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THE RESPONSES OF HUMAN PERIODONTAL LIGAMENT CELLS

TO EXOGENOUS INTERLEUKIN-12

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A Dissertation Submitted in Partial Fulfillment of the Requirements

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การผลิตสารอักเสบในระหว่างการเกิดรอยโรคปริทันต์ถูกเชื่อว่าเป็นกลไกหนึ่งที่มีสำคัญในการธำรงสมดุลของเนื้อเยื่อ อินเตอร์ลิวคิน-12นับเป็นหนึ่งในสารอักเสบที่มีรายงานแสดงการเพิ่มขึ้นในระหว่างการเกิดรอยโรคปริทันต์ โดยพบว่าระดับของอินเตอร์ลิวคิน-12ที่ตรวจพบมีความสอดคล้องกับระดับความรุนแรงของการอักเสบของเนื้อเยื่อ แต่อย่างไรก็ตามกลไกการทำงานหรือบทบาทของอินเตอร์ลิวคิน-12ในรอยโรคปริทันต์ยังไม่ได้รับการค้นพบที่ชัดเจน และเนื่องจากเซลล์เอ็นยึดปริทันต์นับเป็นเซลล์ที่มีบทบาทสำคัญในการตอบสนองต่อสารอักเสบในบริเวณเนื้อเยื่อปริทันต์ การศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาการตอบสนองของเซลล์เอ็นยึดปริทันต์ต่ออินเตอร์ลิวคิน-12 เพื่อศึกษาถึงบทบาทของอินเตอร์ลิวคิน-12ที่มีต่อกระบวนการเกิดรอยโรค และการธำรงสมดุลของเนื้อเยื่อปริทันต์ เพื่อศึกษาถึงบทบาทของอินเตอร์ลิวคิน-12การทดลองนี้จึงดำเนินการโดยศึกษาการตอบสนองของเซลล์เอ็นยึดปริทันต์ เมื่อได้รับการกระตุ้นด้วยอินเตอร์ลิวคิน-12 ในระดับความเข้มข้นและช่วงระยะเวลาที่แตกต่างกัน โดยมุ่งพิจารณาระดับการแสดงออกของสารกระตุ้นการละลายกระดูก (RANKL), ระดับสารต้านการอักเสบ (immunomodulatory molecules) และระดับสารแสดงความเป็นเซลล์ต้นกำเนิด (Stemness markers)ในเซลล์เอ็นยึดปริทันต์ทั้งในระดับmRNAและในระดับโปรตีน หลังจากนั้นจะมีการใช้สารยับยั้งต่อโมเลกุลต่างๆที่เป็นองค์ประกอบในการส่งสัญญาณของอินเตอร์ลิวคิน-12 เพื่อศึกษาถึงกลไกการเปลี่ยนแปลงที่เกิดขึ้นโดยละเอียด ผลการวิจัยพบว่าในสภาวะที่มีอินเตอร์ลิวคิน-12 เซลล์เอ็นยึดปริทันต์จะมีการแสดงออกของ RANKL ที่เพิ่มมากขึ้น ซึ่งกระบวนการนี้ดำเนินผ่านการทำงานของSTAT4และNF-kB นอกจากนี้ยังพบว่ากระบวนการกระตุ้นนี้ไม่ได้เกิดโดยตรงจากการทำงานของอินเตอร์ลิวคิน-12 แต่ผ่านการทำงานของสารตัวกลางที่ได้รับการกระตุ้นให้มีการหลั่งออกมาจากเซลล์เอ็นยึดปริทันต์ หลังจากที่เซลล์เอ็นยึดปริทันต์ได้รับการกระตุ้นด้วยสารอักเสบอินเตอร์ลิวคิน-12 และยังพบว่าแคลเซียมเป็นโมเลกุลที่สำคัญต่อการตอบสนองนี้ นอกจากนี้อินเตอร์ลิวคิน-12 ยังมีบทบาทในการเพิ่มการแสดงออกของ immunomodulatory molecules (ได้แก่IDOและHLA-G) และstemness markersในเซลล์เอ็นยึดปริทันต์ โดยกระบวนการการกระตุ้นนี้ จะเกิดผ่านการทำงานของIFNγ และงานวิจัยนี้ยังพบว่าอินเตอร์ลิวคิน-12ยังมีบทบาทในการยับยั้งความสามารถในการเปลี่ยนแปลงไปเป็นเซลล์สร้างกระดูกของเซลล์เอ็นยึดปริทันต์ และอินเตอร์ลิวคิว-12ยังมีผลช่วยคงความสามารถในการสร้างโคโลนี และการแบ่งตัว ซึ่งเป็นหนึ่งปัจจัยที่แสดงความเป็นเซลล์ต้นกำเนิดของเซลล์เอ็นยึดปริทันต์ จากผลการศึกษาทั้งหมดที่กล่าวมานี้ สามารถแสดงให้เห็นถึงบทบาทของอินเตอร์ลิวคิน-12 ที่มีผลต่อทั้งกระบวนการการเกิดรอยโรคและการธำรงสภาวะสมดุลภายในเนื้อเยื่อปริทันต์

สาขาวิชา ชีววิทยาช่องปาก	ลายมือชื่อนิสิต ....
ปีการศึกษา 2559	ลายมือชื่อ อ.ที่ปรึกษาหลัก ....

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KEYWORDS: IL-12 / RANKL / OSTEOIMMUNOLOGY / INTERFERON GAMMA / IDO / HLA-G / IMMUNOMODULATION / HUMAN PERIODONTAL LIGAMENT CELLS

BENJAR ISSARANGGUN NA AYUTHAYA: THE RESPONSES OF HUMAN PERIODONTAL LIGAMENT CELLS TO EXOGENOUS INTERLEUKIN-12. ADVISOR: PROF. PRASIT PAVASANT, Ph.D., 64 pp.

Presence of cytokines during periodontal inflammation has been proposed as a key factor to modulate homeostasis of periodontal tissue. Interleukin 12 (IL-12) is one of the cytokines, which its expression was found to be increased associated with the severity of periodontal destruction. However, the exact role of IL-12 in periodontitis is still unclear. As human periodontal ligament (hPDL) cells are major local cells that have an ability to respond to many immunological stimuli, this study aimed to investigate the responses of hPDL cells to exogenous IL-12 for determining the osteoimmunological and immunomodulatory effects of IL-12 on hPDL cells. HPDL cells were incubated with IL-12 in a dose and time dependent manner. The expression levels of RANKL, OPG, IFNγ , IDO, HLA-G, as well as stem cell markers were evaluated by quantitative PCR. The protein levels of RANKL, IFNγ , HLA-G and the activity of IDO were measured by ELISA, flow cytometry and enzymatic activity assay, respectively. Chemical inhibitors or neutralizing antibody were used to elucidate underlying pathways. The results of this study showed that, under the influence of IL-12, hPDL cells expressed significantly higher levels of RANKL. This induction occurred indirectly by the activation of intermediate molecule(s). Addition of suramin and EGTA suggest that the nature of the involved intermediate molecule(s) was possibly the ligand that could activate heterodimeric G protein signaling in a calcium dependent pathway. In addition, IL-12 also induced the expression of the immunosuppressive proteins: IDO and HLA-G in hPDL cells via an IFNγ dependent pathway. Moreover, the expression of the stem cells markers was also upregulated in IL-12-treated hPDL cells. Together, these data indicate both pro- and anti-inflammatory effect of IL-12 by inducing expression of RANKL and immunomodulatory properties of hPDL cells. These provide the role of IL-12 in regulating homeostasis of periodontal tissue.

Field of Study: Oral Biology   Student's Signature ....

Academic Year: 2016   Advisor's Signature ....

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CHAPTER I

INTRODUCTION

Background and Rationale

Interleukin 12 (IL-12) is a potent cytokine that plays an important role in immune response by driving the differentiation of naïve T-cells to T helper 1 (Th1) cells and mediating long-term protection of our body against the pathogen [1, 2]. Antigen presenting cells, especially dendritic cells, are the cell type responsible for IL-12 production. The production of IL-12 is stimulated by antigenic signals, particularly lipopolysaccharide (LPS), and activated T-cell signals (mediated by interaction between CD40 and CD40 ligand (CD40L)) [3]. Moreover, IL-12 production is further regulated by the presence of other inflammatory cytokines such as interleukin 1beta (IL-1β), a well-known osteolytic factor [4, 5]. For pathological effect of IL-12, it has been implicated as a destructive stimulator to trigger the development of several inflammatory diseases such as rheumatoid arthritis, periodontitis and periapical lesion [6]. These inflammatory diseases have an identical biological process in bone destruction [6-8]. Clinical studies in rheumatoid arthritis patients showed that the serum level of IL-12 was increased strongly associated with the disease progression [9]. Moreover, injection of recombinant IL-12 resulted in the development of severe joint inflammation in collagen-induced arthritis (CIA) mouse model [10]. In periodontal inflammation, the increased level of IL-12 was found, related to severity of periodontal disease [11, 12]. Promotion of osteoclast differentiation was demonstrated as biological destructive role of IL-12 in bone degradation [13]. Deletion of IL-12 showed the inhibitory effect on bone resorption stimulated by bacterial infection in mice periodontitis model [14]. These data suggest the osteolytic property of IL-12 involved in infection-induced inflammatory disease. In contrast, *in vitro* study of NJ Horwood in 2001 [15] demonstrated an attenuating effect of IL-12 on osteoclast formation during

proliferative phase of osteoclast precursor. Therefore, more studies are required to determine the effect of IL-12 on inflammation-induced bone degradation.

Although, inflammation provides a destructive signal to damage underlining tissues, the presence of inflammatory cytokines has also been proposed as a key activator to stimulate immunomodulatory properties of MSCs [16]. This property is an important characteristic of MSCs that functions to modulate host immune response via the upregulation of immunosuppressive molecules, such as IDO and HLA-G [17]. This capacity is believed to play roles as a protective mechanism of MSCs in order to survive under inflammatory conditions. Evidence indicates that several inflammatory cytokines can modulate the immunomodulatory properties of MSCs [18, 19]. IL-12 is one of the inflammatory cytokines that has been shown to induce the production of IDO (26-27). However, most of the data were collected in studies related to cancer [20, 21]. No studies are available that addressed the question whether IL-12 has a comparable effect on cells in a normal tissue. Taken together, these mentioned studies suggested both destructive and protective roles of IL-12 involved in inflammatory process. As PDL cells are local stem cells resided in periodontium [22, 23] and they are important to maintain tissue homeostasis via an ability to respond to various inflammatory cytokines [24], this study aimed to investigate the response of hPDL cells to exogenous IL-12, to determine the role this cytokine in controlling the pathogenesis and homeostasis of periodontal tissue.

Research question

How does human periodontal ligament cell respond to exogenous IL-12?

Objectives

To investigate the responses of human periodontal ligament cells to exogenous IL-12

Objective 1: to investigate the change in RANKL/OPG expression ratio in human periodontal ligament cells after IL-12 treatment

Experimental design: hPDL cells were incubated with recombinant human IL-12 (p70) in dose (0-10 ng/ml) and time dependent manner. Expression of RANKL and OPG was determined by quantitative polymerase chain reaction (qPCR). Protein level of RANKL was evaluated by western blotting and ELISA. STAT4 and NF-**κ**B inhibitors were used to elucidate underlying signaling pathways of IL-12 activation in hPDL cells. Conditioned medium assay was performed to determine the involvement of intermediate molecule. Series of inhibitors were used in this study to identify the nature of intermediate molecule.

Objective 2: to determine the effect of IL-12 on expression of immunosuppressive molecules in human periodontal ligament cells

Experimental design: hPDL cells were treated with 0-10 ng/ml of exogenous human IL-12 (p70). To investigate the immunomodulatory effect of IL-12, gene expression level of IFN**γ**, IDO, HLA-G, as well as stem cell markers; CD73, CD90, CD105, Nanog, Oct4 and Sox2 were evaluated by qPCR. The protein level of IFN**γ** and HLA-G and the level of activity of IDO were measured by ELISA, flow cytometry and enzymatic activity assay, respectively. To determine the role of IFN**γ** in IL-12 immunomodulation, neutralizing antibody against IFN**γ** and recombinant human IFN**γ** were used in this study. Chemical inhibitors were used to determine the involved signaling pathways.

Hypothesis

IL-12 plays a role to modulate both the expression of RANKL/OPG ratio and immunosuppressive molecules in human periodontal ligament cells, which are important to control the homeostasis of periodontal tissue.

Expected Outcomes

This investigation provides a better understanding of IL-12 biological mechanism involved in the pathogenesis and homeostasis of periodontal tissue,

leading to a new and specific therapeutic approach to regulate the progression of periodontal disease.

Keywords

- IL-12
- RANKL
- Osteoimmunology
- IFNγ
- IDO
- HLA-G
- Immunomodulation
- Human periodontal ligament cells

Research design

Laboratory experimental research

Conceptual Framework
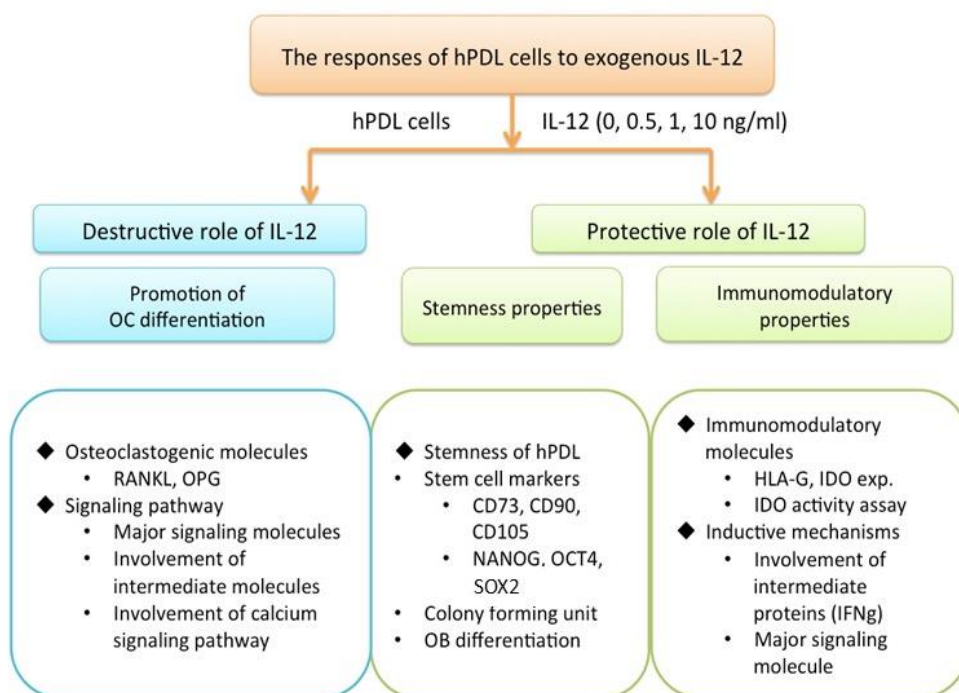
CHAPTER II

LITERATURE REVIEWS

Periodontitis and osteoimmunology

Periodontitis is one of the most prevalent chronic inflammatory diseases, involved in periodontium degradation. A plaque microorganism that migrates and penetrates into the periodontium is proposed as the etiology of periodontal disease formation. However, the presence of these pathogens is not sufficient to trigger the development of periodontal disease. Indeed, the recognition of these pathogen by host immune cells is an essential process in the periodontal pathogenesis[7]. One of the critical recognition components of the host immune response to the pathogenic microorganisms and their products is a family of toll-like receptors (TLRs). The most common TLRs implicated as potent receptors for pathogen associated periodontitis are TLR-2 and TLR-4 [25, 26]. TLR activation stimulates an intracellular signaling cascade that leads to the activation of transcription factors, such as nuclear factor-kB (NF-kB) and activator protein-1 (AP-1)[27], resulting in the production of many pro-inflammatory cytokines that play role in the induction of bone resorption by the process of osteoclastogenesis. Attempts to reduce inflammation could decrease the progression and severity of bone resorption, indicating the importance of inflammatory process in pathogenesis of periodontal disease[28].

The relationship between the immune and skeletal systems is known in term of osteoimmunology. The presence of different inflammatory cytokines has been proposed as key inducers to promote the expression of receptor activator of nuclear factor kappa B ligand (RANKL) by osteoblasts and stromal cells. RANKL and osteoprotegerin (OPG) are the important local factors that tightly control homeostasis of bone [8, 29]. RANKL promotes osteoclast differentiation and activation by stimulating the distinct signaling cascades mediated in osteoclast formation[29]. On the other hand, OPG acts as a decoy receptor against RANKL and prevents binding of RANKL

to its receptor (RANK) [30, 31]. In a normal or healthy condition, the balance between RANKL and OPG is preserved. However, in pathologic or inflammatory conditions, the ratio between RANKL and OPG changes, leading to an increased osteoclast formation and bone resorption [31]. Therefore, the level of RANKL/OPG ratio is considered as a biological marker to determine degree of osteolytic activity.

Interleukin 12

Interleukin 12 (IL-12) is a heterodimeric cytokine that is composed of a 35 kDa light chain (p35) and a 40 kDa heavy chain (p40). They are encoded by two separate genes; IL-12A (for p35) and IL-12B (for p40). It is a potent cytokine that plays an important role in an immune response by driving the differentiation of naïve T-cells to T helper 1 (T_H1) cells and mediating the long-term protection of our body against a pathogen [1, 32]. Thus, this cytokine is known as T cell-stimulating factor. Besides Th1 differentiation, IL-12 also functions to stimulate the production of other cytokines including IFNγ and TNFα from activated T cells and natural killer (NK) cells [33, 34]. Moreover, it also enhances generation of cytotoxic T lymphocytes (CTLs) to further activate cell-mediated immune response. Thus, IL-12 is a pro-inflammatory cytokine that forms a link between innate and adaptive immunity.

For exert IL-12 activity, IL-12 needs to bind to its surface receptor (IL-12R), which mainly expresses on activated T cells following T cell receptor (TCR) activation. Presence of other cytokines such as IFNγ, TNFα, co-stimulatory molecules (CD28) or IL-12 itself is an inducer to stimulate the expression of this receptor. IL-12R is a heterodimeric receptor formed by IL-12R-β1 and IL-12R-β2 [35]. Co-expression of these two components is important to generate high affinity of IL-12 binding site. IL-12R-β2 is considered as a key signal transduction component to activate IL-12 intracellular signaling. Upon binding, IL-12R-β2 becomes tyrosine phosphorylated, and provides the binding sites for involved protein kinases including tyrosine kinase 2 (Tyk2) and Janus kinase 2 (Jak2), leading to the phosphorylation and activation of IL-12 specific transcription factor protein: signal transducer and activator of transcription 4 (STAT4) in JAK-STAT pathway [36] (as shown in figure 2.1). This signaling pathway

are known to promote the development of Th1 cells and also inhibit Th2 cells differentiation.
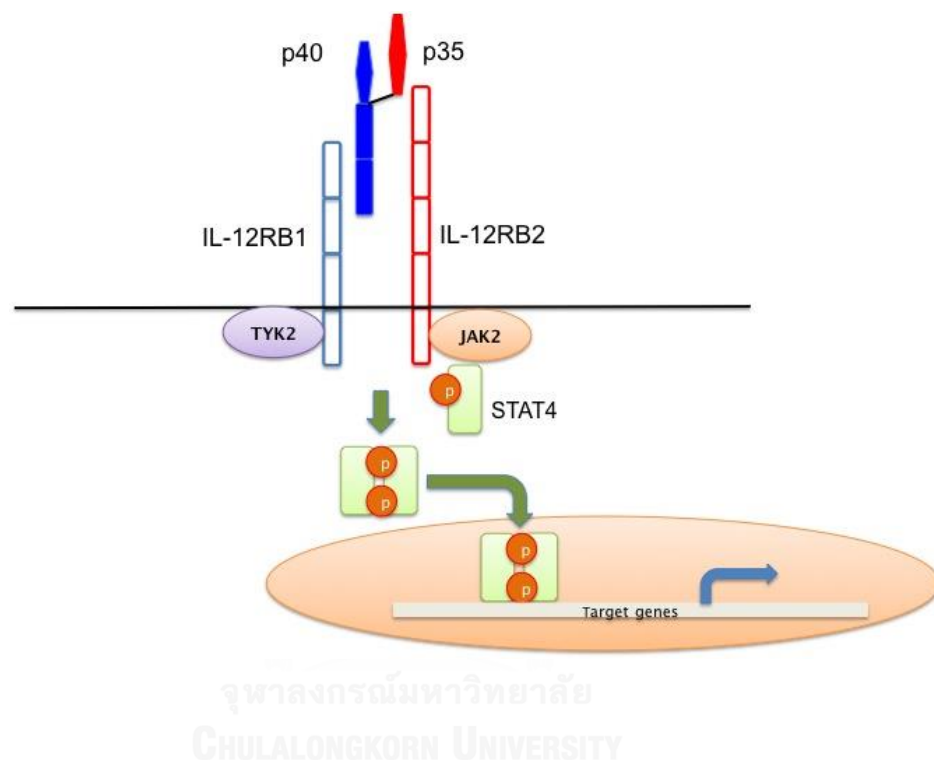
Figure 2.1: Components of IL-12 receptor and its signal transduction (modified from Giorgio Trinchieri, 2003)

Antigen presenting cells, especially dendritic cells, are the primary cell types responsible for IL-12 production [37]. During periodontal inflammation, the number of dendritic cells continue to increase from healthy to periodontitis stage [38]. It has been shown that, at the initiation stage of periodontal inflammation or gingivitis, most of dendritic cells resided in gingival epithelium. However, at the later stage, they moved

into the periodontal tissue and activated the differentiation of adaptive immune cells [38, 39]. During infection, the production of IL-12 is stimulated as a result of the induction by antigenic signals, particularly lipopolysaccharide (LPS), and activated T-cells, mediated by interaction between CD40 and CD40 ligand (CD40L) [3]. Moreover, IL-12 production is further regulated by other cytokines such as interleukin 1beta (IL-1β), a potent osteolytic cytokine [4, 5]. These finding are concordant with the reports from many studies that the level of IL-12 was up regulated associated with severity of inflammatory diseases [9, 11, 12].

IL-12 and osteoimmunological effect

IL-12 has been implicated as a potent destructive stimulator of several inflammatory-induced bone resorption diseases such as rheumatoid arthritis. An increased level of IL-12 was found associated with the level of bone destruction [9]. Injection of IL-12 induced severe joint inflammation in a mouse model of Collagen-Induced Arthritis (CIA) [10]. Moreover, higher sensitivity to IL-12 stimulation and higher level of STAT4 phosphorylation were found in patients with rheumatoid arthritis [9, 40]. Not only systemic autoimmune diseases, IL-12 is also participated in the pathogenesis of oral inflammatory lesions, such as periodontitis and periapical lesion. In periodontal disease, the level of IL-12 in serum and gingival crevicular fluid was found to be increased, correlated with the severity of inflammation [11, 12]. Furthermore, bone resorption induced by *P.gingivalis* infection significantly decreased in IL-12 $^{-/-}$ mice periodontitis model [14].

From the concern in the pathologic role of IL-12, IL-12 inhibiting drugs have been developed continuously. One of the well-known IL-12 inhibition drugs is cyclosporine (CsA), which functions to inhibit the production of IL-12 from activated APCs [41, 42]. In rat periodontitis model, treatment with CsA showed an effective therapeutic outcome to decrease the level of alveolar bone loss [43]. Therefore, these data strongly support the osteolytic role of IL-12 involved in on inflammatory-induced bone degradation.

Induction of osteoclast differentiation was considered to be a major destructive role of IL-12 mediated in bone resorption process [13]. In contrast, *in vitro* study of NJ Horwood in 2001 [15] demonstrated an attenuating effect of IL-12 on osteoclast

formation during proliferative phase of osteoclast precursor. This inhibitory effect of IL-12 couldn't occur directly on osteoclast precursor but rather required the production of inhibitory molecule from T-cell [15]. These showed the dichotomic effect of IL-12 between pro- and anti-inflammatory cytokines, thus more studies are required to clarify the effect of IL-12 on homeostasis of bone tissue.

IL-12 and immunomodulatory effect

In addition to pro-inflammatory function, IL-12 has also been reported in its immunomodulatory effect by stimulating indoleamine-pyrrole 2,3-dioxygenase (IDO) production. IDO is an enzyme that functions to induce intracellular degradation of tryptophan and subsequent inhibiting T cell proliferation [17]. The ability of IL-12 to induce IDO production was demonstrated in various studies related with cancer [20, 21, 44]. Both IFNγ dependent [20, 44] and independent [21] mechanisms has been shown to be involved in IL-12-induced IDO production. This elevated IDO production has been proposed as an escape mechanism of cancer cells to survive under host immune response [44-46]. Therefore, these suggest the immunomodulatory effect of IL-12 to regulate host immune response; however, most of the data were collected in field of cancer. No studies are available that addressed the question whether IL-12 has a comparable effect on cells in a normal tissue.

Mesenchymal stem cells and their immunomodulatory properties

Mesenchymal stem cells (MSCs) are multipotent stem cells that are capable to differentiate to various cell types. They are characterized by expression of stemness cell surface markers (including CD13, CD44, CD73, CD90) and lack of hematopoietic markers (including CD45, CD34, CD80, CD86, HLA molecules). This characteristic distinguishes MSCs from hematopoietic stem cells and positions them close to fibroblast. One of the important characteristics of MSCs is their capacity to modulate an immune response [47-49]. This characteristic is believed to play roles as a protective mechanism of MSCs in order to survive under inflammatory condition, since under such circumstances MSCs are susceptible to be destroyed by activated T cells and NK cells [47, 50, 51]. In dental tissues, evidence suggests that dental stem cells, including

gingival fibroblasts, dental pulp fibroblasts and periodontal ligament cells, can inhibit proliferation of activated peripheral blood mononuclear cells (PBMCs) *in vitro* [52], thereby supporting the immunosuppressive properties of these cells [23, 53].

This immunomodulatory property of MSCs has been proposed by many studies as an ability to modulate host immune response by inhibiting T cell proliferation and cytokine production after mitogen stimulation [54, 55]. Moreover, MSCs were found to promote the generation of regulatory T cells (Tregs) via both direct and indirect mechanisms [48, 56]. For the indirect pathway, MSCs inhibit the generation of antigen presenting cells (APCs) and induce them to acquire a tolerogenic phenotype that supports the differentiation of naïve T cells to Tregs [57, 58]. An ability to switch the phenotype of macrophage from a pro-inflammatory type 1 to an anti-inflammatory type 2 was demonstrated as one of the modulatory mechanisms of MSCs [59, 60]. Besides, down regulation of NK cell activity after IL-2 and IL-15 stimulation has also been reported to be another mechanism played by MSCs to modulate immune response [50, 61].

Production of immunosuppressive molecules, such as human leukocyte antigen (HLA) molecules [16, 51] and indoleamine-pyrrole 2,3-dioxygenase (IDO) enzyme [17, 51, 62], has been demonstrated as an molecular mechanism of MSCs to modulate immune response [17]. HLA molecules attenuate immune cells' activity by signaling via their specific inhibitory receptors [63-65], while IDO enzyme inhibits immune response by degrading tryptophan, an essential amino acid required for T cell growth, resulted in a decrease of T cell proliferation [17, 66]. Not only immunomodulatory molecules, but many kinds of chemokines are also produced by MSCs, to recruit specific immune cells into close proximity to them. This recruitment made those immune cells are more susceptible to the immunosuppressive capacity of MSCs [67]. However, the molecular mechanisms of MSCs are specific and different depended on the type of tissue and local environment, which MSCs are resided [19, 68].

Inflammation and MSCs' immunomodulation

In physiological condition, immunomodulatory property of MSCs is kept inactive and will be activated by inflammatory environment [16]. Indeed, the presence of pro-

inflammatory cytokines has been demonstrated as a key inducer to activate the immunomodulatories property of MSCs [16, 69]. Therefore, MSCs are widely used to treat various inflammatory diseases such as autoimmune and graft versus host diseases (GvHD). In the study of GvHD, a successful outcome occurred when MSCs was administrated during inflammatory process; however, a less effective result found when the administration was done before inflammation has begun [67, 70]. Such findings confirm the role of inflammation on MSC's immunomodulatory properties [18, 71]. In addition, level of inflammation is another crucial factor that affects properties of MSCs. High level of inflammatory cytokines is required to activate the immunomodulatory properties of MSCs [18]. Under low level of inflammation, the production of chemokines was activated, whereas it was not sufficient to induce expression of immunosuppressive molecules in MSCs. Therefore, this condition promoted immune cells' accumulation without attenuating inflammatory response [71].

Among pro-inflammatory cytokines, IFN𝛾 has been proposed as a potent activator to induce the immunomodulatory properties of MSCs. IFN𝛾 mediates the immunosuppressive properties of MSCs by upregulating the expression of immunosuppressive molecules, including HLA class I molecules [16, 51], B7-H1 [62, 72], IDO [16, 51, 62], HGF [73] and TGF𝛽 [51, 73]. Inhibition with antibodies to IFN𝛾 or to IFN𝛾 receptor, as well as the model of IFN𝛾R1-deficient MSCs reversed the modulatory effect of MSCs [16]. In addition to IFN𝛾, other inflammatory cytokine such as TNF𝛼 and IL-17 also play a role to regulate the immunomodulatory properties of MSCs by working synergistically with IFN𝛾 to further boost MSC-mediated immunosuppression *in vitro* and *in vivo* [16, 18, 19] (as shown in figure 2.2).
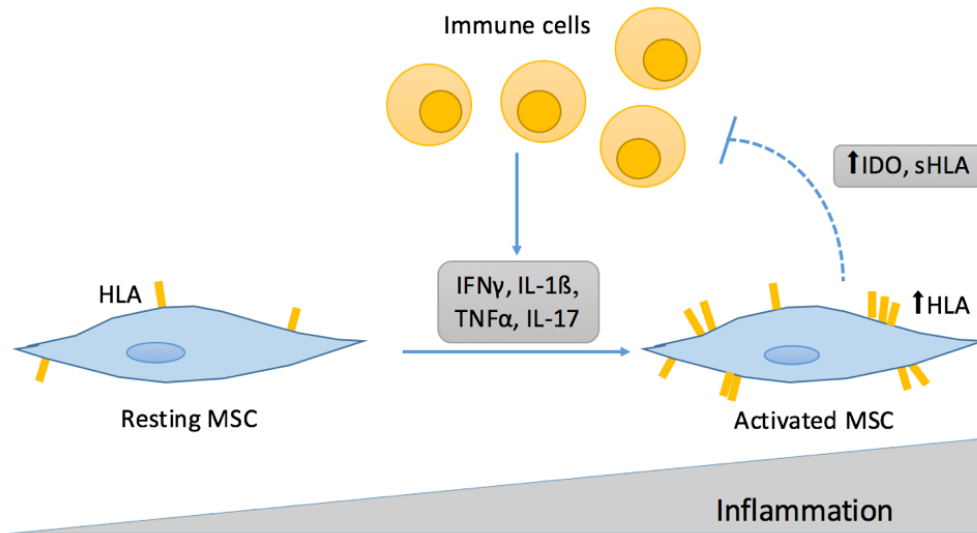
Figure 2.2: Influence of inflammation of immunomodulatory properties of MSCs (modified from James A Ankrum et al., 2003; Ying Wang et al., 2014)

Periodontal ligament cells

Periodontium is the surrounding tissues that support teeth during teeth function. It appears as the widening of periodontal space between tooth and alveolar bone on radiographs. In healthy periodontium, all of the tissue components are firm in texture. An intraoral radiograph with paralleling technique shows a well-defined alveolar bone crest and periodontal space, approximately 0.1-0.2 mm wide along the root.

Periodontal ligament (PDL) cells are fibroblast-like cells that occupy in high numbers in the periodontal ligament. These cells remodel the extracellular matrix, of which, the collagen fibers are the most prominent structures. PDL cells have been proposed as a source of mesenchymal stem cells (MSCs) [23, 74-77]. These cells possess characteristics of other stem cells including (i) a high level of MSC markers such as CD73, CD90 and CD105, (ii) self-renewal capacity, and (iii) multi-lineage

differentiation potential [23, 74-77]. PDL cells have also been demonstrated in their capability to differentiate into periodontal ligaments, alveolar bone, cementum, peripheral nerves, and blood vessels that are the important components of periodontal tissues[75-79]. Among tooth-derived MSCs, PDL cells have been demonstrated to have a very high capacity to differentiate into mineral-secreting cells in the presence of osteoinductive factors [80]. These inducible-osteoblastic cells expressed high level of alkaline phosphatase activity, osteocalcin expression and mineralized matrix formation, which are the major characteristics of osteoblasts. Thus, PDL cells have been chosen to study and look for their responses to any osteoinductive chemicals. Moreover, this cell also plays an important role to regulate osteoclast differentiation by an ability to express RANKL and OPG [81-83]. In addition, PDL cells were shown to respond to an immunological environment presented during periodontal inflammation, due to their expression of various toll like and cytokine receptors [24, 71, 84]. Taken together, these characteristics indicate an essential role of PDL cells to regulate the homeostasis of the periodontal ligament.

CHAPTER III

RESEARCH METHODOLOGY

Cell Culture

All of the experimental protocols were approved by the Ethics Committee of the Faculty of Dentistry, Chulalongkorn University. Human periodontal (hPDL) cells were obtained from non-carious third molars extracted for orthodontic reason with informed consent. The teeth were rinsed with sterile phosphate-buffered saline (PBS); then periodontal tissues were collected from middle third of the root surface. All hPDL cells were cultured in standard medium (Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100U/ml Penicillin, 100mg/ml Streptomycin, 5mg/ml Amphotericin B and 2mM L-glutamine). The cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air. After the cells reached confluency, they were detached with 0.25% trypsin-EDTA and subcultured at a 1:3 ratio. Cells from third-fifth passages of three different donors were used in the experiments.

Cellular Viability

The viability of hPDL cells after treatment with IL-12 was assessed by MTT assay. Briefly, hPDL cells were plated in triplicate into 24 well plates, at initial density of 50,000 cells/well. After allowing the cells to attach the plate for 24 h, the cells were starved in serum free medium for 6 h. The cells were then treated with IL-12 at various concentrations, range from 0, 0.5, 1 and 10 ng/ml, for 24, 48 and 72 h. After the incubation periods, 0.5 mg/ml MTTs solution (Sigma, MO, USA) was added into the culture plates and incubated for 30 min at 37°C. The reaction mixtures were then removed and the crystallized formazans were dissolved with elute solution. The

quantity of formazan was measure at 570nm using microplate reader (BioTek, ELx800, Winooski, VT). The absorbance was taken as proportional to the number of viable cells.

Application of IL-12 and IFNγ

To determine the influence of IL-12 and IFNγ, hPDL cells were plated into 12-well plates, at density of 2×10^5 cells/well. Recombinant human IL-12 (p70) (Peprotech, Rocky Hill, NJ) at concentrations of 0–10 ng/ml or recombinant human interferon gamma (IFNγ) (ImmunoTools, Friesoythe, Germany) at concentrations of 0-20 ng/ml was added into the culture medium for 1-7 days. Culture supernatants and extracted cells were then collected for further analysis. In some experiments, 50µM lisofylline (a STAT4 inhibitor) (Cayman chemical, Ann Arbor, MI, USA), 10nM NF-kB inhibitor (Calbiochem, EMD Chemicals, San Diego, CA, USA), 15µM suramin (G-protein coupled receptor inhibitor) (Calbiochem, San Diego, CA, USA), 7.92µM cyclohexamine (CHX) (Sigma-Aldrich Chemical, USA), 100µM monensin (Sigma-Aldrich Chemical, MO, USA), 10µM ethylene glycol tetraacetic acid (EGTA) (Sigma-Aldrich Chemical, USA), 12.5nM thapsigargin (Sigma-Aldrich Chemical, USA) , 0.5-1µg/ml anti-human IFNγ blocking antibody (eBioscience, San Diego, CA), or 3.75 nM JAK inhibitor I (Calbiochem, EMD Chemicals, San Diego, CA) was added to the culture medium 30 min before the addition of IL-12 or IFNγ.

Determination of IL-12 and IL-12 receptor (IL-12R) mRNA expression in PDL tissue

To determine the involvement of IL-12 in periodontal disease, the expression of IL-12 and IL-12R in periodontal tissue was investigated. Periodontal tissues were collected from the middle third of the root of non-carious third molars as described above. For the inflammatory periodontal tissue, the tissue was collect from extracted third molars with progressive loss of alveolar bone associated with periocoronal infection.

RNA Isolation and Reverse transcription-polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted with Trizol reagent (Molecular Research Center, Cincinnati, OH). Chloroform was then added into the specimens, followed by precipitating in 500μl isopropanol. Then, the RNA pellets were washed with 75% ethanol and dissolved in 10μl of Diethyl Pyrocarbonate (DEPC). Total amount of RNA was quantified using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Wilmington, DE). One microgram of total RNA per sample was converted to complementary DNA (cDNA) by reverse transcriptase (RT) (Promega, Madison, WI). Subsequently, polymerase-chain reactions (PCR) were performed using Taq polymerase (Taq DNA Polymerase, Invitrogen, Brazil) in a DNA thermal cycler (BiometraGmBH, Göttingen, Germany). Glyceraldehyde phosphate dehydrogenase (GADPH) was used as an internal control. The products were electrophoresed on a 2% agarose gel and visualized using ethidium bromide (EtBr; Bio-Rad, Hercules, CA) for fluorostaining. Results obtained from each of experiment groups were showed as band densities, which were then analyzed into the expression ratio compared to control.

Real-Time Polymerase Chain Reaction (Real-Time PCR).

Real-time PCR was performed using the LightCycler1 480 SYBR Green I Master kit (Roche Diagnostic) in a MJ Mini^{TM} Thermal Cycler (Bio-Rad). Gene expression levels were normalized to the expression level of GAPDH. Then, relative gene expression was calculated by CFX Manager^{TM} software (Bio-Rad). The results were shown as fold-change values relative to the control group. The primers sequences were shown in Table 3.1.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA was performed to determine the amount of RANKL and IFN-γ proteins in both culture supernatants and cell extracts. To detect the presence of soluble protein, supernatant were collected and centrifuged at 1,500 rpm for 10 min at 4°C to remove cell debris. Cellular proteins from cell lysate were extracted with

Radioimmunoprecipitation assay buffer (RIPA buffer; 50 mM Tris–HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Nonidet P-40, 0.1% sodium dodecyl sulphate) supplemented with protease inhibitors. Total amount of protein was quantified by a BCA protein assay kit (Pierce, Rockford, IL). To determine the expression levels of RANKL and IFN-γ proteins, human sRANK Ligand ELISA kit and Human IFN-γ Standard TMB ELISA Development Kit (Peprotech, Rocky Hill, NJ) were used, respectively. The absorbance of ELISA reaction product was measured at 405 nm (for RANKL) and 450 nm (for IFNγ), using a microplate reader (BioTek, ELx800, Winooski, VT).

NF-kB Immunofluorescence Staining

To determine the role of NF-kB activation, hPDL cells were seeded into a chamber slide (Lab-TEK® II Chamber slide™ System; Nalge Nunc International Corp., Rochester, NY) at a density of 50,000 cells per chamber. After 6 h starvation in serum free medium, cells were incubated with 1ng/ml of IL-12 for 4 h in presence or absence of STAT4 inhibitor. At the evaluated time, cells were washed twice with PBS and fixed with cold methanol (Merck KGAA, Darmstadt, Germany) for 10 min. Then, the cells were incubated overnight with rabbit-antihuman NF-kB p50 antibody (NLS; dilution 1:200; Santa Cruz Biotechnology), followed by biotinylated goat-anti-rabbit secondary antibody (dilution 1:1000; Zymed Laboratories Inc., South San Francisco, CA). Streptavidin–fluorescein isothiocyanate (FITC) (dilution 1:1000; Sigma-Aldrich Chemical) system was used to detect the localization of NF-kB. Cellular NF-kB translocation was observed using an Apotome photographic system (Zeiss, Germany). The rabbit IgG (Sigma) was used as the negative control in this experiment.

Flow Cytometry

Flow cytometry was performed to detect the expression of HLA-G cell surface protein. HPDL cells ($2×10^6$ cells/sample) were dissociated and resuspended in 200 μl of FACS buffer and stained with purified anti-human HLA-G (eBioscience, San Diego, CA). After incubating in primary antibody, cells were stained with APC-conjugated anti-

mouse IgG2a (Abcam, Cambridge, UK). Stained cells were analyzed according to their fluorescence intensity on a FACSCalibur™ using the CellQuest™ softwere (BD Bioscience, San Jose, CA).

Colony Forming Unit Assay

To examine the self-clonal expansion capacity of hPDL cells, colony forming unit (CFU) assay was perform. HPDL cells were seeded at a low-density of 5,000 cells per 60-mm-diameter culture dishes and maintained in serum-containing growth medium for 14 days, in presence and absence of 1ng/ml IL-12. After 14 days of incubation, cells were wash twice with PBS and fixed with 10% formalin for 10 min. Then, the cultures were stained with Coomassie brilliant blue (Sigma-Aldrich, St. Louis, MO) to visualize the appearance of colonies in cultures. Total number and the size of colonies were counted, compared to the control.

Alizarin red S staining

To examine the osteogenic differentiation potential of hPDL cells, level of *in vitro* calcification was determined by alizarin red s staining. HPDL cells were cultured in osteogenic induction medium (Dulbecco's modified Eagle's medium supplemented with 50 μg/mL of ascorbate-2-phosphate, 10 mM β-glycerophosphate and 100 nM Dexamethasone) in presence and absence of 1ng/ml of IL-12 for 14 days. Then, the cells were fixed with cold methanol for 10 min and washed with deionized water. The cells were stained with 1% alizarin red S solution (Sigma, OM, USA) for 5 min at room temperature. The amount of calcium deposition was quantified by destaining with 10% cetylpyridinium chloride monohydrate (Sigma, OM, USA) in 10 mM sodium phosphate for 15 min at room temperature. The absorbance was evaluated at 570 nm using microplate reader (BioTek, ELx800, Winooski, VT).

IDO Enzymatic Activity Assay

Cellular protein was extracted with 0.1% sodium dodecyl sulfate (SDS) solution.

The amount of protein was quantified by a BCA protein assay kit (Pierce). To determine IDO activity in cell lysates, 50μl of cell lysates were incubated with 2X IDO assay buffer (100 mM phosphate buffer saline, 20 μM methylene blue (Sigma, USA), 40 mM ascorbate (Sigma, USA), 200 μg/ml of catalase (Sigma, USA) and 800 μM L-tryptophan(Sigma, USA)) for 1h at 37°C [85, 86]. The reactions were stopped by incubating with 20μl of 30% trichloroacetic acid (Sigma, USA) for 30 min at 50°C. The samples were then centrifuged at 13,000 g for 10 min at 4°C and the supernatants were collected and mixed with an equal volume of Ehrlich reagent (2% *p*-dimethylbenzaldehyde (Sigma, USA) in glacial acetic acid). The absorbance was measured at 492nm using microplate reader (BioTek, ELx800, Winooski, VT).

Western Blot Analysis

Protein extraction was performed using radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors (Sigma-Aldrich, St. Louis, MO). The protein samples were fractionated by SDS-PAGE (10% gel) and then transferred onto nitrocellulose membranes. The membranes were blocked with 10% FBS in PBS for 1 h. The membranes were then incubated overnight at 4°C with primary antibody to RANKL (Peprotech, Rocky Hill, NJ, USA), pSTAT4 (Invitrogen[TM], Thermo Scientific, Waktham, MA), STAT4 (Santa Cruz Biotechnology, Dallas, TX), or β-actin (Merck Millipore, Darmstadt, Germany). After washing the membrane was incubated with a biotinylated secondary antibody (dilution 1:1,000), followed by an incubation with peroxidase-labeled streptavidin (dilution 1:1,000). The staining was detected using chemiluminescence (Thermo Scientific, Wilmington, DE).

Statistical Analyses

All experimental results were reported as mean ± standard deviation (SD). Statistic analysis was assessed by Kruskal-Wallis test. The differences at $p < 0.05$ were considered as a statistical significant difference.

Table 3.1: Primers used for quantitative PCR

Primers	Forward/ Reverse	Primer sequence (5'-3')	Sequence ID
GAPDH	F	CAC TGC CAA CGT GTC AGT GGT G	NM_002046.5
	R	GTA GCC CAG GAT GCC CTT GAG	
qGAPDH	F	CACTGCCAACGTGTCAGTGGTG	NC_018917.2
	R	GTAGCCCAGGATGCCCTTGAG	
IL-12	F	GGA TGC CCC TGG AGA AAT	NM_002187.2
	R	GCA GGA GCG AAT GGC TTA	
IL-12Rβ2	F	CAG CAC ATC TCC CTT TCT GTT TTC	NC_018912.2
	R	ACT TTA AGG CTT GAA GCC TCA CC	
IFNγ	F	CTA GGC ACG CAA CCT AAG CA	NG_015840.1
	R	CAG GGT CAC CTG ACA CAT TC	
RANKL	F	TCA GCC TTT TGC TCA TCT CAC TAT	NM 033012.3
	R	CCA ACC CCG ATC ATG GT	
OPG	F	TCA AGC AGG AGT GCA ATC G	NM 002546.3
	R	AGA ATG CCT CCT CAC ACA GG	
HLA-G	F	AAG AGG AGA CAC GGA ACA CC	NM_002127.5
	R	TCG CAG CCA ATC ATC CAC TG	
IDO	F	CAT CTG CAA ATC GTG ACT AAG	NG_028155.1
	R	GTT GGG TTA CAT TAA CCT TCC TT	
CD73	F	ATT GCA AAG TGG TTC AAA GTC A	NM_002526.2
	R	ACA CTT GGC CAG TAA AAT AGG G	
CD90	F	GAA GAC CCC AGT CCA GAT CAA	NM_006288.4
	R	TGC TGG TAT TCT CAT GGC GG	
CD105	F	CAT CAC CTT TGG TGC CTT CC	NM_001114753.1
	R	CTA TGC CAT GCT GCT GGT GGA	
Nanog	F	CAG CCC CGA TTC TTC CAC CAG TC	NM_024865.3
	R	CGG AAG ATT CCC AGT CGG GTT CA	

Gene		Primer sequence	Accession number
4-Oct	F	GCA ACC TGG AGA ATT TGT TCC T	NM_002701.4
	R	AGA CCC AGC AGC CTC AAA ATC	
SOX-2	F	ACC AGC TCG CAG ACC TAC AT	NM_003106.3
	R	ATG TGT GAG AGG GGC AGT GT	
ALP	F	CGA GAT ACA AGC ACT CCC ACT TC	NM 000478.3
	R	CTG TTC AGC TCG TAC TGC ATG TC	
Type I collagen	F	GTG CTA AAG GTG CCA ATG GT	NM 000088.3
	R	ACC AGG TTC ACC GCT GTT AC	
Cbfa1	F	ATG ATG ACA CTG CCA CCT CTG A	NM 001024630.2
	R	GGC TGG ATA GTG CAT TCG TG	
BSP	F	ATG GCC TGT GCT TTC TCAATG	NM 004967.3
	R	AGG ATA AAA GTA GGC ATG CTT G	
SOST	F	ACT TCA GAG GAG GCA GAA ATG G	NM_025237.2
	R	CAA GGG GGA ATC TTA TCC AAC TTT C	
TWIST1	F	TCT TAC GAG GAG CTG CAG ACG CA	NM_000474.3
	R	ATC TTG GAG TCC AGC TCG TCG CT	
TWIST2	F	GCT GCG CAA GAT CAT CCC	NM_057179.2
	R	GTA GCT GCA GCT GGT CAT C	

CHAPTER IV

RESULTS

OBJECTIVE 1: to investigate the change in RANKL/OPG expression ratio in human periodontal ligament cells after IL-12 treatment

Expression of IL-12 and IL-12 receptor in inflammatory periodontal tissues and hPDL cells

As IL-12 was found increase during periodontal inflammation, we hypothesize that this cytokine might play a role to regulate homeostasis of periodontal tissue during periodontal inflammation. To determine the possible involvement of IL-12, the presence of IL-12-producing and IL-12-responsive cells in inflammatory periodontal tissues was investigated. Human periodontal tissues were collected from extracted third molars, which had been diagnosed with periodontal disease. The presence of IL-12 and IL-12 receptor (IL-12R) were then examined by RT-PCR. The results shown in Figure 4.1A (lanes A and B) revealed the expression of IL-12 and IL-12R in inflammatory periodontal tissues, suggesting the presence of IL-12-producing and IL-12-responsive cells at the site of periodontal inflammation.

Next, the ability of hPDL cells to respond to exogenous IL-12 was investigated. HPDL cells were obtained from periodontal tissue and cultured with normal growth medium as described in Materials and Methods. The result showed that hPDL cells expressed IL-12R but not IL-12 (Figure 4.1A, lanes C and D). HPDL cells were then treated with 0-10 ng/ml of exogenous IL-12 for 24 hours. The results from RT-PCR analysis showed an upregulation of IFNγ, one of the well-known cytokines known to be modulated by IL-12 [1, 2], in dose-dependent manner (Figure 4.1B and 4.1C).
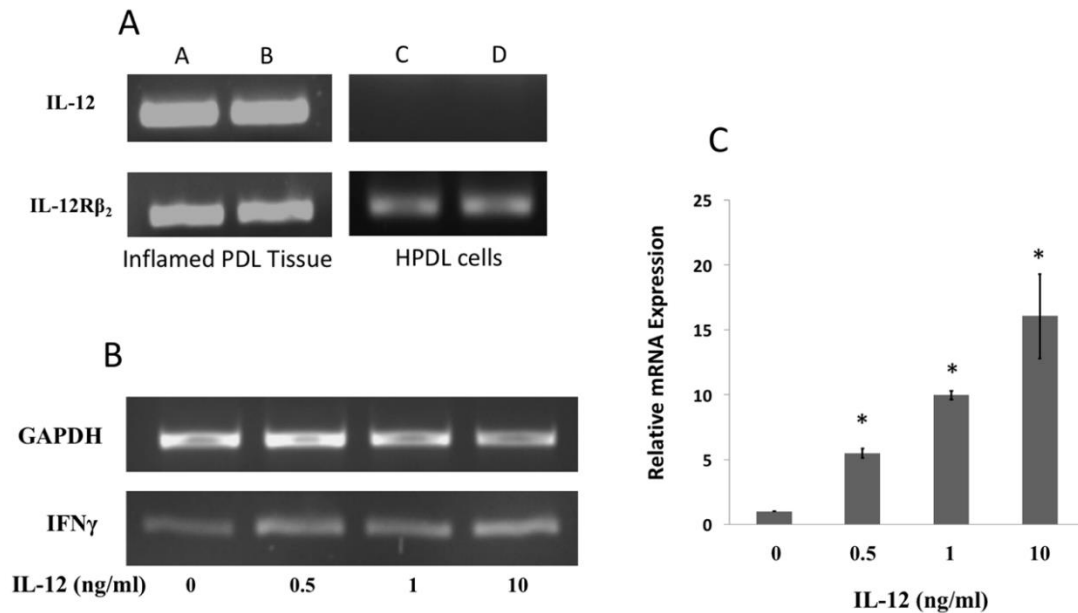
Figure 4.1 Response of hPDL cells to exogenous IL-12.

Expression of IL-12 and IL-12R in both inflamed periodontal tissues (A; lane A and B) and hPDL cells (A; lane C and D) was detected by RT-PCR. The presence of IL-12 was found in inflammatory periodontal tissues. Both inflamed periodontal tissues and hPDL cells expressed IL-12R. HPDL cells were treated with 0-10 ng/ml of recombinant IL-12. After 24h incubation, the expression level of IFNγ, a well-known target gene of IL-12, was detected by RT-PCR (B and C). The expression level of IFNγ was significantly increased in dose-dependent manner after IL-12 treatment. Data were shown as mean ± SD from three triplicate experiments. * indicated significant difference (p<0.05).

RANKL/OPG expression ratio is upregulated in hPDL cells following IL-12 administration.

To investigate the osteoimmunological effect of IL-12, hPDL cells were treated with 0-10 ng/ml of IL-12 for 24h. Total RNA was then extracted and the expression level of RANKL and OPG was examined by RT-PCR. An up-regulation of RANKL expression was detected in a dose-dependent manner, after IL-12 treatment. However, OPG expression was not altered under the influence of IL-12 (Figure 4.2A). This resulted in a significant increase in RANKL/OPG expression ratio in hPDL cells (Figure 4.2B). The concentration of IL-12 at 1 ng/ml was chosen for subsequent experiments.

The protein level of RANKL was evaluated by western blotting and ELISA. After 5 days of IL-12 incubation, in cell lysates a significant increase of RANKL protein was found, both by western blot and ELISA (Fig. 4.2C and 4.2D). No change in RANKL protein level was detected in the supernatant of these cells (Fig. 4.2E). These data indicate an up-regulation of the expression of cell-associated, probably membrane-bound, RANKL.
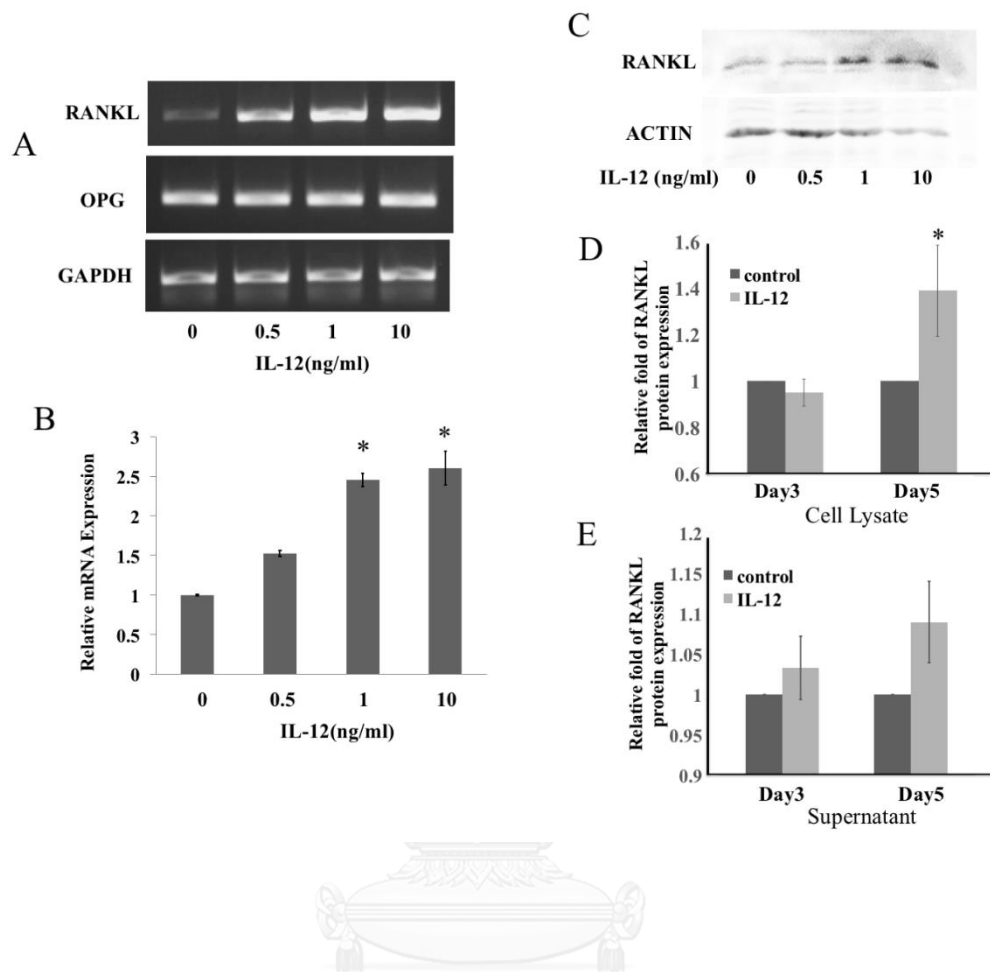
Figure 4.2 The influence of IL-12 on RANKL expression in hPDL cells.

HPDL cells were treated with 0-10 ng/ml of IL-12 for 24h. Then, the expression level of RANKL and OPG was determined by RT-PCR. An upregulation of RANKL mRNA expression was detected in a dose-dependent manner. No change in mRNA expression of OPG was found at any concentration of IL-12 (A). A significant increase in RANKL/OPG ratio was detected in hPDL cells following IL-12 treatment (B). HPDL cells were cultured in presence or absence of 1 ng/ml of IL-12 for 3 and 5 days. Protein level of RANKL was evaluated by western blot analysis (C) and ELISA both in the cell lysate (D) and supernatant (E). A significant increase in RANKL protein was detected by western blotting and ELISA of cell lysates after 5 days of IL-12 incubation. Data were shown as mean ± SD from three triplicate experiments. * indicated significant difference (p<0.05).

IL-12-induced RANKL expression involves STAT4 and NF-kB signaling pathways

STAT4 phosphorylation has been proposed as a major cellular effect of the IL-12 canonical pathway [36], while in dendritic cells and macrophages, NF-kB has been demonstrated as a key transcription factor in response to IL-12 [87]. To determine the role of STAT4 and NF-kB in IL-12 induced RANKL expression, hPDL cells were treated with IL-12 in the presence or absence of specific inhibitors against STAT4 (STAT4i) and NF-kB (NF-kBi). The results shown in Figure 4.3A-B demonstrated that both STAT4i and NF-kBi significantly attenuated the inducing effect IL-12 by reducing the expression level of RANKL to 59% and 70%, respectively. This data indicates that IL-12-induced RANKL expression is mediated by both STAT4 and NF-kB signaling pathways.

To confirm the activation of IL-12-STAT4 signaling pathway in hPDL cells, the level of phosphorylated STAT4 was determined by western blotting. The upregulation of phosphorylated STAT4 was detected within 10 min after IL-12 administration (Fig. 4.3C). The NF-kB translocation was then analyzed by immunofluorescence staining. The results demonstrated the translocation of NF-kB into the nuclei of the hPDL cells after an IL-12 treatment for 4 h (Fig. 4.3D). The presence of STAT4i could not inhibit this translocation process, suggesting the existence of separate pathways: one mediated by STAT4 and one by NF-kB signaling (Fig. 4.3D).
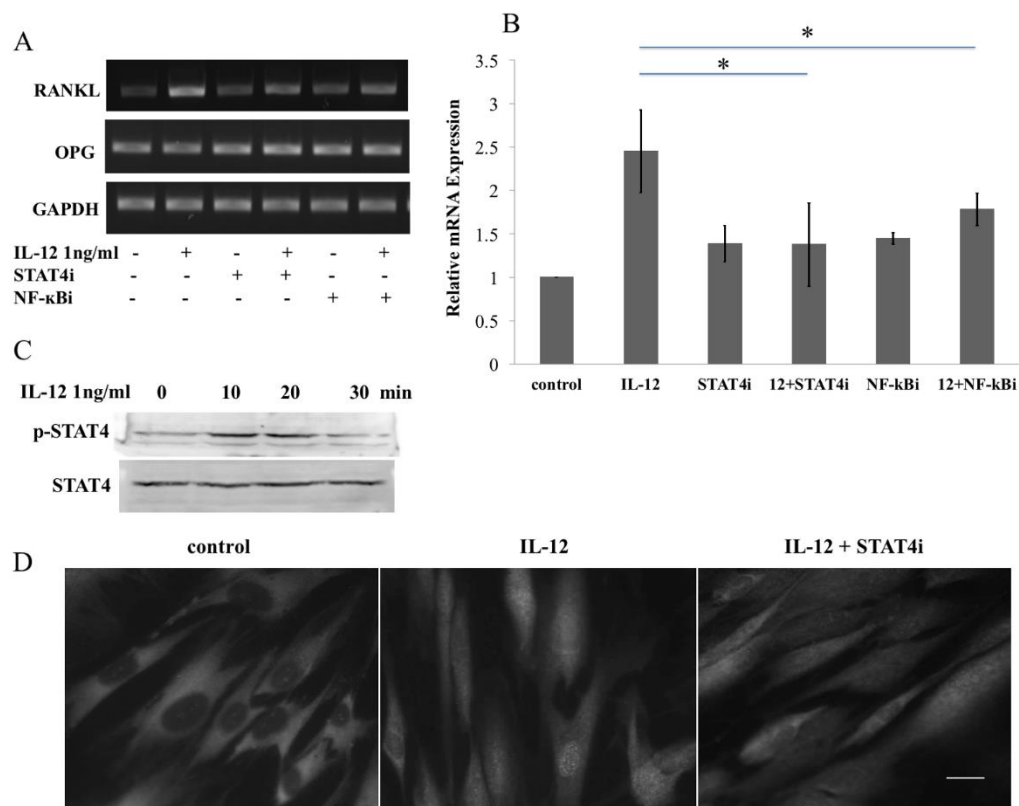
Figure 4.3 IL-12-induced RANKL expression mediated by STAT4 and NF-kB signaling pathways.

HPDL cells were treated with 1 ng/ml of IL-12, in presence or absence of STAT4i and NF-kBi. The expression level of RANKL was analyzed by RT-PCR. Both STAT4i and NF-kBi significantly reduced the promoting effect of IL-12 on RANKL expression by 59% and 70%, respectively (A and B). To determine the activation of STAT4 signaling, the cells were incubated with 1 ng/ml of IL-12 for 0 to 30 min, and the level of p-STAT4 was detected by western blotting (C). Upregulated level of p-STAT4 was found after IL-12 administration. Next, hPDL cells were seeded in chamber slides and treated with IL-12 in the presence or absence of STAT4i for 4h. Immunofluorescence was performed to detect the translocation of NF-kB by using anti-NF-kB p50 (D). Nuclear translocation of NF-kB was observed in presence of IL-12, both IL-12 treated alone and in presence of STAT4i. STAT4i = STAT4 inhibitor, NF-kBi = NF-kB inhibitor, p-STAT4 = phosphorylated

STAT4. Bar = 10 μm. Data were shown as mean ± SD from three triplicate experiments. * indicated significant difference (p<0.05).

The inductive effect of IL-12 on RANKL expression occurs indirectly via promoting secretion of intermediate molecule(s)

To determine the involvement of intermediate molecule(s) in IL-12 induced RANKL expression, conditioned medium (CM) assay was performed. HPDL cells were cultured for 24h in presence or absence of IL-12 as treated or control condition, respectively. The medium in both conditions was then replaced with the fresh medium (without IL-12) for another 24 hours. After 24h, these conditioned mediums were collected from control culture (C-CM) and IL-12 treated cells (IL-12-CM) and then transferred for treating another culture set of hPDL cells. The result showed a significant increase in RANKL expression in cells incubated with IL-12-CM; an increased level was similar to those found in cultures directly incubated with IL-12 (Figure 4.4A). This finding suggests the involvement of intermediate molecule(s) released into the culture medium in the inducing effect of IL-12 on RANKL expression. In addition, NF-kB, but not STAT4, was found as an important signaling molecule mediated in intermediate molecule-induced RANKL expression, since the NF-kBi, but not the STAT4i, significantly inhibited the IL-12-CM-induced RANKL expression (Figure 4.4B).

Surprisingly, addition of cycloheximide (CHX), an inhibitor of protein synthesis, could not inhibit the promoting effect of IL-12 on RANKL expression (Figure 4.4C). However, the expression level of RANKL induced by IL-12 was significantly decreased in presence of monensin. These findings suggest that intracellular protein trafficking rather than de novo synthesis, was important for IL-12 to induce RANKL expression (Figure 4.4C and 4.4D).

Calcium has been proved not only as a necessary molecule for intracellular protein trafficking [88, 89], but calcium ion signaling has been shown also to be an essential mediator for IL-12 activation [90]. Therefore, we hypothesized that calcium signaling might participate in the IL-12-induced RANKL expression. To determine the influence of calcium signaling, EGTA and thapsigargin were used as calcium antagonist

and agonist, respectively. The result in Figure 4.4E demonstrated a significant decrease in RANKL expression in the presence of EGTA, a calcium ion chelator. However, thapsigargin, the drug that increases the level of cytosolic calcium, enhanced the inducing effect of IL-12 on RANKL expression. These findings suggest a role for calcium signaling in the IL-12 induced RANKL expression.

EGTA was then used in IL-12-CM-treated hPDL cells, to determine the role of calcium signaling in intermediate molecule(s)-induced RANKL expression. The results showed that EGTA had no effect on IL-12-CM induced RANKL expression (Figure 4.4F). These data indicate that calcium signaling is not involved in the process of intermediate molecule(s) induced RANKL expression.

A

RANKL
GAPDH

| IL-12 | - | + | - |
| CM | - | - | + |

C

RANKL
GAPDH

control   IL-12   IL-12
+NF-kBi

IL-12
+CHX

IL-12
+Monensin

B

Relative mRNA Expression

*

control   IL-12   CM2(IL-12)   CM2(IL-12)+STAT4i   CM2(IL-12)+NF-kBi

D

Relative mRNA Expression

*

control   IL-12   IL-12
+CHX

IL-12
+Monensin

E

Relative mRNA Expression

*

control   IL-12   IL-12+EGTA   IL12+Thap

F

Relative mRNA Expression

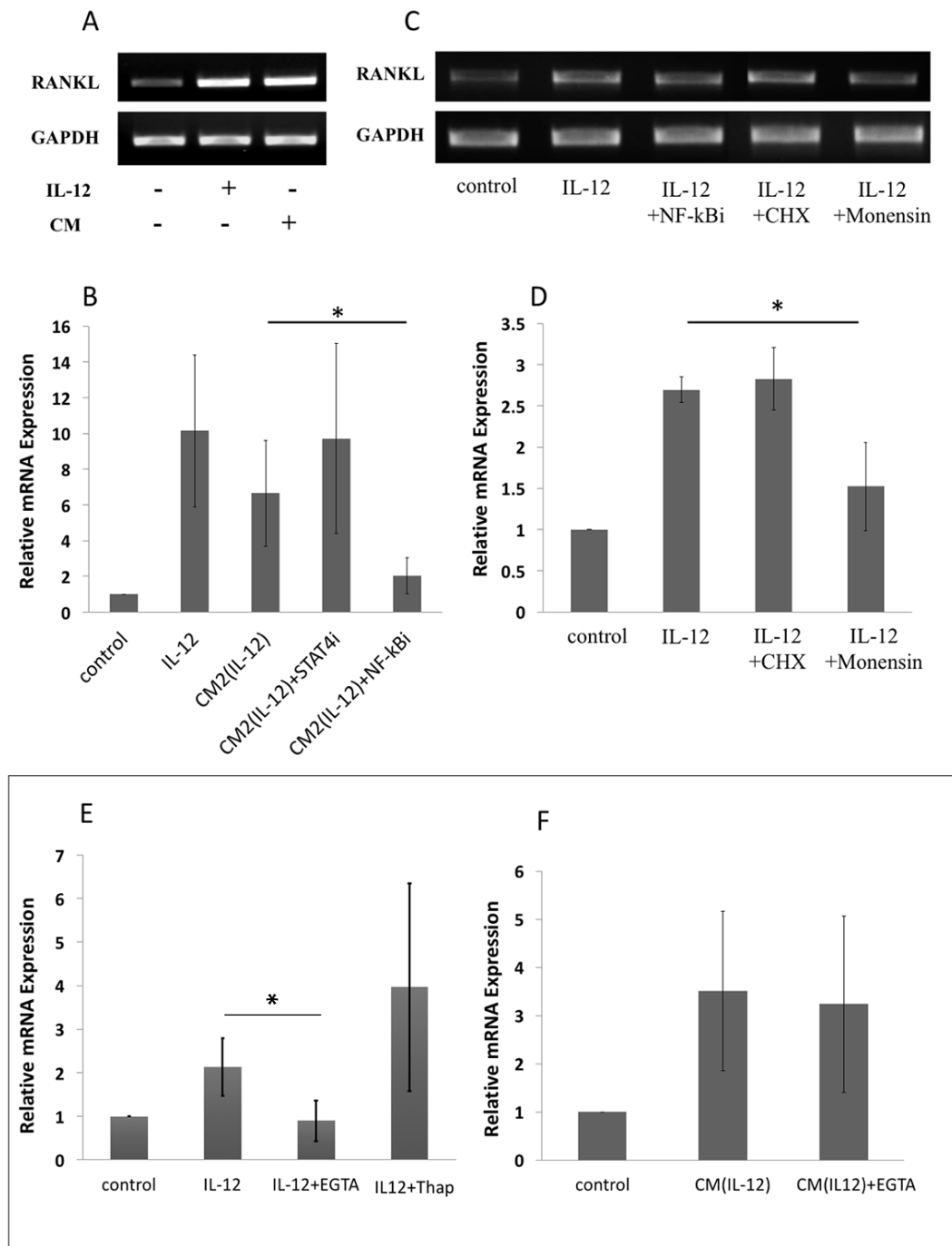control   CM(IL-12)   CM(IL12)+EGTA

Figure 4.4 Intermediate molecule(s) and calcium signaling were required for IL-12-induced RANKL expression.

HPDL cells were cultured with 1 ng/ml of IL-12 and IL-12-CM for 24h. The ability of IL-12-CM to induce RANKL expression was determined by RT-PCR. IL-12-CM significantly increased the expression of RANKL at the same level of IL-12 treatment (A). The IL-12-CM-induced RANKL expression was attenuated by NF-kBi, but not STAT4i (B). Fig. 4C showed the RT-PCR analysis and the graph in Fig. 4D revealed the quantitative analysis. The results showed that cycloheximide (CHX) did not affect the IL-12-induced RANKL mRNA expression, while monensin attenuated this inductive effect. Fig. 4 E and F showed the involvement of calcium on IL-12-induced and IL-12-CM-induced RANKL expression, respectively. HPDL cells were treated with 1 ng/ml of IL-12 for 24 h in presence or absence of EGTA or thapsigargin. The expression level of RANKL was measured by RT-PCR. EGTA significantly reduced the inductive effect of IL-12 on RANKL expression, while thapsigargin enhanced this inductive effect. EGTA had no effect on IL-12CM-induced RANKL expression. Data was shown as mean $\pm$ SD from three separated experiments. * indicated significant difference (p<0.05).

Suramin-sensitive molecule is an intermediate molecule mediated in IL-12 induced RANKL expression

In order to identify the possible intermediate molecule(s) involved in IL-12-induced RANKL expression, the effect was tested of a series of inhibitors. The results showed that addition of suramin significantly inhibited the increase of RANKL expression, induced by both IL-12 and IL-12-CM (Figure 4.5A and 4.5B). However, the level of OPG did not change by suramin in both situations (Figure 4.5C and 4.5D). As suramin functions to inhibit the activation of G-protein coupled receptor (GPCR), it is possible that a member of G protein coupled receptor ligand participated in IL-12-induced RANKL expression in hPDL cells.
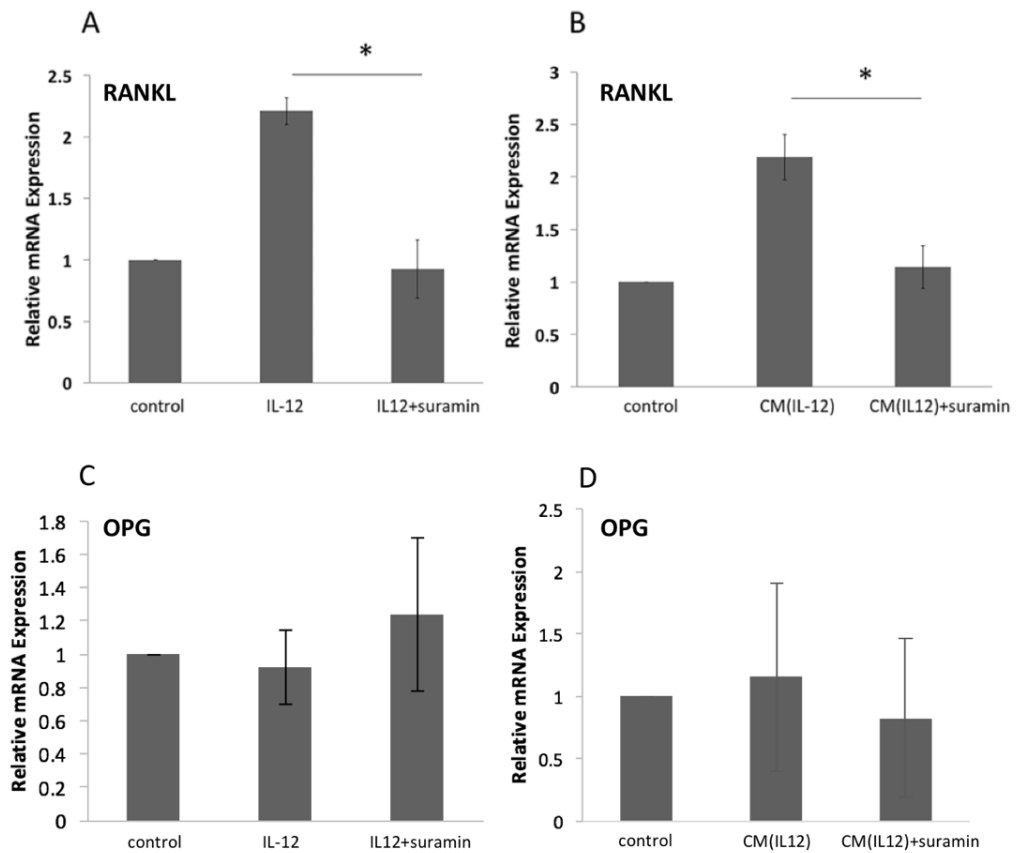
Figure 4.5 Member of G protein coupled receptor ligand might mediate IL-12-induced RANKL expression.

HPDL cells were treated with 1 ng/ml of IL-12 in presence or absence of suramin. Addition of suramin significantly inhibited both IL-12-induced RANKL (A) and IL-12-CM-induced RANKL expression (B). No change in mRNA expression of OPG was found under influence of suramin in both IL-12 (C) and IL-12-CM (D) conditions. * indicated significant difference (p<0.05). Data was shown as mean ± SD from three experiments

OBJECTIVE 2: to determine the effect of IL-12 on expression of immunosuppressive molecules in human periodontal ligament cells

IL-12 promotes immunomodulatory properties of hPDL cells

Since one cytokine can function in both pro- and anti-inflammatory ways as a mechanism to maintain tissue homeostasis, we decided to determine the immunomodulatory effect of IL-12 on hPDL cells. HPDL cells were incubated with exogenous IL-12 from 0 to 10 ng/ml for 24h and the expression level of IFNγ, IDO and HLA-G, which are potent immunosuppressive molecules, was then detected by quantitative real-time PCR. The results demonstrated the promoting effect of IL-12 on IFNγ, IDO and HLA-G mRNA expression (Figure 4.6A-C).
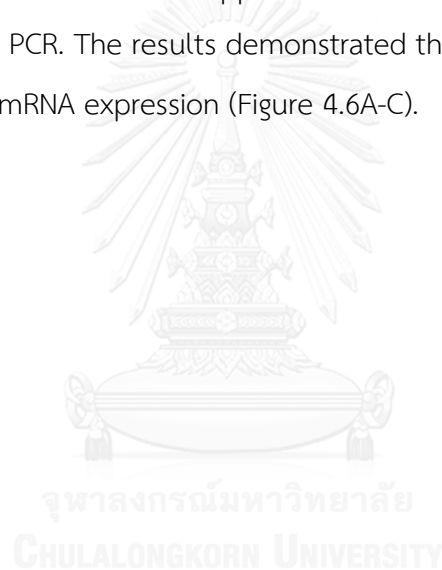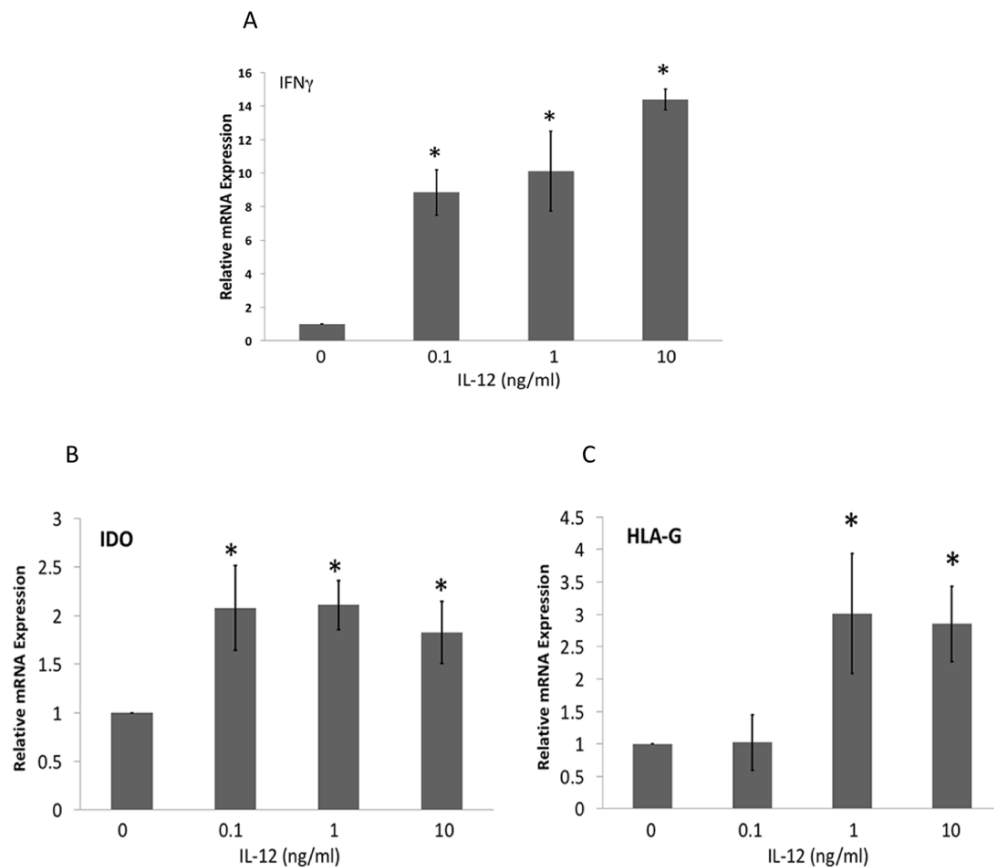
Figure 4.6 IL-12 upregulated the expression of IFNγ, IDO and HLA-G in hPDL cells. HPDL cells were treated with 0-10 ng/ml of IL-12 for 24 hours. The results from real-time PCR analysis (A-C) showed that IL-12 treatment promoted the expression of IFNγ, IDO and HLA-G in hPDL cells. The results were shown as mean ± S.D. form three independent experiments. * indicated the significant different from the control (p<0.05).

Protein analysis by ELISA demonstrated an increased level of IFNγ protein, confirming the inductive effect of IL-12 on IFNγ expression. The increased expression of the protein was detected after an incubation period of 3 and 5 days and it proved to be present only in the cell lysate (Figure 4.7A); no IFNγ was detectable in the conditioned medium.

Moreover, protein expressions of IDO and HLA-G were also induced by IL-12 (Figure 4.7B-C). The analysis of IDO enzymatic activity revealed a significant increase in IDO protein level at day 7 only in the cell lysate. An increased population of HLA-G-positive cells was found at day 5 as detected by flow cytometric analysis.
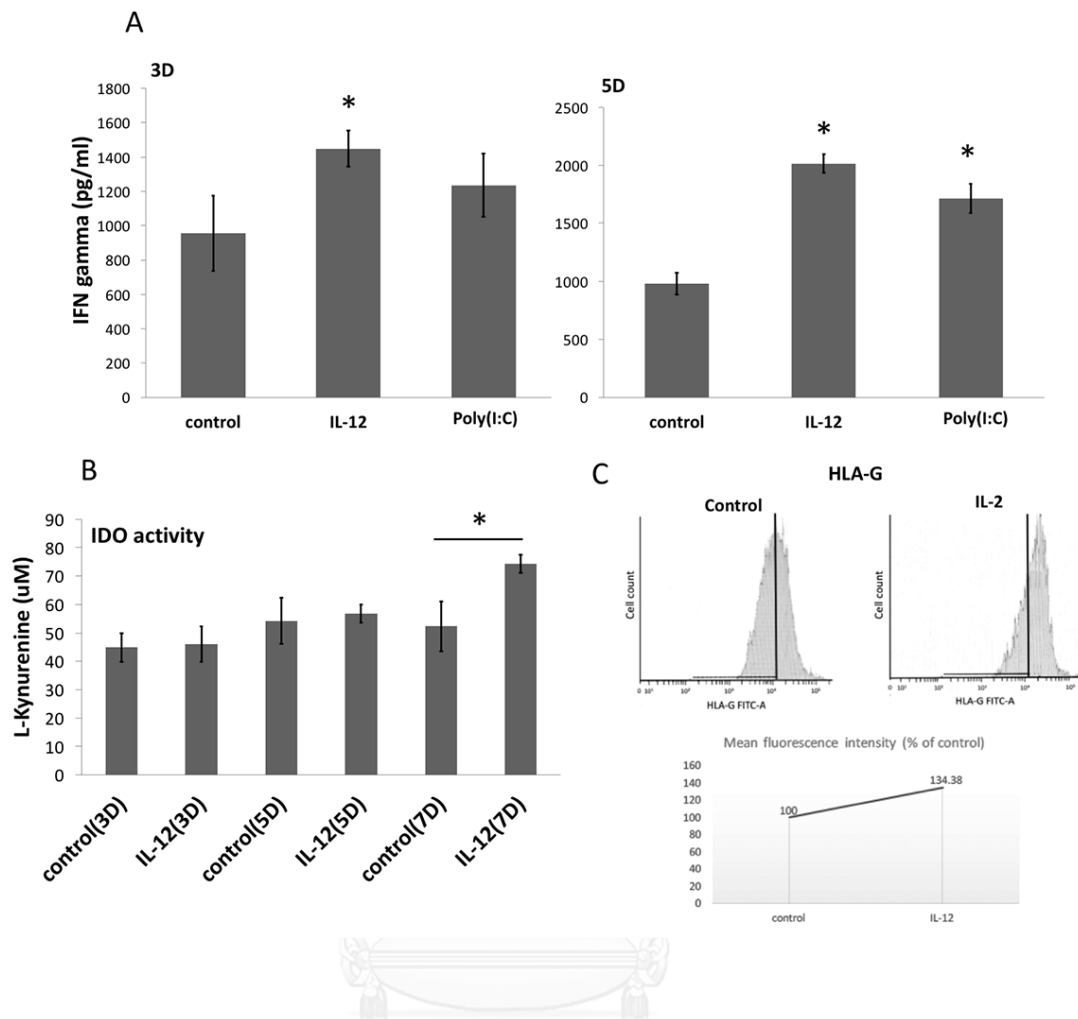
Figure 4.7 Increased protein levels of IFNγ, IDO and HLA-G in HPDL cells after IL-12 treatment.

HPDL cells were treated with 0-10 ng/ml of IL-12 and the expression of IFNγ protein in both conditioned medium and cell extracted was analyzed by ELISA (A). Poly(I:C), a TLR3 agonist, was used as a positive control since IFNγ is a common target of TLR3 activation. The increased level of IFNγ protein was found after treatment with IL-12 for 3 and 5 days. IDO secretion was monitored by enzymatic activity assay (B) and HLA-G expression was monitored by Flow cytometry (C). The significant increased activity of IDO was observed at day 7 under influence of 1 ng/ml of IL-12. Increase population of HLA-G positive was found at day 5 after IL-12 treatment. The results were shown as mean ± S.D. from three independent experiments. * indicated the significant differences as compared to the control (p<0.05).

IL-12 preserves stemness properties of hPDL cells

As immunomodution is an important characteristic of MSCs and IFNγ, HLA-G and IDO are known to be crucial factors involved in the immunomodulatory properties of MSCs, we hypothesize that a similar process might play a role in regulating the stemness properties of hPDL cells. To prove this hypothesis, we investigated the influence of IL-12 on some stem cell/pluripotent markers, the colony forming capacity and the osteogenic differentiation potential of hPDL cells.

After 14 days of incubation in serum containing medium, no significant difference was found in both number and size of colonies in control or IL-12 treated conditions (Figure 4.8A). However, the mRNA expression levels of the stem cell/pluripotent markers; including CD73, CD90, CD105, Nanog, Oct4 and Sox2, were significantly increased, following treatment with IL-12 for 48h (Figure 4.8B).

To determine the effect of IL-12 on osteogenic differentiation of hPDL cells, cells were cultured under osteogenic induction medium in the presence or absence of IL-12. The results shown in Figure 4.8C demonstrated an inhibitory effect of IL-12 on the *in vitro* calcification of hPDL cells. This inhibitory effect coincided with an increased level of SOST, the BMP antagonist, after IL-12 treatment (Figure 4.8D).
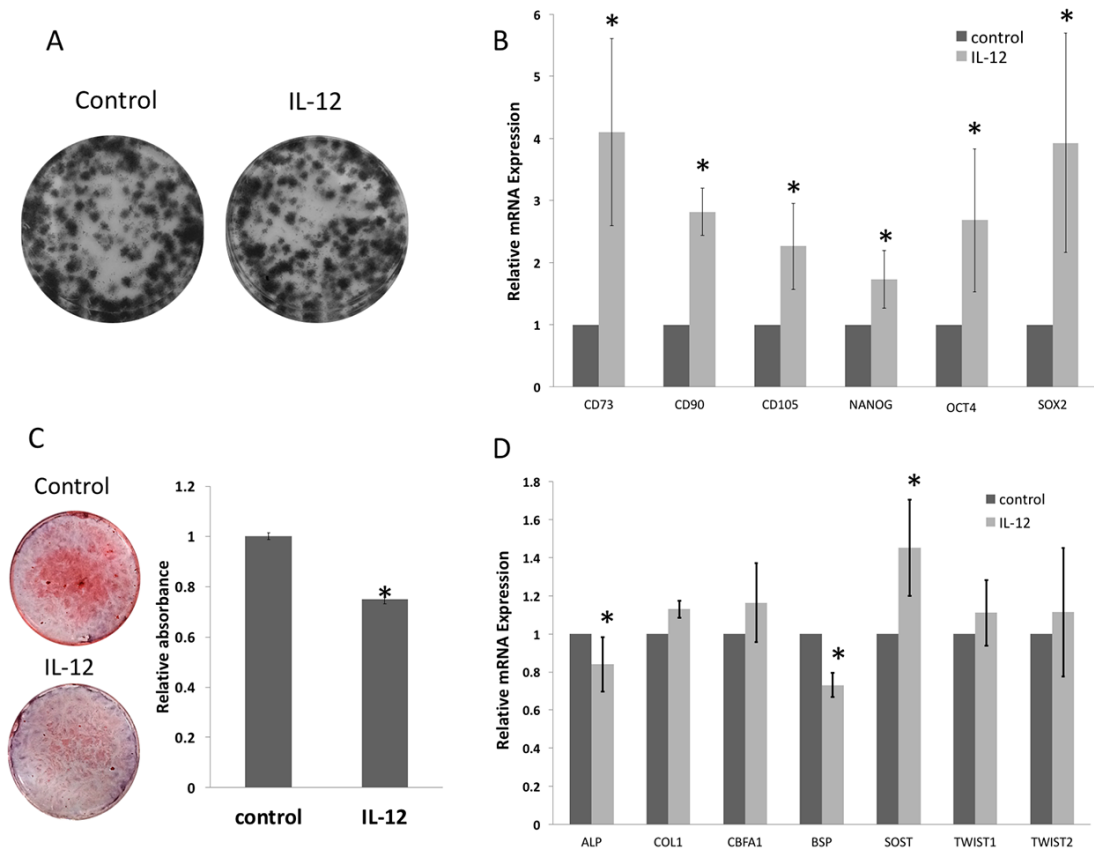
Figure 4.8 IL-12 upregulated stem cell/pluripotent markers and inhibited osteogenic differentiation of hPDL cells.

HPDL cells were treated with 1 ng/ml of IL-12. No significant difference was found in colony forming unit assay (A). Real-time PCR result showed the upregulation of stem cell/pluripotent markers; including CD73, CD90, CD105, nanog, Oct4 and Sox2, after treatment with IL-12 for 48 h (B). IL-12 also inhibited the osteogenic differentiation of hPDL cells cultured under osteogenic induction medium as judged by the alizarin red S staining (C). The inhibitory effect of IL-12 on osteogenic differenitation might due to the upregulation of SOST, the BMP antagonist, as shown in real-time PCR analysis (D). The results were shown as mean $\pm$ S.D. from three independent experiments. * indicated the significant differences as compared to the control ($p < 0.05$).

IL-12 mediated IFNγ promotes immunomodulatory property of hPDL cells

As IFNγ has been demonstrated as a primary inducer to promote IDO expression [16, 51, 62], the possible involvement of IFNγ in the IL-12 induced immunomodulation and stemness properties of hPDL cells was investigated.

In a time course experiment, upregulation of IFNγ was observed as early as 2 hours after IL-12 activation. However, an increased level of HLA-G and IDO was found at a later time point, not before 24 hours (Figure 4.9A). These results suggest that the upregulation of HLA-G and IDO might not be a direct effect of IL-12, but could be caused by IFNγ. In order to clarify this, the activity of IFNγ was inhibited with a neutralizing antibody against IFNγ. The results in Figure 4.9B-C indicated that the inductive effect of IL-12 on IDO and HLA-G expression was abolished in the presence of neutralizing antibody against IFNγ. However, the neutralizing antibody had no effect to down regulate the inductive effect of IL-12 on stem cell/pluripotent markers (Fig. 9D-E).
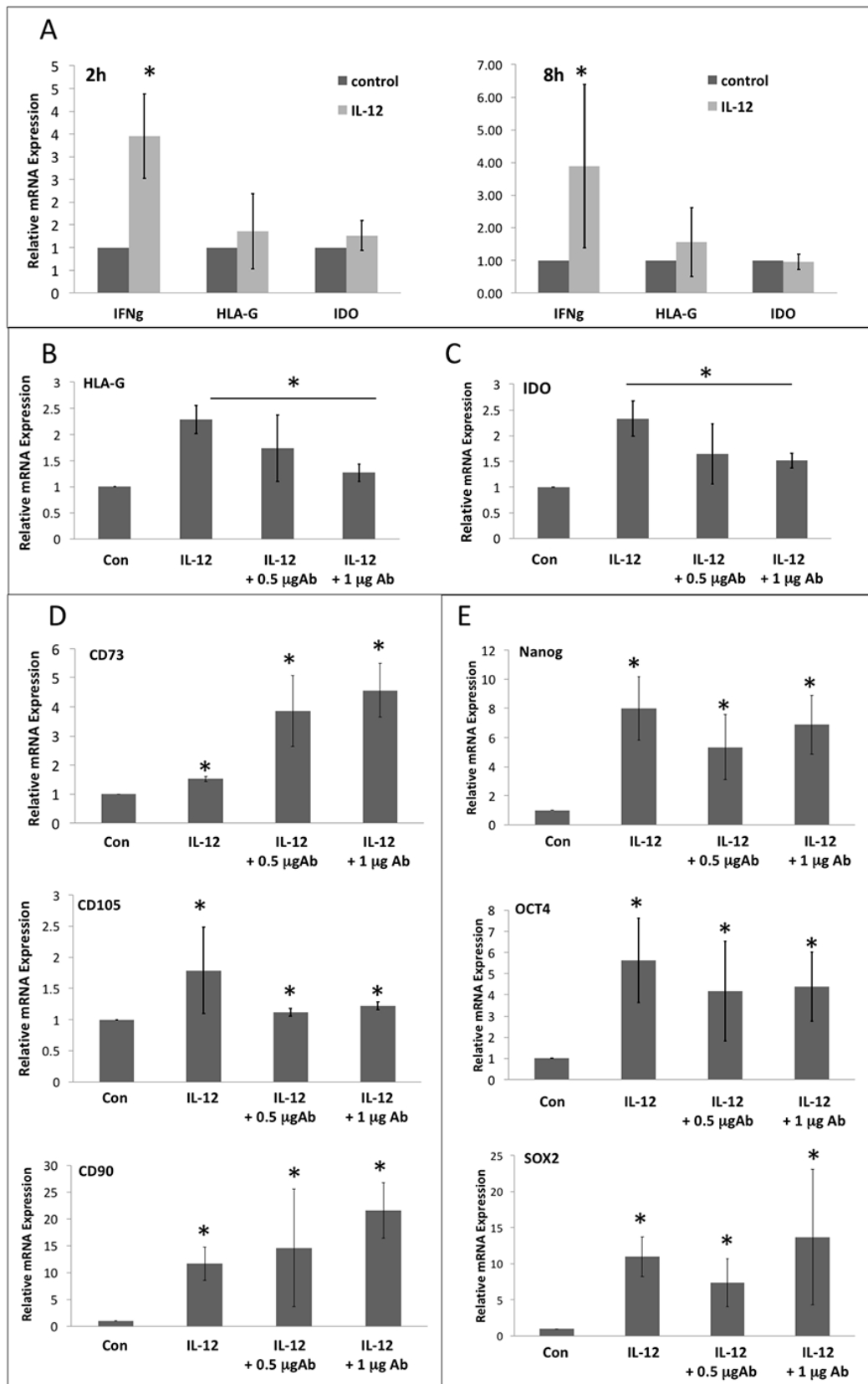
Figure 4.9 IL-12 induced IDO and HLA-G expression occurred via IFNγ pathway.

A time course study revealed that IFNγ expression could be upregulated by IL-12 in hPDL cells within 2 hours (A). However, the expression of both IDO and HLA-G could not be detected at both 2 and 8 hours. HPDL cells were then activated with IL-12 in the presence or absence of the neutralizing antibody against IFNγ. Real-time PCR analysis showed the attenuating effect of IFNγ neutralizing antibody on IL-12-induced HLA-G (B) and IDO (C) expression. However, neutralizing antibody against IFNγ has no effect on the induction of stem cell/pluripotent markers by IL-12 (D and E). The results were shown as mean ± S.D. from three independent experiments. * indicated the significant different from the control (p<0.05).

Inductive effect of IFNγ on HLA-G and IDO expression occurs via different signaling pathways

To determine underlying effect of IFNγ on IDO and HLA-G expression, hPDL cells were incubated with various concentration of IFNγ. Then, mRNA expression level of HLA-G and IDO was examined by real-time PCR. Treatment with exogenous IFNγ significantly increased the mRNA expression of IDO and HLA-G (Figure 4.10A and 4.10B). This finding is in line with the results obtained from IFNγ neutralizing antibody shown above in Figure 4.9B-C.

Since both JAK1/STAT1 and NF-kB have been proposed as the major signaling molecules involved in IFNγ signaling pathway [91], selective inhibitors against JAK1 (JAKi) and NF-kB (NF-kBi) were used to determine the underlying pathways involved in IFNγ-induced HLA-G and IDO expression. Real-time PCR results demonstrated that both JAKi and NF-kBi attenuated the stimulating effect of IFNγ on IDO expression (Figure 4.10C). However, only NF-kBi could inhibit the inductive effect of IFNγ on HLA-G expression (Figure 4.10D). These findings indicate different underlying mechanisms of IFNγ in regulating the expression of HLA-G and IDO by hPDL cells.
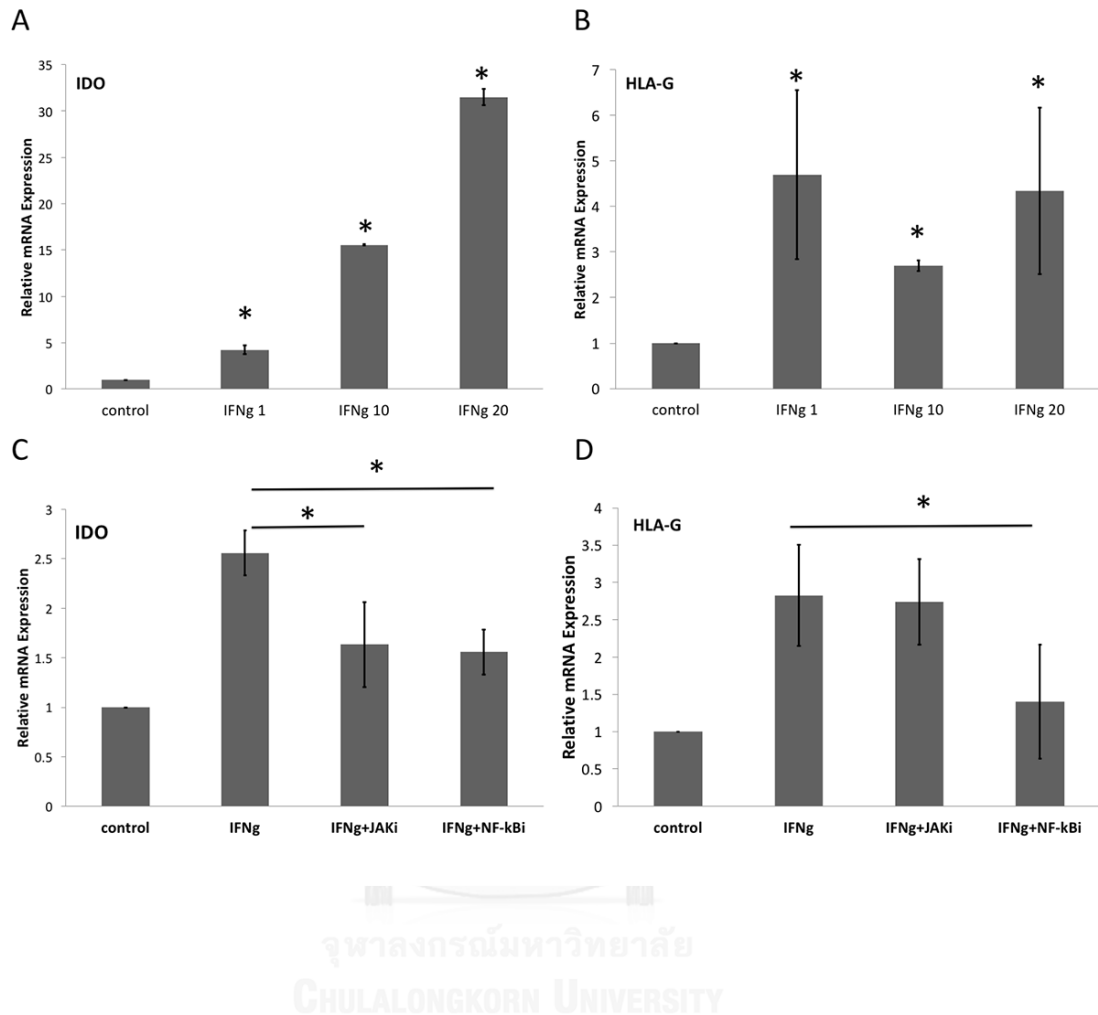
Figure 4.10 IFNγ promoted HLA-G and IDO expression in hPDL cells via different pathways.

HPDL cells were treated with 0-20 ng/ml of IFNγ. Then, the expression level of HLA-G (A) and IDO (B) were examined by real-time PCR. By means of chemical inhibitors (JAKi and NF-kBi), the induction of IDO occurred via both JAK and NF-kB pathways (C), while only NF-kB mediated in the upregulation of HLA-G (D). The results were shown as mean ± S.D. from three independent experiments. * indicated the significant different from the control (p<0.05).

CHAPTER V

DISCUSSION AND CONCLUSION

This study demonstrated the expression of IL-12 and IL-12R in both inflammatory periodontal tissues and hPDL cells. The presence of IL-12 and IL-12R in inflammatory periodontal tissues suggest a presence of IL-12-producing and IL-12-responsive cells at the site of periodontal inflammation. The expression of IL-12R in hPDL cells indicates the ability of hPDL cells to respond to exogenous IL-12, which was confirmed by an increased level of IFNγ, a well-known target of IL-12 [1, 2], after IL-12 administration. Inhibition of STAT4, which is crucial in the IL-12 canonical signaling pathway [36], prevented the cytokine mediated response. Finally, an increased level of phosphorylated STAT4 was also detected, indicating the activation of the IL-12-STAT4 canonical signaling pathway in hPDL cells.

From the first investigation, the osteoimmunological effect of IL-12 was demonstrated by increasing the expression of RANKL, but without affecting level of OPG expression. This resulted in a significant increase in RANKL/OPG expression ratio; thus supporting osteoclast formation. The upregulation of RANKL after IL-12 stimulation suggests a role of IL-12 in bone degradation during periodontitis. These results are in line with data obtained from the IL-12 deficient mice of periodontitis model [14]. The authors reported a significant decrease in bone resorption induced by *P.gingivalis* infection in IL-12 $^{-/-}$ mice [14]. According to this mentioned study, our data strongly suggests that the reduction in bone destruction is a result of the decrease in RANKL expression due to the absence of IL-12.

Not only STAT4 was found involved in IL-12-induced RANKL expression, but NF-kB also mediated in this inductive effect, comparable with the signaling pathway occurred in dendritic cells and macrophages [87]. The increase in RANKL via NF-kB activation is supported by several other studies that demonstrated the role of NF-kB as a major transcription factor to stimulate RANKL expression [92, 93]. Interestingly,

inhibition of STAT4 by lysofylline could not interfere the translocation of NF-kB into the nucleus. Thus, this data indicates two distinct pathways of IL-12 in regulating the expression of RANKL. Further investigation is required to determine the significance of these two different pathways in RANKL regulation.

Conditioned medium assay indicates an involvement of intermediate molecule(s) in the inductive process of IL-12 on RANKL expression. Conditioned medium from IL-12 cultured cells upregulated level of RANKL expression in non-treated cells. Interestingly, we found that de novo synthesis of the intermediate compound(s) was not needed in this inductive effect, as in the presence of cycloheximide (a protein synthesis inhibitor) the inductive effect of IL-12 still occurred. However, blocking intracellular protein trafficking with monensin could attenuate the effect of IL-12 on RANKL expression, thereby, indicating that IL-12 stimulated the secretion of the intermediate compound(s) that activated RANKL expression. Taken together, these findings suggest that the inductive effect of IL-12 on RANKL expression in hPDL cells occurs indirectly via regulating the secretion of yet unknown-intermediate molecule(s). NF-kB was found as key signaling molecule mediated in intermediate molecule-induced RANKL expression. Therefore, it is possible that administration of NF-kB inhibitor in IL-12 treated cells inhibited this inductive effect of intermediated molecule(s), leading to the decreased level of RANKL expression.

Moreover, calcium signaling was found necessary for the IL-12-regulated secretion of the intermediate molecule(s). The results showed that EGTA, a chelating agent, could attenuate the inductive effect of IL-12 on RANKL expression; however, it had no effect on the induction by IL-12-CM. Several studies have demonstrated an importance of calcium signaling in intracellular protein transportation [88, 89, 94]. In presence of EGTA, both endocytosis and exocytosis were inhibited [94, 95]. The role of calcium signaling in IL-12 induced RANKL expression was confirmed by thapsigargin treatment. Thapsigargin is a specific inhibitor of sarco/endoplasmic reticulum Ca^{2+} ATPase [96, 97]. Treatment with thapsigargin rapidly induced the release of stored calcium, leading to a rapid transient increase in cytosolic calcium concentration [98, 99]. EGTA and thapsigargin are usually used together to investigate the involvement of calcium signaling [94, 100]. As expected, thapsigargin administration promoted the

inductive effect of IL-12 on RANKL expression. We assume that the increased level of intracellular calcium induced by thapsigargin enhanced the secretion of IL-12-mediated intermediate molecule(s), resulting in the upregulation of RANKL expression. The role of calcium signaling in IL-12 activation has been reported by Futwan Al-Mohanna et al., 2002 [90]. They revealed that a transient increase in intracellular calcium following IL-12 treatment was necessary for the production of reactive oxygen metabolites in neutrophil [90]. Together, these data validate an important role of calcium signaling in IL-12 activation in hPDL cells.

Nevertheless, the possible nature of IL-12-mediated intermediate molecule required for RANKL expression was also further identified. We found that suramin significantly inhibited the expression of RANKL induced by both IL-12 and IL-12-CM. As suramin is an inhibitor of heterotrimeric G protein activation, this finding implies that a ligand of G protein-coupled receptor is the intermediate molecule induced by IL-12 and responsible for RANKL expression.

Aside from the destructive role of IL-12 in promoting RANKL expression, a modulatory effect of IL-12 was demonstrated in this study. We found that IL-12 acted as a potent inducer to activate the immunomodulatory property of hPDL cells. Treatment with IL-12 upregulated the expression of immunosuppressive molecules including of IDO, IFNɣ and HLA-G in hPDL cells. Moreover, the mesenchymal and pluripotent stem cells markers, including CD73, CD90, CD105, Oct4, Nanog and Sox-2, were also found increased after IL-12 stimulation. Actually, the ability to express immunosuppressive molecules is proposed to be one of the important characteristics of MSC to modulate the immune response. This characteristic plays a role as a protective mechanism of MSCs in order to survive under inflammatory conditions. Several studies reported that the immunosuppressive properties of bone marrow-derived MSCs (BMMSCs) could modulate an immunological reaction [54, 101] and also reduced Graft-versus- host responses *in vivo* [102]. In dental tissues, dental stem cells, including gingival fibroblasts, dental pulp fibroblasts and periodontal ligament cells, have also been demonstrated in their immunomodulatory properties via an ability to inhibit the proliferation of activated peripheral blood mononuclear cells (PBMCs) *in vitro* [52].

One of the immunomodulatory mechanisms of IL-12 was the upregulation of IDO expression in hPDL cells. IDO is a catalyzing enzyme that functions to degrade tryptophan, an essential amino acid for immune cells' growth, resulting in an inhibition of immune cell proliferation [17]. Our results demonstrated an increased level of IDO mRNA expression within 24 h, after IL-12 treatment. However, a significant increase of IDO enzymatic activity was detected after 7 days of IL-12 administration. This relatively late detection of an increase in IDO activity might suggest that a prolonged exposure to IL-12 is necessary for the synthesis of IDO protein. The increased level of IDO production might be a feedback mechanism of IL-12 to modulate the chronic inflammatory reaction, since the major function of IDO is to suppress T-cell proliferation [17].

The ability of hPDL cells to secrete IDO enzyme has been demonstrated previously by Moon et al. [53]. They showed the upregulated level of IDO enzyme in hPDL cells after LPS stimulation. Not only hPDL cells, but gingival fibroblasts were also shown to express IDO enzyme upon LPS activation [53]. An *in vivo* mice model demonstrated that PDL cells expressed much higher levels of IDO than gingival fibroblasts [53], suggesting that PDL cells might be the major cell type responsible for controlling immune response during periodontal inflammation. To activate IDO production, IL-12 has been demonstrated as a stimulating factor to promote IDO expression in cancer cells [44-46]. This upregulation of IDO production induced by IL-12 is believed to act as an escape mechanism of cancer cells to evade host immune system [44-46]. Both IFNγ dependent and independent pathways have been shown to mediate in the inductive effect of IL-12 on IDO production [44-46]. As stem cells and cancer cells possess several similar characteristics, such as a self-renewal property, it is possible that both types of cell share the same underlying mechanism to behave towards the host immune response.

Apart from IDO expression, increased levels of IFNγ and HLA-G were also observed in hPDL cells after IL-12 stimulation. IFNγ is a principle cytokine involved in T_H1-mediated immune response [103]. Moreover, it plays a role as a key inducer to activate the immunomodulatory properties of MSCs by promoting the expression of

immunosuppressive molecules, such as B7-H1 [62, 72] and IDO [16, 51, 62]. HLA-G is a non-classical major histocompatibility complex molecule that its role in immunosuppression has been proposed in a variety of processes involved in immune response [104]. HLA-G exerts its activity by binding to its specific inhibitory receptors, including Ig-like transcripts 2 and 4, leading to inhibition of various immunological processes. Therefore, the upregulation of HLA-G during inflammation might function as a negative regulator to modulate host immune response [63]. This study reported the presence of HLA-G in hPDL cells and demonstrated the stimulating effect of IL-12 on HLA-G expression. The upregulation of both HLA-G and IDO by hPDL cells found in this study indicated the participation of this cell type in regulating host immune reaction during periodontal inflammation.

The results demonstrated that an upregulation of IFNɣ could be detected as early as 2 hours after IL-12 stimulation; however, it took 24 hours before the increased level of HLA-G and IDO was detected. As the inducting effect of IFNɣ on IDO expression was reported previously in hBMMSCs[16], we speculated that IFNɣ might play a similar effect on hPDL cells. In order to clarify the effect of IFNɣ, a neutralizing antibody against IFNɣ was used in this study. We found that treatment with IFNɣ neutralizing antibody indeed inhibited the inducting effect of IL-12 on IDO and HLA-G expression in hPDL cells. Moreover, addition of exogenous IFNɣ also promoted the expression of HLA-G and IDO in hPDL cells. These data suggest that both HLA-G and IDO are downstream targets of IFNɣ in IL-12 treated hPDL cells.

The upregulation of HLA-G and IDO induced by IL-12 was shown to be occured via different underlying mechanisms. Both JAK1 and NF-kB were found as crucial signaling molecules involved in IL-12-induced IDO expression; whereas HLA-G induction was mediated only by NF-kB. The involvement of NF-kB and JAK1 has also been proposed as underlying signaling molecules of IFNɣ-induced IDO expression [91].

Interestingly, the increased level of both mesenchymal and pluripotent stem cells markers in hPDL cells was demonstrated following IL-12 stimulation. Moreover, IL-12 also suppressed the osteogenic differentiation potential of hPDL cells by reducing the mineralization level of the cells cultured under osteogenic induction medium. This

suppression was associated with the increased level of SOST mRNA, the inhibitor of BMP signaling and inhibitor of bone formation [105]. It is possible that the increase of stem cell markers and the inhibition of osteogenic differentiation might be one of the protective mechanisms by which IL-12 preserves the number and stemness properties of hPDL cells. It has been shown that immunomodulatory properties is an important mechanism of MSCs to survive under inflammatory conditions [16, 17, 47]. Therefore, the modulation of IL-12 on immunomodulatory properties and preservation of the stemness properties of hPDL cells might be a crucial mechanism that helps to protect hPDL cells' population under inflammatory environment and to be ready for tissue healing and regeneration.

In conclusion, this study demonstrated the response of hPDL cells to exogenous IL-12 via IL-12R and validated the role of IL-12 in both pro- and anti-inflammatory ways. For the pro-inflammatory reaction, IL-12 was found to induce expression level of RANKL via STAT4 and NF-kB signaling pathways. This inductive effect of IL-12 occurred indirectly by the function of secreted intermediated compound(s), which was identified as a member of G protein coupled receptor ligand. These data suggested the osteoimmunological role of IL-12 might play in the pathogenesis of periodontal disease. Besides, IL-12 also promoted the immunomodulatory properties of hPDL cells by inducing the expression of HLA-G and IDO, which was mediated by the stimulation of up-regulated IFNγ. This indicated a feedback mechanism played by IL-12 to modulate the host immune response during periodontal inflammation. Different signaling pathways were involved in promoting effect of IL-12 on HLA-G and IDO expression. Both JAK1 and NF-kB were found to play a role in IDO induction; however, HLA-G expression was mediated only by NF-kB. Taken together, this study provides a new knowledge on an influencial role of IL-12 in regulating the pathogenesis and homeostasis of periodontal tissue.

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APPENDIX
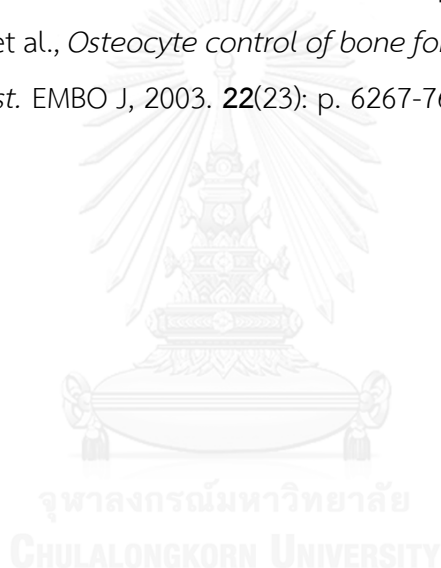
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