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ORAL EPITHELIAL-DERIVED MEDIATORS SUPPRESS RANKL EXPRESSION IN HUMAN MANDIBULAR-DERIVED BONE CELLS

Miss Pakchisa Khonsuphap

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Oral Biology
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By	Miss Pakchisa Khonsuphap
Field of Study	Oral Biology
Thesis Advisor	Dr.Anjalee Vacharaksa
Thesis Co-Advisor	Professor Dr.Prasit Pavasant

Accepted by the Faculty of Dentistry, Chulalongkorn University in Partial Fulfillment of the Requirements for the Doctoral Degree

.....Dean of the Faculty of Dentistry
(Assistant Professor Dr.Suchit Poolthong)

THESIS COMMITTEE

.....Chairman
(Assistant Professor Dr.Jeerus Sucharitakul)

.....Thesis Advisor
(Dr.Anjalee Vacharaksa)

.....Thesis Co-Advisor
(Professor Dr.Prasit Pavasant)

.....Examiner
(Dr.Patcharee Ritprajak)

.....Examiner
(Assistant Professor Dr.Chalida Limjeerajarus)

.....External Examiner
(Associate Professor Dr.Chidchanok Leethanakul)

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พรอสตาแกลนดินอี 2 มีการสะสมบริเวณเนื้อเยื่อที่มีการอักเสบและมีผลกระตุ้นระบบ รีเซบเตอร์แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ (RANKL)/รีเซบเตอร์แอกติเวเตอร์นิวเคลียร์แคปปาบี (RANK)/ออสติโอโพรติจิลิน (OPG) ซึ่งมีบทบาทสำคัญในการควบคุมการละลายตัวของกระดูก เป็นที่ทราบกันดีว่าเยื่อบุผิวในช่องปากมีบทบาทในการควบคุมสมดุลของเนื้อเยื่อในช่องปากแต่ในส่วนที่เกี่ยวข้องกับการควบคุมการละลายตัวของกระดูกยังไม่มีการศึกษา การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาผลของสารตัวกลางจากเยื่อบุผิวช่องปากต่อการควบคุมการแสดงออกของรีเซบเตอร์ แอกติเวเตอร์ นิวเคลียร์แคปปาบีไลแกนด์ในเซลล์เพาะเลี้ยงกระดูกขากรรไกรล่างของมนุษย์ที่ถูกกระตุ้นโดยพรอสตาแกลนดินอี 2 รวมทั้งบทบาทในการกระตุ้นการทำงานของเซลล์ที่ละลายตัวกระดูก เซลล์เพาะเลี้ยงกระดูกขากรรไกรล่างของมนุษย์ซึ่งถูกกระตุ้นด้วยพรอสตาแกลนดินอี 2 ความเข้มข้น 0.1 ไมโครโมลาร์ ที่เวลา 24 ชั่วโมงเพื่อเพิ่มระดับการแสดงออกของ รีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ จากนั้นการแสดงออกระดับเอ็มอาร์เอ็นเอของรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ ถูกตรวจวัดด้วยวิธีรีเวอร์สทรานสคริบชันโพลีเมอร์เรสเชนรีแอคชัน (RT-PCR) และระดับโปรตีนของรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์จะถูกวิเคราะห์ด้วยเทคนิคอีไลซา (ELISA) และวิธีเวสเทิร์น (Western Blot) เซลล์โมโนไซต์ถูกเลี้ยงร่วมกับเซลล์เพาะเลี้ยงกระดูกขากรรไกรล่างของมนุษย์เพื่อศึกษาถึงบทบาทในการเร่งกระตุ้นการพัฒนาไปเป็นเซลล์ออสติโอคลาสต์ ความสามารถในการสลายกระดูกตรวจวัดโดยการใช้วิธีการ Resorption pit ผลการศึกษาในครั้งนี้แสดงให้เห็นว่าพรอสตาแกลนดินอี 2 สามารถเพิ่มระดับการแสดงออกของรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ทั้งในระดับเอ็มอาร์เอ็นเอและโปรตีน แต่ไม่มีผลเปลี่ยนแปลงของออสติโอโพรติจิลินในระดับโปรตีน เซลล์เยื่อบุผิวช่องปากมีการหลั่งอินเตอร์เฟียรอน-แกมมาและเพิ่มการสร้างมากขึ้นภายใต้สภาวะการกระตุ้นด้วยไลโปโพลีแซคคาไรด์ของเชื้อพอร์ไฟโรโมแนสจิงจิวัลลิส พบว่าเซลล์เพาะเลี้ยงกระดูกขากรรไกรล่างของมนุษย์ที่ได้รับสารตัวกลางจากเซลล์เยื่อบุผิวช่องปากที่ถูกกระตุ้นการสร้างอินเตอร์เฟียรอน-แกมมา หรือรีคอมบิแนนท์อินเตอร์เฟียรอน-แกมมา มีการลดลงของระดับการแสดงออกของรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ทั้งในระดับเอ็มอาร์เอ็นเอและโปรตีน แต่ไม่มีผลเปลี่ยนแปลงการแสดงออกของออสติโอโพรติจิลิน ในขณะที่เมื่อใช้แอนติบอดีต่ออินเตอร์เฟียรอน-แกมมา พบว่าสามารถยับยั้งผลของอินเตอร์เฟียรอน-แกมมาที่สร้างจากเซลล์เยื่อบุผิวช่องปากต่อรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ทั้งในระดับเอ็มอาร์เอ็นเอและโปรตีนได้ นอกจากนี้เมื่อศึกษาความสามารถในการละลายตัวของกระดูกของเซลล์โมโนไซต์พบว่าเมื่อเลี้ยงร่วมกับเซลล์เพาะเลี้ยงกระดูกขากรรไกรล่างของมนุษย์ที่ถูกกระตุ้นด้วยพรอสตาแกลนดินอี 2 มีความสามารถในการละลายตัวของกระดูกเพิ่มขึ้นและถูกยับยั้งในสภาวะที่มีอินเตอร์เฟียรอน-แกมมาที่สร้างจากเซลล์เยื่อบุผิวช่องปากร่วมด้วยโดยผ่านทางการยับยั้งการสร้างรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ โดยสรุปการศึกษานี้แสดงให้เห็นว่าพรอสตาแกลนดินอี 2 เพิ่มระดับการแสดงออกของรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ทั้งในระดับเอ็มอาร์เอ็นเอและโปรตีนอย่างมีนัยสำคัญทางสถิติ รีคอมบิแนนท์อินเตอร์เฟียรอน-แกมมา หรือ อินเตอร์เฟียรอน-แกมมาที่สร้างจากเซลล์เยื่อบุผิวช่องปากมีผลยับยั้งการเพิ่มขึ้นของรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ที่เป็นผลจากการกระตุ้นด้วยพรอสตาแกลนดินอี 2 ในเซลล์เพาะเลี้ยงกระดูกขากรรไกรของมนุษย์ กล่าวโดยสรุปคืออินเตอร์เฟียรอน-แกมมาที่สร้างจากเซลล์เยื่อบุผิวช่องปากมีผลยับยั้งการแสดงออกของรีเซบเตอร์ แอกติเวเตอร์นิวเคลียร์แคปปาบีไลแกนด์ในเซลล์เพาะเลี้ยงกระดูกขากรรไกรล่างของมนุษย์และลดความสามารถในการสลายกระดูกของเซลล์ออสติโอคลาสต์

สาขาวิชา ชีววิทยาช่องปาก ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

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KEYWORDS: INTERFERON-GAMMA / RANKL / ORAL KERATINOCYTE / HUMAN MANDIBULAR BONE CELLS

PAKCHISA KHONSUPHAP: ORAL EPITHELIAL-DERIVED MEDIATORS SUPPRESS RANKL EXPRESSION IN HUMAN MANDIBULAR-DERIVED BONE CELLS. ADVISOR: DR.ANJALEE VACHARAKSA, CO-ADVISOR: PROF. DR.PRASIT PAVASANT, 93 pp.

Prostaglandin E_2 (PGE_2) accumulates in inflamed periodontal tissue and induces RANKL/RANK/OPG signaling associated with bone resorption. Although oral epithelial cells maintain tissue homeostasis, the role of oral epithelial cells in RANKL regulation remains unknown. To investigate the epithelial mediators involved in regulating RANKL expression in PGE_2-stimulated human mandibular bone-derived cells (HMBCs) and RANKL driven osteoclastic-cell activity. HMBCs were stimulated with 0.1 μM PGE_2 for 24 h to increase RANKL expression. Concurrently, cells were treated with epithelial supernatant containing constitutively released or *P.gingivalis* Lipopolysaccharide (*Pg*LPS)-stimulated epithelial mediators, or recombinant IFN-γ. Some cells were pretreated with an anti-IFN-γ antibody before PGE_2 stimulation. The expression of RANKL, OPG, and inflammation-related cytokines was assessed by quantitative PCR, and proteins levels were evaluated by ELISA and western blot. THP-1 human monocytes and HMBCs were cocultured to determine the ability of HMBCs to drive THP-1 differentiation into osteoclast-like cells. Osteoclast function was determined using a bone resorption pit assay. PGE_2 significantly increased RANKL mRNA expression and RANKL protein in HMBCs dose-dependently, however, the OPG protein levels remained similar. Epithelial cells constitutively released IFN-γ, which was substantially increased by *Pg*LPS. HMBCs treated with epithelial supernatant or recombinant IFN-γ, concurrently with PGE_2 stimulation, reduced RANKL, but not OPG, expression similar to baseline. In contrast, the anti-IFN-γ antibody reversed the effect of the epithelial mediators on RANKL expression. THP-1 osteoclast activity increased when cocultured with PGE_2-stimulated HMBCs. However, epithelial-derived IFN-γ decreased PGE_2-induced RANKL expression in HmBCs, resulting in decreased THP-1-derived osteoclast activity. PGE_2 stimulation significantly increases RANKL expression in HMBCs. However, recombinant IFN-γ, or IFN-γ derived from oral epithelial cells, suppresses RANKL expression at both the mRNA and protein level. Therefore, oral epithelial cells, by releasing IFN-γ, suppress RANKL upregulation in HMBCs and reduce osteoclast activity.

Field of Study: Oral Biology
Academic Year: 2016

Student's Signature .....
Advisor's Signature .....
Co-Advisor's Signature .....

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CHAPTER I

INTRODUCTION

Background and rationale

Periodontitis is characterized by the inflammation of periodontal tissue inducing alveolar bone loss. Recent studies suggested that microbial challenge in gingival sulci results in upregulation of host immune responses in the mucosal tissue (1). During the interaction of host and pathogens, inflammatory cytokines including Interleukin (IL)-1β, Tumor Necrosis Factor (TNF)-α, prostanoids such as prostaglandin E_2 (PGE_2), or protease enzymes including matrix metalloproteinase (MMPs), may induce excessive inflammatory responses of the local tissue (2-4). These mediators are responsible for the periodontal breakdown leading to clinical signs and symptoms of periodontal disease.

Adult bone is renewed continuously in response to a variety of stimuli through the process of bone remodeling, for which two requirements are essential (5). First of all, bone resorption and formation must occur at the same site. In addition, they must be balanced in terms of time and quantity. For the last 30 years, several studies have shown that osteoblast-lineage cells can control osteoclastogenesis (6). Subsequent studies showed that osteoblastic cell lines or cell preparations rich in osteoprogenitors also support osteoclast formation (6, 7). The rate of bone remodeling is increased in many pathologic conditions affecting the skeleton. Local or systemic alterations in the levels of hormones or pro-inflammatory cytokines can stimulate bone resorption (7). PGE_2 has diverse proinflammatory and immunomodulatory effects (8). High

level of PGE_2 was reported during the progression of periodontal disease in many animal models. In humans, estimation of PGE_2 levels in gingival crevicular fluid (GCF) in healthy and periodontal disease subjects by using calibrated volumetric microcapillary pipettes to collect GCF samples found that the GCF concentration of PGE_2 was significantly increased in chronic periodontitis patients. Moreover, high level of PGE_2 in the GCF is positively correlated with clinical parameters of periodontal disease including gingival index (GI), plaque index (PI), probing depth (PD), clinical attachment loss (CAL) (9-11). These findings emphasize that PGE_2 is a critical host effector commonly appearing in the periodontal tissue during inflammation.

The oral epithelium forms a physical barrier to protect the oral cavity from the outside environment and noxious stimuli. Oral epithelial cells are also capable of releasing certain potent antimicrobial peptides that can directly kill microorganisms, such as β-defensin (12) and LL-37 (13) Oral epithelial cells can produce eicosanoid (14), reactive oxygen species (15) and complement components such as Complement fragment 3b (C3b) (16), Complement fragment 5a (C5a) (17) which initiate and mediate immunoinflammatory responses. These molecules contribute in the host innate immune functions to prevent infection and to control a balance between commensal microbiota and pathogens throughout the oral mucosa. Furthermore, oral commensals play an important role in stimulating immune responses, which are down regulated and reprogrammed by induction of oral tolerance (18, 19). Importantly, induction of immune tolerance toward commensals combined with responsiveness to pathogens is essential for sustaining immune homeostasis while preventing microbial invasion and infection (19).

Beside the presence of PGE_2 in progressive periodontal disease, recent studies demonstrated the potential mechanism of PGE_2 to induce bone resorption through receptor activator of nuclear factor-kappaB ligand (RANKL) upregulation (20-22) and OPG suppression (23). Level of RANKL/OPG in bone tissue was shown to associate with the rate of bone resorption (24). Oral epithelium regulates the host-microbe interactions to maintain tissue homeostasis. Whether oral epithelial cells also contribute in resolving tissue inflammation remains to be elucidated. This study aims to investigate the mediator released by oral epithelial cells that can inhibit PGE_2-induced RANKL expressed by human mandibular-derived bone cells (HMBCs).

Research Question

Do oral epithelial cells play a protective role during PGE_2-induced bone resorption?

Research Hypothesis

Oral epithelial cells can release a mediator(s) that suppresses PGE_2-induced RANKL expression in Human Mandibular-derived Bone Cells (HMBCs).

Objectives

Cells Characterization (HMBCs/NOK-SI)

Before performing the experiments, we characterized bone cell cultures derived from the human mandibular alveolar ridge to confirm that isolating cells from bone explants would have osteogenic characteristics *in Vitro*. Osteogenic gene expression (Col-I, ALP, OCN, BSP, OPN, RUNX2) were analyzed by RT-PCR/Real

time PCR. Surface markers (CD13, CD44, CD90, CD105) were analyzed by flow cytometry and to detect the osteogenic differentiation capacity, cells were stained by Alizarin Red staining, Von Kossa staining and ALP staining/ALP activity. For NOK-SI cells would be confirmed by retaining epithelial morphology, proliferative capacity, and the expression of typical markers such as Cytokeratin 18 and E-Cadherin.

Objective 1: To demonstrate that PGE_2 increases the RANKL expression in HMBCs

Experimental design: HMBCs were treated with a PGE_2. This would be done at doses of 0, 0.1, 1, 10 µM for 24 h for assessment of the optimum dose that can increase the RANKL/OPG ratio and shows no effect on cell viability tested by MTT assay, which would be used in the next step. To investigate the intracellular signaling pathway of RANKL induction in PGE_2 treated HMBCs, HMBCs were pre-treated with 40 nM JNK inhibitor (SP600125; Calbiochem®, Darmstadt, Germany), 2.5 µM ERK inhibitor (Calbiochem®, Darmstadt, Germany), 14 nM PI3K inhibitor (LY294002; Calbiochem®, Darmstadt, Germany), 20 µM PKA inhibitor (Calbiochem®, Darmstadt, Germany) for 30 min and then treated with PGE_2 (at the previously determined optimal concentration for 24 h). HMBCs were collected for RNA and protein analysis, RANKL-, OPG-mRNA were estimated by using reverse transcription polymerase chain reaction (RT-PCR) analysis and/or real-time PCR to compare relative gene expression, and ELISA was performed to compare the soluble RANKL, OPG protein level including Western Blot was performed for RANKL membrane-bound protein.

Objective 2: To investigate whether epithelial-derived supernatant can suppress the RANKL/OPG ratio induced by PGE_2 in HMBCs.

Experimental design: HMBCs were cultured. Bone cells were incubated with PGE_2 at the optimum dose from previous step for 24 h to stimulate RANKL expression. To test the effect of epithelial-derived mediators in inflammatory condition, NOK-SI cells were stimulated in 2.5 µg/mL *P.gingivalis* LPS (InvivoGen, San Diego, CA, USA) in a keratinocyte complete media for 24 h, then supernatant with *Pg*LPS was removed and NOK-SI cells were cultured without *Pg*LPS in growth medium for the next 24 h. Alternatively, to test the effect of constitutive-released mediators, NOK-SI cells were cultured without *Pg*LPS in growth medium for 24 h and NOK-SI cells were cultured in fresh growth medium for the next 24 h. Then, *Pg*LPS-activated or constitutive-released, supernatants collected from epithelial cultures will be added to PGE_2-stimulated bone cells (vary into 3 dilution fraction were 1:3, 1:1, 3:1 between SFM:CM). RANKL-, OPG-specific mRNA was amplified using RT-PCR and/or real-time PCR and ELISA was performed to compare the soluble RANKL/OPG protein level.

Objective 3: To investigate the mediator(s) released by NOK-SI cells that can suppress RANKL expression in HMBCs induced by PGE_2.

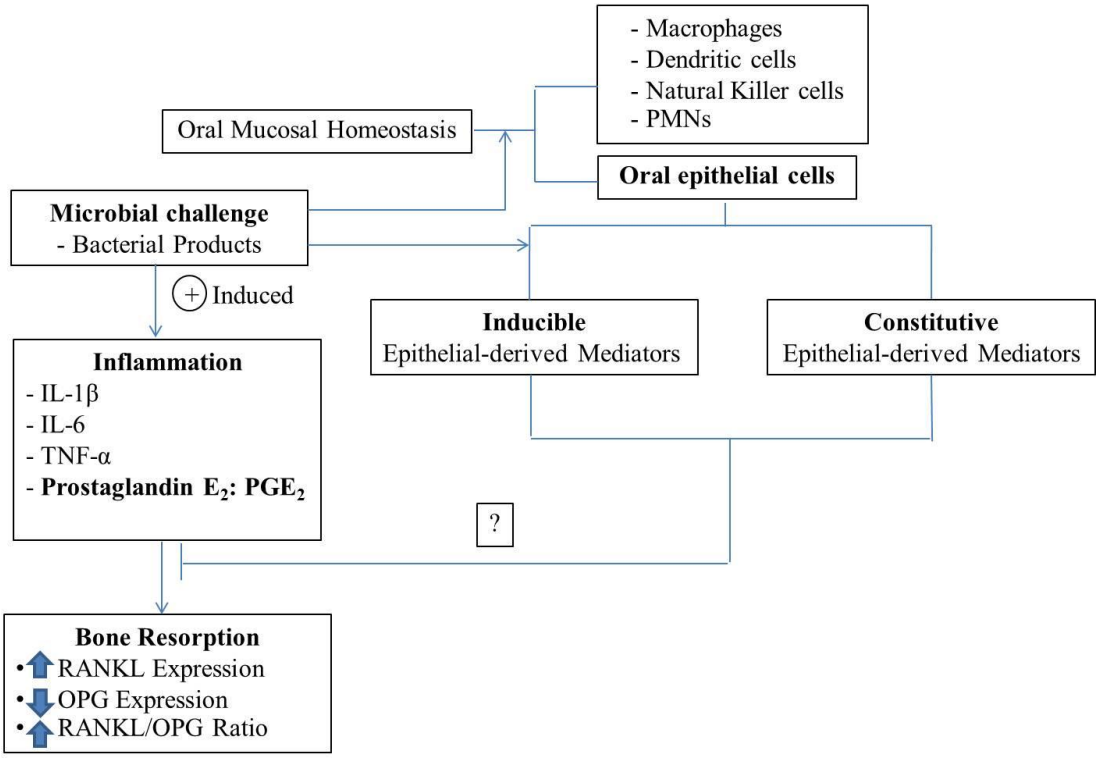
Experimental design: NOK-SI cells were cultured in a keratinocyte complete medium for 24 h NOK-SI cells were challenged with 2.5 µg/mL *Pg*LPS for 24 h NOK-SI cells were washed, and fresh media was added. After 24 h, NOK-SI supernatant was collected for HMBCs treatment/ELISA, and RNA isolation and nuclear protein isolation were performed.

To investigate which epithelial-derived mediators regulate the RANKL expression in HMBCs induced by PGE_2. Based on literature review, Oral epithelium can secretes numerous of cytokines and chemokines when challenged with bacterial products especially LPS, have capacity to synthesize and secrete a wide array of molecules including TNF-α, IL-1β, IL-6, IL-8, IL-10, IL-12, MCP-1, IFN-γ. First, we focus on mRNA profiling that related inflammatory responses in 2 conditions (constitutive and inducible by *Pg*LPS) and then evaluate in proteins level. The total RNA of NOK-SI cells were collected from an epithelial cell culture using RT-PCR /Real-time PCR, ELISA was performed to compare the soluble proteins (follow the results from mRNA expression) in condition medium, and the cell monolayers was lysed to nuclear extracted protein and Quantitative protein was performed by NFκB Transcription factor ELISA kit to detect NFκB p50 and p65 subunits.

Expected Outcomes

The knowledge gained from this study may increase our understanding of the molecular mechanism regarding to the responses of HMBCs to PGE_2 and whether that can be relating to RANKL upregulation in bone resorption process. Moreover, it may explain the protective role of oral epithelium in periodontal tissue to resolve inflammatory reaction and to gain tissue homeostasis.

Conceptual Framework



Keywords

- Human mandibular-derived bone cells
- *Porphyromonas gingivalis* Lipopolysaccharide
- Prostaglandin E_2
- Bone resorption
- RANKL expression
- Oral epithelial cells
- Epithelial-derived mediators
- Interferon-gamma

Research design

Laboratory experiment research

CHAPTER II

REVIEW OF RELATED LITERATURE

Bone Biology

Bone is a specialized tissue, which has multiple functions, such as the protection of vital organs and bone marrow, structural support for muscles and storage many ions such as calcium. Adult bone is renewed continuously in response to a variety of stimuli through the process of bone remodeling, for which two requirements are essential (5). First of all, bone resorption and formation must occur at the same site. In addition, they must be balanced in terms of time and quantity. The processes that drive bone remodeling are still not fully clear, but they include damage of bone in response to normal wear, changes in mechanical force following altering body shape or weight, and local release of cytokines or growth factors.

Bone Remodeling

Bone is a dynamic organ that is continuously remodeled, the process involves break down (resorption) and build-up (formation). Bone remodeling is the metabolic process regulating bone structure throughout life. The concept that osteoblast-lineage cells control osteoclastogenesis originated more than 30 years ago, following the finding that receptors for osteoclastogenic hormones, such as parathyroid hormone (PTH), are present on cells with osteoblastic characteristics (5). Subsequent studies showed that osteoblastic cell lines or cell preparations rich in osteoprogenitors support osteoclast formation (6, 7). The rate of bone remodeling and the amount or

remodeling sites are increased in many pathologic conditions affecting the skeleton, in which local and/or systemic alterations in the levels of hormones or pro-inflammatory cytokines stimulate bone resorption (25). Furthermore, the understanding of molecular mechanisms that regulate osteoclast formation and function has advanced rapidly in the last 18 years since the discovery of RANK/RANKL/OPG signaling (Figure 2.1) (6, 26-29).

Receptor activator of nuclear factor-kappaB (RANK) is a homotrimeric transmembrane protein member of the TNF receptor superfamily. It has shown expression in osteoclast precursors, mature osteoclasts, dendritic cells including mammary glands and cancer cells that have high bone metastatic potential, such as breast and prostate cancer (26, 30, 31).

Receptor activator of nuclear factor-kappaB ligand (RANKL) is a homotrimeric protein and is typical membrane-bound on osteoblastic and activated T cells or secreted by activated T cells (28, 32, 33). The secreted protein is derived from the membrane form as a result of either proteolytic cleavage or alternative splicing (34). Most of the factors known to stimulate osteoblast formation and function induce RANKL expression by osteoblastic stromal cells. RANKL is highly expressed in lymph nodes, thymus, mammary glands and lungs, whereas low levels are seen in the spleen and bone marrow (32). Moreover, it is also expressed by certain malignant tumor cells, which also express RANK; thus, RANKL signaling may regulate tumor cell proliferation, migration and bone metastasis (30, 31).

Osteoprotegerin (OPG) is secreted by a variety of cell types: not only in osteoblasts but also in the heart, kidney, liver and spleen. Most of the factors that induce RANKL expression by osteoblasts also regulate OPG expression (35). However, contradictory results have been reported, showing that when RANKL expression is upregulated, OPG expression is downregulated or not induced to the same degree as RANKL, such that the RANKL/OPG ratio changes in favor of osteoclastogenesis (27, 35). The protective role of OPG in regulating bone homeostasis has been supported by deletion of *OPG* in patients with juvenile Paget's disease, an autosomal recessive disorder in which affected individuals have increased bone remodeling, osteopenia and fracture (36).

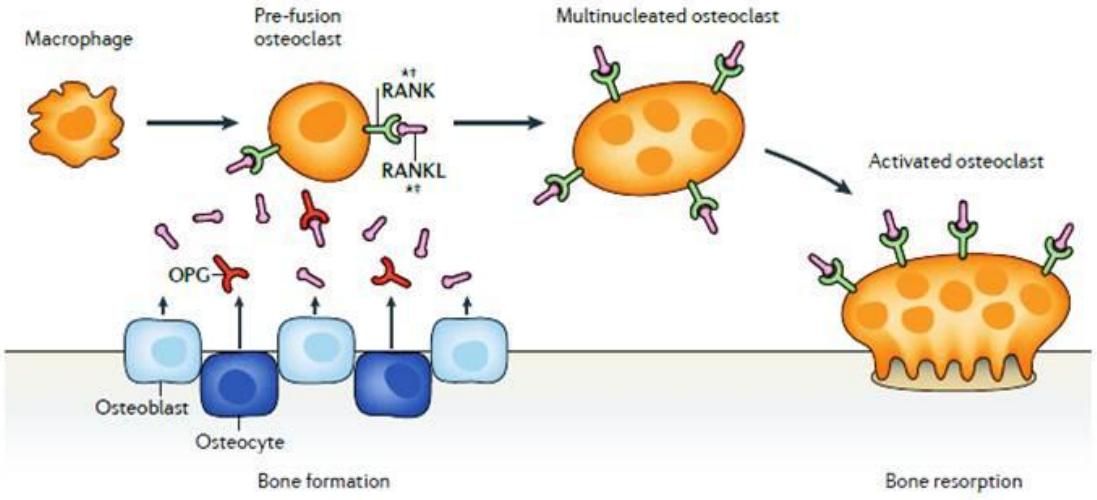


Figure 2.1 The control of bone resorption is dependent on the molecular interplay of the receptor activator of nuclear factor–κB ligand (RANKL) that is express by osteoblasts and stromal cells and osteoprotegerin (OPG). RANKL binding to RANK receptor on the surfaces of pre-osteoclasts drives their maturation and activation, while OPG acts as decoy receptor and inhibits RANK-RANKL engagement. [Modified from (29)]

Osteoclastogenesis

Osteoclasts are derived from mononuclear cells in myeloid lineage of hematopoietic cells that also give rise to macrophages. Since the discovery of the RANK/RANKL signaling system, more understand of the molecular mechanisms that regulate osteoclast formation and activation. Macrophage colony-stimulating factor (M-CSF) was expressed by osteoblastic stromal cells is required for osteoclast precursors cells to differentiate into osteoclasts, but only M-CSF is unable to complete this process. Completion of osteoclast precursors differentiation also requires RANKL expression from osteoblast cells and interaction with RANK is expressed by osteoclast precursor cells (37). Osteoclast precursor cells differentiation is regulated by a numerous of transcription factors and signaling pathways that are activated by RANK/RANKL engagement (Figure 2.2) (38). The completion of osteoclast precursor cells differentiation by RANKL requires the sequential expression of NF-κB (39), c-FOS (40) and NFATc1 (41).

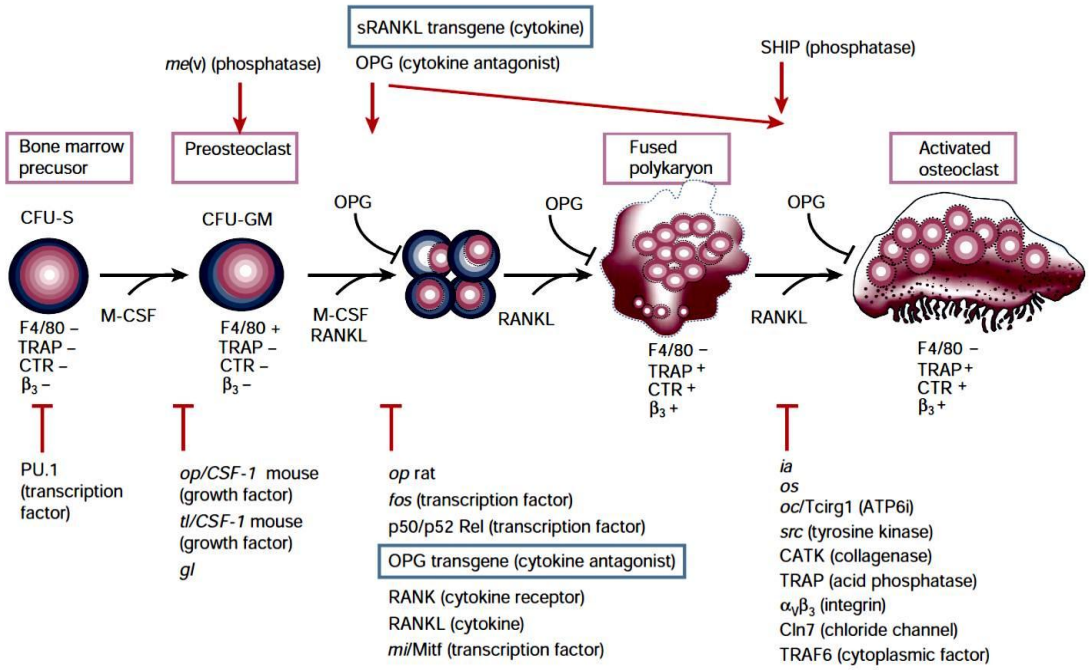


Figure 2.2 Schematic representation of the osteoclast differentiation. Peripheral blood mononuclear cells differentiate into mature multinucleated osteoclasts. M-CSF and RANKL are required to induce expression of osteoclastic genes, including tartrate-resistant acid phosphatase (TRAP), cathepsin K (CATK), calcitonin receptor (CTR) and β_3 integrin leading to the development of mature osteoclasts. [Modified from (38)]

Periodontitis pathogenesis

Periodontitis is a bacterially induced inflammatory disease that destroys the underlying periodontal tissues. It is a multifactorial disease involves in interactions of the biofilm with the host immunomodulatory responses (Figure 2.3) (42) and subsequent alteration in bone and connective tissue homeostasis. *Porphyromonas gingivalis* (*Pg*) is a gram-negative black-pigmenting anaerobe bacteria associated with periodontitis (43). Major virulence factors are lipopolysaccharide (LPS) and gingipain, which cause tissue and bone destruction (44, 45). *Pg* induces RANKL expression in many cell types including osteoblasts, gingival fibroblasts and periodontal ligament cells (46, 47). Furthermore, *Pg* can invade gingival epithelial cells, sulcular epithelial cells and endothelial cells. Invasion into epithelial cells depends on fimbria, which mediate adherence of *Pg* to host cells. Gingipain is a family of proteases which are able to degrade host extracellular matrix protein, and induce detachment of epithelial cells from substratum. Together, these virulence factors may allow *Pg* to invade deeper because of connective tissue destruction.

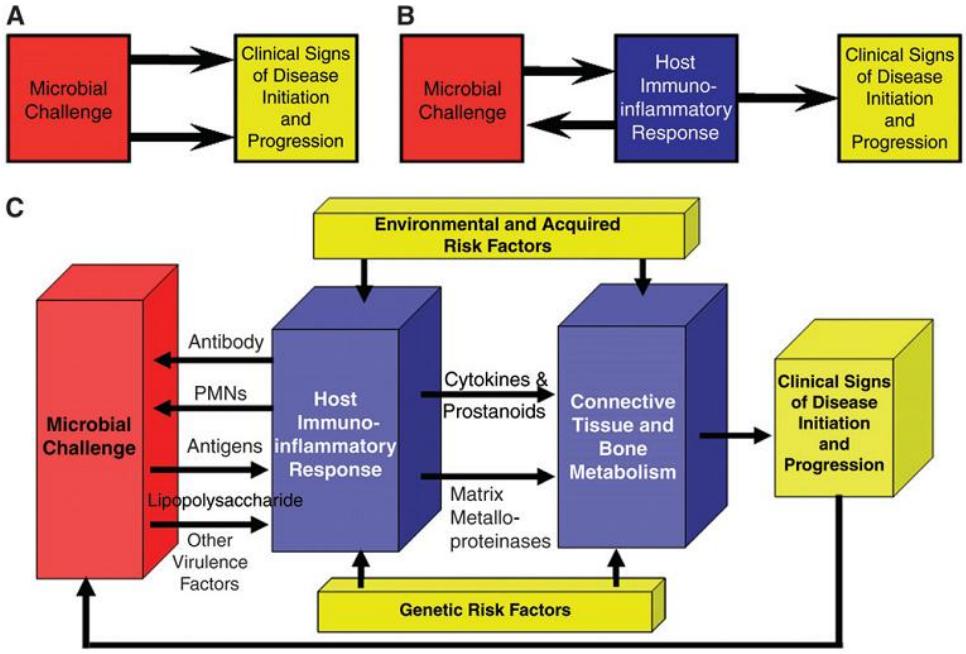


Figure 2.3 The evolution of conceptual models of periodontal disease. A) An early linear model depicting the principal etiologic role for bacteria in the initiation and progression of periodontal disease. B) A 1980s model emphasizing a central role for the host immunoinflammatory response in the clinical development and progression of periodontal disease. C) A 1997 model demonstrating various factors contributing to the pathogenesis of human periodontitis based on pathways and processes known at the time. [Modified from (42, 48)]

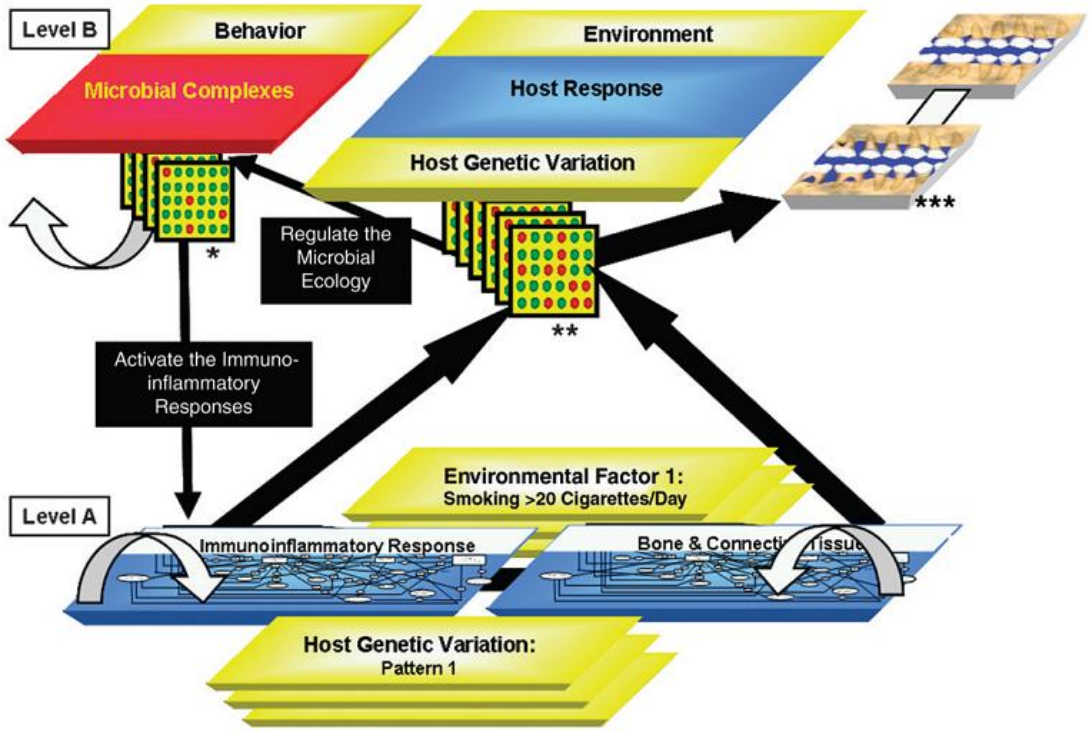


Figure 2.4 The biologic systems model representing the pathogenesis of periodontitis be defined by the bacterial components, environmental factors, and host–genetic variations associated with disease. Level A depicts the biologic mechanisms involved in immunoinflammatory responses and in bone and connective tissue metabolism, and level B depicts the observable clinical parameters and biomarkers. [Modified from (42)]

Oral Mucosal homeostasis

The primary function of immune system in the mouth cavity is to protect the teeth, jaws, gingiva and oral mucosa against infection. The oral immune system is the part of mucosa associated lymphoid tissue (MALT) represented by the oral mucosa, salivary glands/saliva and gingival crevice (49). The intact oral epithelium are supported by lamina propria provide a physical barrier that protects the underlying tissues from endogenous and exogenous invading microorganisms, including commensals and pathogens. This protective mucosa composed of cells in the innate immune system such as macrophages, dendritic cells, natural killer cells and polymorphonuclear leukocytes (Figure 2.5) (14). Oral mucosa immunity neutralizes pathogens by limitation of colonization that continuous shedding by exfoliation of epithelial cells. Moreover, The oral epithelium forms part of an intercommunicating network of the immune system that are exchanged in dynamic interactions that mediates the production of immunoinflammatory responses such as IL-1β, IL-6, TNF-α, GM-CSF, TGF-β and IL-8 (50-52). These cytokines also secreted by macrophages, fibroblast, dendritic cells, mast cells and lymphocytes in the oral mucosa. Oral commensals play an important role in stimulating immune responses which are down regulated and reprogrammed such as by induction of oral tolerance (18). Induction of immune tolerance toward commensals combined with responsiveness to pathogens is essential to sustaining immune homeostasis while preventing life-threatening infections (Figure 2.6).

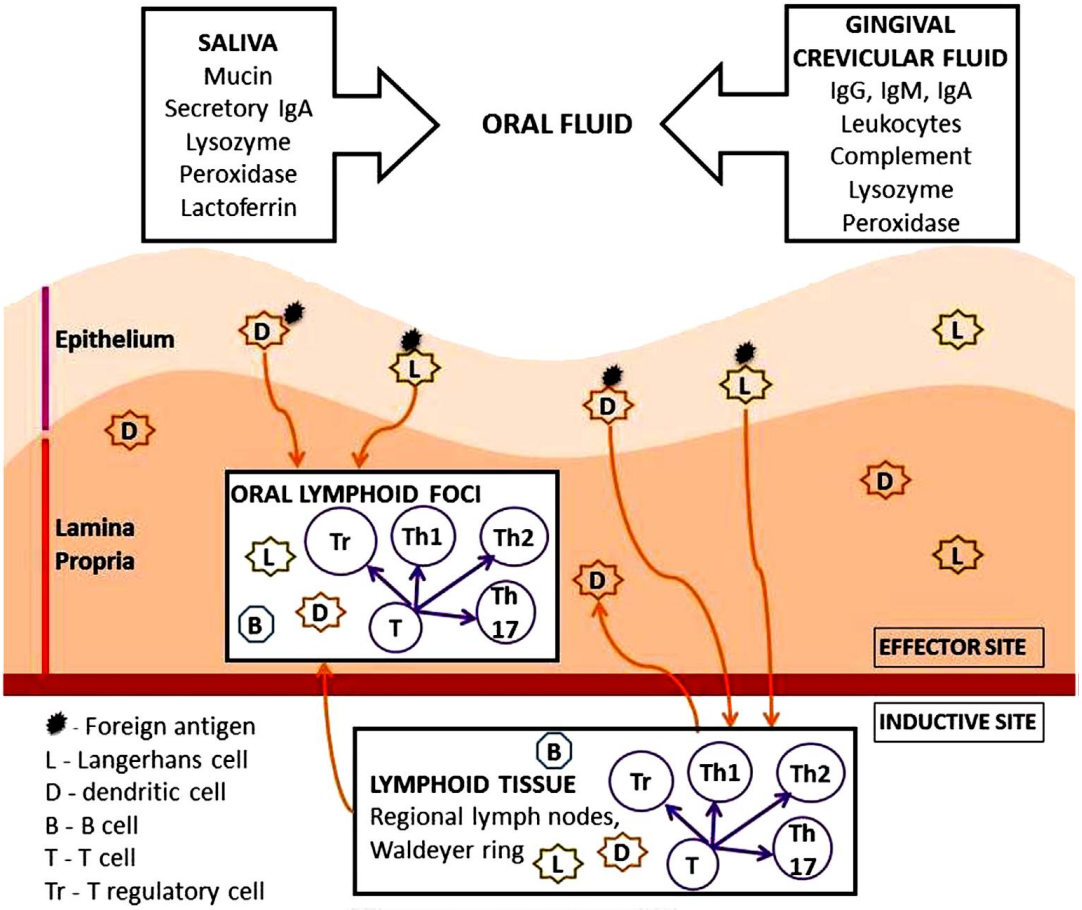


Figure 2.5 Cells and their factors that associated with oral immunity [Modified from (14)]

Appropriate host responses to pathogen and their virulence factors are essential to combat invading bacteria but excessive immune responses can lead to the periodontal tissues destruction (53). Endotoxin tolerance is defined by a reduced capacity of the host to respond to secondary challenge with LPS following an exposure to initial stimulus and is characterized by diminished release of proinflammatory cytokines. Hypo-responsiveness to the same LPS is called to as homotolerance, while that with a different LPS is referred to heterotolerance (54).

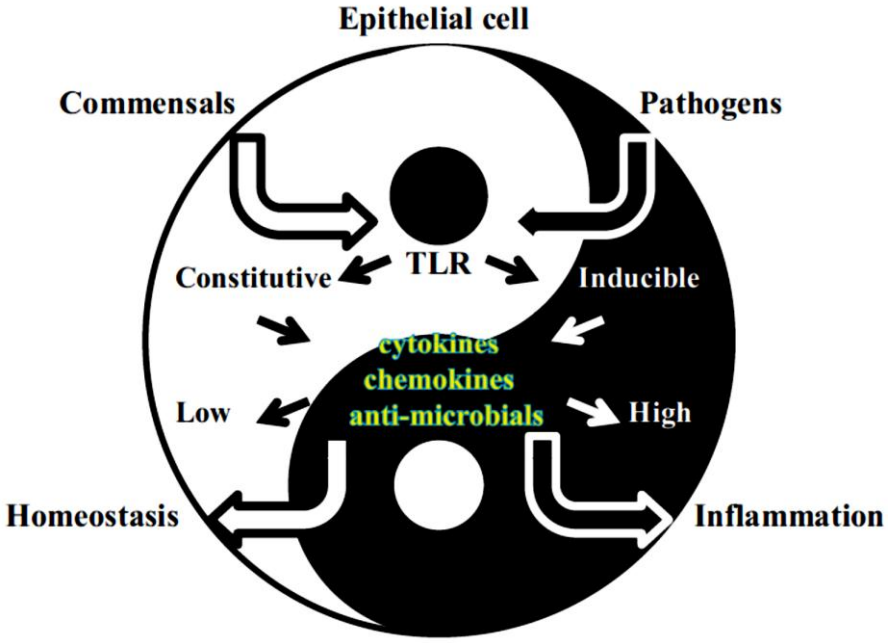


Figure 2.6 Epithelial cell and microorganism dynamic interaction in control oral homeostasis [Modified from (55)]

Toll-like receptors (TLRs)

TLRs are a family of mammalian homologs of Drosophila and are pattern-recognition receptors (PRRs) that sense pathogen-associated molecular patterns (PAMPs) from microorganism. To date ten TLRs have been described in human and 12 TLRs in mice (56). Among them, TLR-2 and -4 function as the principal innate sensors for virulence factors of periodontal pathogen and might be very important in endotoxin tolerance developed in periodontal tissues (57). Many type of human TLR and their ligands are known (Figure 2.7) (58). LPS had been studied as an important component of endotoxin which virulence factor of gram-negative bacteria. TLR signaling initiated by ligand trigger the dimerization of receptors. Follow by the Toll-IL-resistance (TIR) conserved domain that containing of adaptor proteins association.

Lead to the activation of downstream signaling pathway and initiate innate immune responses that critical for adaptive immunity induction. Two major families of transcription factors that are induced downstream of TLR signaling are NFκB and the interferon-regulatory factors (IRFs). A main consequence of TLR signaling is the activation of proinflammatory cytokines (Figure 2.8)

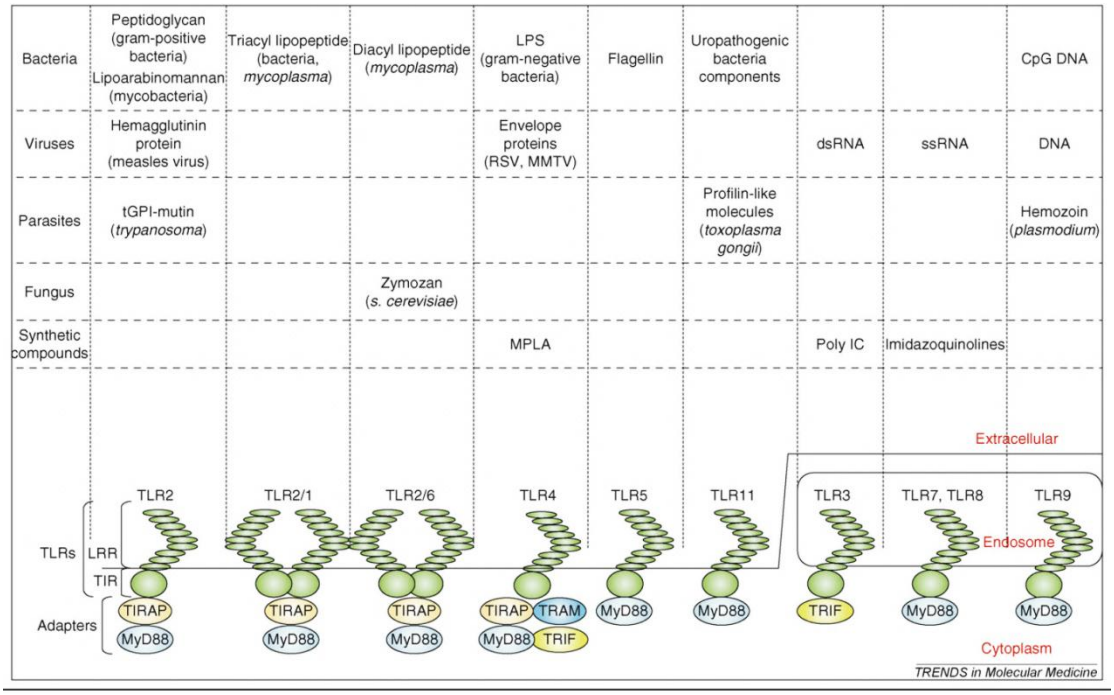


Figure 2.7 Human TLR ligands [Modified from (58)]

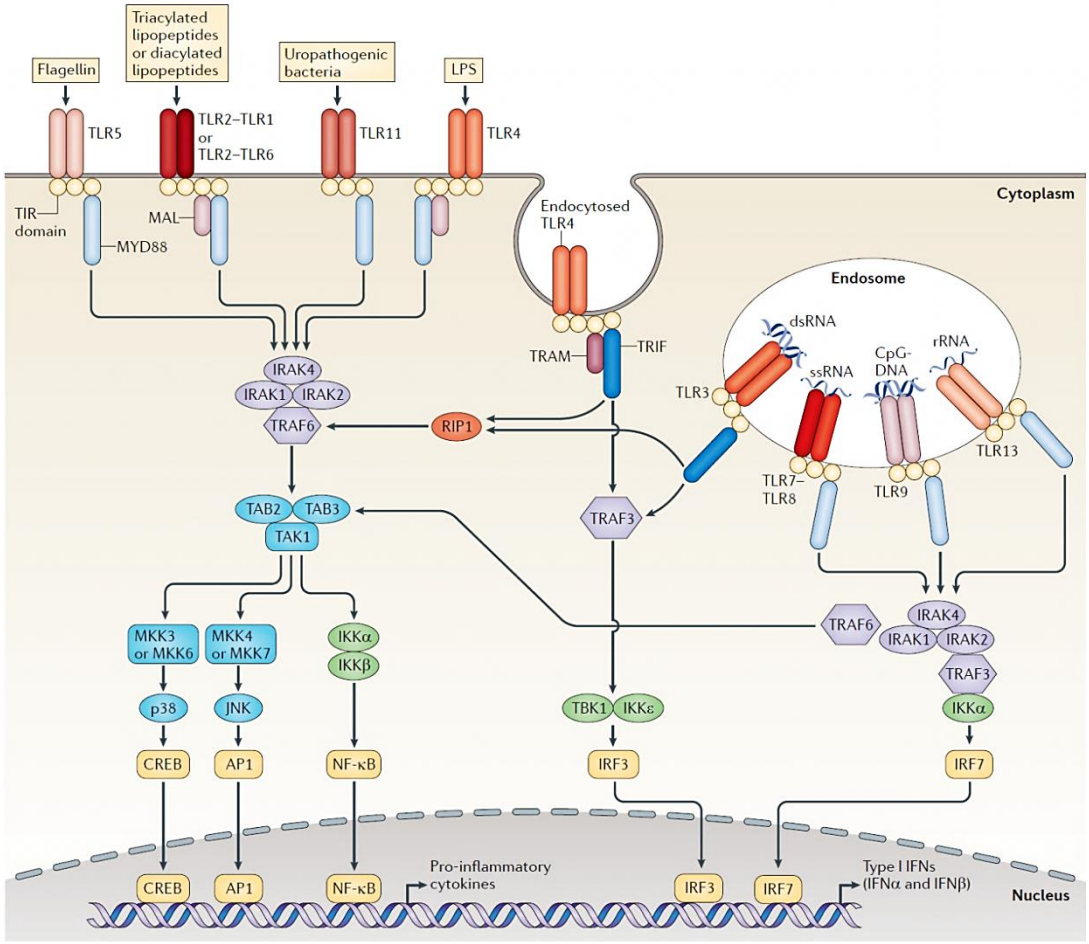


Figure 2.8 Mammalian TLRs signaling pathway [Modified from (57)]

Interferon-gamma (IFN-γ)

The interferon (IFNs) was originally discovered as a cytokine that combat with viral infection (59, 60). IFNs are classified by the receptor specificity and sequence homology into 3 types that are type I, type II and type III (61). The action of the IFNs is mediated through three specific receptor: a heterodimer of IFNAR1 and IFNAR2 bind to type I IFNs, a heterodimer of IL-10R2 and IFNLR1 bind to type III IFNs and a tetramer comprise of two IFNGR2 chains and two IFNGR1 chains bind to type II IFNs. Following binding, signal transduction is initiated and is activated STAT

signaling pathway (Figure 2.9). IFN-γ is the single gene product of type II IFNs and mainly produces by CD4+ T helper cell type 1(Th1) (62). It also secreted by various cell types composed of CD8+ cytotoxic T cells (62), NK cells, B cells (63, 64), NKT cells (65) and professional antigen-presenting cells (macrophage/dendritic cells) (66, 67) (Figure 2.10).

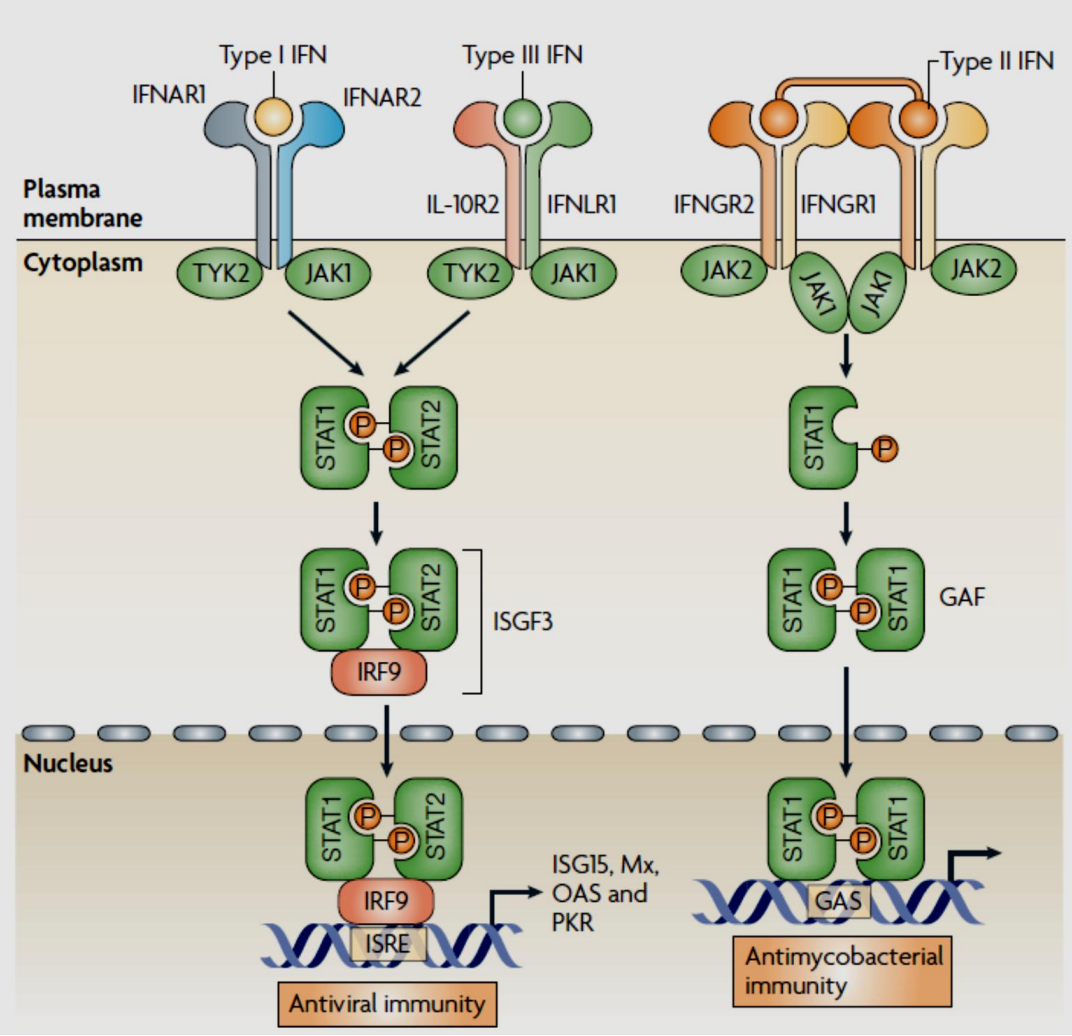


Figure 2.9 The interferon signaling pathway**.** Interferon is mediated via three receptor complex [Modified from (68)]

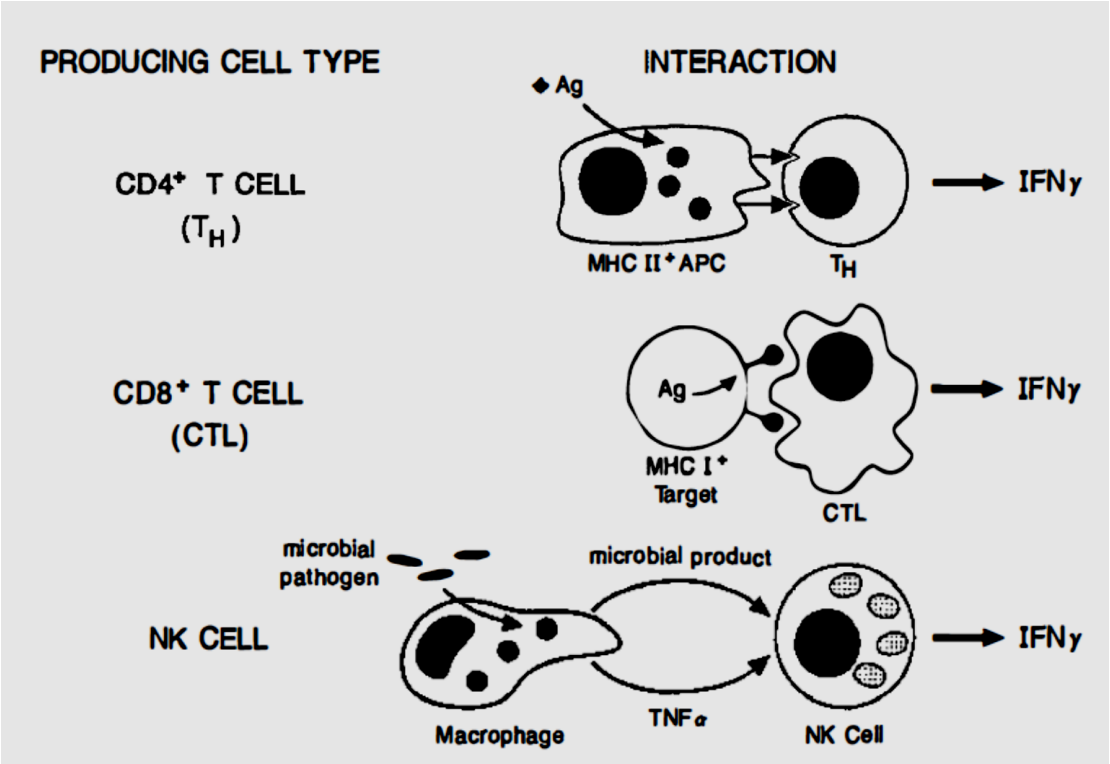


Figure 2.10 IFN-γ is produced by various cell sources [Modified from (69)]

The production of IFN-γ is induced by IL-12 and IL-18 that are secreted by antigen-presenting cells and play a role in cross-link infection with the IFN-γ production in the innate immune responses. IFN-γ is a typical cytokine of Th-1 responses. IFN-γ is described to systematically inhibit osteoclastogenesis (70, 71). In vitro data analysis demonstrated that IFN-γ induces rapid degradation of TRAF6 which are RANK adaptor protein resulting in reduction of RANKL-induced osteoclastogenesis (72) (Figure 2.11). This data supports a hypothesis that Th1 cells are responsible for stable lesion, while Th2 cells are associated with progression stage of periodontal disease (73).

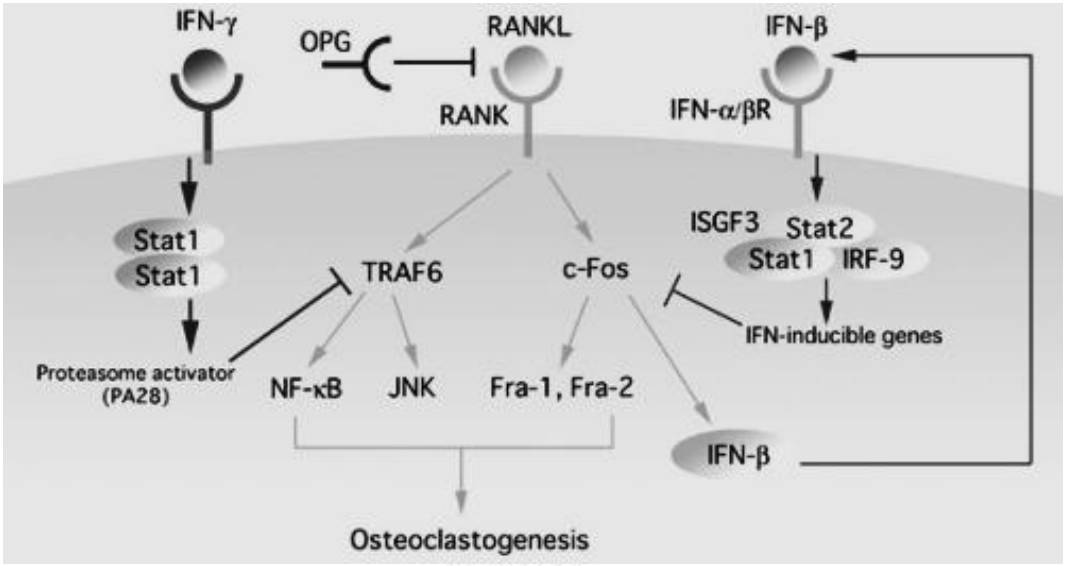


Figure 2.11 IFN-γ induces rapid degradation of TRAF6 resulting in reduction of RANKL-induced osteoclastogenesis [Modified from (41)]

Role of Prostaglandin E_2 in Bone resorption

Prostaglandins are small-molecule derivatives of arachidonic acid, produced by cyclooxygenases and prostaglandin synthases (Figure 2.12) (8). PGE_2 is the most widely produced prostanoids in the human body. It is an endogenous ligand with a high affinity to the prostaglandin E receptor 1, 2, 3 and 4 (EP1, 2, 3, 4) which are present on multiple cell types (Figure 2.13) (8, 74). PGE_2 can be produced by all cell types such as epithelia, fibroblasts and infiltrating inflammatory cells, including osteoblast cells. The receptors belong to the G-protein-coupled seven transmembrane domains and activate either adynylate cyclase or phospholipase C (PLC). In MC3T3-E1 mouse osteoblastic calvaria cells, PGE_2 can stimulate both cAMP and phosphatidylinositol signal transduction pathways (75). Analysis of the role of individual EP subtypes in PGE_2 action on bone has been limited because of the lack of specificity and limited efficacy of agonists and antagonists for these receptors.

However, by using mice deficient in EP receptors, it has been shown that a lacking EP2 or EP4 leads to defects in bone metabolism (formation/resorption) (76). In addition, it has been found that EP4-deficient mice show decreased histomorphometric parameters compared with EP2-deficient mice (77).

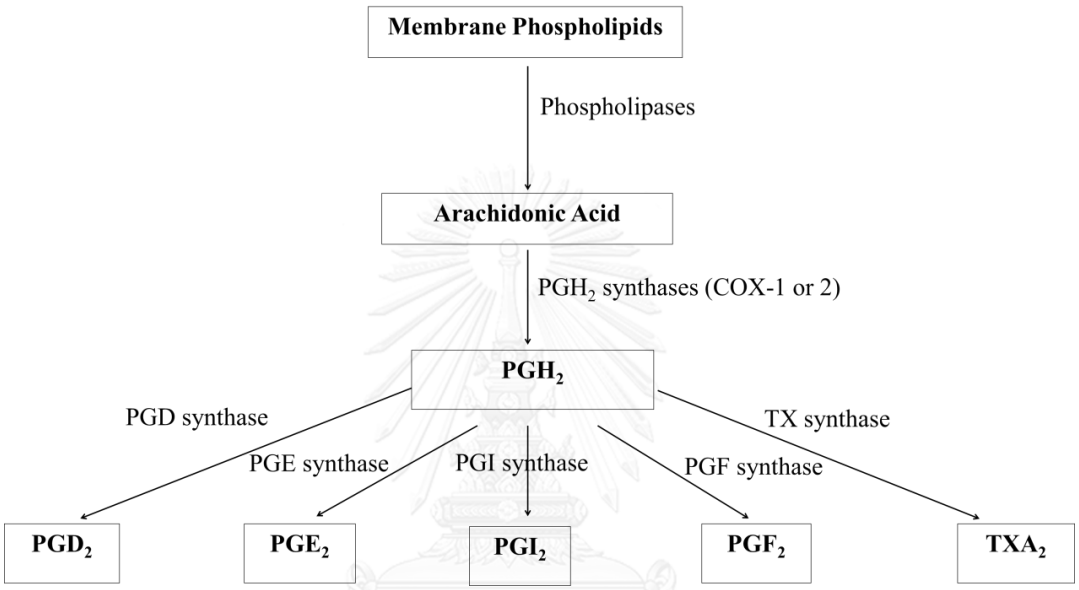


Figure 2.12 Cyclooxygenases metabolize arachidonic acid to five primary prostanoids: PGD_2, PGE_2, PGI_2, PGF_2 and TXA_2 [Modified from (78, 79)]

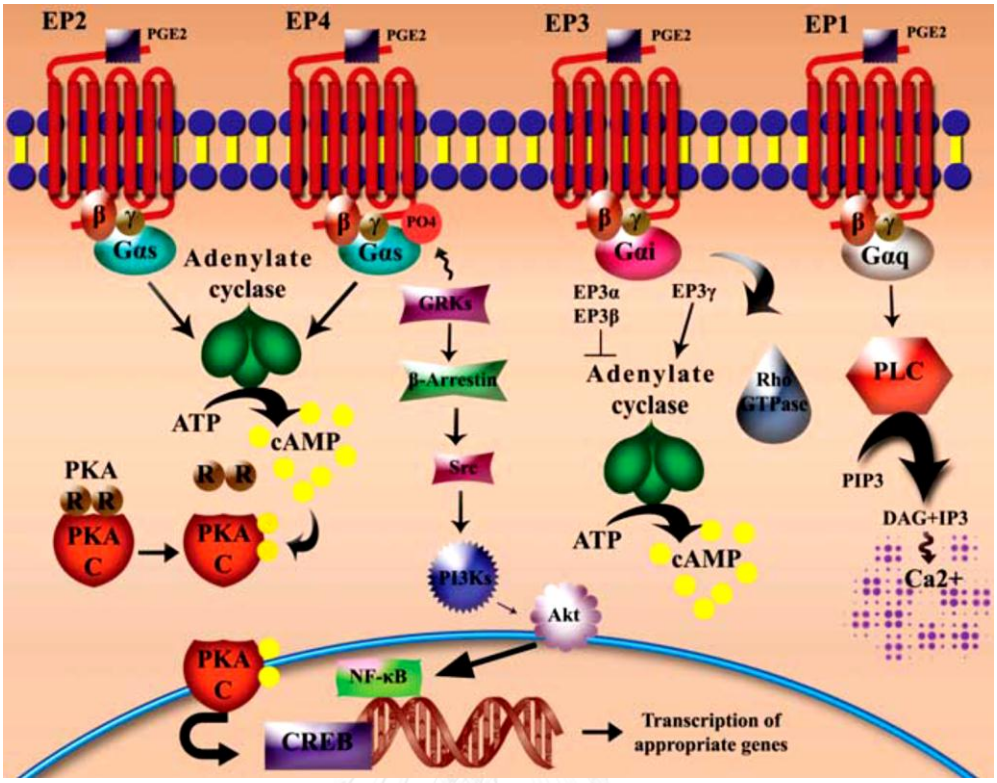


Figure 2.13 PGE_2 Receptor (EP receptors: EP1-4) and signaling pathway [Modified from (80)]

CHAPTER III

RESEARCH METHODOLOGY

Materials and Reagents

All cell culture media were purchased from Gibco by life technologies (Grand Island, NY, USA). Tissue culture plates and plastic wares were purchased from Corning (Grand Island, NY, USA). Ultrapure LPS from *Porphyromonas gingivalis* (Cat # tlrl-pglps) as a TLR2 ligand was purchased from InvivoGen (San Diego, CA, USA). Prostaglandin E_2 (Cat No.14010) was purchased from Cayman Chemical (Ann Arbor, MI, USA). Recombinant human IFN-γ was purchased from BioLegend (San Diego, CA, USA). Purified anti-human IFN-γ (Cat No.507513) was also purchased from BioLegend. IL-1β, IL-6, IL-8 and TNF-α ELISA kits were purchased from R&D System (Quantikine® Human Immunoassay; R&D System, Minneapolis, MN, USA). Human IFN-γ ELISA MAX™ Deluxe set (Cat No.430104) was purchased from BioLegend (San Diego, CA, USA). Human sRANK-Ligand ELISA Development kit (Cat No.900-K142) was purchased from PeproTech (Rocky Hill, USA). Human OPG/TNFRSF11B DuoSet® ELISA Development system (Cat No.DY805) was purchased from R&D Systems (Minneapolis, MN, USA). NFκB p50/p65 Transcription Factor Assay (Cat No.ab133128) was purchased from Abcam (Thermo Scientific, Rockford, IL, USA). Nuclei EZ Prep Nuclei Isolation kit was purchased from Sigma-Aldrich (St. Louis, MO, USA) Ascorbic acid (Cat No.A-4034), Dexamethazone and β-glycerophosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Alizarin S Red staining was also purchased from Sigma-Aldrich.

Patient Selection and Sample Collection

Healthy patients who had a mandibular third molar surgically removed at the Department of Surgery, Faculty of Dentistry, Chulalongkorn University, Thailand, were recruited into this study. The exclusion criteria were having an infection or inflammation at the extraction site as determined by oral or radiographic examination, or having an uncontrolled systemic disease. The patients provided written consent prior to surgery in accordance with the principles of the Declaration of Helsinki of the World Medical Association, and with authorization from the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University (#051/2015). Excess bone tissue was collected from the extraction site using a surgical hand instrument, and bone samples were stored in a sterile tube containing Dulbecco's Modified Eagle's Medium (DMEM; Gibco, NY, USA) containing 10% Fetal Bovine Serum (FBS; Hyclone, Northumberland, UK) for immediate transfer to the laboratory.

Cell cultures

Human Mandibular-derived Bone Cells (HMBCs)

The bone samples were washed three times in phosphate-buffered saline (PBS) containing 100 IU/ml Penicillin, 100 µg/ml Streptomycin, and 0.25 µg/ml Amphotericin B (Multicell, Wisent Inc, Quebec, Canada), cut into small pieces, and placed in a tissue culture dish. The bone tissue was digested in 0.25% Trypsin-EDTA (Gibco) for 1 min at room temperature. The tissue was washed with bone culture medium (DMEM supplemented with 15% heat-inactivated FBS, 1% L-glutamine (Gibco), and 1% Antibiotic-Antimycotic). The bone tissue was cultured in bone culture medium at 37^{o}C in a humidified 5% CO_2 atmosphere for 7 days. When

confluent, the cells were subcultured using 0.25% Trypsin-EDTA and plated at 1:3 ratio. Cells from passages 3–8 were used in the experiments. When needed, 3×10^5 or 1.5×10^5 cells were cultured overnight in 6-well or 12-well plates, respectively, before incubation with 0.1, 1, or 10 μM PGE_2 (Cayman Chemical Corp; Ann Arbor, MI, USA) in bone culture medium for 24 h.

Normal Oral Keratinocyte Spontaneous Immortalized Cell line: NOK-SI

A normal oral keratinocyte spontaneous immortalized (NOK-SI) cell line was derived from the retro-molar area of a healthy volunteer. NOK-SI cells become spontaneously immortalized by escaping cell senescence (81). The NOK-SI cells were cultured at 37^{o}C in a humidified 5% CO_2 atmosphere in keratinocyte complete medium (KCM) consisting of defined keratinocyte serum-free medium (dk-SFM; Gibco) with growth supplement and 100 IU/ml Penicillin, 100 μg/ml Streptomycin, and 0.25 μg/ml Amphotericin B for 3 days. When approximately 70% confluent, the cells were subcultured using 0.25% Trypsin-EDTA and plated at 1:4 ratio. Cells from passages 40–45 were used in the experiments.

NOK-SI cells (2×10^5 cells/well) were plated in 6-well plates overnight, then incubated with 2.5 μg/mL *Pg*LPS (InvivoGen, San Diego, CA, USA) in KCM, with other cells cultured in KCM without *Pg*LPS, for 24 h. The culture medium with or without *Pg*LPS was removed, the cells were washed with PBS, and incubated with fresh KCM. *PgLPS*-stimulated, or untreated, epithelial supernatant as collected after 24 h for use in subsequent experiments. The NOK-SI cells were then collected by trypsinization for quantitative RT-PCR to detect TLR-2 and TLR-4 expression or for the NFκB ELISA.

THP-1 monocytes

The THP-1 human monocytic cell line was originally established from the blood of a one year old boy with acute monocyticleukemia (82). Cells are non-adherent and in a pro-monocytic state**.** The THP-1 cells were maintained at 37^{o}C in a humidified atmosphere of 5% CO_2 atmosphere in RPMI-1640 medium (Gibco), supplemented with 10% FBS (Hyclone), 1% L-glutamine (Gibco), 1% Penicillin (100 IU/ml)/Streptomycin (100 µg/ml)/Amphotericin B (0.25 µg/ml) and 0.1% β-Mercaptoethanol (2-ME; Gibco). Medium was changed every 2-3 days. Cells were passaged weekly before they become confluent to assure optimum cell density that allows proper cell growth. Cells were counted under a microscope using a haemocytometer (Bright-Line, improved Neubauer, Hausser Scientific, PA, USA) and cells were passaged at least one time after thawing before being used for an experiment.

Vitamin D_3, M-CSF and RANKL treatment of THP-1 monocytes

Prior to use in stimulation experiments, THP-1 monocytes (1 x 10^6 cells/ml) were treated with 40 ng/mL 1α, 25$(OH)_2D_3$ (vitamin D_3; Calbiochem, Merck Chemicals, Nottingham, UK) for 24 h. unless otherwise stated, all experiments were conducted in duplicate cultures, on three independent occasions. The treatment with vitamin D_3 induces the cells to differentiate along the myeloid lineage to mature monocyte-like cells which resemble the natural phenotype of primary human monocytes (Kitchens et al., 1992; Schwende et al., 1996). The cells become more adherent and have the capability to release mediators like PGE_2 and TNF-α. Also, the expression of the monocyte marker CD14 is increased. After 24 h of incubation, the

medium was changed to treat with M-CSF and RANKL (PeproTech Inc., USA) at concentration of 25 ng/mL and 40 ng/mL, respectively for 7, 14 and 21 days. The osteoclast differentiated cells were observed under light microscope in randomly selected visual fields in different areas of each well.

Cell viability (Trypan blue staining)

Monitoring of cell viability during routine culture was performed using trypan blue exclusion. Trypan blue is a blue-dye that passes through a damaged cell membrane, thus only staining dead cells (Freshney and Liss, 1987). 10 µl of cell suspension was diluted 1:1 (v/v) with trypan blue. The number of blue-stained cells and unstained cells was counted on a haemocytometer. The number of viable cells was taken as 100 % and the percentage of dead cells was subtracted. Cell viability was found to be > 95 % during routine cell culture.

MTT Assay

The MTT assay is based on the ability of viable cells to reduce a tetrazolium-based compound, 3- (4, 5-dimethyl-2-thiazolyl)-2, 5- diphenyl-2H-tetrazolium bromide (MTT; USB Corporation, OH, USA), to a purplish formazan product. To determine cell viability, the medium was replaced with MTT solution for 30 min at 37^{o}C and the formazan crystals were be solubilized in a detergent solution containing 1: 9 glycine buffer (0.1 M glycine / 0.1 M sodium chloride pH10) and dimethylsulfoxide (DMSO; Sigma-Aldrich, MO, USA). The number of surviving cells is directly proportional to the level of the formazan product created. The

solubilized solution was measured for an absorbance at 570 nm in a microplate reader (ELx800; BioTek, Winooski, VT, USA). The absorbance values were calculated relative to the total cell number using a standard curve. All measurements were done in three wells, representative of three independent experiments.

Flow cytometric analysis

To analyze the surface markers, HMBCs from the third passage were harvested, then $1x10^5$cells were washed twice in 500 µL of 0.1% (w/v) sodium azide dissolved in PBS and supplemented with 1% (v/v) heat-inactivated FBS (NaN_3-FBS buffer; FACS buffer) before incubation with FITC-conjugated anti-human CD44 mouse monoclonal antibody (Cat number 555478, BD Biosciences Pharmingen, USA), PerCP-Cy5.5 (Thy1)-conjugated anti-CD90 mouse monoclonal antibody (Cat number 328117, BioLegend, San Diego, CA, USA), PE-conjugated anti-human CD105 mouse monoclonal antibody (Cat number 560839, BD Biosciences Pharmingen, USA), PerCP-conjugated anti-CD45 mouse monoclonal antibody (Cat number 21810455, Immuno Tools, USA) as a isotype controls for 30 min at room temperature. Stained cells were analyzed on a FACS Calibur (Becton Dickinson, Mountain View, CA, USA) using the CellQuest software (BD Bioscience). Cells were gated on FSC and SSC in order to exclude debris and cell aggregates. Green and red fluorescence emission, FITC and PE, respectively, were detected on a logarithmic scale of four decades of log. Data from donors were collected under identical parameters and analyzed by collecting 25,000 events. Three independent experiments were performed for each sample.

Table 3.1 Specifications for flow cytometry antibodies

Antibodies	Isotype	Cat no.	Company
CD44	IgG1	555478	BD Pharmingen
CD45	IgG1	21810455	Immuno Tool
CD90	IgG1	328117	BioLegend
CD105	IgG1	560839	BD Pharmingen
Cyto18	IgG1	ab668	Abcam

The table shows the clone, Isotype and the origin of flow cytometry antibodies.

Osteogenic differentiation assay

For differentiation experiment, the protocol was performed according to previous reports (83). Briefly, HMBCs from the third passage were seeded in 24-well plate at density 5×10^4 cells in 1 mL bone culture medium per well and cultured overnight. To observe osteogenic differentiation, bone culture medium was changed to osteogenic medium containing DMEM (Gibco®) supplemented with 15% FBS (Hyclone®), 50 µg/mL L-ascorbic acid (Sigma®, St. Louis, MO, USA), 100 nM dexamethasone (Sigma®, St. Louis, MO, USA) and 10 mM β-glycerophosphate (Sigma®, St. Louis, MO, USA). Cells were continued in culture and fresh medium was changed every 2-3 days. Cells cultured in osteogenic medium were used in alkaline phosphatase activity assay, Histochemical staining for alkaline phosphatase

and Mineralization assay. Cells cultured in bone culture medium were added as a control.

Alkaline phosphatase (ALP) activity assay

To determine the osteogenic differentiation, ALP activity of HMBCs were measured. In this reaction, ALP catalyzes the hydrolysis of a colorless organic phosphate ester; p-nitrophenyl phosphate (PNPP, Invitrogen, Frederick, MD, USA) as a substrate. The cells were extracted in alkaline extracted buffer and incubated at -20 ^{o}C for 30 minutes. The cell lysate were incubated with a substrate solution containing PNPP 2 mg/ml, 0.1 M 2-amino-2-methyl-1-propanol and 2 mM $MgCl_2$ for 30 minutes at 37 ^{o}C. The reaction was stopped by the addition of 0.1 M NaOH. The absorbance of product was measured using a microplate reader (ELx800; BioTek) at a wavelength of 410 nm. The total cellular protein level will be determined using bicinchoninic acid; BCA Protein Assay (Pierce, Thermo Scientific, Rockford, IL, USA), is a detergent-compatible formulation based on BCA for the colorimetric detection and quantitation of total protein. This method combines the well-known reduction of Cu^{+2} to Cu^{+1} by protein in an alkaline medium (Biuret reaction) with the highly sensitive and selective colorimetric detection of the Cu^{+1} using a unique reagent containing bicinchoninic acid. The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with Cu^{+1}. The cells are treated in the same manner as in the ALP assay until the cells are frozen. After freezing and thawing cycle, BCA solution was added to the samples. The samples will be incubated at 37 ^{o}C for 15 min. The absorbance of the medium will be measured at 562 nm with the microplate reader. The amount of the total protein will be calculated against a

standard curve. The value of ALP activity will be normalized to the total protein level of the cells (U/µg)

Histochemical staining for ALP

After 7 days in osteogenic medium, HMBCs were stained for ALP by using TRACP&ALP double-stain kit (Takara Bio Inc, Otsu, Shiga, Japan). Briefly, culture media were removed and cells were washed once with PBS. Subsequently, cells were fixed for 5 min at room temperature in a 250 µL fixation solution containing citrate buffer (pH 5.4), 45% acetone and 10% methanol. Cells were carefully washed once with PBS, mixed substrate was added to the cells, then cells were incubated at 37^{o}C in a humidified atmosphere of 5% CO_2 for 30 min. The staining solution was removed and washed three times with distilled water to terminate the reaction. The presence of ALP was examined under light microscopy. Photographs were taken by Fujifilm camera; FinePix S100FS.

Alizarin Red S Staining

Calcium mineral deposition was stained using Alizarin Red S staining solution. Two grams of Alizarin Red S (Sigma-Aldrich, MO, USA) were dissolved in 100 ml distilled water, mixed and adjusted to a pH of 4.1-4.3 with 0.1% ammonium hydroxide to prepare the Alizarin Red S staining solution. HMBCs were grown in monolayer for 14-21 days in growth medium (GM) and osteogenic medium (OM). The cells were washed with PBS and were fixed with cold methanol (Merck KGaA, Darmstadt, Germany) for 10 min, washed with distilled water and stained with 1% Alizarin Red S solution for 3 min at room temperature. The Alizarin Red S staining

solution was carefully aspirated and the cell monolayer was washed with distilled water. To quantify the amount of calcium deposition, the staining was eluted with 10% cetylpyridinium chloride monohydrate in 10 mM sodium phosphate at room temperature for 15 min, and the absorbance was read at 570 nm.

Von Kossa Staining

Phosphate mineral deposition was stained using Von Kossa staining solution. Five grams of silver nitrate ($AgNO_{3;}$ BDH Laboratory Supplies, Poole, UK) were dissolved in 100 mL distilled water. HMBCs were grown in monolayer for 14-21 days in GM and OM. The cells were washed once in cold PBS. Fixation was carried out for 10 min in 4% paraformaldehyde in PBS at room temperature and rinsed with distilled water. Distilled water was removed and cells were covered with 5% silver nitrate for 45 min under a UV light. After UV exposure, to stop the silver nitrate development, the silver nitrate solution was removed and the cell monolayer was washed three times with distilled water and treated with a 5% solution of sodium thiosulfate. Following another water wash and allowed to air-dry overnight. Phosphate deposits were visualized as dark staining patches using a light microscope.

RANKL regulation by epithelial-derived mediator

HMBCs were plated in 6-well plates at a density of 3×10^5 cells/well and cultured overnight. The constitutively-released or *Pg*LPS-stimulated epithelial supernatant from NOK-SI cells were prepared as described above. The HMBCs were incubated in serum-free bone culture medium for 4 h, and then stimulated with 0.1 μM PGE_2 and epithelial supernatant (50% volume) for 24 h. To identify if the effect of the epithelial-derived mediators was due to IFN-γ, the epithelial supernatant was

incubated with 0.1, 1, or 10 µg/mL of an anti-IFN-γ antibody (BioLegend, San Diego, CA, USA) 30 min before adding the supernatant to the HMBCs. In some experiments, HMBCs were incubated with 0.1, 1, or 10 ng/mL recombinant IFN-γ (BioLegend), instead of epithelial supernatant, for 24 h. Subsequently, HMBCs RNA, intracellular and secreted protein were collected.

mRNA Analysis

RNA Isolation

When needed, the total cellular RNA was extracted with Isol-RNA lysis reagent (S Prime Inc., Gaithersburg, MD, USA) according to the manufacturer's instructions. First, 1 ml of Isol-RNA lysis reagent was added to each well. After scraping the cell layer and incubating for 5 min, Isol-RNA lysis reagent was transferred into a 1.5 ml tube. Next, 200 µl of chloroform was added, after which the mixture was shaken vigorously and centrifuged at 14,000 rpm for 15 min. The aqueous phase was collected and 500 µl Isopropanol was added to precipitate the RNA. After centrifugation, the RNA pellet was dissolved in nuclease-free water and the amount of RNA was determined at an absorption of 260/280 nm using a micro-volume UV-Vis spectrophotometer for nucleic acid and protein quantitation (NanoDrop2000; Thermo Scientific, Wilmington, DE, USA).

Reverse transcription (RT) and Polymerase Chain reaction (PCR)

For each RNA sample, One microgram was converted to cDNA using the Improm-II reverse transcriptase system (Promega, Madison, WI, USA) for 1 h 30 min at 42ºC and 2 min at 99ºC. Subsequent to reverse transcription, PCR was performed.

Primers were prepared following the reported sequences from GenBank. Table 3.2 and Table 3.3 show the sequences of PCR primers that were used in this study.

Semi-quantitative polymerase-chain reaction (PCR) was performed in a DNA thermal cycler (Biometra, Gottingen, Germany) using Taq polymerase (Taq polymerase enzyme kit, Invitrogen, Federick, MD, USA) with a PCR mix volume of 25 µl. The amplification profile was one cycle at 94ºC for 45 s, annealing at 60ºC for 1 min, and extended at 72ºC for 1 min 30 s, followed by one extension cycle at 72ºC for 10 min. The amplified DNA was separated by electrophoresis on a 1.8% (w/v) agarose gel and visualized using ethidium bromide fluorostaining (Sigma-Aldrich, St. Louis, MO, USA). The band density was measured using Bio1D software analysis (VilberLourmat, Marne la Vallée, France).

Quantitative polymerase chain reaction (qPCR) was performed using the SYBR green detection system (FastStart Essential DNA Green Master kit; Roche Diagnostic, Indianapolis, IN, USA) on a MiniOpticon™ Real–Time PCR Detection System detection system (Bio-Rad, Singapore). The sequences of the primers of the genes analyzed in both PCR types are shown in Table 1. The qPCR was performed at 95°C for 1 min followed by 40 amplification cycles consisting of 95°C for 45 s, 60°C for 60 s, 72°C for 90 s, and one extension cycle at 72ºC for 10 min. The reactions were performed in duplicate, and the average values were used for gene expression analysis. GAPDH mRNA expression was used as an internal control. The PCR products were stained with ethidium bromide on a 1.8% agarose gel to confirm the specific product size.

Table 3.2 Sequences of semi-quantitative PCR primers (following the reported sequences from GenBank)

Gene	Primer Sequence 5′→ 3′	Accession No.	bp
ALP	F: CGA GAT ACA AGC ACT CCC ACT TC R: CTG TTC AGC TCG TAC TGC ATG TC	NM 000478.3	121
BSP	F: GAT GAA GAC TCT GAG GCT GAG A R: TTG ACG CCC GTG TAT TCG TA	NM 004967.3	514
COL-I	F: GCA AAG AAG GCG GCA AA R: CTC ACC ACG ATC ACC ACT CT	NM000088.3	500
GAPDH	F: TGA AGG TCG GAG TCA ACG GAT R: TCA CAC CCA TGA CGA ACA TGG	NM 002046.3	396
RANKL	F: CCA GCA TCA AAA TCC CAA GT R: CCC CTT CAG ATG ATC CTT C	NM 033012.2	602
RUNX2	F: ATG ATG ACA CTG CCA CCT CTG A R: GGC TGG ATA GTG CAT TCG TG	NM 001024630.3	167
OCN	F: ATG AGA GCC CTC ACA CTC CTC R: GCC GTA GAA GCG CCG ATA GGC	NM 199173.2	293
OPG	F: TCA AGC AGG AGT GCA ATC G R: AGA ATG CCT CCT CAC ACA GG	NM 002546.3	341

Gene	Primer Sequence 5′→ 3′	Accession No.	bp
OPN	F: AGT ACC CTG ATG CTA CAG ACG R: CAA CCAGCA TAT CTT CAT GGC TG	NM 001040060.1	320
M-CSF	F: CTA AGC TGG ACG CAC AGA ACA R: TCT CAG GCT GCA CAC CTT	NM 000757.3	538
IL-1β	F: GGA GCA ACA AGT GGT GTT CT R: AAA GTC CAG GCT ATA GCC GT	NM 000576.2	458
IL-6	F: CCT GAA CCT TCC AAA GAT GGC R: CTG ACC AGA AGA AGG AAT GCC	NM 000600.2	423
IL-8	F: CGA TGT CAG TGC ATA AAG ACA R: TGA ATT CTC AGC CCT CTT CAA AAA	NM 000584.3	200
IL-12	F: GGA TGC CCC TGG AGA AAT R: GCA GGA GCG AAT GGC TTA	NM 002187.2	191
IFN-γ	F: CTA GGC AGC CAA CCT AAG CA R: CAG GGT CAC CTG ACA CAT TC	NM 000619.2	180
MCP-1	F: GCC TCC CAG CAT GAA AGT CTC R: CTT GGG TTC TGG AGT GAG TG	NM 002982.3	326
TLR-2	F: TGC ATTT CCC AAG ACA CTG GA R: TCA ATA CCA CAG GCC ATG GA	NM 003264.3	495
TLR-4	F: ACC AGA GTT TTC CTG CAA TGG A R: AAA CTG CCA GGT CTG AGC AA	NM 138554.3	517

Gene	Primer Sequence 5′→ 3′	Accession No.	bp
E-cad	F: CAA CAG CTG TGA TCA CAG TCA R: CAG TTG GCA GTG TCT CTC CA	NM 004360.3	520
EP1	F: CAT CCT ACT GCG CCA GGC CG R: CCA GGC GCT CGG TGT TAG GC	NM 000955.2	106
EP2	F: CGC AGC TGC AGC TGT ACG R: GAT GGC AAA GAC CCA AGG	NM 000956.3	368
EP3	F: GAG CAC TGC AAG ACA CAC AC R: GAT CTC CAT GGG TAT TAC TGA CAA	NM 198719.1	398
EP4	F: CCT GCA GCA CGT CGG ATG CT R: GGG CCT CTG CTG TGT GCC AA	NM 000958.2	166

Table 3.3 Sequences of quantitative PCR primers (reported from GenBank)

Gene	Primer Sequence 5′→ 3′	Accession No.	bp
GAPDH	F: CAC TGC CAA CGT GTC AGT GGT G R: GTA GCC CAG GAT GCC CTT GAG	NM 002046.4	121
RANKL	F: ATA CCC TGA TGA AAG GAG GA R: GGG GCT CAA TCT ATA TCT CG	NM 033012.3	202
OPG	F: AGC TGC AGT ACG TCA AGC AGG A R: TTT GCA AAC TGT ATT TCG CTC TG	NM 002546.3	164
M-CSF	F: AGT CTG TCT TCC ACC TGC TG R: TTC CAC CTG TCT GTC ATC CT	NM 000757.5	170
IL-1β	F: GTC ATT CGC TCC CAC ATT CT R: ACT TCT TGC CCC CTT TGA AT	NM 000576.2	105
IL-6	F: ATG CAA TAA CCA CCC CTG AC R: AAA GCT GCG CAG AAT GAG AT	NM 000600.3	110
IFN-γ	F: CTA GGC AGC CAA CTT AAG CA R: CAG GGT CAC CTG ACA CAT TC	NM 000619.2	180
MCP-1	F: CAT TGT GGC CAA GGA GAT CTG R: CTT CGG AGT TTG GGT TTG CTT	NM 002982.3	91
COL-I	F: GTG CTA AAG GTG CCA ATG GT R: ACC AGG TTC ACC GCT GTT AC	NM 000088.3	128

Western blot

Protein samples (40 µg) were extracted from HMBCs using radioimmunoprecipitation assay (RIPA) buffer containing 1 mM PMSF (Merck Millipore, Darmstadt, Germany). The protein concentration of each sample was measured using the BCA Protein Assay Kit (Pierce, Rockford, IL, USA). The protein samples were fractionated by SDS-PAGE (12.5%). The separated proteins were blot transferred onto a nitrocellulose membrane. The membrane was blocked using 1% Tween 20 and 5% non-fat dry milk in Tris-buffered saline (TBS) at room temperature for 1 h and washed three times with TBS-Tween 20 for 5 min. The membrane was incubated overnight at 4°C in primary antibody: 2 µg/mL RANKL (Peprotech, Rocky Hill, NJ, USA) or β-actin (Cell Signaling, Danvers, MA, USA) (1:1000) as an internal control. The membrane was then washed three times with TBS-Tween 20 for 5 min. The membrane was incubated with secondary antibody: horseradish peroxidase-conjugated anti-rabbit IgG (Enzo, Farmingdale, NY, USA) (1:2000) at room temperature for 1 h and staining was detected using the enhanced chemiluminescence (ECL) Western Blot System (Thermoscientific, Rockford, IL, USA).

Enzyme-linked immunosorbent assays (ELISA)

Development of sRANKL, OPG and IFN-γ ELISA

Sandwich ELISAs were used to detect IFN-γ as a protocol according to manuals from manufacturing. Briefly, 96-well ELISA microplates with high protein binding properties (NUNC Maxisorp^{TM}) were coated overnight with Capture antibody (Cat No. 430104, BioLegend, San Diego, CA, USA) 1:200 in 1X coating buffer. After

washing with PBS added 0.05% Tween-20 and blocking non-specific binding with 1X Assay Diluent A for 1 h with shaking at 200 rpm on a plate shaker, plates were again washed, samples and IFN-γ standard (rhIFN-γ ; Cat No. 79103) were added. Samples were incubated for 2 h and then washed in PBS added 0.05% Tween-20 and the detection antibody added at a 1:200 dilution in Assay Diluent A and incubated for 1 h. After washing, the bound antibody was detected with 1:200 diluted Avidin-HRP solution that was incubated for 30 min. A final wash was then performed. Freshly mixed Tetramethylbenzidine (TMB) solution was added to each well and incubated in the dark for 20 min. Color development was stopped with 2 N H_2SO_4,(Stop solution) and the resulting absorbance was read at 450 nm on a FL600 Microplate fluorescence reader (BioTek). To correct for the background absorbance of the plate, a second reading at 570 nm was subtracted. Concentrations were determined by 4-parameter standard curve-fitting procedures using KC4 Kineticalc 2.7 for windows.

IL-1β, IL-6, IL-8, MCP-1 and TNF-α ELISA

The amount of secreted protein (IL-1β, IL-6, IL-8, MCP-1 and TNF-α) in culture medium was determined by ELISA methods according to manuals of ELISA kit protocol (Quantikine® Human Immunoassay; R&D System, Minneapolis, MN, USA). Briefly, a conditioned medium was added to plates pre-coated with antibodies and was incubated at room temperature for 2 h. Following a wash to remove excess conjugate and unbound sample, a substrate solution was added to the wells to determine the bound enzyme activity for 20 min. The color development was stopped and the absorbance was measured by using microplate reader at wavelength 450 nm.

The intensity of the color is proportional to the concentration of cytokines or chemokines in the samples. A standard curve was generated by plotting the mean absorbance for each cytokine's standard on the Y-axis against the concentration on the X-axis and a linear equation was determined. Data within the range of the standard curves were used to calculate the concentration of each protein. The concentration based on the standard curve was adjusted by the dilution factor.

Nuclei Protein Isolation

Nuclei protein was extracted with Nuclei EZ Prep Nuclei Isolation kit (Sigma Aldrich, Saint Louis, Missouri, USA) according to the manufacturer's instructions. Briefly, NOK-SI cells were washed with ice-cold PBS, and then ice-cold nuclei EZ lysis buffer was added. The cells were harvested by scraping, and the cell lysate was transferred into a separate tube Vortex briefly and set on ice for 5 min and then the nuclei was collected by centrifugation at 5000 rpm for 5 min at 4 ^{o}C. The clear supernatant was carefully aspirated from each tube and the nuclei pellet was set on ice and resuspended with cold Nuclei EZ lysis buffer. Finally, the nuclei pellet was resuspended again in ice cold Nuclei EZ storage buffer. Nuclei protein was used immediately or stored at 80 ^{o}C.

Detection of Transcription Factor NFκB p50 and p65 activation by ELISA

The active form of NFκB was determined using NFκB p50 and p65 ELISA kit (Thermo Scientific, Rockford, IL, USA) followed the manufacturer's instruction. Briefly, The NOK-SI nuclear extract was added to plates pre-coated with NFκB

consensus duplex, incubated with primary antibody specific for NFκB p50 or p65, and then incubated with a secondary HRP-conjugated antibody. A chemiluminescent substrate was added and the signal was detected using a luminometer (Synergy^{TM} H1; BioTek, Winooski, VT, USA).

Resorption activity analysis

To observe THP-1 osteoclastic activity, cells were cultured in 24-well plates at a density of 2.5×10^5 cells/well in RPMI-1640 complete medium for 24 h, and the cells were then incubated with RPMI-1640 containing 40 ng/mL vitamin D_3, 40 ng/mL RANKL and 25 ng/mL M-CSF for 21 days. The culture medium was replaced with fresh RPMI-1640 complete medium supplemented with vitamin D_3, RANKL, and M-CSF every 3 days. In some wells, THP-1 cells were co-cultured with untreated, PGE_2-stimulated, or *Pg*LPS epithelial supernatant and PGE_2 stimulated HMBCs using a transwell cell culture system (Corning Inc, New York, USA). On day 21, the THP-1 cells were harvested, and total RNA was extracted for RT-PCR. In some experiments, the co-cultured THP-1 cells were evaluated for their osteoclastic activity on Corning® osteo assay surfaces (Corning Inc). To visualize the resorption pits, the medium was removed and 300 µL of bleaching solution was added for 5 min at room temperature. The wells were washed twice with 500 µL of distilled water, and allowed to air dry for 5 h. Under an inverted phase contrast microscope, pit formation appeared as individual or multiple clusters at the bottom of the wells. Three visual fields were randomly selected from each well, and the pit numbers were counted. For SEM sample preparation, the bottom of the wells were cut using a steel round bur to make the samples the appropriate size to fit in the specimen chamber, rinsed with distilled

water, and dried at room temperature. The samples received an ultrathin gold coating. The resorption lacunae morphology was observed using a SEM (Quanta 250, Fei Company, Eindhoven, Netherland).

Statistical analyses

HMBCs derived from three to five individuals were used in repeated independent experiments, and each experiment was performed in triplicate, except for the quantitative PCR assays that were performed in duplicate. Means (±standard deviation) representing the summative data from at least 3 independent experiments are shown. All statistical analyses were performed using SPSS software version 20.0 (SPSS Inc, Chicago, IL, USA). One-way ANOVA followed by Bonferroni post-hoc analysis was used and a significant difference between control and experimental groups was determined at $p < 0.05$.

CHAPTER IV

RESULTS

PART I: Characteristics of human mandibular-derived bone cells (HMBCs) and normal oral keratinocytes (NOK-SI)

We began by characterizing the cells isolated from the human mandibular alveolar ridge (Fig. 4.1) and the normal oral keratinocytes (NOK-SI) *in vitro*. The HMBCs were analyzed by flow cytometry for surface marker expression. Immunophenotypically, the cells were positive for the mesenchymal stromal cell surface markers CD44, CD90 and CD105 (Fig. 4.2A), and negative for the hematopoietic surface marker CD45 (data not shown). To evaluate the HMBCs osteogenic differentiation capability, the mRNA expression of the osteogenic markers alkaline phosphatase (ALP), bone sialoprotein (BSP), collagen type I (Col I), osteocalcin (OC), osteopontin (OPN), and Runt-related transcription factor 2 (RUNX2) were determined by RT-PCR. HMBCs cultured in osteogenic medium for 7 days upregulated their expression of ALP, BSP, OC, and OPN, however, Col I and RUNX2 expression was not affected (Fig. 4.2B). We used ALP staining and calcification assays to confirm the osteoblastic nature of the HMBCs. HMBCs cultured in osteogenic medium demonstrated increased ALP staining compared with control cultures in bone culture medium after 7 days (Fig. 4.2C). HMBCs cultured in osteogenic medium had increased calcium mineral deposition after 21 days compared with control cultures (Fig. 4.2C). The HMBCs also had increased phosphate mineral deposition as shown by Von Kossa staining when cultured in osteogenic medium compared with the control group (Fig. 4.2C).

NOK-SI cells, derived from the retro-molar area of the oral cavity, and retained an epithelial morphology (Fig. 4.3A). *Pg*LPS stimulation significantly upregulated TLR-2 mRNA (p=0.038), while TLR-4 mRNA was slightly increased (Fig. 4.3B). The cells were analyzed for the expression of cytokeratin 18, a typical epithelial cell marker, using flow cytometry (Fig. 4.3C). We also found that the NFκB transcription factors, p50 (p=0.004) and p65 (p=0.001), were significantly increased in the nuclear fraction when the NOK-SI cells were cultured with 2.5μg/mL *Pg*LPS for 24 h (Fig. 4.3D).

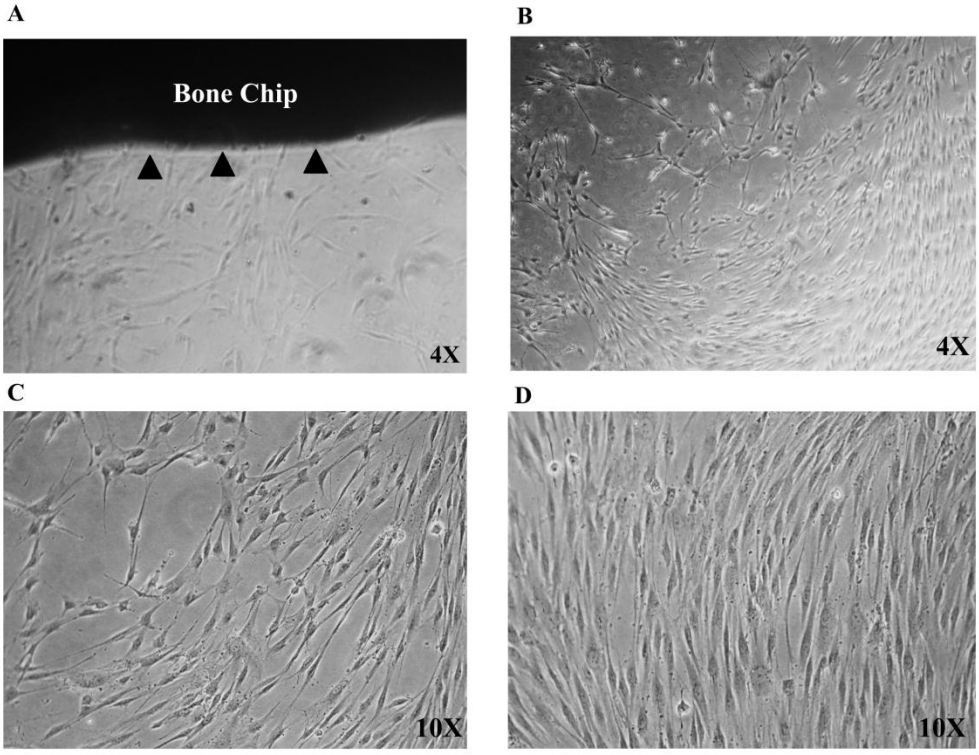


Figure 4.1 Phase-contrast photomicrographs of HMBCs growing out from mandibular-derived bone explant after 7 days in culture (A), confluent monolayer of HMBCs after approximately 3-4 weeks of explant culture (B), and appearance of passage 1 HMBCs 3 days and 5 days after subcultured (C, D), respectively. Photomicrophaphs are represented for 3 individual explant culture, magnification 4X and 10X

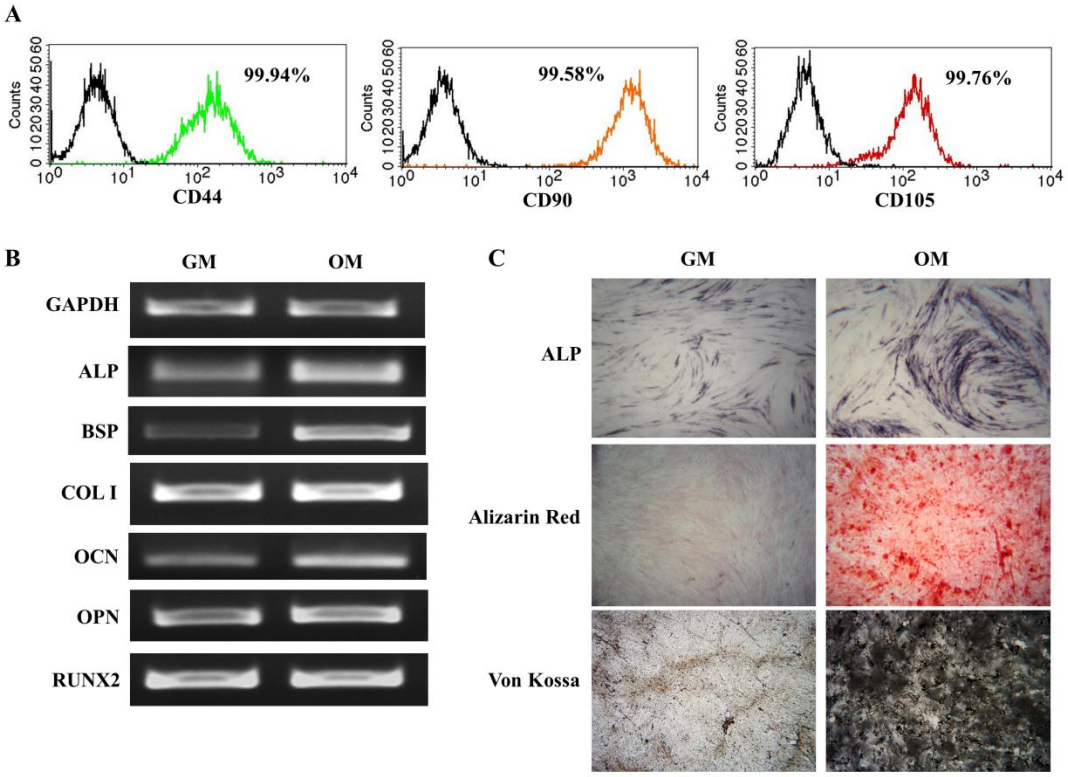


Figure 4.2 HMBCs characterization. The bone cells derived from the human mandibular alveolar ridge were characterized to confirm their osteogenic characteristics. (A) Expression of the surface markers CD44, CD90, and CD105, as analyzed by flow cytometry. (B) Osteogenic gene expressions in HMBCs cultured in growth medium (GM), or osteogenic medium (OM) for 7 days by RT-PCR. (C) Osteogenic differentiation and mineralization on day21 evaluated by ALP staining, Alizarin Red S staining and Von Kossa staining.

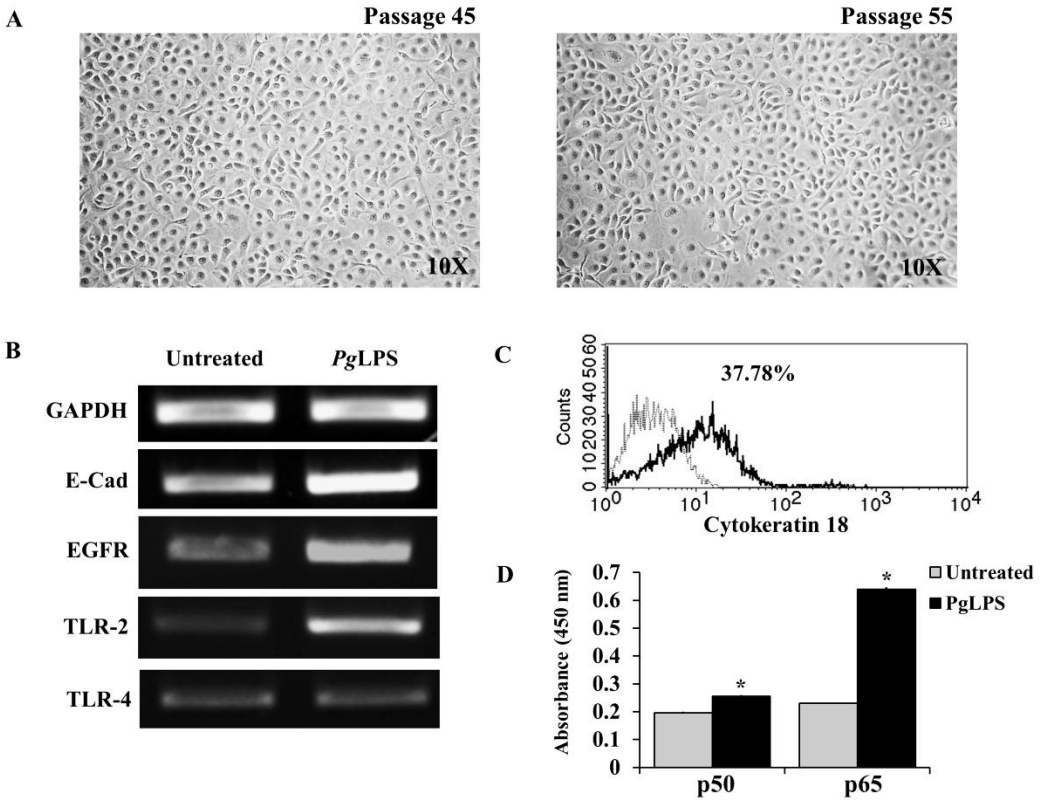


Figure 4.3 NOK-SI cells characterization. (A) NOK-SI cell morphology visualized under the light microscope. NOK-SI cells are incubated with 2.5µg/mL *Pg*LPS for 24 h demonstrate (B) E-Cadherin, EGFR, TLR-2 and TLR-4 mRNA are determined by RT-PCR. (C) The epithelial cell marker cytokeratin 18, analyzed by flow cytometry. (D) NFκB transcription factors, p50 and p65, in the nuclear fraction by ELISA All experiments were performed in triplicate. *indicates a significant difference compared with the untreated group using paired t-test.

PART II:

Epithelial supernatant suppresses RANKL expression in HMBCs

The basal level of RANKL mRNA expression in HMBCs was relatively low, and the results from 10 individuals showed that the RANKL basal level varied among individuals (data not shown). To imitate periodontal tissue inflammation, HMBCs were treated with 0, 0.1, 1, or 10 μM PGE_2 for 24 h, and the RANKL and OPG mRNA expression was analyzed by qRT-PCR. We found increased RANKL mRNA expression (Fig. 4.4A), however, the OPG mRNA level significantly decreased (Fig. 4.4B). The amount of soluble RANKL (sRANKL) (Fig. 4.4C), and OPG protein (Fig. 4.4D) in the culture medium were determined by ELISA. One-way ANOVA followed by Bonferroni post-hoc analysis revealed a significantly increased fold change in RANKL mRNA and soluble RANKL protein by 0.1, 1, and 10 μM PGE_2 treatment compared with untreated control cells ($p<0.05$), however the decrease in OPG protein was not significant. The lowest PGE_2 concentration, 0.1 μM, which significantly up-regulated RANKL levels in HMBCs, was used in subsequent experiments.

The effect of epithelial supernatant on HMBCs was then investigated. The constitutively-released or *Pg*LPS-stimulated epithelial supernatant from the NOK-SI cells were collected. HMBCs were incubated with 0.1 μM PGE_2 and a 50% volume of the epithelial supernatant. After 24 h, PGE_2-stimulated bone cells expressed RANKL mRNA more than 10-fold higher than non-stimulated cells, and (Fig. 4.6A). RANKL expression decreased by approximately 50% when incubated with conditioned medium from untreated epithelial cells and approximately 75% when incubated with conditioned medium from *Pg*LPS-stimulated epithelial cells. These results were consistently found in experiments using cells from 3 different donors. As in our

earlier experiments, incubating HMBCs with 0.1 μM PGE_2 for 24 h significantly decreased OPG mRNA expression. The OPG expression levels were similar after the addition of conditioned medium from epithelial cells (Fig. 4.6B). RANKL protein was detected as both membrane-bound (Fig. 4.6C) and soluble RANKL (Fig. 4.6D) using western blot analysis and ELISA, respectively. The results revealed that epithelial supernatant suppressed both RANKL isoforms (Fig. 4.6C, D), but not OPG protein, from HMBCs. One-way ANOVA followed by Bonferroni analysis showed that the RANKL mRNA expression ($p<0.001$) and sRANKL protein ($p<0.001$) in cells treated with *Pg*LPS-activated epithelial supernatant was significantly decreased when compared with KCM. Moreover, one-way ANOVA followed by Bonferroni analysis showed that RANKL mRNA expression ($p=0.039$) and sRANKL protein ($p=0.001$), was significantly decreased in HMBCs treated with constitutively-released epithelial supernatant when compared with cells cultured in KCM alone. Our observation that the epithelial supernatant from *Pg*LPS-stimulated cells suppressed the RANKL mRNA and protein levels more effectively compared with constitutively-released conditioned medium from untreated cells, led us to evaluate the effect *Pg*LPS had on epithelial cell cytokine production.

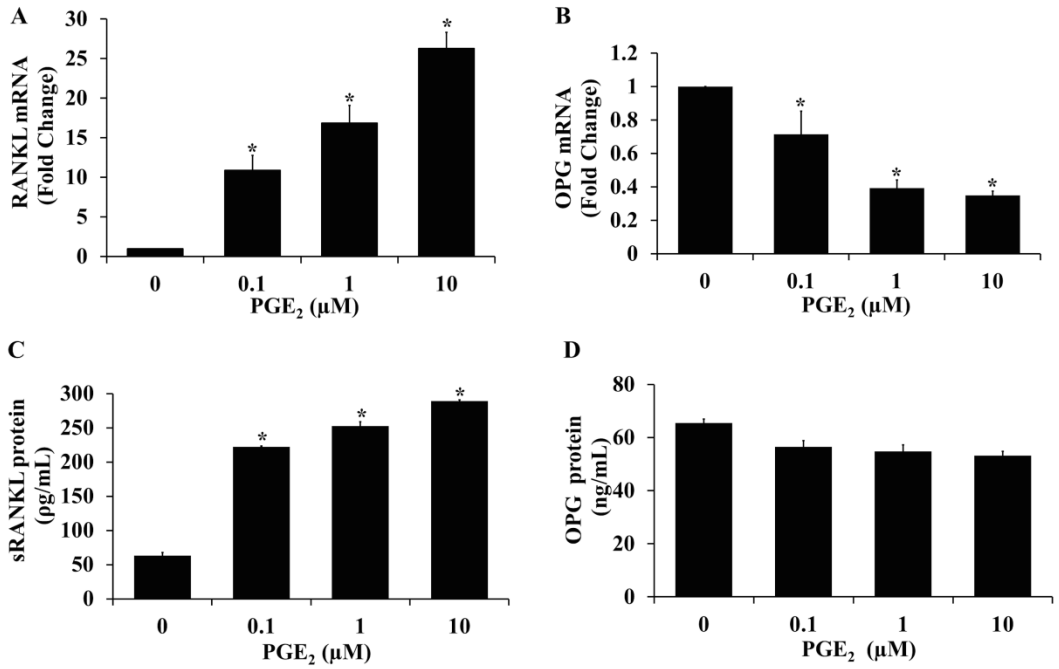


Figure 4.4 PGE_2 induced RANKL expression in HMBCs. HMBCs were incubated with 0.1, 1, or 10 μM PGE_2 for 24 h. (A) RANKL, and (B) OPG mRNA expression as shown by qRT-PCR. (C) Soluble RANKL protein (sRANKL) and (D) OPG protein released in culture medium are demonstrated by ELISA. HMBCs cultured in serum-free growth medium served as a control. All experiments were performed in triplicate. * indicates a significant between control and PGE_2 treated cell groups ($p<0.05$).

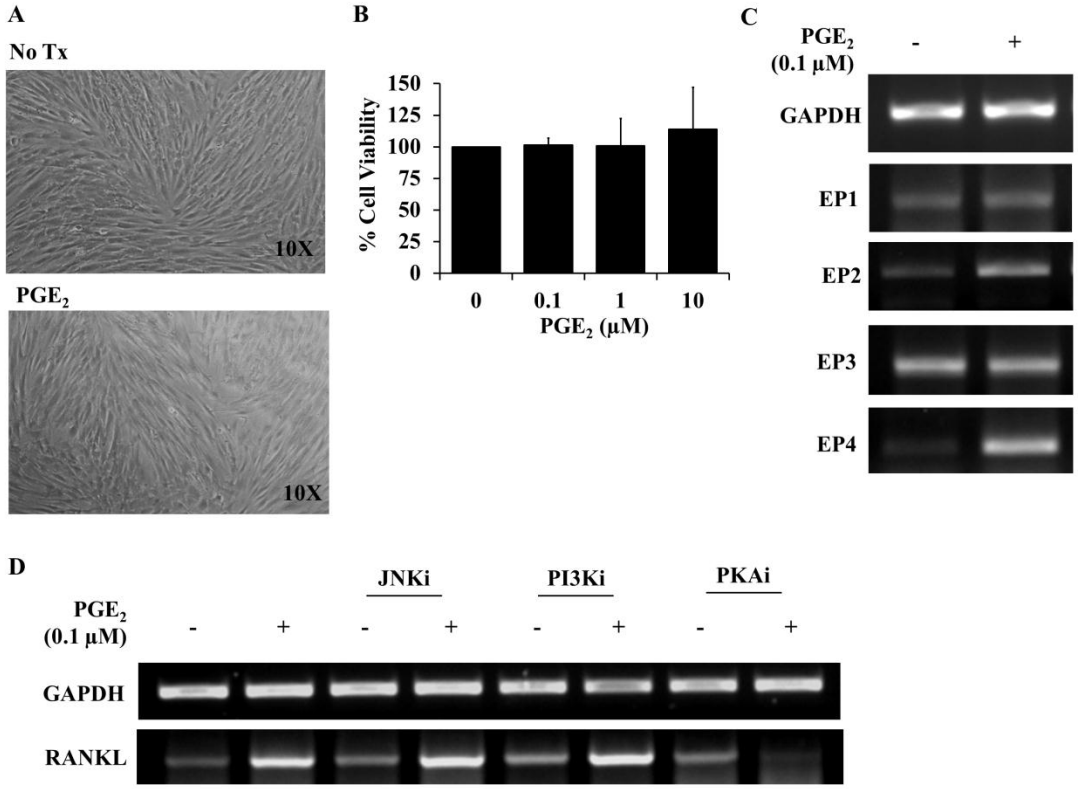


Figure 4.5 PGE_2 stimulation for 24 h in HMBCs (A) Morphological analysis of cells was evaluated using light microscope (10X) upon cultured cells with and without PGE_2 treatment. (B) Cell viability tested by MTT assay. (C) EP1-EP4 mRNA expression induced by PGE_2 in HMBCs. (D) Molecular mechanism of PGE_2-induced RANKL in HMBCs.

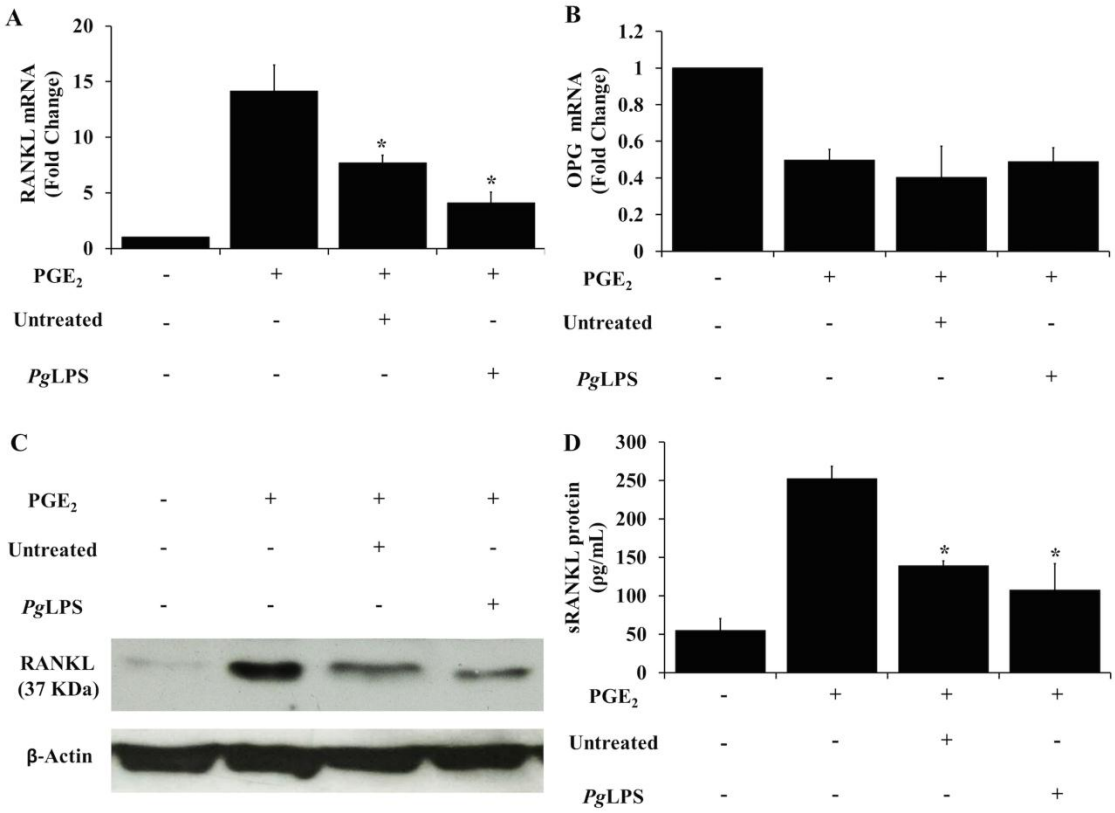


Figure 4.6 Epithelial supernatant suppressed PGE_2-induced RANKL expression by HMBCs. HMBCs were simultaneously stimulated with 0.1 μM PGE_2 and supernatant from epithelial cells stimulated by *P. gingivalis* LPS (*Pg*LPS), or constitutively-released supernatant from untreated epithelial cells (untreated), for 24 h. A significant decrease in (A) RANKL and (B) OPG mRNA in HMBCs after epithelial supernatant treatment was determined by qRT-PCR. (C) RANKL protein assayed by western blot decreased with epithelial supernatant treatment. β-actin as internal control. All experiments were performed in triplicate. * indicates a significant difference using one-way ANOVA followed by Bonferroni analysis ($p < 0.05$) comparing HMBCs treated with keratinocyte complete medium to HMBCs treated with the supernatant from untreated NOK-SI and the supernatant from *Pg*LPS-stimulated NOK-SI.

Epithelial cells release an increased amount of inflammatory mediators after *Pg*LPS stimulation

The constitutively-released or *Pg*LPS-stimulated epithelial mediators were investigated. Visualization and densitometry of the RT-PCR results revealed the mRNA expression of inflammatory mediators, including IL-1β, IL-6, IL-8, TNF-α, and IFN-γ, in untreated or *Pg*LPS-stimulated NOK-SI cells (Fig. 4.8A and 4.8B). *Pg*LPS stimulation significantly increased IL-1β ($p<0.001$), IL-6 ($p=0.001$), IL-8 ($p =0.006$), and IFN-γ ($p<0.001$) mRNA levels (the slight change in TNF-α mRNA was not significant), as shown by paired t-test analysis. We also found that IL-12 and IL-13 mRNA levels were unchanged and IL-4 and IL-10 expression was not detectable (data not shown). Inflammatory mediators were detected in the epithelial supernatant using ELISA (Fig. 4.8C). However, only IL-1β ($p=0.019$) and IFN-γ ($p=0.009$) showed significantly increased protein levels after *Pg*LPS stimulation.

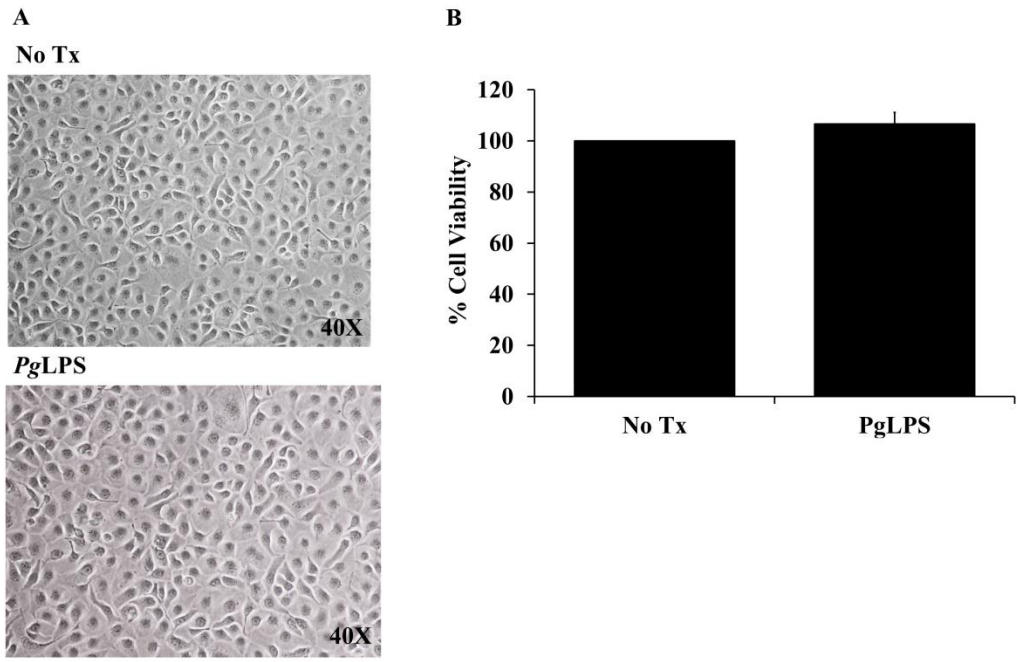


Figure 4.7 *Pg*LPS stimulation for 24 h in NOK-SI cells (A) Morphological analysis of cells was evaluated using light microscope (40X) upon cultured cells with and without *Pg*LPS treatment. (B) Cell viability tested by MTT assay**.**

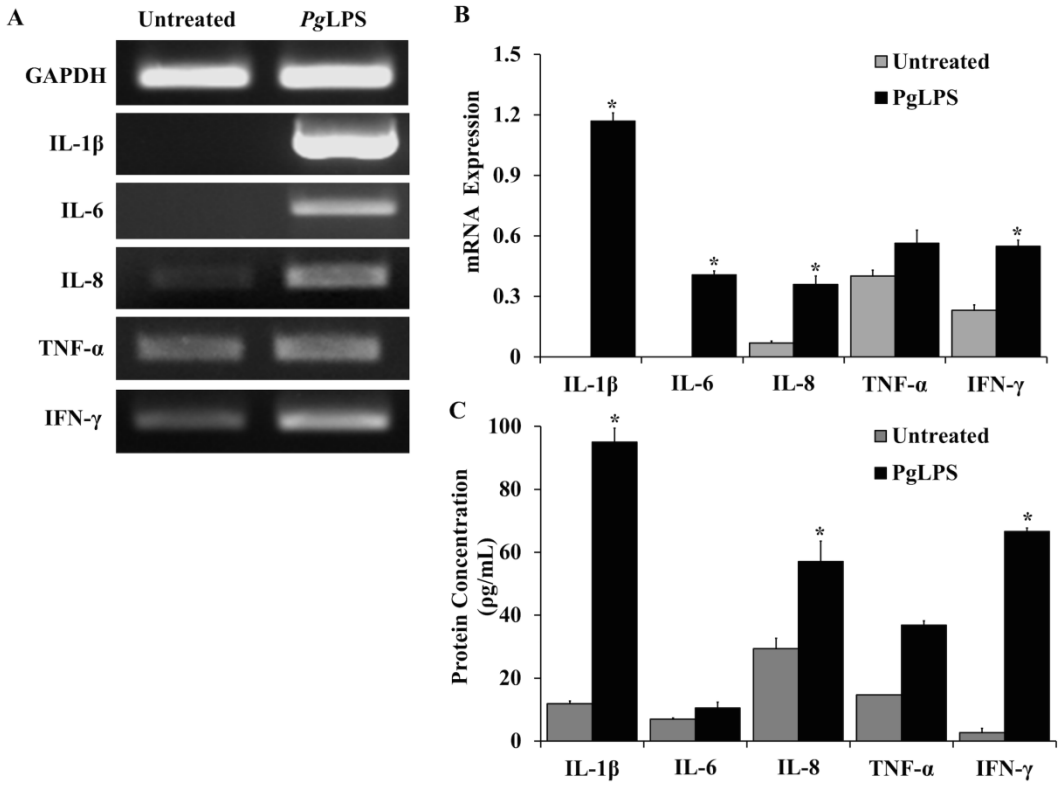


Figure 4.8 *Pg*LPS upregulated IL-1β, IL-8 and IFN-γ expression by oral epithelial cells. NOK-SI cells were challenged with 2.5 µg/mL *Pg*LPS for 24 h, and total RNA was harvested for RT-PCR assay. (A) The PCR bands represent the products of IL-1β, IL-6, IL-8 and IFN-γ mRNA. (B) The band densities analyzed using Bio1D software. (C) Protein release in NOK-SI culture supernatant assayed by ELISA. All experiments were performed in triplicate. * indicates a significant difference significance using t-test analysis ($p < 0.05$) when comparing the supernatant from untreated cells and the supernatant from *Pg*LPS-stimulated cells.

Neutralizing antibody against human IFN-γ recovers the level of RANKL expression in HMBCs

Based on our PCR and ELISA results, we hypothesized that IFN-γ might be the cytokine in the epithelial cell conditioned medium regulating RANKL expression in HMBCs. Therefore, RANKL mRNA expression was analyzed in PGE_2-stimulated HMBCs incubated with 0.1, 1, or 10 ng/mL recombinant IFN-γ. Recombinant IFN-γ attenuated PGE_2-induced RANKL mRNA expression in a concentration-dependent manner(Fig. 4.9A). When stimulated by *Pg*LPS, NOK-SI cells released approximately 60 pg/mL IFN-γ in the epithelial supernatant as shown by ELISA (Fig. 4.9B). The IFN-γ protein level was significantly reduced by pretreatment with 0.1, 1, or 10 μg/mL of an anti-IFN-γ antibody (Fig. 4.9B), with 1 and 10 μg/mL of anti-IFN-γ antibody resulting in similar levels of IFN-γ protein in the supernatant.

To examine the role of IFN-γ released by epithelial cells, HMBCs were pretreated with the anti-IFN-γ antibody 30 min before stimulation by PGE_2 and epithelial supernatant containing *Pg*LPS-stimulated or constitutively-released mediators. Pre-treatment with 0.1 or 1 μg/mL of the anti-IFN-γ antibody reversed the effect of the epithelial supernatant, increasing RANKL upregulation in a concentration-dependent manner (Fig. 4.9C). The effect of IFN-γ was nearly abolished when 1 μg/mL of the antibody was used. Decreased RANKL protein, consistent with mRNA level, was demonstrated by western blot (Fig. 4.9D). The RANKL protein level was restored when the antibody was added to neutralize the IFN-γ in the epithelial supernatant.

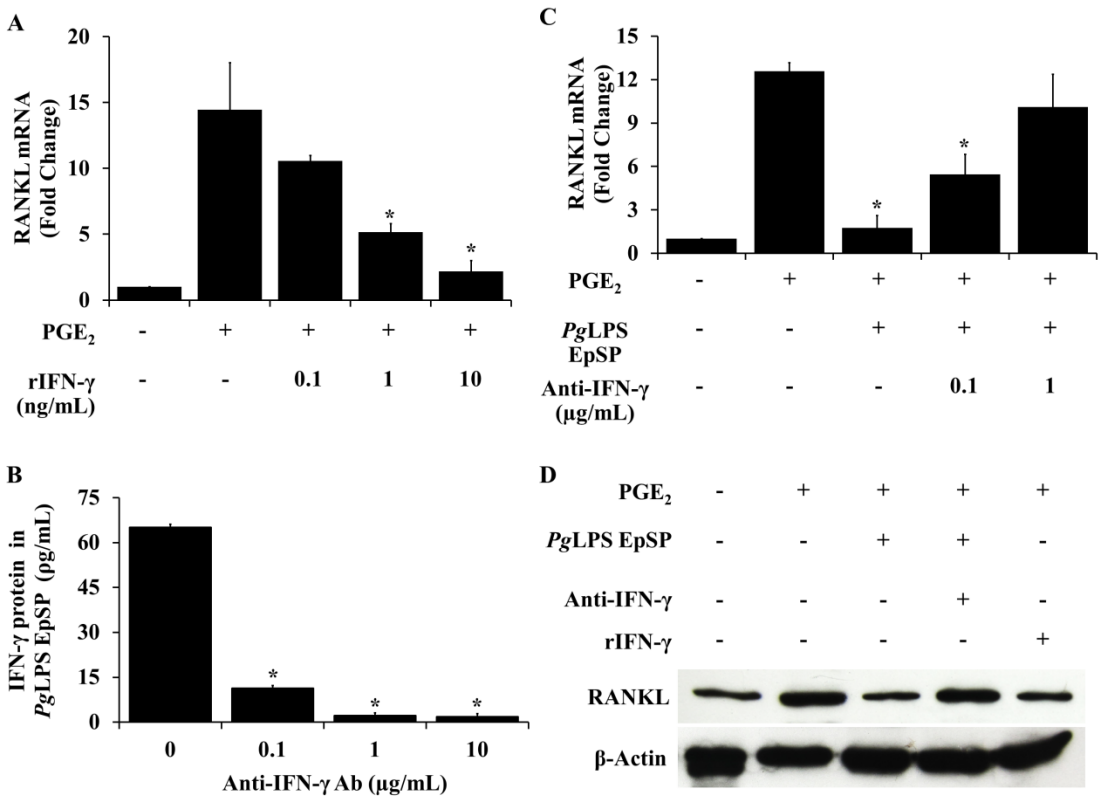


Figure 4.9 IFN-γ-derived from oral epithelial cells exerted a suppressive effect on RANKL upregulation in HMBCs. HMBCs was stimulated with 0.1 μM PGE_2 for 24 h to increase RANKL. Concurrently with PGE_2 stimulation, HMBCs were treated with epithelial supernatant containing *Pg*LPS-activated or constitutively-released mediators or recombinant IFN-γ. In some wells, epithelial supernatant was incubated with an anti-IFN-γ antibody before being added to HMBCs. (A) RANKL mRNA expression in HMBCs treated with recombinant IFN-γ at dose 0.1, 1, or 10 ng/mL. (B) IFN-γ protein level in the supernatant from *Pg*LPS-stimulated NOK-SI (*Pg*LPS EpSP) incubated with anti-IFN-γ antibody at 0.1, 1, or 10 μg/mL. (C) RANKL mRNA expression in HMBCs treated with *Pg*LPS EpSP or *Pg*LPS EpSP neutralized with 0.1, or 1 μg/mL anti- IFN-γ antibody. (D) RANKL protein from HMBCs treated with *Pg*LPS EpSP, or *Pg*LPS EpSP neutralized with 1 μg/mL anti- IFN-γ antibody, or

1 ng/mL recombinant IFN- γ, assayed by Western Blot. β-actin served as internal control. All experiments were performed in triplicate. * indicates a significant difference using one-way ANOVA followed by Bonferroni analysis ($p < 0.05$).

IFN-γ derived from epithelial supernatant suppresses osteoclastic activity in THP-1 cells

The THP-1 human monocyte cell line was demonstrated as an osteoclastic precursor that can undergo differentiation when treated with Vitamin D_3, M-CSF and RANKL (84) (Fig.4.10). To assess the plausibility of THP-1 differentiation the differentiation of THP-1 cells into osteoclasts, THP-1 cells were co-cultured with PGE_2-stimulated HMBCs for 21 days (Fig. 4.11). HMBCs increased RANKL release in the co-culture after PGE_2 stimulation, thus, THP-1 cells showed increased mRNA expression of the osteoclastic related genes TRAP, NFATc1, and cathepsin K, while decreasing that of the of monocyte marker, CD14 (Fig. 4.12A). The effect of the *Pg*LPS-stimulated epithelial mediators on THP-1 osteoclastic differentiation was then investigated. HMBCs were incubated with 0.1 μM PGE_2 and 50% volume of the epithelial-conditioned medium. After 24 h, the HMBCs and THP-1 cells were cocultured using a transwell system on the Corning® osteo assay surface and pit formation was investigated by observation and counting using light and electron microscopy. The THP-1 cells without vitamin D_3 and RANKL treatment failed to show any pit formation (data not shown). However, THP-1 cells cocultured with PGE_2-stimulated HMBCs created a significantly higher number of pits formed compared with THP-1 cells cocultured with untreated HMBCs (data not shown), suggesting that RANKL protein released from HMBCs drives THP-1 osteoclastic

activity. When THP-1 cells were co-cultured with HMBCs treated with both PGE_2 and epithelial supernatant from *Pg*LPS-stimulated cells, the number of pits formed decreased compared with when co-culturing with HMBCs treated with PGE_2 alone (Fig. 4.12B). The effect of medium conditioned by epithelial cells on HMBCs induced THP-1 osteoclast differentiation was quantified. HMBCs treated with PGE_2 and epithelial supernatant from *Pg*LPS-stimulated cells failed to drive THP-1 osteoclastic activity, as demonstrated by a decreased number of resorption pits compared with THP-1 cells co-cultured with HMBCs treated with PGE_2 and unstimulated epithelial supernatant (Fig. 4.12C). Thus, adding *Pg*LPS-activated epithelial supernatant reversed the effect of PGE_2-stimulated HMBCs on osteoclast function.

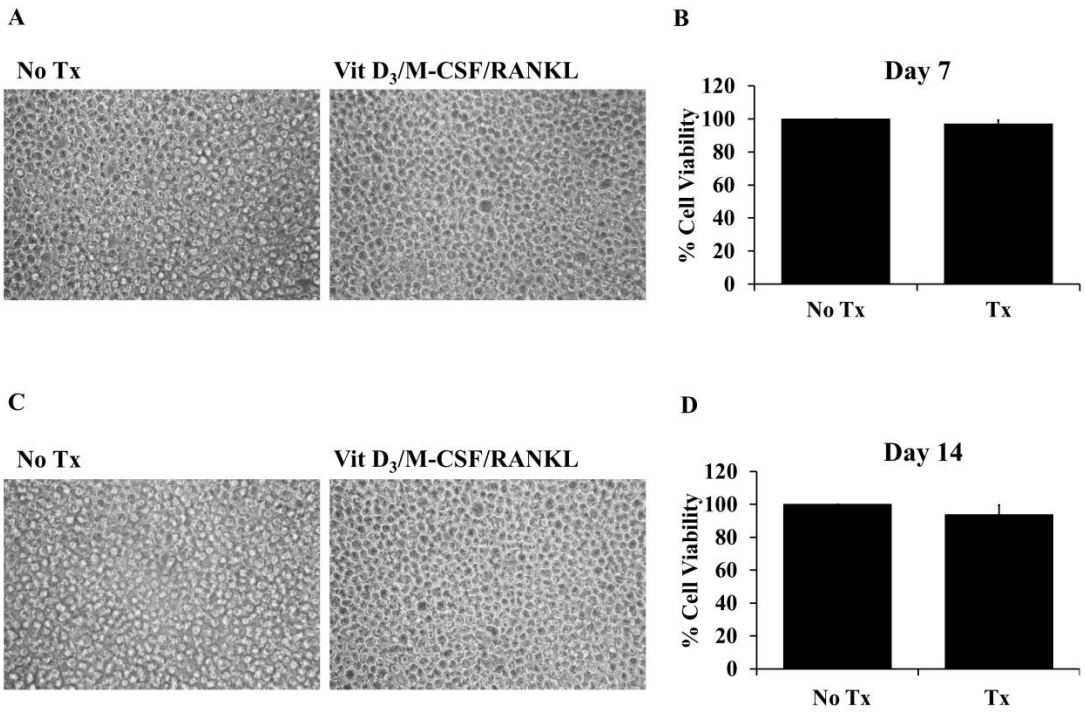


Figure 4.10 Vitamin D_3, M-CSF and RANKL treatment in THP-1 cells. Morphological analysis of cells was evaluated using light microscope (40X) upon cultured cells with and without treatment day 7 (A) and day 14 (B). Cell viability tested by MTT assay day 7 and day 14 (C, D), respectively.

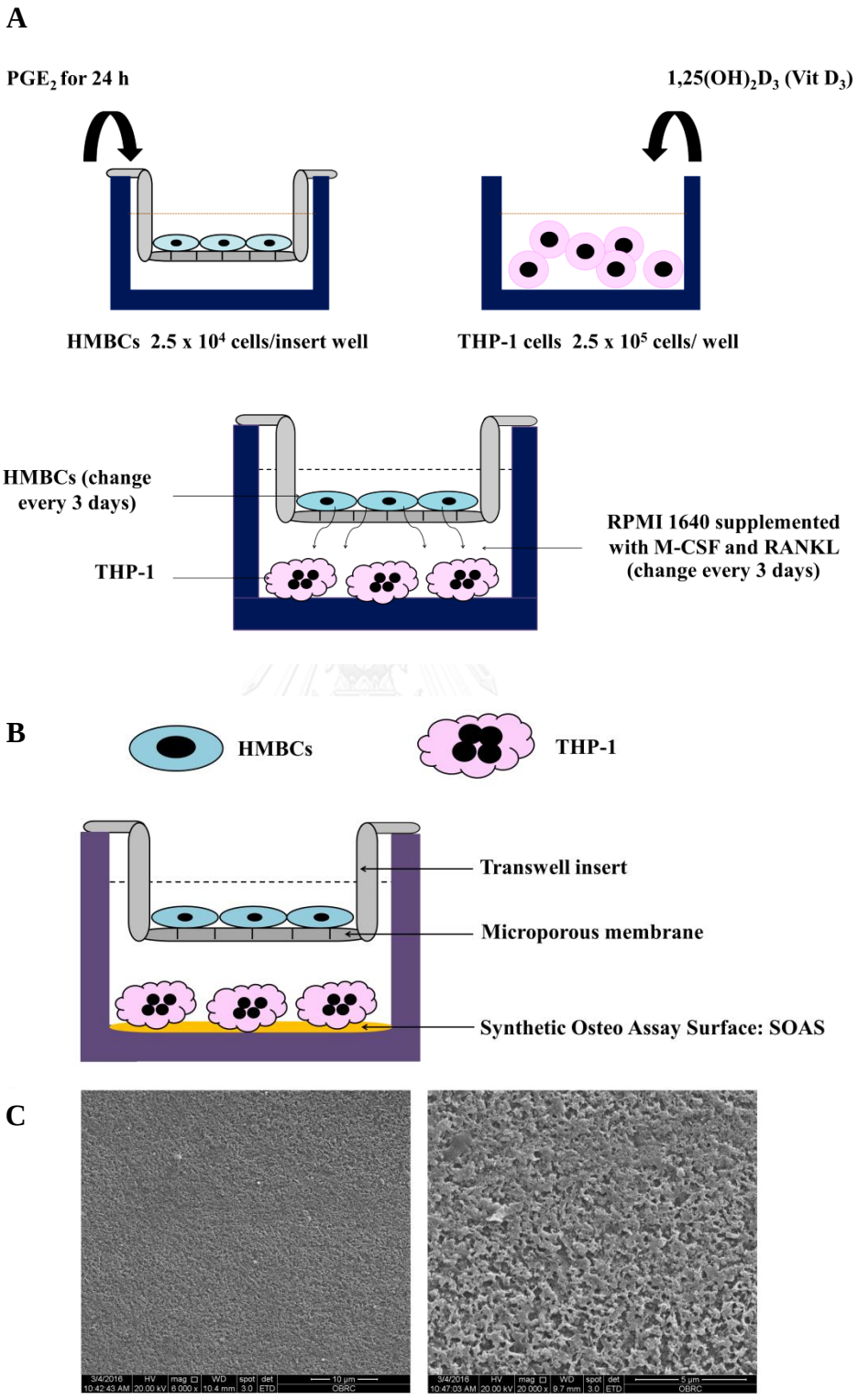


Figure 4.11 Co-cultured experimental design. (A, B) Co-cultured model by using Transwell system/Osteo Assay (B) Synthetic osteo assay surface: SOAS under SEM

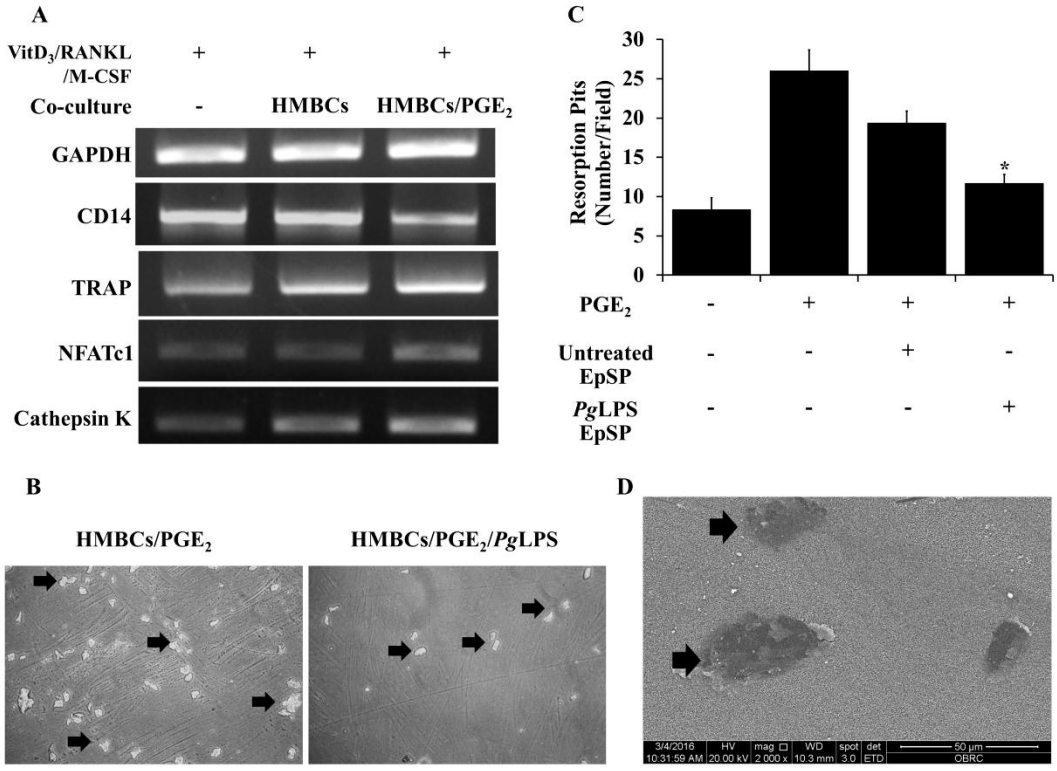


Figure 4.12 Epithelial supernatant suppressed PGE_2-induced osteoclast resorption. THP-1 cells were cultured with or without vitamin D_3, RANKL, and M-CSF for 21 days. Unstimulated THP-1 cells were cocultured with untreated HMBCs or PGE_2-stimulated HMBCs (HMBCs/PGE_2) in transwell cultures. (A) Osteoclastic gene expression in THP-1, CD14, TRAP, NFATc1, Cathepsin K, was detected by RT-PCR. (B) Osteoclastic activity was visualized on the Corning® osteo assay surface by observation of the bone resorption pits (arrows) under light microscope at 10X magnification. Resorption pits formed by THP-1 cells co-cultured with PGE_2-stimulated HMBCs (HMBCs/PGE_2), or with HMBCs treated with epithelial supernatant from *Pg*LPS-stimulated NOK-SI cells (HMBCs/PGE_2/*Pg*LPS) are shown (C) Resorption pits were counted, and the number per field is shown. The error bar indicates mean of three fields from each group. (D) Resorption pits on the Corning®

osteo assay surface were visualized using scanning electron microscope (arrows). All experiments were performed in triplicate. The photographs represent data from two experiments. * indicates a significant difference using one-way ANOVA followed by Bonferroni analysis ($p < 0.05$).

CHAPTER V

DISCUSSION AND CONCLUSION

It has long been questioned whether the oral epithelium can modulate alveolar bone remodeling. This question was based on the observation that a relatively constant distance is maintained between the epithelial layer of the gingival sulcus and the alveolar bone, known as the biological width (85). One explanation for this maintenance is a cross-talk between epithelial and bone cells. Our present findings support the presence of this cross-talk mechanism. The finding that epithelial supernatant obtained from both non-stimulated and *Pg*LPS stimulated cells, could suppress RANKL, a cytokine essential for osteoclast differentiation and maturation, in bone cells, strongly suggest that oral epithelial cells may play a crucial role in modulating bone remodeling by releasing suppressive mediator(s). Thus, we propose that the function of oral epithelial cells is not limited only as a physical barrier, but are also capable of releasing mediators that modulate the homeostasis of the underlying bone tissue. This role of epithelial cells has never been reported, and could be one of the important defended activities for microbial invasion as well as for the maintenance tissue homeostasis.

Numerous studies have shown the epithelial secretes potent antimicrobial peptides and many cytokines/chemokines (18, 19, 50-52) that act as host defense molecules to combat infection and control the balance between normal flora and pathogens in the oral cavity. Oral epithelial cells act as a physical protective barrier to defense with pathogen and their virulence factors including the outer membrane of

P.gingivalis LPS (*Pg*LPS), one of the most important periodontal pathogenic bacteria (86), by secreting several proinflammatory cytokines, anti-inflammatory cytokines and chemokines to maintain oral mucosal homeostasis (87). Optimum host responses to *Pg*LPS are important to resist invading bacteria and lead to balance of normal flora and pathogen. One of essential characteristic of epithelium in combat with the infection, endotoxin tolerance, a hypo response to subsequent challenge lead to diminished releasing of proinflammatory cytokines that contributed to limit inflammatory destruction of periodontal tissue (88). However, molecular mechanism of endotoxin tolerance still needs to be fully elucidated.

The TLR family plays a pivotal role in recognizing potentially harmful microorganisms and activating the NFκB signaling pathway (89). Oral epithelial cells produce IL-12 and IFN-γ, the T helper (Th) type 1 cytokines and IL-10, TGF-β, MCP-1, and IL-8, Th2 cytokines in response to microbial invasion (55, 90). The proinflammatory cytokines, IL-8 and IL-1β, was elevated in gastroesophageal epithelium after the NFκB activation (91). Consistent with previous reports, we have shown that *Pg*LPS upregulated TLR-2 mRNA expression and NFκB signaling pathway activation in NOK-SI cells leading to the production of proinflammatory cytokine, including IL-1β, IL-8, and IFN-γ protein.

The epithelial cells appear to grow in monolayer that differs from multilayer (stratified epithelium) in oral cavity (92). Epithelium rests on basement membrane, which acts as scaffold on which epithelial cells can grow including a selectively permeable membrane that determines which enter of substances through the epithelium. Moreover, the epithelial cells are polarized cells that specialization makes

the epithelium secretes a plethora of cytokine/chemokine in an apical and basolateral direction (93). Therefore, additional studies are necessary to detect the cellular function. We applied Transwell® cell culture insert assay to create an environment that resembles the in *Vivo* condition as closely as possible to enable the growth of epithelial cells and to allow polarized epithelial cells to be grown on plasma membrane that acts as basement membrane and determines the effect of *Pg*LPS in secretion under more natural environment. Cellular differentiation can also proceed resulting in cells that morphologically and functionally better represent their in *Vitro* counterparts (are in progress).

The present study demonstrated that the epithelial mediators suppressed RANKL expression in HMBCs, therefore suggesting a role for epithelial cells in alveolar bone remodeling. IL-1β is potent cytokine that induces pathologic bone resorption (94). IL-1β plays a role in both RANKL-induced and RANKL-independent osteoclast formation (95). IL-1β increased RANKL production in pre-stimulated T-cells (96). IL-8 is a chemokine originally identified as a neutrophil chemoattractant (97) and stimulates osteoclast differentiation and bone resorption by both RANKL dependent and independent pathways (98). Involving in the inflammatory reaction and bone resorption, IFN-γ has been shown to interfere with the RANK-RANKL signaling pathway by inducing the rapid degradation of tumor necrosis factor receptor-associated factor 6 (TRAF6) followed by decreasing RANKL-induced NFκB activation in osteoclast precursor (72, 99) and osteoclastogenesis (100). However, its role in regulating RANKL expression in osteoblasts has never been reported.

Osteoclast differentiation requires RANKL provided by osteoblasts or immune cells (101).

Several factors, including $1\alpha,25(OH)_2D_3$, parathyroid hormone, and PGE_2, stimulated RANKL upregulation and increased pit-forming activity when osteoblasts and osteoclasts from mouse bone marrow were cocultured (102). IFN-γ is commonly found in Th-1 type response associating with stable periodontal disease (73), but absent in progressive periodontitis, we therefore investigate whether IFN-γ is the cytokine responsible for the observed reduction in RANKL expression and its role in modulating bone homeostasis. Such a hypothesis was supported; we now show that IFN-γ is the cytokine released by epithelial cells responsible for the down-regulation of RANKL expression by HMBCs. Using a specific activity-blocking antibody against IFN-γ, the down-regulating effect of the conditioned medium was nullified. We also observed that HMBCs-released RANKL significantly increased osteoclastic gene expression in the osteoclast precursor THP-1, and stimulated pit-forming activity on the bone-mimicking Corning® osteo assay surface. Adding epithelial supernatant containing IFN-γ to HMBCs significantly reduced the number of resorption pits. Our findings strongly suggest that IFN-γ may play an inhibitory role in the formation of osteoclasts leading to the regulation of bone resorption through suppression of RANKL expression by HMBCs. A schematic of our proposed mechanism is shown in Figure 5.1. The present study is the first to explore the cross-communication between oral epithelial cells and mandibular bone cells with respect to regulating bone remodeling through IFN-γ and RANKL. It suggests that the epithelial activity may reduce bone loss by inhibiting osteoclast formation. This mechanism may indicate a role for osteoimmunology in regulating alveolar bone homeostasis, a more

understanding of relationship between the epithelial cells and alveolar bone cells, one of the key for maintenance of periodontal health and the opportunity for the development of new therapeutic approaches by preventing impingement of the periodontal attachment apparatus. Whether this action can inhibit bone resorption to maintain tissue homeostasis in vivo remains to be investigated.

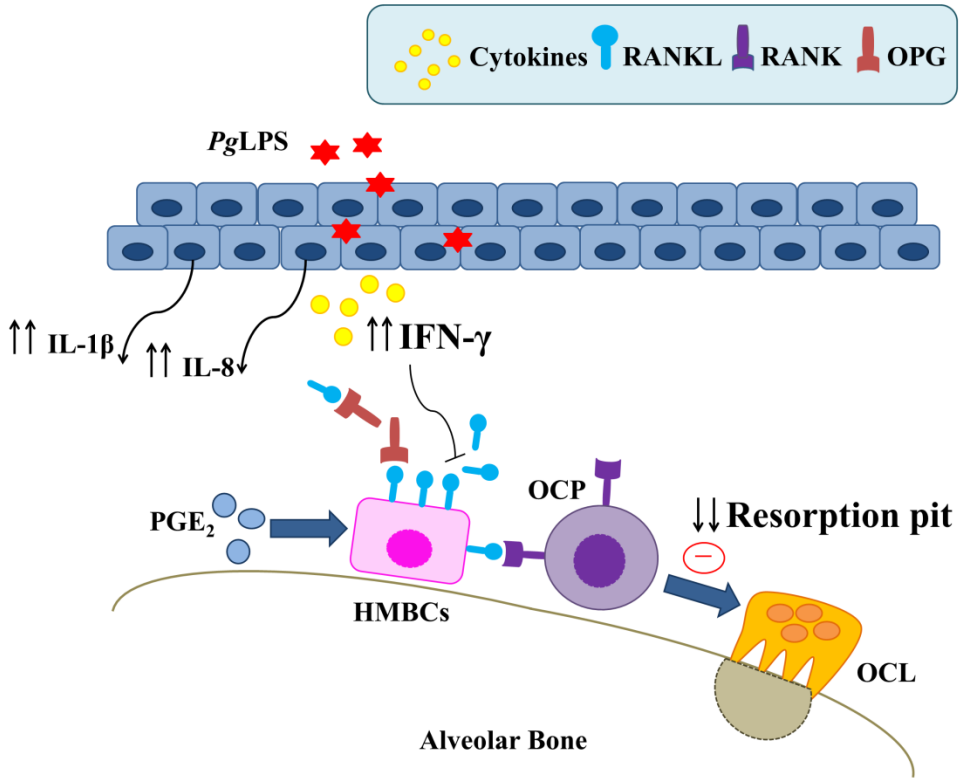


Figure 5.1 Schematic model of the proposed signaling mechanism by which IFN-γ secreted from oral epithelial cells stimulated by bacteria inhibits RANKL expression by HMBCs and inhibits osteoclastogenesis/osteoclastic activity by osteoclast precursor cells (OCP)/osteoclast like cells (OCL). *Pg*LPS represents bacterial challenge and PGE_2 represents inflammatory conditions.

Conclusions: In this study we found that PGE_2 significantly increased the expression of RANKL while decreased the expression OPG in HMBCs. Addition of IFN-γ, or IFN-γ derived from oral epithelial cells, could suppressed PGE_2-induced RANKL expression at both mRNA and protein level. IFN-γ, or IFN-γ derived from oral epithelial cells, could also suppress the osteoclast activity as determined by the resorption pit assay. Therefore, oral epithelial cells can regulate bone resorption by modulating RANKL/OPG ratio in HMBCs via IFN-γ signaling pathway.

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APPENDIX

VITA

Miss Pakchisa Khonsuphap was born on May 1, 1984 in Nakhon Si Thammarat, Thailand. She graduated the degree of Doctor of Dental Surgery (D.D.S) with second class honors from Faculty of Dentistry, Prince of Songkla University, Songkla, Thailand in 2008. After graduation, she enrolled as a lecturer in Dental Public Health Program, Sirindhon College of Public Health, Trang from May 2009- May 2011. She started her study for the Degree of Doctor of Philosophy Program in Oral Biology at the Faculty of Dentistry, Chulalongkorn University in 2011. The research component of this degree was performed at the Research Unit of Mineralized Tissue (RUMT), Faculty of Dentistry, Chulalongkorn University. At the present, she returns to work in Dental Public Health Program, Sirindhon College of Public Health, Trang, Thailand.