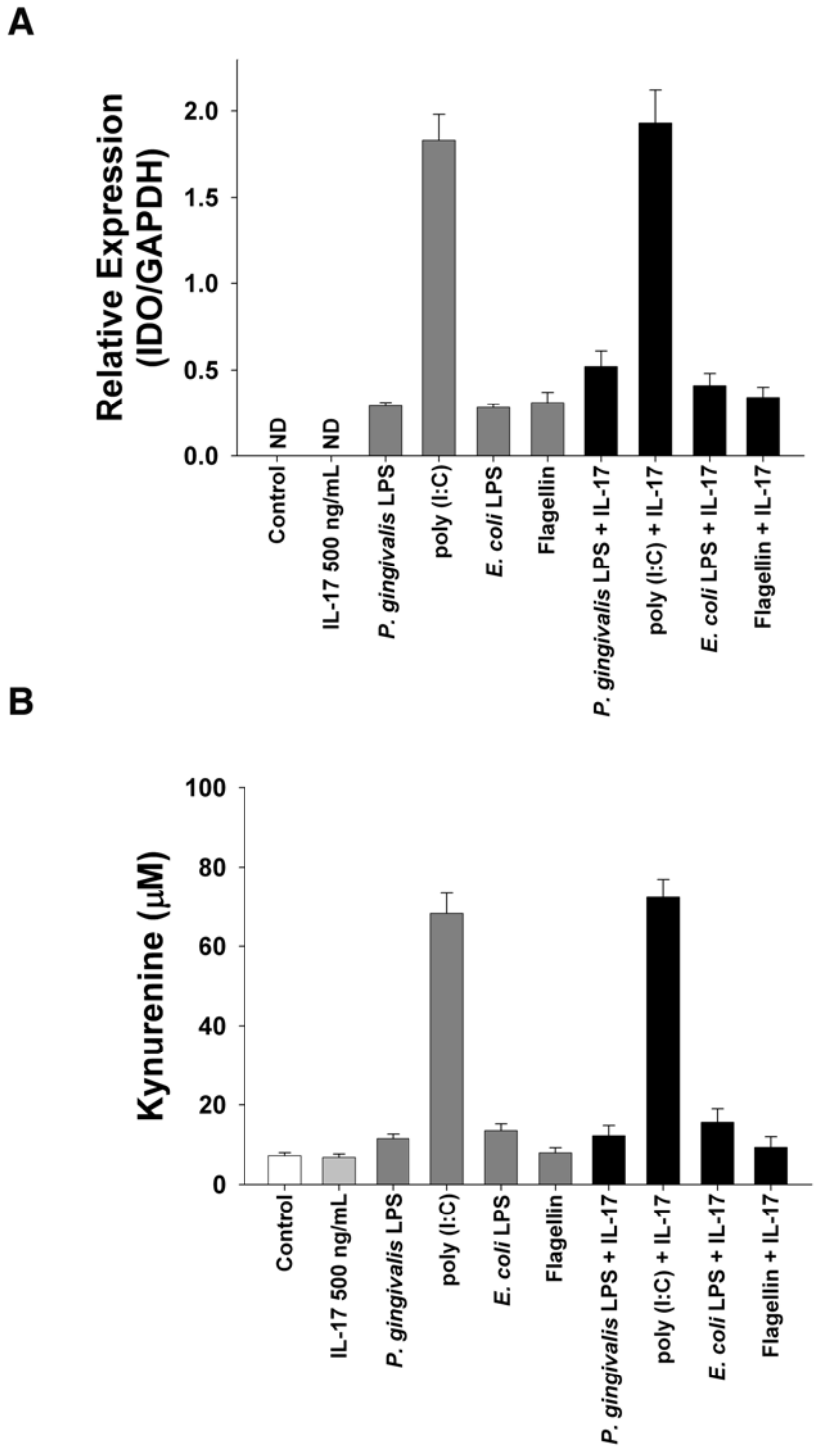

Figure 9. Expression of IDO in HGFs after stimulation with TLR ligand and cytokine combination. HGFs were cultured in a 24-well plates and stimulated with the following ligand and cytokine combination: TLR2 ligand *(P. gingivalis* LPS) + IL-17; TLR3 ligand (poly (I:C)) + IL-17; TLR4 ligand (*E.coli* LPS) + IL-17; TLR5 ligand (*S.*

typhimurium flagellin) + IL-17. Culture medium was used as a control. For semiquantitative analysis of IDO expression, stimulated HGFs were harvested after 12 hr and mRNA expression of IDO was analyzed by RT-PCR. Data shown are the ratio ± SEM of IDO:GAPDH from 4 separate experiments (A). To asseaa IDO activity, the concentrations of kynurenine in culture supernatants were determined. Data shown are mean concentrations of kynurenine ± SEM from 4 separate experimant (B)

The infiltration of plasmacytoid dendritic cells in gingival tissue from healthy and periodontitis subjects.

We investigated the infiltration of plasmacytoid dendritic cells in gingival tissue section from healthy and periodontitis subjects. BDCA-2 is a characteristic marker of plasmacytoid dendritic cells. Figure 10 shows positve cells in the connective tissue in periodontitis patient.. We found the infiltration of plasmacytoid dendritic cells in seven of the nine periodontitis specimens whereas found in only one of the eight healthy specimens.

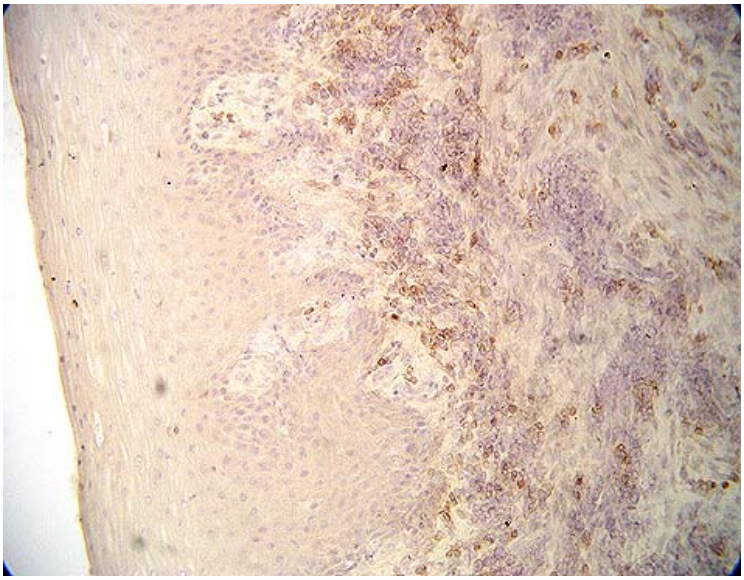

Figure 10. Expression of BDCA2 in periodontitis lesions. Single immunohistochemical staining was performed via an avidin-biotin-complex-immunoperoxidase (ABC-PO) system (Vector, Burlingame, CA, USA) on serial frozen gingival sections with anti-BDCA2 (to identify plasmacytoid dendritic cells) (Miltenyi, Biotec, Germany). Control staining with isotype-matched mouse IgG as a primary mAb yield negative results (data not shown). Original magnification 200x

Effects on plasmacytoid dendritic cells by the periodontopathic bacterial DNA

We next investigated the effects on IFN-a prodution of plasmacytoid dendritic cells by the periodontopathic bacterial DNA, specifically *P. gingivalis, Actinobacillus actinomycetemcomitans* and *Tanerella forsythia*. Fig. 11 demonstrates that, different DNAs from periodontopathic bacterial had defferent ability to stimulate plasmacytoid dendritic cells to produce IFN-alpha, *P. gingivalis* DNA had most potent stimulate.

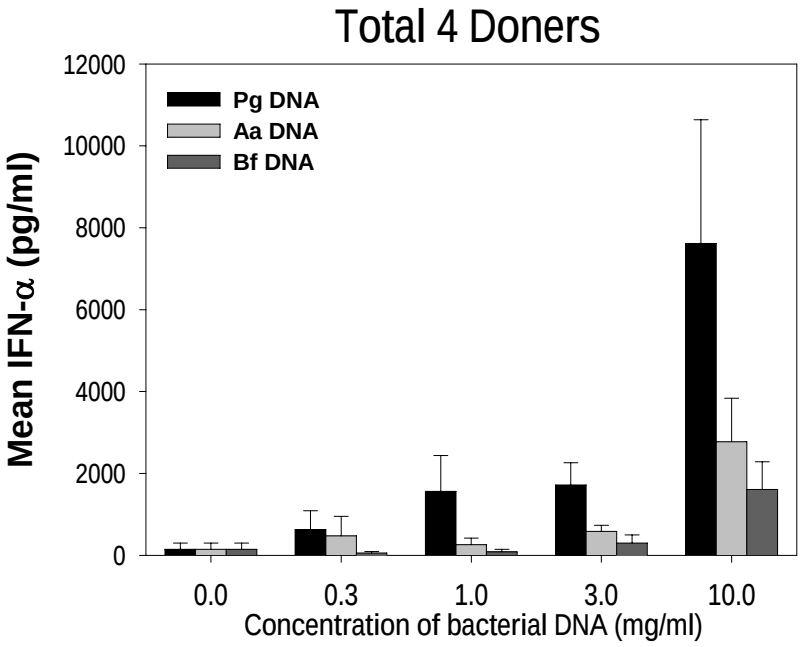

Figure 11. Production of IFN-α in plasmacytoid dendritic cells after stimulation with different DNAs from periodontopathic bacterial.

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Discussion

It is known that host immune response employs TLR and non-TLR pathways to recognize pathogens and commensal bacteria (Akira et al., 2004; Rakoff-Nahoum et al., 2004). This recognition leads to expression of mediators which limit microbial invasion. When gingival epithelium is ruptured, HGFs can be exposed to many bacterial pathogen-associated molecular patterns (PAMPs). Clinical observations demonstrated the presence of periodontopathic bacteria in epithelial and connective tissue layer of periodontitis lesions (Gillett and Johnson 1992; Allenspach-Petrzillka and Guggenheim 1983; Manor et al., 1984; Christersson et al., 1987; Saglie et al., 1988; Lamont et al., 1995; Meyer et al., 1996; Cutler et al., 1999). The pathogens such as *P. gingivalis, A. actinomycetemcomitans ,* and *Fusobacterium nucleatum* were also shown to invade human gingival epithelial cells and fibroblasts *in vitro* as well as *in vivo* (Sandros et al., 1994; Han et al., 2000; Amornchat et al., 2003; Rautemaa et al., 2004). The ability of HGFs to recognize and respond to such patterns renders them crucial in

dealing with microbial invasion. In this study, we evaluated the expression of TLRs and their role in signaling by HGFs. Our results demonstrate that HGFs derived from healthy gingival tissues expressed mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9 but not TLRs 7, 8, and 10. This is similar to observations in nasal fibroblasts (Takahashi et al., 2006). Recent study showed that fibroblasts from human foreskin and lung expressed cell surface TLR3 (Matsumoto et al., 2002). However, our study, using the same clone of mAb, demonstrated the presence of intracellular TLR3, but not cell surface TLR3, on HGFs. Thus, fibroblast TLR expression may differ across anatomic sites. Further study is needed to identify and compare the physiologic significance of intracellular and cell surface TLR3.

In line with the TLR mRNA expression, HGFs expressed IL-8 and IDO in response to *P. gingivalis* LPS, poly I:C, *E. coli* LPS, and *S. typhimurium* flagellin, respective ligands for TLRs 2, 3, 4, and 5. It has been shown that highly purified *P. gingivalis* LPS possess lipid A heterogeneity which may contribute to the ability to interact with either TLR2 or TLR4 (Darveau et al., 2004). *P. gingivalis* LPS at concentration of 50 μg/ml used in our study predominantly stimulated HGFs via TLR2 and to a lesser extend via TLR4 (InvivoGen product information). Poly I:C appeared to induce higher IL-8 and IDO expression than did other TLR ligands. Surprisingly, CpG ODN 2006, a potent ligand for TLR9 did not induce IL-8 or IDO expression. Similarly, purified DNA derived from either *E. coli, P. gingivalis, or A. actinomycetemcomitans*, which is also recognized as a TLR9 ligand, did not induce either of these mediators (data not shown). Our findings may indicate a non-functional TLR9 in HGFs. (Gingival epithelial cells also express TLR9 mRNA, but do not respond to CpG ODN 2006 : R. Mahanonda and S. Pichyangkul, unpublished observations). In contrast, some recent studies showed that DNA preparation from periodontopathic bacteria activated HGFs via TLR9 to produce IL-6 or IL-8 (Nonnenmacher et al., 2003; Takeshita et al., 1999). This inconsistency requires further investigation.

The finding that HGFs expressed TLRs 2, 4, and 5 supports their role in the innate immune response against bacteria. Oral plaque bacteria are known to have PAMPs that are recognized by TLRs 2, 4, and 5. For example, *P. gingivalis* LPS and *P. gingivalis* fimbriae are recognized by TLR2 (Hirschfeld et al., 2001; Asai et al., 2001; Zhou et al., 2005); LPS from *Actinobacillus actinomycetemcomitans* and *Bacteroides fragalis* are recognized by TLR4 (Erridge et al., 2004; Yoshimura et al., 2002; Gutierrez-Venegas et al., 2006). Flagellin of *Treponema denticola* is most likely recognized by TLR5. The expression of TLR3 in HGFs is interesting since TLR3 recognizes double stranded RNA, a by-product of viral replication and transcription (Alexopoulou et al., 2001). Possible role of herpesviruses in etiology and severity of periodontal diseases has been reported (Amit et al., 1992; Contreras et al., 1999; Slots 2005). The presence of TLR3 thus suggests a role of HGFs in antiviral response.

The effects of TLR ligand combinations on IL-8 and IDO expression by HGFs were not significantly different from those of single ligands or the sum of individual ligands, except for the combination of CpG ODN 2006 with poly I:C. Addition of CpG ODN 2006 markedly inhibited poly I:C-induced IL-8 and poly I:C-induced IDO

expression. CpG ODN2006, by itself had no effect on the expression of either IL-8 or IDO. The inhibitory effect of CpG ODN2006 on poly I:C-stimulated HGFs is unlikely to be limited to the early phase, since the addition of CpG ODN2006 at 6 h after poly I:C treatment still completely suppressed IL-8 production (data not shown). Further studies will be needed to understand the inhibitory effect of CpG ODN on poly I:C-induced HGF activation.

Previous studies demonstrated that different cytokines have different effects on HGFs in IL-8 production (Takashiba et al., 1992; Takigawa et al., 1994). TNF-α but not IFN-γ induced IL-8 production from HGFs, the observations agree with those previous studies (Takashiba et al., 1992; Takigawa et al., 1994; Tamura et al., 1992). Combinations of cytokines and bacterial PAMPs are known to modulate cytokine production from different cell types (Uehara et al., 2002; Matikainen et al., 2006). A high level of IL-8 as well as the increased presence of IL-8 secreting fibroblasts has been detected in periodontitis lesions (Wang et al 2003; Dongari-Bagtzoglou and Ebersole 1998). Our data demonstrate that stimulation of HGFs with TNF-α, combined with TLR ligands 2, 4, or 5, synergistically enhanced IL-8 production. The IL-8 response in periodontal tissue could have both beneficial and deleterious effects. IL-8 is important in neutrophil activation and recruitment. On one hand, undue down-regulation of this function could compromise anti-microbial defense. On the other hand, unduly vigorous or sustained IL-8 response could cause chronic inflammatory tissue destruction.

It is reported that skin fibroblasts can dampen local immune cell responses via IDO. In this study we demonstrated that HGFs were also able to induce IDO expression in response to *P. gingivalis* LPS, poly I:C, *E. coli* LPS, and *S. typhimurium* flagellin. IDO expression was synergistically enhanced when HGFs were treated with the combination of some PAMPs (TLR ligands 2, 4, or 5) and IFN-γ. It is interesting that TNF-α which enhanced TLR ligand-induced IL-8 production has negligible effect on TLR ligand-induced IDO expression of HGFs. Marked suppression of T cell proliferation in MLRs was mediated by IFN-γ and *P. gingivalis* LPS-treated HGFs. The suppression was reversible with the addition of either 1-Methyl-DL-tryptophan or L-tryptophan, thus confirming that stimulated HGFs suppressed T cell response via induced IDO.

Recent studies demonstrate the presence of IL-17, a product of Th17, in periodontal lesions, and suggest a possible role of this cytokine in disease severity (Oda *et al*., 2003; Johnson *et al*., 2004; Takahashi *et al*., 2005; Vernal *et al*., 2005). From our observation, HGFs expressed IL-17 receptor. In this study we systematically investigated the effects of IL-17, and of combined stimulation with IL-17 and IFN-γ on HGFs. We have previously shown that IFN-γ and *P. gingivalis* LPS enhanced IDO expression in HGFs and suppress T cell proliferation (Mahanonda *et al*., 2007). In this study we found that IL-17, by itself, had no effect on IDO expression in HGFs, however, when combined with IFN-γ, significant enhancement of IDO mRNA expression as well as its biological activity was detected. These findings therefore suggest a role of IL-17 in immune suppression when it is present with IFN-γ.

In conclusion, our study demonstrates that HGFs express mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9. Triggering with *P. gingivalis* LPS, poly I:C, *E. coli* LPS, and *S. typhimurium* flagellin, ligands specific for TLRs 2, 3, 4, and 5 respectively, led to the expression of IL-8 and IDO. In contrast, the potent TLR9 ligangd CpG ODN 2006 did not induce IL-8 and IDO expression. Moreover, it specifically inhibited poly I:C-induced HGF activation. The ability to induce IL-8 and IDO expression in ligand-stimulated HGFs was enhanced when combined with cytokine TNF-α and IFN-γ respectively. Finally, that HGFs can enhance IDO expression and down-regulate T cell response, when stimulated with some PAMP-cytokine combinations, suggests that these strategically-placed cells have an important role in modulating the unwelcome hyper-reactive inflammatory reaction that periodontitis often entails. Furthermore, we observed diverse effects of IL-17 especially in the combined stimulation with IFN-γ via HGF activation. Apparently, IL-17 or IFN-γ differentially controls HGF activation, possibly via different intracellular signaling pathways. These effects of individual cytokine are potentiated by their combination. Perhaps the most important finding of this investigation was that IL-17 not only preferentially stimulates pro-inflammatory reaction as previously shown in many diseases, but also functions as a negative feedback inhibitor of inflammatory T cell response in the presence of IFN-γ. Hence the role of IL-17 in periodontal disease should be carefully interpreted, and the full complexity of cytokine-mediated disease like periodontitis remains to be unraveled.

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Appendices

Reprint from Periodontology 2000

Periodontology 2000, Vol. 43, 2007, 41–55
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PERIODONTOLOGY 2000

Toll-like receptors and their role in periodontal health and disease

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Periodontitis 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. It is now known that the immune response applies a family of pattern-recognition receptors called Toll-like receptors as a tool to trigger an inflammatory response to microbial invasion(109). These data suggest the emerging role of Toll-like receptors in periodontitis.

Toll gene products were first discovered in 1985 and were described as being critical for the embryonic development of dorsal–ventral polarity in the fruit fly, *Drosophila* (8, 9). In addition, the protein Toll mediates the immune response to fungal infection in *Drosophila*, and binding to this protein can induce the release of antimicrobial proteins (68). In 1991, the sequence of the cytoplasmic domain of the Toll protein and the interleukin 1-receptor were reported to be similar, which is consistent with involvement in the immune response (35). This cytoplasmic domain is called the Toll–IL-1 receptor domain. Subsequently, homologues of *Drosophila* Toll, the so-called Toll-like receptors, were identified in mammals (73). The discovery of mouse Toll-like receptor 4 acting as a receptor for lipopolysaccharide (92) linked Toll-like receptor 4 to the innate immune system. Now it is clear that Toll-like receptors function as key pattern-recognition receptors of the innate immune system (54). They recognize and distinguish highly conserved structures present in large groups of microorganisms. The structures are referred to as pathogen-associated molecular patterns. Examples of pathogen-associated molecular patterns are bacterial lipopolysaccharide, peptidoglycan, lipoproteins, bacterial DNA, and double-stranded RNA. In the innate immune system, Toll-like receptors sense invasion by microorganisms such as bacteria, viruses, fungi, and protozoa, and trigger immune responses to clear such pathogens. To date, 10 Toll-like receptors in humans and 12 Toll-like receptors in mice have been described (19). Upon interaction with pathogen-associated molecular patterns, Toll-like receptors transmit this information through intracellular signaling pathways, resulting in activation of innate immune cells. The Toll-like receptor-mediated innate immune response is also critical for the development and direction of the adaptive immune system. Today, Toll-like receptor signaling has become a central topic in immunology.

Toll-like receptor ligands and signaling

Ilya Mechnikov, an immunologist and Nobel Prize winner in 1908, first recognized that upon rupture of skin, phagocytes rapidly move to the affected area and take up foreign substances. He would have appreciated knowing that this phenomenon is most likely triggered by Toll-like receptors. Innate immunity represents the first line of immunological defense. The main distinction between the innate and the adaptive immune systems lies in the receptors used for immune recognition. The antigen receptors of T and B cells are generated somatically, whereas the innate pattern-recognition receptors are encoded in the germ line (3). Historically, innate immunity has been suggested to mediate non-specific immune responses as a consequence of ingestion and digestion of microorganisms and foreign substances by macrophages and neutrophils. However, innate immunity is now recognized as showing remarkable specificity by means of discriminating between the host and pathogens, through a sophisticated Toll-like receptor-based system.

Although Toll-like receptor and interleukin 1-receptor cytoplasmic domains are homologous, the Toll-like receptor extracellular domains differ. The interleukin 1-receptors contain three

immunoglobulin-like domains, whereas the Toll-like receptor extracellular domains are characterized by the frequency of leucine-rich repeats. The number of leucine-rich repeats in each of the 10 known human Toll-like receptors respond to distinctive pathogen-associated molecular patterns that characterize a microbial infection. Specificity for pathogen-associated molecular patterns is provided by a relatively limited repertoire of Toll-like receptors; combinations of Toll-like receptors are generally required for recognition of certain pathogen-associated molecular patterns (38, 89). For example, Toll-like receptor 2 forms heterophilic dimers with Toll-like receptor 1 and Toll-like receptor 6 to recognize triacyl and diacyl lipopeptides, respectively (110). Many other human Toll-like receptors and their ligands are known (Table 1). For example Toll-like receptor 2 recognizes peptidoglycan, Toll-like receptor 3 recognizes viral double-stranded RNA, Toll-like receptor 4 recognizes lipopolysaccharide, Toll-like receptor 5 recognizes flagellin, Toll-like receptor 8 recognizes viral single-stranded RNA, and Toll-like receptor 9 recognizes bacterial DNA. It should be noted that cell surface Toll-like receptors (TLR1, TLR2, TLR4, TLR5, and TLR6) seem to recognize microbial products whereas intracellular Toll-like receptors (TLR3, TLR7, TLR8, and TLR9) recognize nucleic acids. Much research is underway, to identify additional ligands recognized by Toll-like receptors, and to identify accessory molecules that may aid in the recognition of

Table 1. Human Toll-like receptor (TLR) ligands

Receptor	Ligand	References
TLR1	Triacyl lipopeptides	110
TLR2	Lipoprotein/lipopeptides	6
	Peptidoglycan/lipoteichoic acid	100
	Mycobacterial lipoarabinomannan	72
	Zymosan	118
	Porphyromonas gingivalis lipopolysaccharide	49
	Porphyromonas gingivalis fimbriae	11
	Bacteroides fragilis lipopolysaccharide	30
	Capnocytophaga ochracea lipopolysaccharide	128
TLR3	Double-stranded RNA	5
	Polyinosine-polycytidylic acid	5
TLR4	*Escherichia coli* lipopolysaccharide	114
	Porphyromonas gingivalis lipopolysaccharide	28
	Actinobacillus actinomycetemcomitans lipopolysaccharide	75
	Fusobacterium nucleatum lipopolysaccharide	128
TLR5	Flagellin	36
TLR6	Peptidoglycan/lipoteichoic acid	100
	Diacyl lipopeptides	110
	Zymosan	89
TLR7	Imidazoquinoline	46
TLR8	Single-stranded RNA	44
	Imidazoquinoline	56
TLR9	Bacterial DNA	17
	CpG oligodeoxynucleotide	45
TLR10	Not determined	

pathogen-associated molecular patterns by Toll-like receptors.

Upon ligand binding, Toll-like receptor-mediated signaling activates signal transduction, leading to transcription of pro-inflammatory cytokines that initiate innate immune responses critical for the induction of adaptive immunity. Investigation of the Toll-like receptor signaling pathway is an important step to understanding how Toll-like receptor stimulation determines the outcome of immune responses. Toll-like receptor ligation initiates the interaction between Toll-IL-1-receptor domains and cytoplasmic adaptor molecules (Fig. 1). Myeloid differentiation primary-response protein 88, a key adaptor molecule, is used by most Toll-like receptors. Myeloid differentiation primary-response protein 88 mediates the Toll-like receptor-signaling pathway that activates interleukin 1-receptor-associated kinase. Interleukin 1-receptor-associated kinase then associates with tumor-necrosis-factor-receptor-associated factor 6, leading to the activation of two distinct signaling pathways. One pathway leads to activation of activator protein-1 through activation of mitogen-activated protein kinase. The other pathway activates the transforming growth factor-β-activated kinase/transforming growth factor-β-activated kinase-1-binding protein complex, which enhances activity of the inhibitor of nuclear factor-κB kinase complex. Once activated, this complex phosphorylates and induces subsequent degradation of the inhibitor of nuclear factor-κB and releases nuclear factor-κB, which translocates into the nucleus and induces expression of cytokines and chemokines (4).

Toll-like receptor signaling cascades are separated into two groups: the Myeloid differentiation primary-response protein 88-dependent pathway and the Myeloid differentiation primary-response protein 88-independent pathway (Fig. 1). The Myeloid differentiation primary-response protein 88-dependent pathway is essential for most Toll-like receptor-mediated cell activation. Stimulation of Toll-like receptor 3 or Toll-like receptor 4, however, results in induction of type I interferon through interferon-regulatory factor-3 in a Myeloid differentiation primary-response protein 88-independent manner (5, 29).

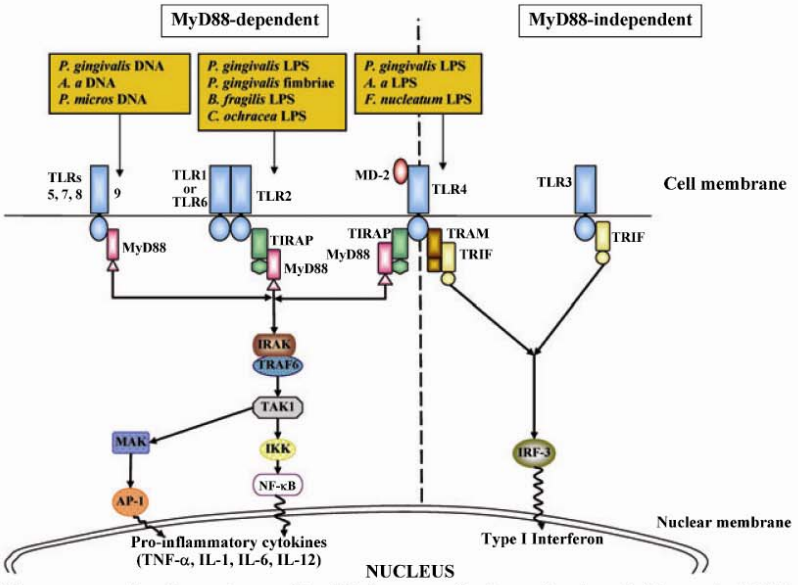

Fig. 1. Toll-like receptors signaling pathways (simplified diagram) and TLR ligands derived from bacterial plaque microorganisms. Abbreviations: AP-1, activator protein-1; IKK, inhibitor of nuclear factor-κB kinase; IRAK, interleukin (IL)-1-receptor-associated kinase; IRF-3, interferon (IFN)-regulatory factor-3; MAK, mitogen-activated protein kinase; MyD88, Myeloid differentiation primary-response protein 88; NF-κB, nuclear factor-κB; TAK1, transforming growth factor-β-activated kinase 1; TIRAP, Toll-IL-1 receptor domain-containing adaptor protein; TLR, Toll-like receptors; TRAF6, tumor-necrosis-factor-receptor-associated factor 6; TRAM, TRIF-related adaptor molecules; TRIF, Toll-IL-1 receptor domain-containing adaptor inducing IFN-β; A.a = Actinobacillus actinomycetemcomitans; LPS = lipopolysaccaride.

A database search for molecules that are structurally related to Myeloid differentiation primary-response protein 88 led to identification of other adaptors including:

- Toll-IL-1 receptor domain-containing adaptor protein/Myeloid differentiation primary-response protein 88-adaptor-like (TIRAP / MAL) (33, 50).
- Toll-IL-1 receptor domain-containing adaptor inducing interferon-β (TRIF) (123).
- TRIF-related adaptor molecules (TRAM) (34, 124).

Current information suggests that specific Toll-like receptor-mediated signaling pathways differentially select adaptors to initiate the Myeloid differentiation primary-response protein 88-dependent or Myeloid differentiation primary-response protein 88-independent pathway. A limited number of Toll-like receptors, acting through a restricted portal of only four adaptors, might differentially signal in response to a wide array of microbial products. As a result, clusters of many genes are selectively regulated to control needed immune processes (4).

Toll-like receptors in innate and adaptive immunity

Toll-like receptors are predominantly expressed on cells of the innate immune system, including neutrophils, monocytes/macrophages, and dendritic cells. These cells express different Toll-like receptors, allowing them to induce a wide variety of immune responses to specific pathogens. Neutrophils, the predominant innate immune cells in blood, express Toll-like receptor 1, Toll-like receptor 2, and Toll-like receptor 4 to Toll-like receptor 10, but not Toll-like receptor 3 (43). Being the first innate immune cells to migrate to the site of infection, neutrophils utilize relevant Toll-like receptors 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. Monocytes/macrophages express Toll-like receptor 1, Toll-like receptor 2, and Toll-like receptor 4–Toll-like receptor 8 (53). The binding of a pathogen-associated molecular pattern to monocyte Toll-like receptor can influence the type of adaptive immune response. A recent report shows that activation of Toll-like receptor 2/1 on monocytes leads to their differentiation into macrophages rather than dendritic cells, resulting in a poor antigen-specific Th1 response (64).

During the past decade, there have been major advances in our understanding of the generation of the antigen-specific immune response. It is now clear that development of adaptive immunity to pathogens is controlled through activation of innate immune cells, especially antigen-presenting dendritic cells. These 'professional' antigen-presenting cells are derived from bone marrow and deployed throughout the body as immature cells. During infection, Toll-like receptors of resident immature dendritic cells detect the pathogen-associated molecular patterns on or released from invading microorganisms. Upon interaction with a pathogen, Toll-like receptors transmit information about the encounter through signaling pathways, resulting in activation of dendritic cells. This activation involves expression of co-stimulatory molecules and production of cytokines and chemokines, all of which are critical for T-cell priming and differentiation (15). Dendritic cells express several Toll-like receptors. In human skin, two subsets of immature dendritic cells are found: Langerhans cells in the epidermis and interstitial dendritic cells in the dermis. In human blood, two subpopulations of dendritic cell precursors are present, which can be identified by cell phenotype and morphology. $CD11c^+$ dendritic cells are myeloid in appearance and express myeloid markers (CD13 and CD33) (61, 83, 115), whereas $CD11c^-$ dendritic cells have negligible expression of myeloid markers and express high levels of the interleukin-3R (CD123) (61). As a result of their plasma cell-like morphology, $CD11c^-$ dendritic cells have been called plasmacytoid dendritic cells (20, 61, 93). Human myeloid dendritic cells express Toll-like receptor 1–Toll-like receptor 6, Toll-like receptor 8, and Toll-like receptor 10. In contrast, human plasmacytoid dendritic cells express Toll-like receptor 1, Toll-like receptor 6, Toll-like receptor 7, Toll-like receptor 9 but not Toll-like receptor 2–Toll-like receptor 5, or Toll-like receptor 8 (51, 57). It is increasingly recognized that dendritic cells use Toll-like receptors to distinguish between different pathogens and initiate appropriate, effective types of immune responses. The Toll-like receptor expression profiles of distinct dendritic cell subsets suggest a differential function in sensing pathogens and influencing an adaptive immune response. Inflammatory cytokines can trigger dendritic cell maturation, but without direct Toll-like receptor stimulation, the dendritic cells fail to induce T helper cell differentiation (103). Different Toll-like receptor ligands instruct dendritic cells to stimulate distinct T helper cell responses. *Escherichia coli*

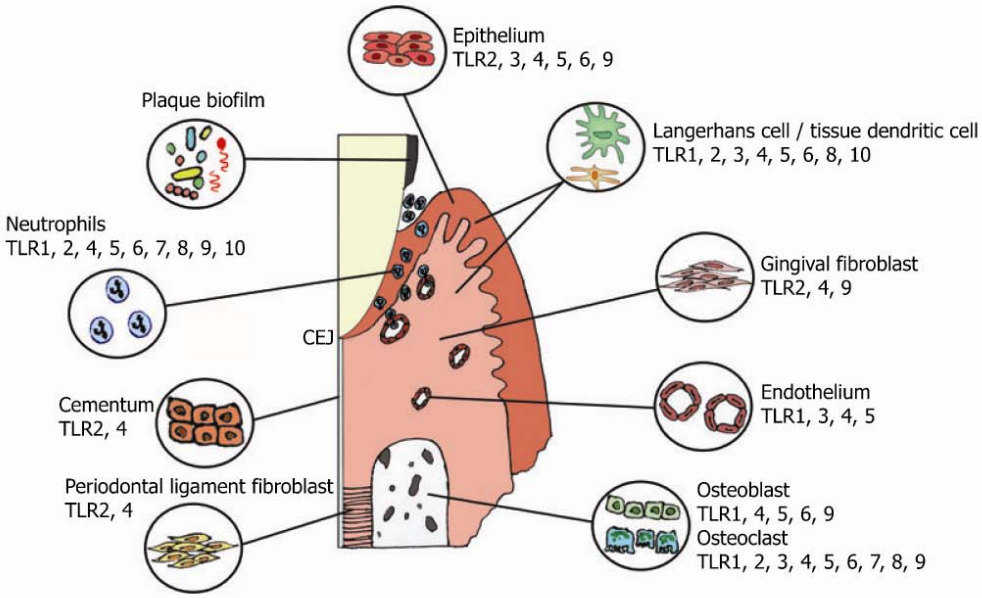

Fig. 2. Messenger RNA expression of Toll-like receptors (TLR) on different cell types of periodontium (CEJ = cemento-enamel junction).

lipopolysaccharide and flagellin, which trigger Toll-like receptor 4 and Toll-like receptor 5, respectively, cause human dendritic cells to induce a Th1 response via interleukin-12 production. In contrast, the Toll-like receptor 2 ligand, Pam3cys, and *Porphyromonas gingivalis* lipopolysaccharide cause induction of a T helper type 2 response (2, 95).

Toll-like receptors in periodontal tissue

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 very low numbers of immune cells such as macrophages, Langerhans cells, tissue dendritic cells, and migratory neutrophils in gingival crevicular fluid and the epithelial cell layer. In addition to immune cells, cells of the periodontium express Toll-like receptors (Fig. 2). Since the gingiva is consistently exposed to bacterial pathogen-associated molecular patterns, Toll-like receptor sensing and signaling in periodontal tissue could indeed play an 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 etiological agents. The disease initiation and progression results from the host response to plaque bacteria. In healthy periodontal tissue, low amounts of gram-positive aerobes and facultative anaerobes, such as species of *Streptococcus* and *Actinomyces*, are found supragingivally (77). 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 interleukin-1β, tumor necrosis factor-α, prostaglandin E_2, and interferon-γ in tissues and gingival crevicular fluid (90). B cells and plasma cells are the dominant cell type in periodontitis lesions, and numerous gram-negative anaerobes are found in subgingival biofilms (101). The differences in microbial compositions and quantities between health/gingivitis and periodontitis may influence the local inflammatory response. Key periodontal pathogens, *P. gingivalis, Actinobacillus actinomycetemcomitans*, and *Tannerella forsythia*, which are frequently detected in deep periodontal pockets, are well recognized for their virulence as

Table 2. Comparison between gingival and gut epithelial cells

Characteristics	Gingival epithelial cells	References	Gut epithelial cells	References
Morphology	Multiple layer	16	Single layer	70
TLR expression	2, 3, 4, 5, 6, 9	66	1, 2, 3, 4, 5, 6, 8, 9	88
Bacterial exposure	>500 species	91	>500 species	18
Innate immunity	β-defensins	26	β-defensins	87
	Cathelicidin LL-37	26	Cathelicidin LL-37	116
	Calprotectin	98	Calprotectin	99
	Interleukin-8	39, 66	Interleukin-8	25

etiological agents in human periodontitis (10). Chronic Toll-like receptor stimulation in periodontal tissues by bacterial pathogen-associated molecular patterns can lead to excessive production of pro-inflammatory mediators, resulting in tissue destruction.

Gingival epithelial cells and gingival fibroblasts

Gingival epithelium protects the underlying periodontal tissues from microorganisms and other harmful agents. It plays an important role in the innate immune response and homeostasis. Gingival epithelial cells are multi-layered whereas gut epithelial cells form a single layer. However, both epithelia are continually exposed to large numbers of commensal and pathogenic bacteria (Table 2). How the normal gingiva and gut respond to bacteria via Toll-like receptor signaling and maintain homeostasis, without clinical expression of inflammation, is a subject of intense investigation.

Toll-like receptor expression by human gingival epithelial cells was investigated in gingival biopsies obtained during periodontal surgery. The cells constitutively expressed a repertoire of Toll-like receptors: Toll-like receptor 2–Toll-like receptor 6, and Toll-like receptor 9 (66). Although gingival epithelial cells express low levels of Toll-like receptor 4, their expression can be enhanced by treatment with interferon-γ (117). Toll-like receptor 2 expression is denser in the spinous epithelial layer than in the basal epithelial layer (66). An abundance of Toll-like receptor 2-positive cells was also observed in connective tissue subjacent to the pocket epithelium (78). Thus, Toll-like receptor 2 may be especially important given its strategic location in the outermost layer where continual direct contact with oral bacterial products occurs.

Toll-like receptor 2 signaling appears to contribute to the production of interleukin-8 by gingival epithelial cells in response to *P. gingivalis*, but the mechanism is unclear. Using immortalized human gingival epithelial cells, Asai et al. (11) demonstrated that *P. gingivalis* fimbriae and *Staphylococcus aureus* peptidoglycan induced interleukin-8 production via Toll-like receptor 2 expressed on epithelial cells. In another study, however, *P. gingivalis* fimbriae (prepared similar to the first study) induced negligible production of interleukin-8 from human gingival epithelial cells. However, a sonic extract of *P. gingivalis* strongly induced interleukin-8 and monocyte chemoattractant protein-1 production (66). In our research, Toll-like receptor 2 ligands, such as *P. gingivalis* lipopolysaccharide and *S. aureus* peptidoglycan, activated human gingival epithelial cells derived from healthy gingival biopsies to produce interleukin-8 (R. Mahanonda, Chulalongkorn University, Bangkok, unpublished data). Gingival epithelial cells express Toll-like receptor 3 and Toll-like receptor 9 (66). The presence of these Toll-like receptors provides the ability for epithelial cells to respond to both viral and bacterial nucleic acids. Further studies are needed to ascertain their specific functions in the oral cavity.

Gingival fibroblasts are the major constituents of periodontal connective tissue. They maintain gingival tissue integrity by regulating collagen and proteoglycan metabolism. They also produce various inflammatory cytokines such as interleukin-1, interleukin-6, and interleukin-8 upon stimulation by bacteria and their components (107, 112, 125). Human gingival fibroblasts constitutively express mRNA of Toll-like receptor 2, Toll-like receptor 4, and Toll-like receptor 9 (82, 106, 120) and other Toll-like receptor-related molecules, e.g. CD14 (a co-receptor for lipopolysaccharide) and Myeloid differentiation primary-response protein 88

(42, 48, 106). In addition, Toll-like receptor 3 mRNA expression on human gingival fibroblasts has recently been observed in our laboratory (unpublished data). DNA microarray analysis demonstrated that expressed levels of Toll-like receptor 2, Toll-like receptor 4, and CD14 in the human gingival fibroblasts were higher in patients with periodontitis than in healthy individuals (121). Interestingly, upon *in vitro* stimulation with *P. gingivalis* lipopolysaccharide, human gingival fibroblasts showed increased expression of Toll-like receptor 2, Toll-like receptor 4, CD14, and MD-2 (a co-receptor for Toll-like receptor 4) (106). The data suggest that *P. gingivalis* lipopolysaccharide may be responsible for the observed up-regulation of Toll-like receptor 2, Toll-like receptor 4, and CD14 in periodontitis.

Fibroblasts isolated from gingiva and periodontal ligaments show heterogeneous expression of CD14 (105). The CD14 heterogeneity may contribute to differential cell activation. Similar to *P. gingivalis* lipopolysaccharide, interferon-γ produced in inflamed gingival tissues can up-regulate CD14 expression in human gingival fibroblasts (75). Interferon-γ markedly up-regulated CD14 and MyD88 of $CD14^{high}$ but not of $CD14^{low}$ human gingival fibroblasts. Upon stimulation with *Salmonella enterica* lipopolysaccharide, the interferon-γ-primed $CD14^{high}$ human gingival fibroblasts, showed increased interleukin-8 production via the enhanced CD14–Toll-like receptors 4 system (111). Similar findings of enhancement of cytokine production in interferon-γ-primed human gingival fibroblasts were observed after stimulation with *A. actinomycetemcomitans* lipopolysaccharide (75). The finding that *P. gingivalis* lipopolysaccharide and interferon-γ enhance Toll-like receptor 2, Toll-like receptor 4, CD14, and MD-2 expression suggests that these two molecules may have critical roles in amplifying the inflammatory reaction in periodontal connective tissue by increasing the response to Toll-like receptor 2 and Toll-like receptor 4 ligands derived from oral plaque bacteria.

Other cell constituents of periodontium

So far, there has been little information of Toll-like receptor signaling in other cell types of periodontium besides gingival epithelial cells and gingival fibroblasts. Similar to human gingival fibroblasts, periodontal ligament fibroblasts, which are a major cellular component of the periodontal ligament, constitutively express mRNA for Toll-like receptor 2, Toll-like receptor 4, CD14, MD-2, and Myeloid differentiation primary-response protein 88. When periodontal ligament fibroblasts and human gingival fibroblasts isolated from the same donor were compared, the periodontal ligament fibroblasts were found to express weaker CD14 but stronger levels of Toll-like receptor 2 expression than the human gingival fibroblasts (42). This differential expression of CD14 and Toll-like receptor 2 may suggest different functions of both cell types in response to plaque bacteria.

Cementum, the mineralized cellular tissue of the root surface, plays important roles in mineralization and, possibly, bone resorption. In periodontitis, cementum can be invaded by biofilms comprising many gram-negative pathogenic bacteria. The study of murine cementoblast cell lines demonstrated that they express mRNA for Toll-like receptor 2, Toll-like receptor 4, CD14, and MD-2. The *P. gingivalis* lipopolysaccharide up-regulated osteopontin and osteoprotegerin but down-regulated receptor activator of nuclear factor-κB ligand. This effect was partially inhibited by antibodies against Toll-like receptor 4/MD-2, suggesting that Toll-like receptor 4 signaling is involved (81). It is noteworthy that most lipid A species derived from *P. gingivalis* lipopolysaccharide are capable of activation via Toll-like receptor 2 signaling (14, 49, 130). It is also conceivable that the altered gene expression observed by Nociti et al. (81) could have resulted from Toll-like receptor 4 activation by other lipid A species present in the preparation (28).

Osteoblasts, the bone-forming cells, play a central role in modulating the differentiation and activity of osteoclasts, the bone-resorbing cells. Bone remodeling is regulated by the balance of osteoblasts and osteoclasts. Bacterial products and host mediators (interleukin-1, tumor necrosis factor-α, prostaglandin E_2) inhibit bone formation by osteoblasts and stimulate osteoclast activity, leading to bone resorption (67, 80). The increased receptor of receptor activator of nuclear factor-κB ligand and tumor necrosis factor-α expression of osteoblasts is involved in osteoclast formation (55, 60). Both Toll-like receptor 4 and Toll-like receptor 9 signaling stimulated mouse ostoblasts to produce tumor necrosis factor-α. Unlike Toll-like receptor 4 signaling, Toll-like receptor 9 signaling minimally induced receptor activator of nuclear factor-κB ligand expression (58, 131). The study by Asai et al. (12) demonstrated that human osteoblastic cell line, SaOS-2, constitutively expressed mRNA of Toll-like receptor 1, Toll-like receptor 4–Toll-like

receptor 6, Toll-like receptor 9, MD-2, CD14 and Myleoid differentiation primary-response protein 88. They produced interleukin-8 and increased expression of receptor activator of nuclear factor-κB ligand in response to Toll-like receptor 4 ligand (*Escherichia coli*-type synthetic lipid A). Osteoclasts from mouse bone marrow cells were shown to express Toll-like receptor 2, Toll-like receptor 4, and CD14 mRNA (52). Signaling via Toll-like receptor 2 and Toll-like receptor 4 on mouse osteoclasts enhanced the survival of mature osteoclasts (52, 104). The presence of Toll-like receptors on osteoblasts and osteoclasts suggests that both cell types can directly respond to bacterial products. It is too early to conclude the effect of Toll-like receptor stimulation on bone resorption. Although most studies imply that osteoclast activities are enhanced via Toll-like receptor stimulation, recent observations demonstrated that Toll-like receptor stimulation inhibited osteoclast differentiation (108). To date, Toll-like receptor signaling studies have been mainly evaluated in the murine model. More investigations in humans are required to precisely understand the role of Toll-like receptors in bone remodeling.

The periodontium is a highly vascularized tissue surrounding the root surface of the tooth. Human microvascular endothelial cells were traditionally regarded as a relatively passive, inert vascular lining. It is now clear that they play an important active role in regulation of immune and inflammatory responses. These cells constitutively express mRNA for Toll-like receptor 1, and Toll-like receptor 3–Toll-like receptor 5, but express little or no mRNA for Toll-like receptor 2. Thus, as expected, they respond to *E. coli* lipopolysaccharide in a Toll-like receptor 4 and soluble CD14-dependent manner, but do not respond to *Mycobacterium tuberculosis* 19-kDa lipoprotein, which is a Toll-like receptor 2 ligand (31). Activation of vascular endothelium by Toll-like receptor 4 signaling results in the production of various pro-inflammatory cytokines and chemokines (47, 129). Toll-like receptor 2 expression on human endothelial cells (dermal microvessel and umbilical vein) could be up-regulated by Toll-like receptor 4 ligand, lipopolysaccharide, and pro-inflammatory cytokines such as interferon-γ and tumor necrosis factor-α (32). Lipopolysaccharide and interferon-γ also up-regulate Toll-like receptor 4 mRNA expression in endothelial cells (32). The up-regulation of Toll-like receptor 4 by interferon-γ may constitute a novel mechanism for the well-described synergy between interferon-γ and lipopolysaccharide in cell activation (76, 94).

Bacterial plaque microorganisms and their pathogen-associated molecular patterns

Unlike classic enterobacterial *E. coli* lipopolysaccharide (a well-known Toll-like receptor 4 ligand), highly purified native *P. gingivalis* lipopolysaccharide and lipid A preparations consistently demonstrate Toll-like receptor 2 activity (14, 49, 130). Consistent with this is the ability of *P. gingivalis* lipopolysaccharide to stimulate tumor necrosis factor-α production from both lipopolysaccharide-non-responsive C3H/HeJ (Toll-like receptor 4 mutant) and lipopolysaccharide-responsive C3H/HeN macrophages (85, 113). This suggests that the tumor necrosis factor-α response to *P. gingivalis* stimulation may be irrelevant to Toll-like receptor 4 signaling and may involve other Toll-like receptor(s).

Lipopolysaccharide is a complex glycolipid composed of a hydrophilic polysaccharide portion and a hydrophobic domain known as lipid A. Lipid A is an immunostimulatory principal moiety of lipopolysaccharide responsible for most lipopolysaccharide-induced biological effects. The lipid A structure of *P. gingivalis* is composed of unique branched fatty acids longer than those in enterobacterial lipid A and lacking a phosphoryl group at position 4′ of the non-reducing glucosamine (65, 84). The different Toll-like receptor activities of *E. coli* lipopolysaccharide and *P. ginvivalis* lipopolysaccharide may be the result of the unique structure of *P. gingivalis* lipopolysaccharide and its lipid A, which differs from *E. coli* lipopolysaccharide and its lipid A (84). *P. gingivalis* lipopolysaccharide is also highly heterogeneous, consisting of multiple lipid A species, even when obtained from a single purified preparation (28, 65). Lipid A heterogeneity could contribute to the ability of *P. gingivalis* lipopolysaccharide to interact with either Toll-like receptor 2 or Toll-like receptor 4 (28), and may explain the seemingly conflicting results obtained concerning Toll-like receptor utilization (14, 41, 49, 86, 106, 120).

Like *P. gingivalis* lipopolysaccharide, *Bacteroides fragilis* lipopolysaccharide (30) and *Capnocytophaga ochracea* lipopolysaccharide bind Toll-like receptor 2 (128). In contrast, the lipopolysaccharides of *A. actinomycetemcomitans* and *Fusobacterium nucleatum* bind to Toll-like receptor 4 (75, 128). The differential ability of oral bacterial lipopolysaccharide to stimulate and signal through Toll-like receptors may induce host immune responses selectively. The *A. actinomycetemcomitans* lipopolysaccharide-stimulated

human monocyte-derived dendritic cells stimulated T helper type 1 differentiation via interleukin-12 production (59). In contrast, *P. gingivalis* lipopolysaccharide-stimulated dendritic cells induced a T helper type 2 response (95). The level of interleukin-10 and interleukin-12 production was shown to be regulated via a Toll-like receptor-mediated pathway. It was demonstrated that *P. gingivalis* lipopolysaccharide activated the phosphatidylinositol-3-kinase-Akt pathway via Toll-like receptor 2 in human monocytes, enhancing interleukin-10 levels and down-regulating interleukin-12 levels (71).

Other plaque bacterial components, such as DNA derived from *P. gingivalis, A. actinomycetemcomitans*, and *Peptostreptococcus micros*, were shown to activate human gingival fibroblasts to produce interleukin-6, possibly via Toll-like receptor 9 activation (82). However, our own observation using synthetic Toll-like receptor 9 ligand (CpG DNA), showed no effect on human gingival fibroblasts. The issue of Toll-like receptor 9 signaling in human gingival fibroblasts requires further investigation. A summary of pathogen-associated molecular patterns derived from bacterial plaque microorganisms and their specific Toll-like receptors is shown in Fig. 1. Indeed, many pathogen-associated molecular patterns remain to be investigated. To date, most studies of Toll-like receptor signaling by pathogen-associated molecular pattern components from oral plaque bacteria have evaluated these components individually. In future research, it will surely be important to assess the effects of combinations of pathogen-associated molecular patterns and whole bacterial cells.

Viruses in periodontitis

Oral plaque bacteria play a key role in causing periodontitis. During the past decade, it has become apparent that viral infection may also be involved in the development of periodontitis. Several lines of evidence link herpesviruses, especially human cytomegalovirus and Epstein-Barr virus to the severity of periodontitis. These evidences include:

- The detection of viral DNA in gingival tissue (7).
- The presence of higher frequency of viral DNA in periodontitis tissue than in healthy periodontal tissue (23, 74).
- The detection of active human cytomegalovirus replication in periodontal tissue (22).

Herpesviruses may directly cause the breakdown of periodontal tissue. Several studies demonstrate that the presence of subgingival human cytomegalovirus or Epstein-Barr virus-1 DNA is associated with an increased presence periodontopathic bacteria (24, 40, 102). The findings suggest that local immune responses against oral plaque bacteria could be suppressed as a result of herpesvirus infection.

Recently, progress has been made in understanding the antiviral innate immune response. Viral components such as envelope protein(s), genomic DNA and RNA or double-stranded RNA (dsRNA) produced in infected host cells can be recognized by host Toll-like receptors and non-Toll-like receptors. More specifically, Toll-like receptors 2 and 9 have been demonstrated to sense herpesvirus-associated molecules and initiate antiviral responses. Recognition of human cytomegalovirus virions by Toll-like receptor 2 and CD14 leads to cytokine production (21). In a mouse model, recognition of DNA viruses by Toll-like receptor 9 is required for interferon-α production (62, 63, 69). Toll-like receptor 3 has been shown to recognize dsRNA (5), a product of viral replication. Host cells also express cytoplasmic RNA helicases that can recognize dsRNA via Toll-like receptor-independent mechanisms (126, 127).

Although, there is evidence to support the role of viruses in periodontitis, there is limited information on the understanding of interaction between viruses and periodontal pattern recognition receptors. The presence of mRNA expression of Toll-like receptors 2, 3 and 9 in human gingival epithelial cells and fibroblasts may suggest the role of periodontal tissue in antiviral responses. There is still much work ahead not only to firmly establish the role of viruses in periodontitis, but also to understand the crucial role of periodontal tissue in innate immune recognition of viral infection.

Conclusion

It is clear that periodontal cells actively participate in the innate immune response against dental plaque bacteria. They express different types of Toll-like receptors (Fig. 2). Periodontitis, a chronic inflammation of the periodontium, provides a unique opportunity to investigate the host–microbe interactions. Gingival epithelium is continually exposed to at least 500 species of both oral commensal and pathogenic bacteria (91). How the gingival epithelial cells orchestrate their response via Toll-like receptor signaling, and thereby maintain themselves without severe, chronic inflammation is a very interesting issue. Some workers have recently suggested that the oral mucosa develops tolerance after repeated expo-

sure to bacterial products (37, 79). Down-regulation of Toll-like receptor expression and inhibition of intracellular signaling may represent the underlying mechanism of tolerance. This speculation is consistent with how gut epithelium applies tolerance to attenuate the inflammatory response to commensal bacteria (1, 88). However, this hypothesis has been challenged. Recent research indicates that activation of Toll-like receptors by commensal bacteria, under steady-state conditions, is critical to maintaining intestinal epithelial homeostasis and protecting against gut injury (97).

Gingival epithelial cells express mRNAs of Toll-like receptor 2–Toll-like receptor 6, and Toll-like receptor 9 (66), allowing them to recognize the different repertoire of pathogen-associated molecular patterns that are encountered. As a result of the specificity of the Toll-like receptor–ligand interaction, gingival epithelial cells are probably unable to differentiate between commensal and pathogenic bacteria with regard to their responses. For example, lipopolysaccharide from pathogenic *P. gingivalis* and peptidoglycan from *S. aureus* are able to stimulate gingival epithelial cells to produce interleukin-8 via Toll-like receptor 2 signaling (R. Mahanonda, Chulalongkorn University, Bangkok, unpublished data). We hypothesize that in the steady-state condition, Toll-like receptors expressed on gingival epithelium (especially in the dentogingival region) continually interact with components of oral plaque bacteria that form biofilms attached to the tooth surface. This Toll-like receptor signaling results in innate immune responses involving the release of antibacterial peptides (β-defensins, cathelicidin, and calprotectin) and neutrophil recruiting chemokine (interleukin-8). Toll-like receptor signaling, therefore, serves to limit pathogenic infection and to prevent commensal organisms from breaching the epithelial barrier. This hypothesis is based on two previous observations. First, continuous migration of large numbers of neutrophils into the oral cavity has been detected (13, 96). This may reflect recruitment of neutrophils by interleukin-8 released from gingival epithelial cells in response to commensal bacteria. Second, β-defensin-2, the inducible antibacterial peptide, has been detected in healthy gingival epithelial cells (122). In contrast, epithelial cells in healthy skin, trachea, and gut do not express β-defensin-2, but they do express this molecule when the tissues become inflamed or infected (27). The expression of β-defensin-2 in gut epithelium is dependent on Toll-like receptor signaling (119). This suggests that β-defensin-2 is possibly induced by Toll-like receptor signaling of gingival epithelial cells, in response to commensal bacteria in the steady-state. Further investigation of dentogingival epithelial cells and neutrophils, and of their cross-talk in response to oral plaque bacteria in the steady-state, should greatly enhance our understanding of how the periodontium exerts an innate immune response through Toll-like receptor signaling without severe inflammation.

A possible scenario for bacterial plaque-induced periodontitis may start with disruption and penetration of the dentogingival epithelial barrier after confrontation with high levels of invasive pathogenic bacteria (*P. gingivalis, A. actinomycetemcomitans*, and *T. forsythia*) and/or with their cytotoxic products (gingipains, cysteine proteinases, major surface protein, and cagE). Invasion would enable bacterial components to activate other cells deeper in the epithelium and in connective tissue via Toll-like receptor signaling. These cells include antigen-presenting cells (Langerhans and interstitial dendritic cells), macrophages, fibroblasts, endothelial cells, osteoblasts, and osteoclasts. These stimulated cells could produce an array of cytokines, chemokines, and other mediators that promote inflammation and immune cell infiltration. Additional cytokines produced by infiltrated memory T cells would amplify the inflammatory reaction, leading to destruction of connective tissue and bone. The chronic inflammation and bone loss, induced by both innate and adaptive immune responses, abate once the bacterial stimulus is removed.

In summary, periodontitis is highly complex and multi-factorial. Further investigation into Toll-like receptor signaling in periodontal tissue should provide new insight into the role of the immune system in maintaining health and combating disease, not only in the periodontium but in other tissues as well.

Acknowledgements

We thank Drs M. Herzberg and R. Giacaman-Sarah of the University of Minnesota, USA for constructive editorial comments. We also thank the Thailand Research Fund and Ratchadapisek Endowment.

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Reprint from Journal of Immunology

IL-8 and IDO Expression by Human Gingival Fibroblasts via TLRs[1]

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Human gingival fibroblasts (HGFs), a predominant cell type in tooth-supporting structure, are presently recognized for their active role in the innate immune response. They produce a variety of inflammatory cytokines in response to microbial components such as LPS from the key periodontal pathogen, *Porphyromonas gingivalis*. In this study, we demonstrated that HGFs expressed mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9, but not TLRs 7, 8, and 10. Stimulation of HGFs with highly purified TLR2 ligand (*P. gingivalis* LPS), TLR3 ligand (poly(I:C)), TLR4 ligand (*Escherichia coli* LPS), and TLR5 ligand (*Salmonella typhimurium* flagellin) led to expression of IL-8 and IDO. A potent TLR 9 ligand, CpG oligodeoxynucleotide 2006 had no effect, although HGFs showed a detectable TLR9 mRNA expression. No significant enhancement on IL-8 or IDO expression was observed when HGFs were stimulated with various combinations of TLR ligands. Surprisingly, the TLR9 ligand CpG oligodeoxynucleotide 2006 was able to specifically inhibit poly(I:C)-induced IL-8 and IDO expression. TNF-α enhanced TLR ligand-induced IL-8 production in HGFs, whereas IFN-γ enhanced TLR ligand-induced IDO expression. HGF production of IDO in response to *P. gingivalis* LPS, IFN-γ, or the two in combination inhibited T cell proliferation in MLRs. The observed T cell inhibition could be reversed by addition of either 1-methyl-DL-tryptophan or L-tryptophan. Our results suggest an important role of HGFs not only in orchestrating the innate immune response, but also in dampening potentially harmful hyperactive inflammation in periodontal tissue. ***The Journal of Immunology,*** **2007, 178: 1151–1157.**

Periodontitis is a chronic bacterial infection of tooth-supporting structures. It causes destruction of periodontal connective tissue and bone and, in severe cases, tooth loss. Key oral plaque bacteria including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Tanerella forsythia* are recognized as etiologic agents in periodontitis. The disease initiation and progression results from the host response to plaque bacteria. Immunohistochemistry studies reveal dense cellular infiltration, including numerous T and B cells in periodontitis lesions. In addition, high levels of inflammatory mediators such as IL-1β, TNF-α, PGE_2, IFN-γ, and IL-8 can be detected in inflamed gingival tissues and gingival crevicular fluid (1, 2).

Gingival fibroblasts, the major cell type in periodontal connective tissues, provide a tissue framework for tooth anchorage. Until recently, they were presumed to be immunologically inert. Currently, however, researchers recognize their active role in host defense. Upon stimulation with bacterial pathogens and their products, as well as with cytokines, gingival fibroblasts secrete various soluble mediators of inflammation such as IFN-γ, PGE_2, IL-1, IL-6, and IL-8 (3–6) and up-regulate expression of HLA-DR and ICAM-1 (7). These fibroblast-derived mediators and surface Ags are thought to play an important role in periodontal inflammatory response. Recently, human gingival fibroblasts (HGFs)[3] have been demonstrated to express TLRs 2, 4, and 9 (8–10). TLRs are recognized as key pathogen recognition receptors that sense microbial invasion. TLR ligation triggers inflammatory innate immune response, which is critical for pathogen elimination (11). It is likely that the release of inflammatory mediators from HGFs in response to microbial stimuli occurs via TLR triggering.

Recent findings also suggest that fibroblasts play an important role in negative feedback inhibition of inflammatory T cell response. IFN-γ-treated dermal fibroblasts express IDO (12, 13). IDO is known as an enzyme that catabolizes tryptophan, an essential amino acid. Immune inhibitory effects by IDO is due to tryptophan depletion and/or cytotoxic effects by the tryptophan metabolites, such as kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid (14, 15). Recent observation showed that a synthetic derivative of the tryptophan metabolite anthranilic acid reversed paralysis in mice with experimental autoimmune encephalomyelitis by suppression of myelin-reactive T cell responses (16). It is becoming clear that IDO can act as a critical immune suppressive molecule responsible for the attenuation of T cell hyperactivity.

Gingiva, the outer layer of the oral cavity, is consistently exposed to ~500 bacterial species of both commensal and pathogenic bacteria (17). How the oral tissues orchestrate their response to bacterial stimuli via TLR signaling, and thereby either maintain homeostasis or mediate expression of disease, is thus a very important research topic. We investigated the local innate immune

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Received for publication July 20, 2006. Accepted for publication November 2, 2006.

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[1] This work was supported by the Thailand Research Fund (BRG-4980006) and Ratchapisek Endowment.

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[3] Abbreviations used in this paper: HGF, human gingival fibroblast; PAMP, pathogen-associated molecular patterns; ODN, oligodeoxynucleotide.

response and immune regulation by focusing on TLR expression of HGFs and on their function after triggering by their specific ligands.

Materials and Methods

Reagents and Abs

Medium for HGF cultures was DMEM that was purchased from Invitrogen Life Technologies. The medium was supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml), Fungizone (2.5 μg/ml), and 10% heat-inactivated FCS. Highly purified TLR ligands including LPS from *Porphyromonas gingivalis* (TLR2 ligand), poly(I:C) (TLR3 ligand), LPS from *Escherichia coli* K12 strain (TLR4 ligand), flagellin from *Salmonella typhimurium* (TLR5 ligand), loxoribine (guanosine analog, TLR7 ligand), and single-stranded poly(U) oligonucleotide complexed with LyoVec (TLR8 ligand) were obtained from InvivoGen. CpG ODN 2006 (TLR9 ligand) was obtained from Coley Pharmaceutical Group. IFN-γ and TNF-α were purchased from R&D Systems. Anti-human TLR3 mAb (TLR3.7) was obtained from eBioscience. Both 1-methyl-DL-tryptophan and L-tryptophan were purchased from Sigma-Aldrich.

Human gingival fibroblasts

Gingival tissue samples were collected from subjects who had clinically healthy periodontium and no history of periodontitis. The gingival biopsies were obtained at the time of the crown-lengthening procedure for prosthetic reasons from the Periodontal Clinic, Faculty of Dentistry (Chulalongkorn University). Before operation, ethical approval was obtained from the ethics committee of the Faculty of Medicine, Chulalongkorn University, and informed consent was obtained from each subject. Fibroblasts from the gingival tissues were obtained following established procedure (18). In brief, the excised tissue was immediately washed and then minced with scissors into fragments (1–3 mm^2) and placed in 60-mm tissue culture dishes. After a confluent monolayer of cells was reached, HGFs were trypsinized, washed twice, and then transferred to new tissue culture flasks. The HGF cultures at passages four to eight were used throughout the study.

Preparation of P. gingivalis *sonicates*

P. gingivalis FDC-381 was cultured in trypticase soy broth (Sigma-Aldrich) at 37°C under an anaerobic chamber (Thermo Electron). The bacteria were harvested by centrifugation (Beckman Coulter) at 2000 $\times$ *g* for 15 min and washed twice in PBS. The purity was assessed by Gram staining and colony morphology on trypticase soy agar. The microorganisms were subjected to sonication with high-density ultrasonication (High Intensity Ultrasonic Processor, microprocessor controlled 600-W model; Sonics and Material) at 4°C for 20 min elapsed time, with pulse on 2.5 s and pulse off 2 s. The sonicated extracts were examined microscopically for complete breakage of cells. Protein concentration of the sonicates was determined by using a Bio-Rad protein assay. The bacterial stock was aliquoted and stored at −20°C until use.

mRNA expression of TLRs in HGFs

Total RNA from HGFs was isolated by using a RNeasy Mini Kit from Qiagen. One microgram of DNase I-treated total RNA was reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR (Promega). TLRs 1–10 and GAPDH were amplified using specific primers purchased from Sigma-Aldrich as described as follows and the PCR conditions were described as in previous studies (19, 20): *TLR1* (5′-CGTAAA ACTGGAAGCTTTGCAAGA-3′/5′-CCTTGGGCCATTCCAAATAAGT CC-3′); *TLR2* (5′-GGCCAGCAAATTACCTGTGTG-3′/5′-CCAGGTAG GTCTTGGTGTTCA-3′); *TLR3* (5′-ATTGGGTCTGGGAACATTTCTC TTC-3′/5′-GTGAGATTTAAACATTCCTCTTCGC-3′); *TLR4* (5′-CTG CAATGGATCAAGGACCA-3′/5′-TCCCACTCCAGGTAAGTGTT-3′); *TLR5* (5′-CCTCATGACCATCCTCACAGTCAC-3′/5′-GGCTTCAAGG CACCAGCCATCTC-3′); *TLR6* (5′-ACTGACCTTCCTGGATGTGG-3′/ 5′-TGGCACACCATCCTGAGATA-3′); *TLR7* (5′-ACAAGATGCCTT CCAGTTGC-3′/5′-ACATCTGTGGCCAGGTAAGG-3′); *TLR8* (5′-CA GAATAGCAGGCGTAACACATCA-3′/5′-AATGTCACAGGTGCATTC AAAGGG-3′); *TLR9* (5′-GCGAGATGAGGATGCCCTGCCCTACG-3′/ 5′-TTCGGCCGTGGGTCCCTGGCAGAAG-3′); *TLR10* (5′-GGCCAG AAACTGTGGTCAAT-3′/5′-AACTTCCTGGCAGCTCTGAA-3′); and *GAPDH* (5′-TCATCTCTGCCCCCTCTGCTG-3′/5′-GCCTGCTCACCACC TTCTTG-3′).

Flow cytometric analysis of TLR3 expression

The specific localization of TLR3 of the HGFs was investigated by flow cytometry. For surface TLR3 staining, HGFs were incubated with PE-conjugated anti-human TLR3 mAb (clone TLR3.7, 1 μg) for 30 min at 4°C. For intracellular staining, cells were pretreated with fixation/permeabilization solution (BD Pharmingen) for 20 min at 4°C, washed once with PBS, and then incubated with the same mAb for 1 h at 4°C. Mouse isotype mAbs conjugated with PE was used as control. The stained cells were then analyzed on a FACSCalibur (BD Biosciences).

TLR ligation on HGFs after stimulation with TLR ligand(s) and/or cytokine

HGFs (1.5 $\times$ 10^5 cells/ml) in 96-well plates or 24-well plates (Corning Glass) were treated with either 1) various single TLR ligands: *P. gingivalis* LPS (50 μg/ml), poly(I:C) (100 μg/ml), *E. coli* LPS (10 μg/ml), *Salmonella typhimurium* flagellin (5 μg/ml); loxoribine (100 μM), ssPolyU (5 μg/ml), and CpG ODN 2006 (10 μg/ml); 2) TLR ligand combinations: *P. gingivalis* LPS (50 μg/ml) plus poly(I:C) (100 μg/ml), *P. gingivalis* LPS (50 μg/ml) plus *E. coli* LPS (10 μg/ml), *P. gingivalis* LPS (50 μg/ml) plus *S. typhimurium* flagellin (5 μg/ml), *P. gingivalis* LPS (50 μg/ml) plus CpG ODN 2006 (10 μg/ml), poly(I:C) (100 μg/ml) plus *E. coli* LPS (10 μg/ml), poly(I:C) (100 μg/ml) plus *S. typhimurium* flagellin (5 μg/ml), poly(I:C) (100 μg/ml) plus CpG ODN 2006 (10 μg/ml), *E. coli* LPS (10 μg/ml) plus *S. typhimurium* flagellin (5 μg/ml), *E. coli* LPS (10 μg/ml) plus CpG ODN 2006 (10 μg/ml), or *S. typhimurium* flagellin (5 μg/ml) plus CpG ODN 2006 (10 μg/ml); 3) cytokines: IFN-γ (100 U/ml) and TNF-α (50 ng/ml); or 4) TLR ligand and cytokine combinations: *P. gingivalis* LPS (50 μg/ml) plus IFN-γ (5 U/ml), poly(I:C) (10 μg/ml) plus IFN-γ (5 U/ml), *E. coli* LPS (10 μg/ml) plus IFN-γ (5 U/ml), *S. typhimurium* flagellin (5 μg/ml) plus IFN-γ (5 U/ml), *P. gingivalis* LPS (50 μg/ml) plus TNF-α (1 ng/ml), poly(I:C) (10 μg/ml) plus TNF-α (1 ng/ml), *E. coli* LPS (10 μg/ml) plus TNF-α (1 ng/ml), and *S. typhimurium* flagellin (5 μg/ml) plus TNF-α (1 ng/ml).

After stimulation with TLR ligand(s) and/or cytokine for 12–24 h, the cells and culture supernatants were collected for measurement of IL-8 and IDO expression.

Determination of IL-8

The supernatants of HGFs after stimulation with TLR ligand(s) and/or cytokine were harvested and assessed for IL-8 production by ELISA (R&D Systems).

mRNA expression of IDO

The kinetics study of IDO mRNA expression (6, 12, and 24 h) was conducted using IFN-γ- or TNF-α-treated HGFs. Twelve-hour-treated cells showed optimal mRNA expression of IDO. The total RNA of HGFs after 12 h of stimulation with TLR ligand(s) and/or cytokine was reverse transcribed and treated with DNase I as previously mentioned. IDO was amplified using specific primer (5′-CTTCCTGGTCTCTCTATTGG-3′/5′-GAAGTTCCTGTGAGCTGGT-3′; Sigma-Aldrich) (21). The expected size of the PCR product was 430 bp. For semiquantitative RT-PCR analysis, band intensities on scanned gels were analyzed (GeneTools; Synoptics) using specific bands of the housekeeping gene GAPDH as a reference.

IDO activity: kynurenine assay

IDO-dependent catabolism of tryptophan produces kynurenine. Hence, the biological activity of IDO was evaluated by measuring the level of kynurenine in HGF culture supernatants (22). One hundred microliters of culture supernatants of HGFs after stimulation with TLR ligand(s) and/or cytokine was mixed with 50 μl of 30% trichloroacetic acid, vortexed, and centrifuged at 8000 $\times$ *g* for 5 min. Then, 75 μl of the supernatant was added to an equal volume of Ehrlich reagent (100 mg of *p*-dimethylbenzaldehyde in 5 ml of glacial acetic acid) in a 96-well microtiter plate, and the absorbance was read at an OD of 492 nm. A standard curve of defined kynurenine concentration (0–100 μM) permitted analysis of unknowns.

Suppression of T cell response in MLR

To assess whether IDO-expressed HGFs inhibit allogeneic T cell responses, MLRs were performed on a layer of HGFs that had been treated for 2 days with either *P. gingivalis* LPS (50 μg/ml), IFN-γ (5 U/ml), or the combination of *P. gingivalis* (50 μg/ml) and IFN-γ (5 U/ml). PBMC were isolated from the blood of healthy human donors. MLCs were performed by mixing PBMC (each at 4 $\times$ 10^7 cells/ml in PBS) from two healthy donors. A total of 4 $\times$ 10^5 mixed PBMC in 10 μl of PBS was cocultured with a confluent layer of stimulated HGFs (200 μl/well) in 96-well plates. ^{3}[H]Thymidine (0.5 μCi/200 μl/well) was added on day 5 and cell cultures were incubated for another 18 h. Cells were harvested onto glass filter paper and radioactivity was measured (beta plate; PerkinElmer Wallac). To

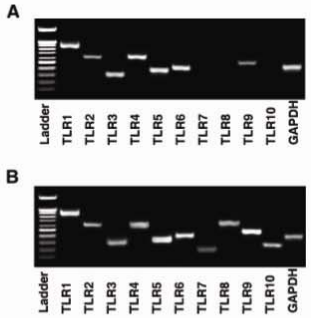

FIGURE 1. TLR expression in HGFs. *A*, TLRs 1–10 mRNA expression was measured in cultured HGFs by RT-PCR. *B*, PBMC mRNA was used as positive control. GAPDH mRNA was used as an internal control. Data are representative of four separate HGF lines and PBMC.

confirm the inhibitory effect of IDO, 1-methyl-DL-tryptophan (1000 μg/ml) or L-tryptophan (1000 μM) was added during the coculture of the stimulated HGFs with mixed PBMC.

Statistical analysis

Statistical comparisons among treatment conditions with respect to production of IL-8 and IDO and to inhibition of the T cell response were analyzed using SigmaStat (Jandel). The parametric Student's t test was used for normally distributed data, and the nonparametric Mann-Whitney U rank-sum test was used for nonnormally distributed data. A value of $p < 0.05$ was considered statistically significant.

Results

mRNA expression of TLRs on HGFs

TLRs have been found on many cell types and are known to play a central role in pathogen recognition in the innate immune system. To evaluate the expression of TLRs in HGFs, total RNA from HGFs was analyzed by RT-PCR using a panel of specific primers of TLRs 1–10. We found the mRNA expression of TLRs 1, 2, 3, 4, 5, 6, and 9 on HGFs but not TLRs 7, 8, and 10 (Fig. 1*A*). The results were reproducible in all four HGF lines. Human PBMC were used as a positive control and shown to express all mRNA of TLRs 1–10 (Fig. 1*B*).

TLR3 is generally recognized as an intracellular receptor, but a recent study showed a surface TLR3 on human skin and lung fibroblasts (23). Thus, we analyzed the specific location of TLR3 expression in HGFs. Using the same mAb specific to TLR3 (clone TLR 3.7), we demonstrated the detection of intracellular TLR3 on HGFs but not on the cell surface (Fig. 2).

We next evaluated whether TLR expression could be modulated by oral bacterial products. Coculture of HGFs with sonicated extracts of *P. gingivalis* for 24 h did not lead to a significant upregulation of any TLR mRNA expression (data not shown).

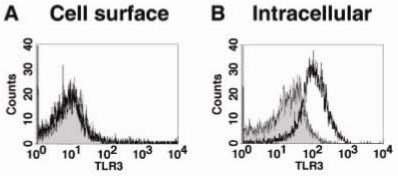

FIGURE 2. Flow cytometric analysis of cell surface and intracellular TLR3 expression in HGFs. HGFs were stained for cell surface (*A*) or intracellular TLR3 (*B*) with mAb against human TLR3 (clone TLR3.7, open histograms). Shaded histograms represent cells stained with isotype-matched control Abs.

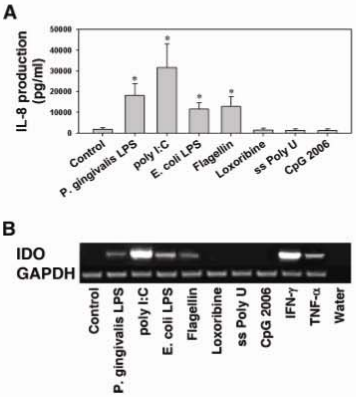

FIGURE 3. Expression of IL-8 and IDO in HGFs after stimulation with various TLR ligands. HGFs were cultured in 96-well plates or 24-well plates and stimulated with the following ligands: *P. gingivalis* LPS (TLR2 ligand), poly(I:C) (TLR3 ligand), *E. coli* LPS (TLR4 ligand), *S. typhimurium* (TLR5 ligand), loxoribine (TLR7 ligand), poly(U) (TLR8 ligand), and CpG ODN 2006 (TLR9 ligand). Culture medium was used as a control. For assessment of IL-8 production, culture supernatants of stimulated HGFs were harvested after 24 h and then assayed by ELISA. Data shown are mean ± SEM of four separate experiments (*, $p < 0.05$, compared with unstimulated control, *A*). To measure IDO expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. IFN-γ- and TNF-α-stimulated HGFs were used as positive controls. GAPDH mRNA was used as an internal control. Data are representative of four separate experiments (*B*).

TLR ligands stimulate expression of IL-8 and IDO

To characterize the functional relevance of TLRs in HGFs, expression of IL-8 and IDO was determined after stimulation with highly purified TLR ligand(s). IL-8 production coincided with mRNA expression of TLRs (i.e., TLRs 2, 3, 4, and 5; Fig. 3*A*). On the contrary, no IL-8 production was observed in HGFs stimulated with CpG ODN 2006, even though the cells expressed TLR9 mRNA (Fig. 3*A*).

Skin fibroblasts are known to express IDO when treated with IFN-γ (12, 13). In this study, the IDO expression of HGFs after TLR ligation was also evaluated. Similar to IL-8 production, *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin, respective ligands for TLRs 2, 3, 4, and 5, induced IDO mRNA expression. IFN-γ- and TNF-α-treated HGFs were used as positive controls (Fig. 3*B*).

TLR ligand combinations (*P. gingivalis* LPS plus poly(I:C), *P. gingivalis* LPS plus *E. coli* LPS, *P. gingivalis* LPS plus *S. typhimurium* flagellin, *P. gingivalis* LPS plus CpG ODN 2006, poly(I:C) plus *E. coli* LPS, poly(I:C) plus *S. typhimurium* flagellin, poly(I:C) p;us CpG ODN 2006, *E. coli* LPS plus *S. typhimurium* flagellin, *E. coli* LPS plus CpG ODN 2006, and *S. typhimurium* flagellin plus CpG ODN 2006) did not lead to a significant enhancement of IL-8 production (Fig. 4*A*) or IDO expression (Fig. 4*B*), as compared with the sum of individual ligands. Surprisingly, CpG ODN 2006 specifically inhibited poly(I:C)-induced IL-8 production ($p < 0.05$) and poly(I:C)-induced IDO expression ($p < 0.05$; Fig. 4).

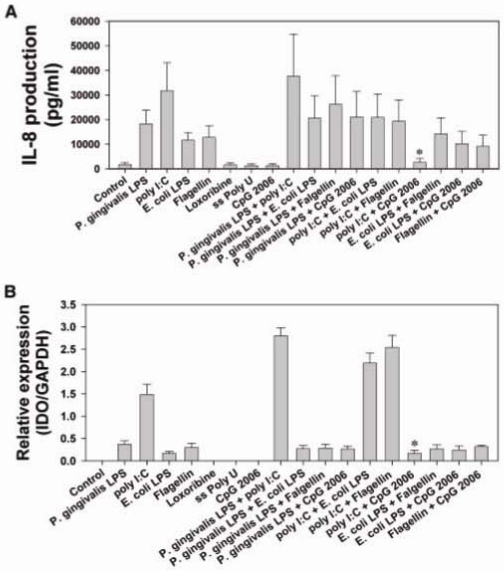

FIGURE 4. Expression of IL-8 and IDO in HGFs after stimulation with TLR ligand combination. HGFs were cultured in 96-well plates or 24-well plates and stimulated with the following ligand combinations: *P. gingivalis* LPS + poly(I:C), *P. gingivalis* LPS + *E. coli* LPS, *P. gingivalis* LPS + *S. typhimurium* flagellin, *P. gingivalis* LPS + CpG ODN 2006, poly(I:C) + *E. coli* LPS, poly(I:C) + *S. typhimurium* flagellin, poly(I:C) plus CpG ODN 2006, *E. coli* LPS + *S. typhimurium* flagellin, *E. coli* LPS + CpG ODN 2006, and *S. typhimurium* flagellin + CpG ODN 2006. Culture medium was used as a control. Culture supernatants of stimulated HGFs were harvested after 24 h and IL-8 production was determined by ELISA. Data shown are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with poly(I:C) treatment, *A*). For semiquantitative analysis of IDO mRNA expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. IFN-γ- and TNF-α-stimulated HGFs were used as positive controls. Data shown are the mean ratio ± SEM of IDO:GAPDH from four separate experiments (*, $p < 0.05$, compared with poly(I:C) treatment, *B*).

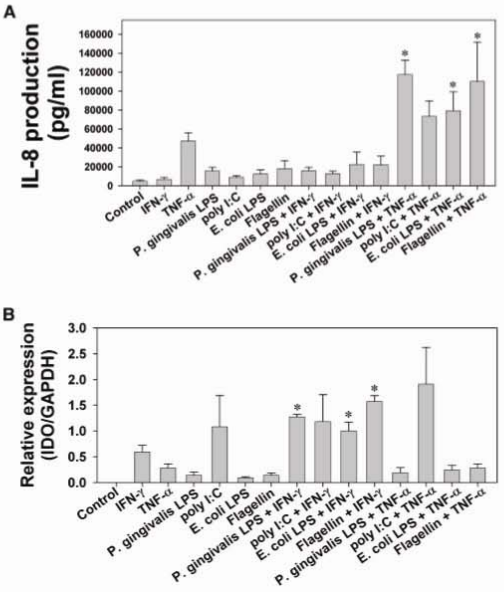

FIGURE 5. Expression of IL-8 and IDO in HGFs after stimulation with TLR ligand and cytokine combination. HGFs were cultured in 96-well plates or 24-well plates and stimulated with the following ligand and cytokine combinations: *P. gingivalis* LPS + IFN-γ, poly(I:C) + IFN-γ, *E. coli* LPS + IFN-γ, *S. typhimurium* flagellin + IFN-γ, *P. gingivalis* LPS + TNF-α, poly(I:C) + TNF-α, *E. coli* LPS + TNF-α, and *S. typhimurium* flagellin + TNF-α. Culture medium was used as a control. Culture supernatants of stimulated HGFs were harvested after 24 h and IL-8 production was determined by ELISA. Data shown are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with the sum of two individual stimulators, *A*). For semiquantitative analysis of IDO expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. Data shown are the mean ratio ± SEM of IDO:GAPDH from four separate experiments (*, $p < 0.05$, compared with the sum of two individual stimulators, *B*).

Combination of TLR ligand and cytokine stimulates expression of IL-8 and IDO

Cytokines IFN-γ, and TNF-α have been consistently detected in periodontitis lesions (6, 7). We next investigated the effects on HGF production of IL-8 and IDO by either cytokine or by the combination of cytokine with different TLR ligands, specifically *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, or *S. typhimurium* flagellin. Fig. 5*A* demonstrates that unlike IFN-γ, TNF-α, when combined with *P. gingivalis* LPS, *E. coli* LPS, or *S. typhimurium* flagellin, significantly induced more IL-8 from HGFs than the additive ($p < 0.05$). Interestingly, the results of IDO mRNA expression were quite different. IFN-γ, but not TNF-α when combined with *P. gingivalis* LPS, *E. coli* LPS, or *S. typhimurium* flagellin significantly induced IDO mRNA expression greater than the sum of individual stimulators ($p < 0.05$; Fig. 5*B*).

P. gingivalis *LPS and/or IFN-γ-treated HGFs induce suppression of T response via IDO*

According to the enhancement of IDO mRNA expression on HGFs after stimulation with the combination of *P. gingivalis* LPS and IFN-γ, we next assessed the biological activity of IDO by measuring the kynurenine concentration in those cultured supernatants. Fig. 6*A* demonstrates that the kynurenine could be detected within 24-h culture supernatants of stimulated HGFs. The levels of kynurenine continued to increase up to 72 h in cultures.

We further evaluated whether HGFs, which were stimulated with *P. gingivalis* LPS, IFN-γ, or the two in combination could inhibit T cell response in MLRs. Cocultures of mixed PBMC with 2-day *P. gingivalis*- or IFN-γ-stimulated HGF showed 10–32% inhibition of the T cell proliferative response (*, $p < 0.05$) as compared with unstimulated HGFs. However, the combination of *P. gingivalis* and IFN-γ-stimulated cells markedly inhibited T cell proliferation by 62% (**, $p < 0.001$; Fig. 6*B*). The response of T cells was restored when 1-methyl-DL-tryptophan or L-tryptophan was added to the cultures (Fig. 6*B*).

Discussion

It is known that host immune response employs TLR and non-TLR pathways to recognize pathogens and commensal bacteria (24, 25). This recognition leads to expression of mediators that limit microbial invasion. When gingival epithelium is ruptured, HGFs can be

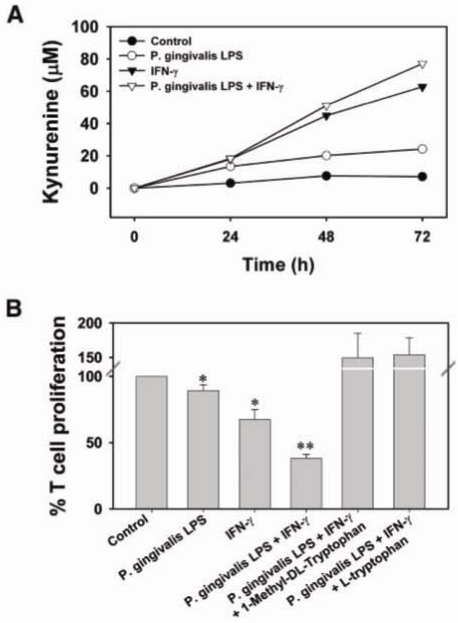

FIGURE 6. Kinetics of IDO activity and suppression of T cell proliferation in MLRs. HGFs were stimulated with either IFN-γ, *P. gingivalis* LPS, or the combination of IFN-γ and *P. gingivalis* LPS. Culture supernatants were harvested at different time points (0, 24, 48, and 72 h) and the concentration of kynurenine was determined (*A*). Suppression of T cell proliferation in MLR was assessed by coculturing mixed PBMC from two donors with *P. gingivalis* LPS, IFN-γ, or the combination of *P. gingivalis* LPS and IFN-γ-stimulated HGFs. Either 1-methyl-DL-tryptophan or L-tryptophan was added to the stimulated HGF cultures at the same time as mixed PBMC. After 6 days of incubation, T cell proliferative response was determined by tritiated thymidine uptake. T cell proliferation was calculated as a percentage of control. Data shown are mean ± SEM from four separate experiments (*, $p < 0.05$; **, $p < 0.001$, compared with unstimulated control, *B*).

exposed to many bacterial pathogen-associated molecular patterns (PAMPs). Clinical observations demonstrated the presence of periodontopathic bacteria in epithelial and connective tissue layers of periodontitis lesions (26–33). The pathogens such as *P. gingivalis, A. actinomycetemcomitans,* and *Fusobacterium nucleatum* were also shown to invade human gingival epithelial cells and fibroblasts *in vitro* as well as *in vivo* (34–37). The ability of HGFs to recognize and respond to such patterns renders them crucial in dealing with microbial invasion. In this study, we evaluated the expression of TLRs and their role in signaling by HGFs. Our results demonstrate that HGFs derived from healthy gingival tissues expressed mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9, but not of TLRs 7, 8, and 10. This is similar to observations in nasal fibroblasts (38). A recent study showed that fibroblasts from human foreskin and lung expressed cell surface TLR3 (23). However, our study, using the same clone of mAb, demonstrated the presence of intracellular TLR3, but not cell surface TLR3, on HGFs. Thus, fibroblast TLR expression may differ across anatomic sites. Further study is needed to identify and compare the physiologic significance of intracellular and cell surface TLR3.

In line with the TLR mRNA expression, HGFs expressed IL-8 and IDO in response to *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin, respective ligands for TLRs 2, 3, 4, and 5. It has been shown that highly purified *P. gingivalis* LPS possess lipid A heterogeneity, which may contribute to their ability to interact with either TLR2 or TLR4 (39). *P. gingivalis* LPS at a concentration of 50 μg/ml used in our study predominantly stimulated HGFs via TLR2 and to a lesser extend via TLR4 (InvivoGen product information). Poly(I:C) appeared to induce higher IL-8 and IDO expression than did other TLR ligands. Surprisingly, CpG ODN 2006, a potent ligand for TLR9, did not induce IL-8 or IDO expression. Similarly, purified DNA derived from either *E. coli*, *P. gingivalis*, or *A. actinomycetemcomitans*, which is also recognized as a TLR9 ligand, did not induce either of these mediators (data not shown). Our findings may indicate a nonfunctional TLR9 in HGFs. (Gingival epithelial cells also express TLR9 mRNA, but do not respond to CpG ODN 2006; R. Mahanonda and S. Pichyangkul, unpublished observations.) In contrast, some recent studies showed that DNA preparation from periodontopathic bacteria activated HGFs via TLR9 to produce IL-6 or IL-8 (10, 40). This inconsistency requires further investigation.

The finding that HGFs expressed TLRs 2, 4, and 5 supports their role in the innate immune response against bacteria. Oral plaque bacteria are known to have PAMPs that are recognized by TLRs 2, 4, and 5. For example, *P. gingivalis* LPS and *P. gingivalis* fimbriae are recognized by TLR2 (41–43); LPS from *A. actinomycetemcomitans* and *Bacteroides fragilis* are recognized by TLR4 (44–46). Flagellin of *Treponema denticola* is most likely recognized by TLR5. The expression of TLR3 in HGFs is interesting because TLR3 recognizes dsRNA, a by-product of viral replication and transcription (47). A possible role of herpesviruses in etiology and severity of periodontal diseases has been reported (48–50). The presence of TLR3 thus suggests a role of HGFs in antiviral response.

The effects of TLR ligand combinations on IL-8 and IDO expression by HGFs were not significantly different from those of single ligands or the sum of individual ligands, except for the combination of CpG ODN 2006 with poly(I:C). Addition of CpG ODN 2006 markedly inhibited poly(I:C)-induced IL-8 and poly(I:C)-induced IDO expression. CpG ODN 2006, by itself, had no effect on the expression of either IL-8 or IDO. The inhibitory effect of CpG ODN 2006 on poly(I:C)-stimulated HGFs is unlikely to be limited to the early phase, because the addition of CpG ODN 2006 at 6 h after poly(I:C) treatment still completely suppressed IL-8 production (data not shown). Further studies will be needed to understand the inhibitory effect of CpG ODN on poly(I:C)-induced HGF activation.

Previous studies demonstrated that different cytokines have different effects on HGFs in IL-8 production (3, 51). TNF-α, but not IFN-γ, induced IL-8 production from HGFs; these observations agree with those of previous studies (3, 51, 52). Combinations of cytokines and bacterial PAMPs are known to modulate cytokine production from different cell types (53, 54). A high level of IL-8 as well as the increased presence of IL-8-secreting fibroblasts has been detected in periodontitis lesions (6, 55). Our data demonstrate that stimulation of HGFs with TNF-α, combined with TLR ligands 2, 4, or 5, synergistically enhanced IL-8 production. The IL-8 response in periodontal tissue could have both beneficial and deleterious effects. IL-8 is important in neutrophil activation and recruitment. On one hand, undue down-regulation of this function could compromise antimicrobial defense. On the other hand, unduly vigorous or sustained IL-8 response could cause chronic inflammatory tissue destruction.

It is reported that skin fibroblasts can dampen local immune cell responses via IDO. In this study, we demonstrated that HGFs were also able to induce IDO expression in response to *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin. IDO

expression was synergistically enhanced when HGFs were treated with the combination of some PAMPs (TLR ligands 2, 4, or 5) and IFN-γ. It is interesting that TNF-α, which enhanced TLR ligand-induced IL-8 production, has a negligible effect on TLR ligand-induced IDO expression of HGFs. Marked suppression of T cell proliferation in MLRs was mediated by IFN-γ and *P. gingivalis* LPS-treated HGFs. The suppression was reversible with the addition of either 1-methyl-DL-tryptophan or L-tryptophan, thus confirming that stimulated HGFs suppressed T cell response via induced IDO.

In conclusion, our study demonstrates that HGFs express mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9. Triggering with *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin, ligands specific for TLRs 2, 3, 4, and 5, respectively, led to the expression of IL-8 and IDO. In contrast, the potent TLR9 ligand CpG ODN 2006 did not induce IL-8 and IDO expression. Moreover, it specifically inhibited poly(I:C)-induced HGF activation. The ability to induce IL-8 and IDO expression in ligand-stimulated HGFs was enhanced when combined with cytokine TNF-α and IFN-γ, respectively. Finally, that HGFs can enhance IDO expression and down-regulate T cell response when stimulated with some PAMP-cytokine combinations suggests that these strategically placed cells have an important role in modulating the unwelcome hyperreactive inflammatory reaction that periodontitis often entails.

Acknowledgments

We thank Dr. Kitti Torrungruang and Dr. Kanokwan Nisapakultorn for their technical assistance. We also thank Pimprapa Rerkyen for manuscript preparation.

Disclosures

The authors have no financial conflict of interest.

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J Dent Res 87(3):267-272, 2008

Effects of IL-17 on Human Gingival Fibroblasts

ABSTRACT

Interleukin (IL)-17 is present in inflammatory periodontal lesions, thus suggesting a role in mediating inflammation. We tested the hypothesis that IL-17, especially when combined with interferon (IFN)-γ, may modulate the responses of human gingival fibroblasts (HGFs). IL-17 induced IL-8 and minimal intercellular adhesion molecule (ICAM)-1 expression. It had no effect on expression of HLA-DR, CD40, or the immune-suppressive enzyme indoleamine 2,3-dioxygenase (IDO). The effects of IL-17 on HGFs were compared with those of IFN-γ. Unlike IL-17, IFN-γ augmented the expression of HLA-DR, ICAM-1, and IDO, but not IL-8. Thus, IL-17 and IFN-γ induce different HGF responses when administered separately. Interestingly, when IL-17 and IFN-γ were combined, marked enhancement of ICAM-1, IL-8, and IDO expression by HGFs was observed. These findings suggest that IL-17, especially when combined with IFN-γ, could play an important role in immune modulation through stimulation of HGFs in periodontal disease. *Abbreviations*: Interleukin (IL), T-helper (Th), human gingival fibroblasts (HGFs), indoleamine 2,3-dioxygenase (IDO), monoclonal antibodies (mAbs), intercellular adhesion molecule (ICAM)-1, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, mean fluorescence intensity (MFI).

KEY WORDS: human gingival fibroblasts, IL-17, IFN-γ, IL-8, IDO.

Received April 20, 2007; Last revision October 3, 2007; Accepted November 27, 2007

A supplemental appendix to this article is published electronically only at http://jdr.iadrjournals.org/cgi/content/full/87/3/267/DC1.

INTRODUCTION

Periodontal disease results from immune response to bacterial pathogens. The disease causes gingival inflammation, destruction of alveolar bone, and occasionally tooth loss. The lesion is characterized by dense lymphoid infiltrates that contain activated $CD4^+$ T-cells as well as their cytokines, such as interferon (IFN)-γ (Page *et al.*, 1997). These infiltrating T-cells are located adjacent to gingival fibroblasts, the most abundant cells in periodontal connective tissue (Seymour and Greenspan, 1979). It is likely that cytokines released from activated T-cells may directly modulate human gingival fibroblast (HGF) functions.

HGFs play an active role in host defense. They can recognize and respond to many pathogens and cytokines, and can release a variety of pro-inflammatory mediators, such as IL-8, tumor necrosis factor (TNF)-α, IL-1, IL-6, and prostaglandin E_2 (Page *et al.*, 1997; Wang *et al.*, 2003; Mahanonda and Pichyangkul, 2007; Mahanonda *et al.*, 2007). All of these mediators can promote periodontal inflammation. Conversely, HGFs are also involved in negative feedback inhibition of inflammatory T-cell responses, *via* the release of indoleamine 2,3-dioxygenase (IDO) (Mahanonda *et al.*, 2007). IDO is an enzyme that catabolizes tryptophan, an amino acid essential in T-cell proliferation (Mellor and Munn, 2004). It acts as an important immune-suppressive molecule responsible for attenuation of T-cell hyperactivity.

IL-17, a product of T-helper (Th) 17, has emerged as a crucial regulator of inflammatory responses (Dong, 2006). It is known to activate fibroblasts, epithelial cells, endothelial cells, and osteoblasts to produce pro-inflammatory cytokines such as IL-6, IL-8, granulocyte colony-stimulating factor, and matrix metalloproteinases (Yao *et al.*, 1995; Fossiez *et al.*, 1996). Overexpression of IL-17 is associated with inflammatory diseases in humans, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, psoriasis, and allograft rejection (Witowski *et al.*, 2004; Dong, 2006). In chronic periodontal lesions, IL-17 has been detected in gingival tissue biopsies and gingival crevicular fluid (Oda *et al.*, 2003; Johnson *et al.*, 2004; Takahashi *et al.*, 2005; Vernal *et al.*, 2005). IL-17 treatment of HGFs has also been observed to lead to IL-6 production, which may contribute to local tissue inflammation (Takahashi *et al.*, 2005). Even so, the underlying mechanisms through which IL-17 influences development and severity of periodontal disease remain unclear. We hypothesized that IL-17, especially when combined with IFN-γ, may modulate the responses of HGFs.

MATERIALS & METHODS

Reagents and Monoclonal Antibodies (mAbs)

For HGF cultures, we used DMEM (Gibco Laboratory, Grand Island, NY, USA), supplemented with penicillin G (50 U/mL), streptomycin (50 μg/mL),

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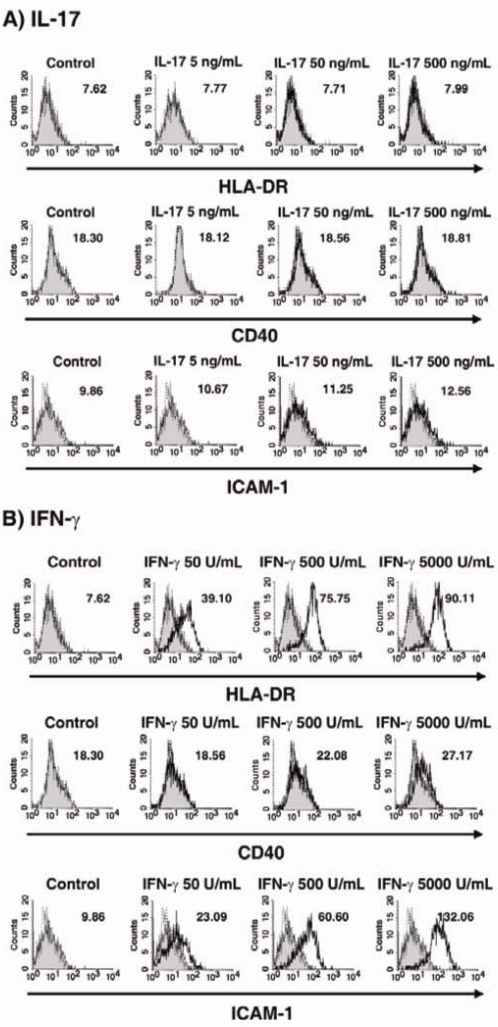

Figure 1. Flow cytometric analysis of HLA-DR, CD40, and ICAM-1 expression by HGFs after single stimulation with IL-17 or IFN-γ. HGF cultures were stimulated with indicated concentrations of IL-17 **(A)** and IFN-γ **(B)** for 5 days. Culture medium was used as a control. The surface molecule expression was determined by flow cytometry. Dotted lines are isotype controls, shaded areas are unstimulated HGFs, and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each histogram indicates mean fluorescence intensity (MFI). Data are representative of 13 separate experiments.

fungizone (2.5 μg/mL), and 10% heat-inactivated fetal calf serum. Recombinant human IL-17 and IFN-γ were obtained from R&D Systems Inc. (Minneapolis, MN, USA). Monoclonal antibodies (mAbs) against ICAM1 and HLA-DR were obtained from BD PharMingen (San Diego, CA, USA). mAb against CD40 and mouse isotype control antibodies were obtained from Becton Dickinson (San Jose, CA, USA).

Human Gingival Fibroblasts

Gingival tissue samples were collected from 15 persons with clinically healthy periodontium and no history of periodontitis. The gingival biopsies were obtained at the time of crown-lengthening procedures, for prosthetic reasons, at the Periodontal Clinic, Faculty of Dentistry, Chulalongkorn University. The experimental procedure was approved by the ethics committee of the Faculty of Medicine, Chulalongkorn University, and informed consent was obtained from all persons. Fibroblasts from gingival tissues were obtained according to established procedures (Mahanonda *et al.*, 2007). Briefly, the excised tissue was washed, then minced with scissors into fragments (1-3 mm^2), and placed in 60-mm tissue culture dishes. The HGF cultures at passage 4 were used throughout the study.

Stimulation of Human Gingival Fibroblasts with IL-17, IFN-γ, or Combined Cytokines

HGFs (1 x 10^5 cells/mL) in 48-well plates (Corning Inc., Corning, NY, USA) were stimulated with predetermined concentrations of IL-17 and IFN-γ, separately and in combination. The HGF responses were monitored by their expression of surface molecules (HLA-DR, CD40, and ICAM-1), IL-8, and IDO. Based on preliminary kinetic experiments, we selected five-day incubation for surface molecule analysis by flow cytometry, two-day incubation for assessment of IL-8 production by ELISA, and 12-hour incubation for determination of IDO mRNA expression by RT-PCR. The cytokine concentrations for HGF stimulation used were as follows: (1) surface molecule expression assay, IL-17 at 5, 50, and 500 ng/mL; IFN-γ at 50, 500, and 5000 U/mL; or combined cytokines at 500 ng/mL IL-17 + 50 U/mL IFN-γ, 500 ng/mL IL-17 + 500 U/mL IFN-γ, and 500 ng/mL IL-17 + 5000 U/mL IFN-γ; (2) IL-8 assay, IL-17 at 5, 50, and 500 ng/mL; IFN-γ at 50, 500, and 5000 U/mL; or combined cytokines at 500 ng/mL IL-17 + 50 U/mL IFN-γ and 500 ng/mL IL-17 + 500 U/mL IFN-γ; and (3) IDO mRNA and activity assay, IL-17 at 5, 50, and 500 ng/mL; IFN-γ at 1, 5, and 25 U/mL; or combined cytokines at 500 ng/mL IL-17 + 1 U/mL IFN-γ, 500 ng/mL IL-17 + 5 U/mL IFN-γ, and 500 ng/mL IL-17 + 25 U/mL IFN-γ.

Flow Cytometric Analysis of HLA-DR, CD40, and ICAM-1

The stimulated cells were stained with mAbs anti-ICAM1, anti-CD40, and HLA-DR at 4°C for 30 min, washed in PBS, treated with 1% paraformaldehyde, and analyzed by FACSCalibur (BD Biosciences, Mountain View, CA, USA). Mouse isotype mAbs were used as control. The levels of surface molecule expression were measured by mean fluorescence intensity (MFI).

Determination of IL-8

The supernatants of HGFs after stimulation with cytokine(s) were harvested and assessed for IL-8 production by ELISA (R&D Systems).

Detection of IDO Expression and Activity

mRNA Expression of IDO

The total RNA of stimulated HGFs was isolated by means of an RNeasy Mini kit (Qiagen, Chatsworth, CA, USA). One μg of

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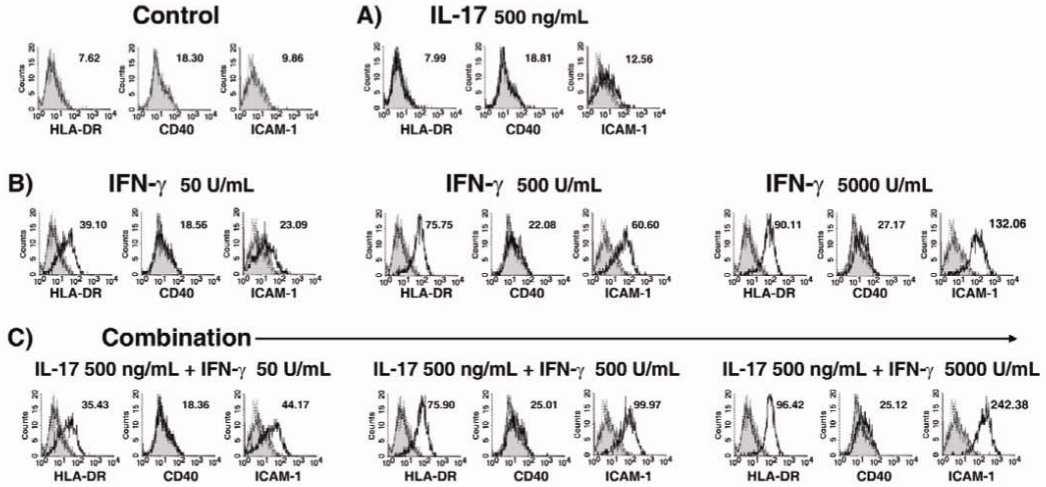

Figure 2. Flow cytometric analysis of HLA-DR, CD40, and ICAM-1 expression by HGFs after combined stimulation with IL-17 and IFN-γ. HGF cultures were stimulated with indicated concentrations of IL-17 **(A)**, IFN-γ **(B)**, or combined cytokines **(C)** for 5 days. Culture medium was used as a control. The surface molecule expression was determined by flow cytometry. Dotted lines are isotype controls, shaded areas are unstimulated HGFs, and solid lines represent cytokine stimulation. The x-axis and the y-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each histogram indicates mean fluorescence intensity (MFI). Data are representative of 13 separate experiments.

DNase I-treated total RNA was reverse-transcribed *via* the ImProm-II Reverse Transcription System for RT-PCR (Promega, Madison, WI, USA).

IDO was amplified with specific primer (5′-CTTCCTGGT CTCTCTATTGG-3′/5′-GAAGTTCCTGTGAGCTGGT-3′) (Proligo, Singapore) (von Bubnoff *et al.*, 2002). The expected size of the PCR product was 430 bp. For semi-quantitative RT-PCR analysis, band intensities on scanned gels were analyzed (GeneTools, Syngene, Cambridge, UK) and compared with specific bands of the housekeeping gene GAPDH as a reference.

IDO Activity: Kynurenine Assay

We evaluated the biological activity of IDO by measuring the level of kynurenine in HGF culture supernatants (Mahanonda *et al.*, 2007). A 100-μL quantity of culture supernatants of stimulated HGFs was mixed with 50 μL of 30% trichloroacetic acid, vortexed, and centrifuged at 8000 *g* for 5 min. Then, a 75-μL quantity of the supernatant was added to an equal volume of Ehrlich reagent (100 mg p-dimethylbenzaldehyde in 5 mL glacial acetic acid) in a 96-well microtiter plate, and the absorbance was read with OD at 492 nm. A standard curve of defined kynurenine concentration (0-100 μM) permitted us to analyze unknowns.

Statistical Analysis

Statistical comparisons among treatment conditions with respect to expression of surface molecule expression, IL-8, and IDO were conducted with SigmaStat (Jandel Scientific, San Rafael, CA, USA). The parametric paired Student's *t* test was used for normally distributed data, and the non-parametric Wilcoxon signed-rank test was used for non-normally-distributed data. A value of $P < 0.05$ was considered statistically significant.

RESULTS

Effects of IL-17 and IFN-γ on Expression of ICAM-1, HLA-DR, and CD40 by HGFs

Previous studies demonstrated that stimulation of HGFs with IFN-γ- induced surface molecule expression, ICAM-1, and HLA-DR (Shimabukuro *et al.*, 1996). In this study, we investigated the capacity of IL-17 to induce surface molecule expression by flow cytometry and used IFN-γ-treated cells as positive controls. HGF cultures were derived from healthy gingival biopsies. Treatment of HGFs with IL-17 (5, 50, 500 ng/mL) induced only a minimal expression of ICAM-1 and had no effect on HLA-DR or CD40 (Fig. 1A). In contrast, treatment of HGFs with IFN-γ (50, 500, 5000 U/mL) dose-dependently induced expression of ICAM-1 and HLA-DR, but only weak expression of CD40 (Fig. 1B). Next, we evaluated the combined effect of IL-17 and IFN-γ. Cells were treated with either IL-17 (500 ng/mL), IFN-γ (50, 500, 5000 U/mL), or the combination. The responses of HGFs to the combined cytokine treatment (Fig. 2C) were then compared with those to the single cytokine treatment (Figs. 2A, 2B). IL-17 consistently enhanced IFN-γ-induced ICAM-1 expression on HGFs (n = 13), but not HLA-DR or CD40 expression (Fig. 2).

Effects of IL-17 and IFN-γ on IL-8 Production by HGFs

IL-17 has been reported to induce IL-8 release by skin and rheumatoid synovial fibroblasts (Yao *et al.*, 1995; Fossiez *et al.*, 1996). In this study, we evaluated the effect of IL-17 on IL-8 production by HGFs. IL-17 (5, 50, 500 ng/mL) dose dependently augmented IL-8 production by HGFs (Fig. 3A), whereas IFN-γ (50, 500, 5000 U/mL) did not (Fig. 3B). We

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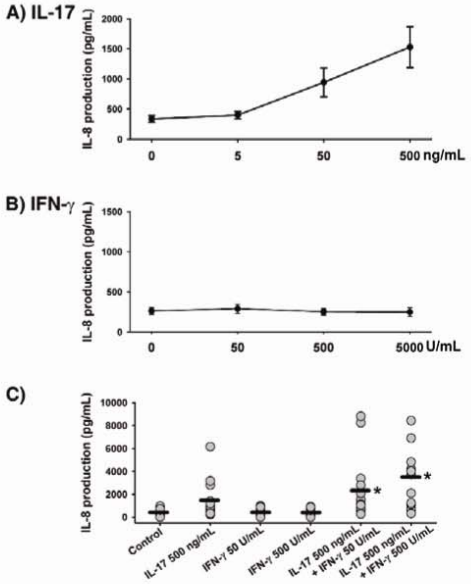

Figure 3. IL-8 production by HGFs after single stimulation with IL-17, IFN-γ, and combined cytokine stimulation. HGF cultures were stimulated with indicated concentrations of IL-17 **(A)**, IFN-γ **(B)**, and the combined cytokines **(C)** for 2 days. Culture medium was used as a control. The culture supernatants were collected, and IL-8 concentrations were determined by ELISA. Data in (A) and (B) are mean ± SEM of 15 separate experiments. Each symbol in (C) represents IL-8 production of each individual culture. Horizontal lines are means of 15 separate experiments (**P* < 0.05, compared with the sum of two individual cytokine stimulations).

next evaluated the combined effect of IL-17 (500 ng/mL) and IFN-γ (50, 500 U/mL). Together, these substances exerted a synergistic effect on IL-8 production—that is, the combined effect was statistically significantly higher than the sum of the individual effects ($P < 0.05$, n = 15) (Fig. 3C).

Effects of IL-17 and IFN-γ on IDO Expression by HGFs

We previously showed that HGFs expressed IDO when treated with IFN-γ (Mahanonda *et al.*, 2007). In this study, we investigated IDO mRNA expression by HGFs upon stimulation with IL-17 by RT-PCR. Unlike IFN-γ, IL-17 did not induce IDO mRNA expression at any concentration tested (5, 50, 500 ng/mL) (Fig. 4A). The combined effect of IL-17 (500 ng/mL) and IFN-γ (1, 5, 25 U/mL) was then investigated. The combined cytokine stimulation led to a significant enhancement of IDO mRNA expression ($P < 0.05$, as compared with the sum of two individual cytokines' stimulation) (Fig. 4B). We also assessed the biological activity of IDO by measuring kynurenine concentrations in cultured supernatants. A significant increase in kynurenine levels was observed in the combined treatment cultures (Fig. 4C) ($P < 0.05$, as compared with the sum of the effects of individual treatments), thus exhibiting consistency with the mRNA expression results.

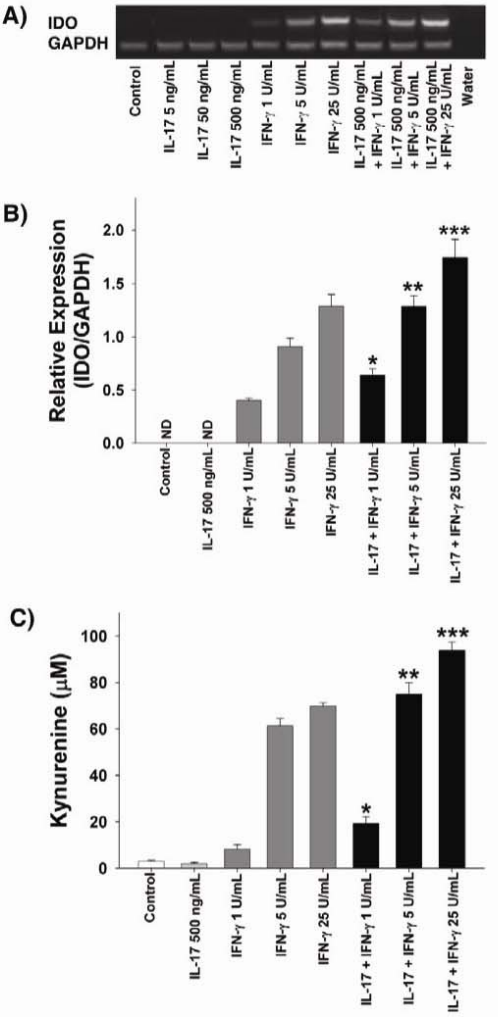

Figure 4. Expression of IDO in HGFs after stimulation with IL-17, IFN-γ, or combined cytokines. HGF cultures were stimulated with indicated concentrations of IL-17, IFN-γ, and combined cytokines. Culture medium was used as a control. Stimulated HGFs were harvested, and mRNA expression of IDO was analyzed by RT-PCR. GAPDH mRNA was used as an internal control. Data are representative of 4 separate experiments **(A)**. Mean relative expression of IDO:GAPDH ± SEM from 4 separate experiments is shown (**P* < 0.05, compared with the sum of IL-17 and IFN-γ [1 U/mL] stimulation; ***P* < 0.05, compared with the sum of IL-17 and IFN-γ [5 U/mL] stimulation; ****P* < 0.05, compared with the sum of IL-17 and IFN-γ [25 U/mL] stimulation) **(B)**. To assess IDO activity, we determined the concentrations of kynurenine in culture supernatants. Data shown are mean concentrations of kynurenine ± SEM from 4 separate experiments (**P* < 0.05, compared with the sum of IL-17 and IFN-γ [1 U/mL] stimulation; ***P* < 0.05, compared with the sum of IL-17 and IFN-γ [5 U/mL] stimulation; ****P* < 0.05, compared with the sum of IL-17 and IFN-γ [25 U/mL] stimulation) **(C)**.

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DISCUSSION

Periodontal disease is characterized by high levels of cytokines, cellular infiltration, and tissue destruction. Recent studies have demonstrated the presence of IL-17, a product of Th17, in periodontal lesions, and suggested a possible role of this cytokine in disease severity (Oda *et al.*, 2003; Johnson *et al.*, 2004; Takahashi *et al.*, 2005; Vernal *et al.*, 2005). From our observations, HGFs expressed IL-17 receptor (APPENDIX Fig. 1). In this study, we systematically investigated the effects of IL-17, and of combined stimulation with IL-17 and IFN-γ, on HGFs. Treatment of HGFs with IL-17 induced production of pro-inflammatory cytokine IL-8. This is similar to previous observations in IL-17-treated synoviocytes and foreskin fibroblasts (Yao *et al.*, 1995; Fossiez *et al.*, 1996). In contrast, a well-known Th1 cytokine, IFN-γ, the expression of which was concomitant with that of IL-17 in inflamed gingival tissues (Takahashi *et al.*, 2005), had no effect on IL-8 production by HGFs. Also, our observation that IFN-γ induced no IL-8 production was consistent with previous reports (Tamura *et al.*, 1992; Takigawa *et al.*, 1994).

The combination of IL-17 and IFN-γ significantly enhanced the secretion of IL-8 from HGFs. In addition, we observed a similar synergistic effect on IL-6 production from HGFs by these two cytokines (APPENDIX Fig. 2). Early studies of human keratinocytes derived from skin and intestine also demonstrated synergistic effects on IL-8 production induced by these two cytokines (Teunissen *et al.*, 1998; Albanesi *et al.*, 1999; Andoh *et al.*, 2001). IL-8 is known as a key chemokine involving neutrophil activation and recruitment to the site of inflammation (Kobayashi, 2006). High levels of IL-8 have been associated with periodontal disease (Dongari-Bagtzoglou and Ebersole, 1998). HGFs are considered to be a major source of this cytokine, since periodontitis lesions contain abundant IL-8-expressing HGFs (Dongari-Bagtzoglou and Ebersole, 1998; Wang *et al.*, 2003). The vigorous or sustained IL-8 response in periodontal tissue induced by the combination of IL-17 and IFN-γ could plausibly contribute to chronic inflammatory tissue destruction.

Treatment of HGFs with IL-17 induced only minimal ICAM-1 expression and had no effect on expression of HLA-DR and CD40. In contrast, IFN-γ induced substantial expression of HLA-DR and ICAM-1, and only a minimal effect on CD40 expression. Unlike dendritic cells, fibroblasts are recognized as non-professional antigen-presenting cells and cannot 'prime' antigen-specific naïve T-cells (Murakami and Okada, 1997). Our observation that HGFs were induced to markedly up-regulate HLA-DR and minimal CD40 expression by IFN-γ may suggest that these activated HGFs could serve as alternative antigen-presenting cells for antigen-specific memory T-cells, which require a lower threshold of co-stimulatory molecule signaling. Consistent with previous observations on human keratinocytes (Albanesi *et al.*, 1999), analysis of our data indicated that IL-17, when combined with IFN-γ, markedly up-regulated ICAM-1 expression on HGFs. ICAM-1 belongs to the Ig superfamily of adhesion molecules, which promotes retention of leukocytes (Buck, 1992). In periodontitis tissues, up-regulation of ICAM-1 expression on HGFs was greater than that in healthy gingiva (Hayashi *et al.*, 1994). Interaction between ICAM-1-positive fibroblasts and the leukocyte-function-associated antigen-1-positive lymphocytes mutually influences functional activities of each cell type, to include expression of inflammatory cytokines by HGFs, and lymphocyte activation and retention at sites of periodontally diseased tissues (Murakami and Okada, 1997). Therefore, the observed increase in ICAM-1 expression in stimulated HGFs by IL-17 and IFN-γ may contribute to the typical sustained inflammatory reaction in periodontal lesions.

We have previously shown that IFN-γ and *P. gingivalis* LPS enhanced IDO expression in HGFs and suppressed T-cell proliferation (Mahanonda *et al.*, 2007). In this study, we found that IL-17, by itself, had no effect on IDO expression in HGFs; however, when combined with IFN-γ, significant enhancement of IDO mRNA expression, as well as its biological activity, was detected. These findings therefore suggest a role for IL-17 in immune suppression when it is present with IFN-γ.

In summary, we observed diverse effects of IL-17, especially in the combined stimulation with IFN-γ *via* HGF activation. Apparently, IL-17 or IFN-γ differentially controls HGF activation, possibly *via* different intracellular signaling pathways. IL-17 triggers IL-8 production, whereas IFN-γ triggers ICAM-1 and HLA-DR, as well as IDO. These effects of individual cytokines are potentiated by their combination. Perhaps the most important finding of this investigation was that IL-17 not only preferentially stimulates a pro-inflammatory reaction, as previously shown in many diseases, but also functions as a negative feedback inhibitor of inflammatory T-cell responses in the presence of IFN-γ. Hence, the role of IL-17 in periodontal disease should be carefully interpreted, and the full complexity of cytokine-mediated diseases such as periodontitis remains to be unraveled.

ACKNOWLEDGMENTS

The investigation was supported by the Rachadapisek Endowment (R.34/2548) and the Thailand Research Fund (BRG4980006 & MRG4980177).

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