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THE EFFECTS OF IL-17 AND IFN-GAMMA ON HUMAN GINGIVAL FIBROBLASTS

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โรคปริทันต์อักเสบ คือโรคติดเชื้อแบคทีเรียชนิดหนึ่ง ซึ่งมีลักษณะการอักเสบเรื้อรังของเหงือก อันนำไปสู่การสูญเสียของอวัยวะรองรับฟัน ในรอยโรคปริทันต์มักตรวจพบทีเซลล์ที่สามารถจดจำแอนติเจนและถูกกระตุ้นแล้วเป็นจำนวนมาก รวมทั้งไซโตไคน์ที่ทีเซลล์เหล่านี้หลั่งออกมาในปริมาณสูงเสมอ ถึงแม้ว่าทีเซลล์จะได้รับการพิจารณาว่าเป็นศูนย์กลางในการควบคุมและการดำเนินโรคของโรคปริทันต์อักเสบชนิดเรื้อรัง แต่บทบาทในแง่การควบคุมระบบภูมิคุ้มกันเฉพาะที่ยังไม่เป็นที่ทราบแน่ชัด การศึกษาเมื่อไม่นานมานี้ตรวจพบว่ามีอินเตอร์ลิวคิน-17 ซึ่งเป็นไซโตไคน์ของทีเซลล์ชนิดใหม่ และอินเตอร์เฟียรอน-แกมมาในเนื้อเยื่อปริทันต์ที่อักเสบ ดังนั้นการศึกษานี้จึงมีวัตถุประสงค์เพื่อศึกษาบทบาทในการกระตุ้นระบบภูมิคุ้มกันเฉพาะที่ของอินเตอร์ลิวคิน-17 และอินเตอร์เฟียรอน-แกมมาต่อเซลล์ไฟโบรบลาสต์จากเหงือกของคนซึ่งมีอวัยวะปริทันต์สุขภาพดี เซลล์ไฟโบรบลาสต์จะถูกกระตุ้นด้วยอินเตอร์ลิวคิน-17 และอินเตอร์เฟียรอน-แกมมาในความเข้มข้นต่าง ๆ รวมทั้งส่วนผสมของไซโตไคน์ทั้งสองชนิด ตรวจวัดระดับการแสดงออกของซีดี 40 และ เอชแอลเอ-ดีอาร์ ด้วยการวิเคราะห์โดยใช้โฟล โซโตเมทรี และวัดระดับการผลิตอินเตอร์ลิวคิน-8 ด้วยวิธีอีไลซ่า ผลการทดลองแสดงให้เห็นว่า อินเตอร์เฟียรอน-แกมมาสามารถส่งเสริมการแสดงออกของเอชแอลเอ-ดีอาร์บนเซลล์ไฟโบรบลาสต์ได้อย่างชัดเจน และมีผลต่อการแสดงออกของซีดี 40 เพียงเล็กน้อย ส่วนอินเตอร์ลิวคิน-17 นั้น ไม่มีผลต่อการแสดงออกของแอนติเจนทั้งสองชนิด อีกทั้งยังไม่มีผลส่งเสริมอินเตอร์เฟียรอน-แกมมาในการกระตุ้นการแสดงออกของซีดี 40 และ เอชแอลเอ-ดีอาร์บนเซลล์ไฟโบรบลาสต์ ในทางตรงกันข้ามกับอินเตอร์เฟียรอน-แกมมา อินเตอร์ลิวคิน-17 สามารถกระตุ้นให้เซลล์ไฟโบรบลาสต์ผลิตอินเตอร์ลิวคิน-8 ได้ และเมื่อกระตุ้นด้วยไซโตไคน์ทั้งสองชนิดร่วมกัน อินเตอร์เฟียรอน-แกมมามีผลส่งเสริมอินเตอร์ลิวคิน-17ในการกระตุ้นการผลิตอินเตอร์ลิวคิน-8 ผลในการส่งเสริมนี้พบในเซลล์เพาะเลี้ยงทั้งหมดเมื่อกระตุ้นด้วยอินเตอร์ลิวคิน-17ที่ความเข้มข้นสูง (500 นาโนกรัม/มิลลิลิตร) ในขณะที่ความเข้มข้นต่ำ (5 และ 50 นาโนกรัม/มิลลิลิตร) นั้นเราพบการตอบสนองที่แตกต่างกัน ความแตกต่างในการผลิตอินเตอร์ลิวคิน-8ของเซลล์ไฟโบรบลาสต์ที่ตอบสนองต่อการกระตุ้นด้วยส่วนผสมของอินเตอร์ลิวคิน-17 และอินเตอร์เฟียรอน-แกมมาเป็นสิ่งที่น่าสนใจ และข้อมูลนี้อาจจะสามารถอธิบายความหลากหลายในการตอบสนองของร่างกาย ความไวต่อการเกิดโรค และการดำเนินของโรคปริทันต์อักเสบได้ สำหรับกลไกที่แน่ชัดนั้นยังต้องได้รับการศึกษาต่อไป

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PAIBOON JITPRASERTWONG: THE EFFECTS OF IL-17 AND IFN-GAMMA ON HUMAN GINGIVAL FIBROBLASTS. THESIS ADVISOR: ASSISTANT PROFESSOR RANGSINI MAHANONDA, Ph.D, THESIS CO-ADVISOR : SATHIT PICHYANGUL, Ph.D, 72 pp. ISBN 974-17-6072-8.

Periodontitis is a bacterial infection characterized by chronic gingival inflammation, which leads to the loss of tooth-supporting tissues. Dense infiltration of activated memory T cells and high levels of their cytokines were consistently detected in periodontal lesions. Although T cells have been considered to be central to both progression and control of chronic inflammatory periodontal diseases, the definite contribution in local immunoregulation has not been fully clarified. Recent observations showed the presence of IL-17, a novel T cell cytokine, and IFN-γ in the inflamed periodontal tissues. Therefore, in this present study we investigated the immunostimulatory role of IL-17 and IFN-γ on human gingival fibroblasts (HGF) which were obtained from clinically healthy periodontal tissues. Various concentrations of IL-17, IFN-γ, or the combination of these two cytokines were added to HGF cultures. The expression of CD40 and HLA-DR was assessed by flow cytometry and IL-8 production was determined by ELISA. Our results demonstrated that IFN-γ markedly up-regulated HLA-DR and minimally up-regulated CD40 expression on HGF. IL-17 did not induce the expression of both molecules and did not enhance IFN-γ-induced CD40 and HLA-DR expression on HGF. Unlike IFN-γ, IL-17 induced IL-8 production from HGF. When combined, IFN-γ synergistically enhanced IL-17-induced IL-8 production. This enhancement was detected in all HGF cell lines at a higher dose of IL-17 (500 ng/ml) whereas at lower doses (5 and 50 ng/ml), heterogeneous response was observed. The findings of heterogeneity in IL-8 production by HGF in response to the combination of IL-17 and IFN-γ are interesting and may explain the variation in host response in disease susceptibility or disease progression in periodontitis. Clearly, further investigation into this issue is needed.

Department Periodontology Student's signature.....

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

Academic year 2004 Co-advisor's signature.....

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LIST OF ABBREVIATIONS

A. actinomycetemcomitans	*Actinobacillus actinomycetemcomitans*
Ab	antibody
APC	Antigen presenting cell
CD	Cluster of differentiation
DMEM	Dulbecco's modified Eagle's medium
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
HGF	human gingival fibroblasts
HLA	human leukocyte antigen
ICAM	intercellular adhesion molecule
IFN	interferon
Ig	immunoglobulin
IL	interleukin
LPS	lipopolysaccharide
mAbs	monoclonal antibodies
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MMPs	matrix metalloproteinases
NK cell	natural killer cell
OPG	osteoprotegerin
P. gingivalis	*Porphyromonas gingivalis*
PBS	phosphate buffered saline
PerCP	peridinin chloropyll protein
PGE	prostaglandin
PMNs	polymorphonuclear leukocytes
RANKL	receptor activator of nuclear factor-kappa B ligand
T. forsythia	*Tannerella forsythia*
TNF	tumor necrosis factor

CHAPTER 1

INTRODUCTION

1.1 Background of the present study

The periodontal diseases are defined as a group of *chronic* inflammatory conditions that result in the destruction of the supporting structures of the dentitions. Periodontitis is the severe form of periodontal diseases which constitutes one of the most common causes of adult tooth loss (Papapounou, 1996). The etiology of periodontitis is specific Gram-negative microorganisms in plaque biofilm such as *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans and Tannerella forsythia* (The American Academy of Periodontology (AAP), 1996). To date it is becoming clear that the pathogenesis of the disease is caused by the complex interaction between host defense mechanisms and bacterial plaque pathogens and their products (Offenbacher et al., 1993; Van Dyke and Serhan, 2003). This interaction leads to the release of pro-inflammatory mediators and cytokines e.g. interleukin (IL)-1β, interferon (IFN)-γ, tumor necrosis factor (TNF)-α, IL-6, IL-8, matrix metalloproteinases (MMPs) and prostaglandin E_2 (PGE_2) (Kornman et al., 1997). High levels of these mediators have been identified in periodontitis lesions and thought to play a pivotal role in degradative pathways of periodontal tissue destruction (Socransky and Haffajee, 1991). However, the precise pathological mechanisms underlying tissue destruction are still poorly understood.

The majority of cells in the periodontal connective tissues are the fibroblasts (Hassell, 1993). Although gingival fibroblasts have classically been described as engineers of the extracellular connective tissue matrix, recent observations have

expanded their functions to include the synthesis of various soluble mediators which include immunoregulatory cytokines, chemokines and chemical mediators. These findings suggest that gingival fibroblasts could play important roles not only in maintaining periodontal tissue integrity or wound healing, but also in regulating local inflammatory responses. In inflamed periodontal tissues, dense accumulations of inflammatory cellular infiltrates such as lymphocytes (both T and B cells) were detected histopathologically and so were their cell-derived cytokines. These locally infiltrated cells were located adjacent to the periodontal fibroblasts (Okada et al., 1983; Murakami et al., 1999). It is, therefore, reasonable to speculate that these immunocompetent cells and their cytokines may have an opportunity to directly interact with the resident fibroblasts and are mutually affected through heterotypic cell-cell interaction as well as cell-cytokine interaction. To date, little is known about such interaction. In this study, we will focus on the role of T cell-derived cytokines, IL-17 and IFN-γ, on human gingival fibroblasts. Both cytokines are identified as pro-inflammatory cytokines and predominantly produced by activated T cells. IFN-γ is a classic Th1 cytokine which enhances phagocytic activity of macrophages (Morris, 1988; Fujihashi et al., 1996). IL-17, a more recently identified cytokine, whose function is quite similar to IL-1 and TNF-α in inflammatory responses and bone destruction. (Kotake et al., 1999; Van Bezooijen et al., 1999; Van Bezooijen et al., 2002). Very recent observation demonstrated the presence of IL-17 in the inflamed gingival tissue (Oda et al., 2003). To date the role of this cytokine alone or in combination with others on human gingival fibroblasts have not yet been fully explored. Therefore, we analyzed the effects of IL-17 and IFN-γ or in combination on human gingival fibroblast with regard to co-stimulatory molecule (CD40 and HLA-DR) expression as well as IL-8 production.

1.2 Objectives

In the present study we examined the effects of IL-17 and IFN-γ on human gingival fibroblasts in terms of

1.2.1 Expression of CD40 and HLA-DR.

1.2.2 IL-8 production.

1.3 Hypothesis

IL-17 and IFN-γ synergistically enhance expression of CD40 and HLA-DR as well as IL-8 production by human gingival fibroblasts.

1.4 Field of research

To investigate the enhanced inflammatory response of HGF by IL-17 and IFN-γ.

1.5 Criteria inclusions

1.5.1 HGF were obtained from healthy adult subjects.

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

1.5.3 Analysis of CD40 and HLA-DR expression was determined by flow cytometry.

1.5.4 IL-8 production was measured by enzyme-linked immunosorbent assay (ELISA).

1.6 Limitation of research

This study could not use many samples due to high expenses.

1.7 Application and expectation of research

In periodontitis, dense infiltration of activated memory T cells and high levels of T cell cytokines were consistently reported in extravascular connective tissues. Although T cells have been implicated in the pathogenesis and are considered to be central to both progression and control of chronic inflammatory periodontal diseases, the precise contribution of T cells to tissue destruction has not been fully clarified. In this study, we focused on the role of T cell-derived cytokines, IL-17 and IFN-γ, on human gingival fibroblasts. These two cytokines have been reported in inflamed gingival tissues. It may be possible that during periodontal disease progression, the presence of the local cytokines may drive inflammatory responses of gingival fibroblasts contributing to the pathological mechanisms of the disease. The induction of IL-8 production and the expression of co-stimulatory molecule, CD40 and HLA-DR on the cells were monitored after stimulation with IL-17, IFN-γ, and the combination.

We anticipate that the results from this line of investigation would provide a better understanding in the immunopathogenesis of periodontal diseases. With the proper knowledge of the pathological process, manipulation of the response of the cellular network or the inflammatory cytokines may be possible, thus leading us to new and specific therapies for periodontal diseases in the future.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Periodontal diseases are chronic inflammatory diseases that affect supporting tissues of the dentitions. Epidemiological studies have shown that some forms of periodontal diseases in adult can remain stable over many years and not endanger the life of the dentition, whereas other forms, despite extensive treatment, continue to break down, leading ultimately to tooth loss (Seymour and Gemmell, 2001). Gingivitis and periodontitis are the two common forms of periodontal diseases. Gingivitis, a more stable form, is an inflammatory condition of the soft tissue surrounding the teeth (the gingiva) without involvement of the attachment apparatus whereas periodontitis involves deeper periodontium resulting in the clinical attachment loss with the destruction of gingiva, periodontal ligament, cementum and alveolar bone (Kinane, 2001). Periodontal diseases are found worldwide and are a global public health problem (Albandar and Rams, 2002), particularly periodontitis, which has long been implicated as a major cause of adult tooth loss (Papapanou, 1996).

2.2 Immunopathogenesis

2.2.1 Plaque and host response

It is now accepted that periodontitis is a multi-factorial disease with microbial dental plaque as the initiator (Kinane, 1999). Although bacteria are essential, they

are insufficient for the disease to occur. The pathogenesis of periodontal disease is modulated by host responses to dental plaque. In addition, a wide variety of determinants and factors, either environmental or acquired, e.g. smoking, stress, systemic diseases, genetic factors, microbial composition of dental plaque (Nunn, 2003) are known to influence the host response. Therefore, these factors could subsequently have certain effects on the disease initiation and progression.

The bacterial profile of plaque associated with periodontitis is a specific group of predominantly Gram-negative, anaerobic or microaerophilic bacteria. They colonize on the root surface in the subgingival area (Page and Kornman, 1997). The key periodontal pathogens in chronic periodontitis are *P. gingivalis, and T. forsythia* whereas those in localized aggressive periodontitis are *A. actinomycetemcomitan*s (Van Dyke and Serhan, 2003). Lipopolysaccharide (LPS) is well known Gram negative bacterial product found in dental plaque biofilms. If the bacterial biofilms are left undisturbed in periodontal pockets, they continue releasing their products, particularly LPS, into pocket junctional epithelium, blood vessels and deeper connective tissues of periodontium (Socransky and Haffajee, 1991; Slots and Ting, 1999). LPS has the potential to interact with nearly all cell types in periodontal tissues including immune cells (lymphocytes, polymorphonuclear leukocytes (PMNs), macrophages) and resident cells (fibroblasts, epithelial and endothelial cells). The binding of LPS to its receptor on host cells leads to cell activation and the release of mediators and cytokines, all of which could contribute to the local inflammatory response in the periodontal tissues.

Current knowledges suggest the involvement of many cell types and mediators in the immunopathogenesis of periodontal disease. Histological findings show dense cellular infiltrates of both T and B cells in periodontitis lesions as

compared to minimal number of infiltrates found in gingivitis or healthy tissues. In addition, higher levels of inflammatory mediators and cytokines are observed in gingival crevicular fluid and inflamed tissue in periodontitis than gingivitis (Seymour, 1993). Phenotypic analysis of infiltrated T-lymphocytes in periodontitis lesions reveal the profiles of activated memory helper T cells ($CD45RO^{+}HLA\text{-}DR^{+}CD4^{+}$) (Cole et al., 1987; Gemmell et al., 1992; Yamasaki et al., 1993). T cell-derived cytokines were also reported to be present. These are IFN-γ, IL-4, IL-5, IL-6, IL-10 as well as a recently identified IL-17 (Yao et al., 1995; Aarvak, 1999). Locally, these infiltrated immune cells were located adjacent to the resident fibroblasts (Murakami and Okada, 1997). It is, therefore, reasonable to speculate that the immunocompetent cells and their cytokines may have an opportunity to interact with the fibroblasts. Thus, the heterotypic cell-cell interaction and cell-cytokine interaction are indeed needed to be explored.

2.2.2 Gingival fibroblasts

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

Gingival fibroblasts could secrete various soluble mediators of inflammation including PGE_2, IL-1, IL-6 and IL-8 in response to extrinsic environmental factors such as plaque bacterial pathogens and their products and cytokines. These

fibroblast-derived mediators are thought to play important role in inflammatory response in local periodontal lesions. Many plaque bacterial products e.g. LPS derived from *P. gingivalis, A. actinomycetemcomitans* and *Prevotella intermedia* were shown to enhance IL-6 and IL-8 production from gingival fibroblasts (Ohmori, 1987; Takada et al., 1991; Tamura et al., 1992; Dongari-Bagtzoglou and Ebersole, 1996a, 1996b; Imatani, 2001;). Fibroblasts are considered to be major sources of these IL-6 and IL-8 cytokines which are secreted in high amount both constitutively (Bartold and Haynes, 1991; Dongari-Bagtzoglou and Ebersole, 1998) and in response to bacteria, IL-1, TNF-α (Takashiba et al., 1992) and IFN-γ (Takashiba et al., 1992; Sakuta et al., 1998; Daghigh et al., 2002).

Fibroblasts not only respond to extrinsic environmental signals, but are also able to receive and process signals from other host cells by direct contact and through cell-cell receptors and ligands (Fries et al., 1995). Lymphocyte-HGF interaction in inflammatory gingival tissue was first descried morphologically by Schroeder and Page (1972), who reported that lymphocytes were frequently observed in intimate contact with damaged fibroblasts in the infiltrated connective tissue of human gingival biopsy specimens and speculated that lymphocytes sensitized to dental plaque may exert a marked cytotoxic effect on HGF through heterotypic direct interaction. Recent studies of cell adhesion molecules have revealed the molecular mechanisms of such adhesive interactions between various cell types. Among these, the adhesive interactions between lymphoid cells and fibroblasts have been suggested to play important roles in lymphocyte retention and lodging in inflamed connective tissues (Buckley et al., 2001; Murakami and Okada, 1997; Murakami et al., 1997).

Cellular activation, which could lead to a local inflammatory response in periodontal tissues, results from lymphocyte-HGF interaction through a cell receptor and ligand such as CD40 and CD40 ligand (CD40L) interaction. CD40 is recognized as one of the activation antigens on fibroblast and also as one of the co-stimulatory molecules for T cell activation. It is expressed on many cell types, including monocytes/macrophages, dendritic cells, epithelial cells and fibroblasts. The counter receptor for CD40, CD40L, is expressed on activated T cells and mast cells (Banchereau et al., 1994). Immunohistochemical staining of gingival biopsies demonstrates expression of CD40 in both inflamed and non-inflamed tissues (Sempowski et al., 1997). Gingival fibroblast activation via CD40 was able to produce pro-inflammatory cytokines IL-6 (Sempowski et al., 1997) and IL-8 (Dongari-Bagtzoglou et al., 1997, Dongari-Bagtzoglou and Ebersole, 1998).

2.3 HLA-DR and CD40 on human gingival fibroblasts

2.3.1 HLA-DR

Major histocompatibility complex (MHC) class II antigens are important recognition elements regulating cell-cell interactions in immune response. These molecules are necessary for antigen recognition and presentation to T cells and for T cell activation. In contrast to earlier reports describing HLA-DR, a subclass of MHC class II antigens, expression as restricted to lymphoids cells including B cells, activated T cells and monocytes/macrophages, several studies demonstrated that these antigens can also be induced on endothelial cells, keratinocytes and fibroblasts (Takahashi et al., 1994). Among various cytokines, IFN-γ is an immunoregulatory protein produced by activated T cells and is known as the most potent inducer or enhancer of MHC class II expression on non-lymphiod cell types

including fibroblasts. Interestingly, IFN-γ treated HGF induced proliferation of primed allo-reactive CD4+ T cells in a HLA-DR dependent manner. This finding suggested that expression of HLA-DR on fibroblast subsets may cause pre-activated T-lymphocytes to proliferate and release inflammatory cytokines at the sites of inflammation (Shimabukuro et al., 1996). The association between HLA-DR expression and antigen presenting function of HGF was further investigated using superantigen and bacterial antigens (Wassenaar et al., 1997). However, the results are still inconclusive (Murakami and Okada, 1997).

2.3.2 CD40

CD40 is a 50 kDa transmembrane protein, which is constitutively expressed by many cells of hematopoietic origin including B lymphocytes, follicular dendritic cells, and monocytes/macrophages. Cellular responses mediated by CD40 are naturally triggered by CD40L which is principally displayed on activated T cells. The CD40-CD40L interaction is considered a crucial step in T cell-dependent B cell co-stimulatory pathways and in the priming of T helper cell functions. Fibroblasts, which are traditionally thought of as non-immune cells, were shown to express CD40 both *in vivo* and *in vitro* and its expression was up-regulated by IFN-γ (Fries et al., 1995; Sempowski et al., 1997). In addition, a direct functional relationship between CD40 expression on HGF and its IL-6 or IL-8 secretion was demonstrated. It was shown that ligation of CD40 on HGF with CD40L resulted in cytokine secretion *in vitro* (Dongari-Bagzoglou et al., 1997; Sempowski et al., 1997). It is possible therefore that CD40 expression on HGF may play an important role in inflammatory response in local periodontal lesions.

2.4 Cytokines in periodontitis and related cytokines in the present study

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

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

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

2.4.1 Interleukin-8

IL-8 is a chemoattractant cytokine produced by a variety of tissue and blood cells, It is formerly known as neutrophil-activating peptide-1 (NAP-1), It has a distinct target specificity for the neutrophil, with weaker effect on other blood cells (Bickel, 1993; Baggiolini, 1994). IL-8 attracts neutrophils by inducing neutrophil extravasation at the site of inflammation. It then activates the cells to undergo the metabolic burst and to degranulate on arrival at the site of the challenge (Kornman et al., 1997). This cytokine has been thought to play a significant role in various forms of periodontitis (Takashiba, 1992; Bickel, 1993; Fitzgerald, 1995; Gainet, 1998).

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

cellular/cytokine network in the lesion. Therefore, additional work is required to determine the significance of this cytokine in periodontal disease.

2.4.2 Interferon-γ

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

High levels of IFN-γ mRNA are detectable in inflammed gingival tissues (Shimabukuro et al., 1996; Takeichi et al., 2000). Lundqvist et al. (1994) reported that not only αβ T cells but also γδ T cells from adult periodontitis patients expressed mRNA for IFN-γ. In addition, IFN-γ could be demonstrated in supernatant of gingival mononuclear cells from rapidly progressive periodontitis patients. Furthermore, IFN-γ was detected in gingival crevicular fluid of periodontitis patients (Salvi et al., 1998). The presence of IFN-γ is likely to prime local HGF and these primed HGF could subsequently induce further immune reaction. For example, T cell proliferation could be induced *in vitro* by IFN-γ treated HGF, as mentioned.

2.4.3 Interleukin-17

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

It was shown that IL-17 stimulated transcriptional NF-kB activity and IL-6 and IL-8 secretion in mouse fibroblasts, endothelium and epithelial cells and also induced T cell proliferation (Rouvier et al., 1993). Futhermore, IL-17 stimulates stromal cells to secrete cytokines and growth factors (Fossiez et al., 1996). It enhances IL-1 mediated-IL-6 production by rheumatoid arthritis synoviocytes *in vitro* (Chaubaud et al., 1998). In combination with IFN-γ, IL-17 showed a synergism in the stimulation of IL-8 secretion and the induction of intercellular adhesion molecule-1 (ICAM-1) and HLA-DR expression by keratinocytes (Teunissen et al., 1998; Albanesi et al., 1999). Moreover, IL-17 stimulates granulopoiesis (Schwarzenberger et al., 1998) and is a strong inducer of neutrophil recruitment through chemokines release (Laan et al., 1999). Apart from its role in inflammatory responses, IL-17 has potential to induce bone destruction. A recent work showed that IL-17 gene transfer strongly

up-regulated the synovial receptor activator of nuclear factor-kappa B ligand (RANKL) / osteoprotegerin (OPG) ratio and enhanced the formation of osteoclast-like cells and bone erosion compared with the control groups (Lubberts et al., 2003). RANKL and the decoy receptor OPG has been identified as an important positive and negative regulator of osteclastogenesis and bone erosion (Kong et al., 1999). Periodontitis is a chronic inflammatory disease, which involves alveolar bone destruction. Even though, the presence of IL-17 in gingivitis and periodontitis tissue has been recently demonstrated, the role of this cytokine in periodontitis is still largely unknown.

In periodontitis, dense infiltration of activated memory T cells and high levels of T cell cytokines were consistently detected in extravascular connective tissues. Although T cells have been implicated in the pathogenesis and are considered to be central to both progression and control of chronic inflammatory periodontal disease, the precise contribution of T cells to tissue destruction has not been fully clarified. In this study, we investigated the effects of T cell-derived cytokines IL-17 and IFN-γ on HGF with regard to co-stimulatory molecule expression (CD40 and HLA-DR) and cytokine production (IL-8).

CHAPTER 3

MATERIALS AND METHODS

3.1 Medium and monoclonal antibodies

Dulbecco's modified Eagle's medium (DMEM) supplemented with gentamycin (20 μg/ml), penicillin G (50 U/ml), streptomycin (50 μg/ml) and fungizone (2.5 μg/ml) and 10% heat-inactivated fetal calf serum (FCS) (Gibco Laboratory, Grand Island, NY) was used throughout the study. Recombinant human IL-17 and IFN-γ were obtained from R&D system Inc., Minneapolis, MA, USA.

The monoclonal antibodies (mAbs) that were used either labeled with peridinin chloropyll protein (PerCP) or fluorescein isothiocyanate (FITC). Monoclonal antibodies against HLA-DR were obtained from Becton Dickinson (San Jose, CA, USA), while monoclonal antibodies against CD40 and mouse isotype control monoclonal antibodies (FITC, PerCP) were obtained from BD Phar Mingen (San Diego, CA, USA).

Table 1: Monoclonal antibodies used for flow cytometry analysis

Monoclonal antibodies	Specificity
Anti-CD40 (FITC)	Co-stimulatory molecule
Anti-HLA-DR (PerCP)	Major histocompatibility complex Class II
Mouse IgG1 (FITC, PerCP)	-

3.2 Fibroblast preparation and cell culture

3.2.1 Gingival tissue samples

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

3.3.2 Fibroblast cell culture

The method to obtain fibroblasts from the gingival tissues was that described by Murakami et al. (2000). Briefly, the biopsies were washed twice with culture medium to remove blood clots and adherent erythrocytes. Then, the biopsies were minced with scissors into fragment of 1-3 mm^2 and placed in 60 mm. tissue culture dishes. These tissue explants were cultured with culture medium supplemented with 10% heat-inactivated FCS at 37 °C in humidified atmosphere of 5% CO_2 in air. Culture medium was changed twice a week. After a confluent monolayer of cells was reached, HGF were trypsinized, washed twice and then transferred to new tissue culture dishes (ratio 1:3). 24 hours before stimulation with cytokine (s), HGF ($1x10^5$ cells/ml) in DMEM with 5% heat-inactivated FCS were seeded in 48-well plates. The HGF at passage 4 were used throughout the study.

3.3 Stimulation of HGF with IFN- γ and IL-17

HGF ($1x10^5$ cells/ml) in 48-well plates (Corning Inc. Corning, NY, USA) were stimulated with various concentrations of IFN-γ, IL-17, or the combinations. The cells were harvested at different time points to measure the expression of CD40 and HLA-DR using flow cytometry. Culture supernatants were assayed for IL-8 production.

3.4 Flow cytometry analysis

HGF were gently trypsinized and washed twice with phosphate buffered saline (PBS). The cells were then stained with mAbs anti-CD40 (FITC) and HLA-DR (PerCP). Mouse isotype mAbs conjugated with FITC and PerCP were used as control. HGF were stained at 4°C for 30 min, washed in PBS, and reconstituted with 1% paraformaldehyde. Normally, 1,000-2,000 cells were analyzed by FACSCalibur (Becton Dickinson, Mountain View, CA). The levels of surface molecule expression were presented by mean fluorescence intensity (MFI).

3.5 Cytokine determination

Production of IL-8 in supernatants of IFN-γ and IL-17 stimulated HGF was measured by commercially available ELISA kit (R&D system Inc., Minneapolis, MA, USA.). The assay was performed according to the manufacturer's protocol. The detection limit of ELISA assay for IL-8 is 3.5 pg/ml.

3.6 Statistical analysis

The data were analyzed using the computer program SPSS version 10.0 for window. Student's *t* test was used to determine the difference between the effects of single cytokine stimulation and combined stimulation of IL-17 and IFN-γ on co-stimulatory molecule expression. *P* values less than 0.05 was regarded as significant. The results of IL-8 production in response to the combination of IL-17 and IFN-γ were presented using the index of synergy.

$$\text{Index of synergy} = \frac{\text{Cytokine production}_{(\text{IL-17+IFN-}\gamma)}}{\text{Cytokine production}_{(\text{IL-17})} + \text{Cytokine production}_{(\text{IFN-}\gamma)}}$$

The difference of the combined effect was also analyzed by statistical test using Wilcoxon matched pairs signed-ranks test. An index of synergy > 1 and $P < 0.05$ was considered as significantly synergistic (Woltman et al., 2000).

3.7 Budget

1.	DMEM	10,000	Baht
2.	Fetal calf serum	1,400	Baht
3.	Trypsin-EDTA, Antibiotic, Plastic ware	3,000	Baht
4.	IFN-γ and IL-17	40,000	Baht
5.	Monoclonal antibodies	40,000	Baht
6.	IL-8 ELISA kits	33,000	Baht
	Total	127,400	Baht

CHAPTER 4

RESULTS

4.1 The effects of IL-17 and IFN-γ on HLA-DR and CD40 expression

Our preliminary study indicated the induced-expression of HLA-DR and CD40 on HGF was varied at different time points of incubation, therefore, we first evaluated the kinetics of the effects of IL-17 and IFN-γ on HLA-DR and CD40 expression on HGF. HGF were cultured with IFN-γ (5000 U/ml), and IL-17 (500 ng/ml) for different time intervals. The expression of HLA-DR and CD40 on the cells was then analyzed by flow cytometry. As compared with un-stimulated HGF, IFN-γ markedly up-regulated HLA-DR expression on HGF. The IFN-γ–induced HLA-DR expression was early detected at day 2 after the incubation and then was increased over time (MFI = 15.74 on day 1 to 78.98 on day 5) (Figure 1A). In addition, IFN-γ also up-regulated CD40 expression on HGF, but the induced effect was lower than HLA-DR expression (Figure 1B). IL-17 had no effect on the expression of both HLA-DR and CD40 on HGF (Figure 1A, 1B).

Dose response effect of IFN-γ on HGF was evaluated. HGF cultures were stimulated with various concentrations of IFN-γ (50, 500, and 5000 ng/ml). Figure 2 shows representative histograms of HGF cultures measured after 5 days of incubation. The clear effect of HLA-DR expression was detected at 500 U/ml of IFN-γ, whereas, higher concentration of IFN-γ (5000 U/ml) was required to induce CD40 expression.

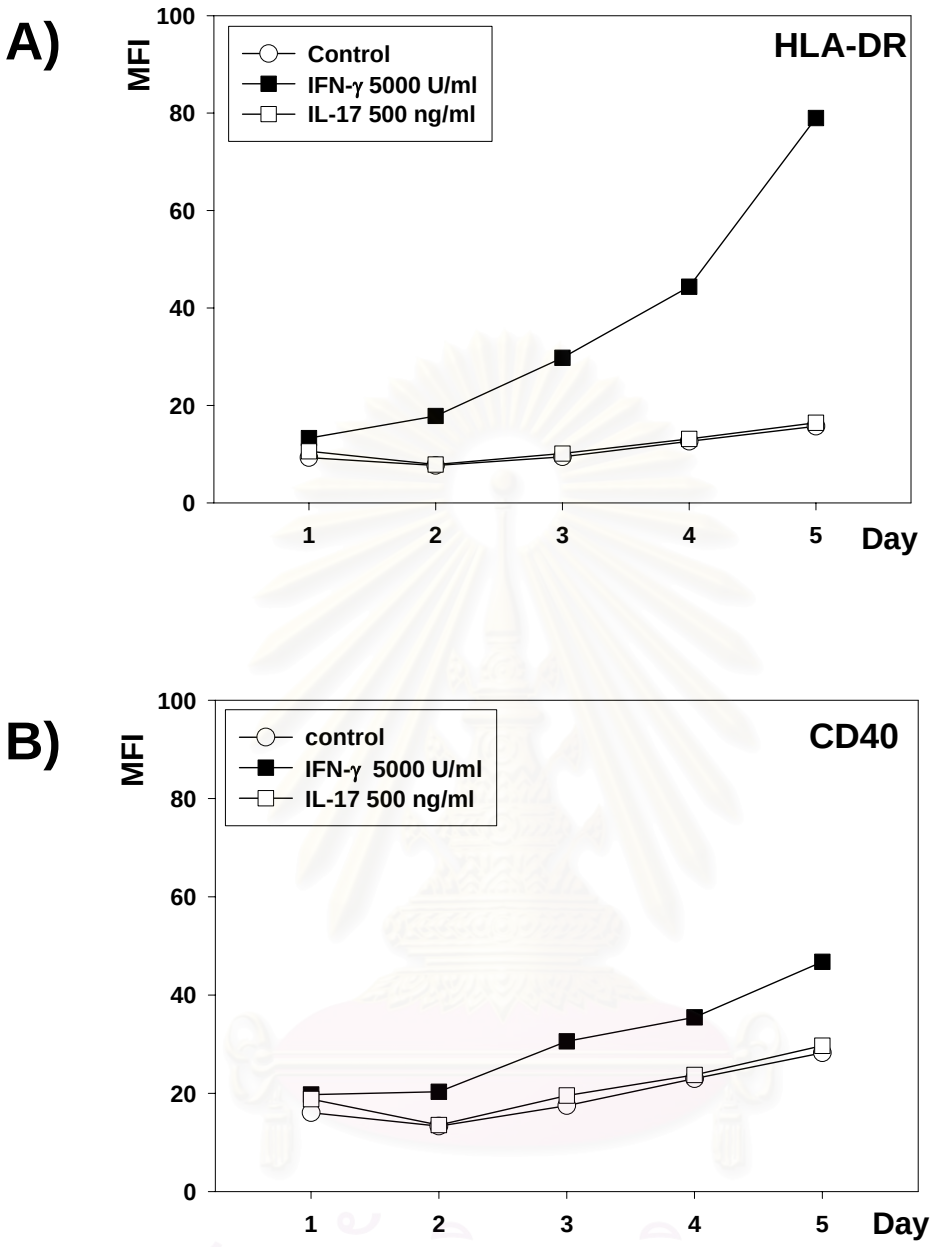

Figure 1. Kinetics of HLA-DR (**A**) and CD40 expression (**B**) on HGF. HGF (1×10^5 cells/ml) were stimulated with IFN-γ (5000 U/ml), and IL-17 (500 ng/ml) for various periods of time and the expression of HLA-DR and CD40 was determined by flow cytometry.

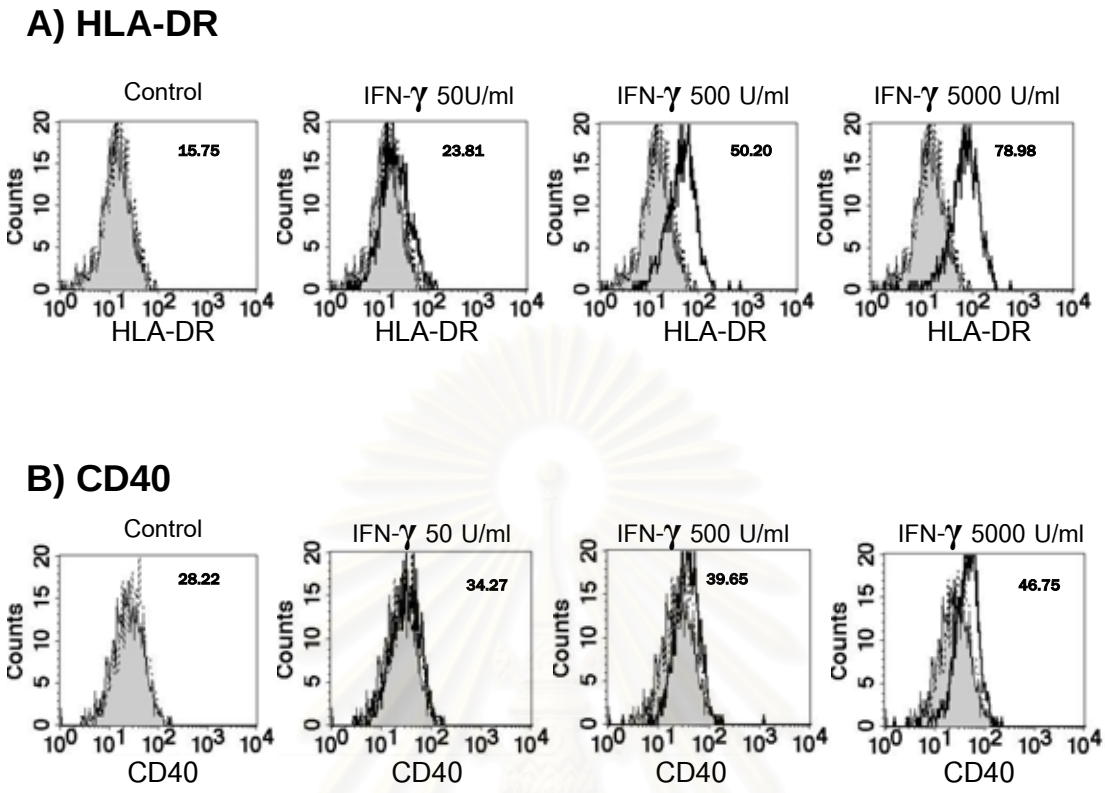

Figure 2. Representative histograms showing dose response analysis of HLA-DR (**A**) and CD40 expression (**B**) on HGF (n=12). HGF cultures were stimulated with various dose of IFN-γ (50, 500, 5000 U/ml) for 5 days. The cells were harvested and stained with 1) FITC-conjugated anti-CD40, 2) PerCP-conjugated anti-HLA-DR antibodies. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each histogram indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

4.2 The combination effects of IFN-γ and IL-17 on HLA-DR and CD40 expression on HGF

We evaluated the combination effects of IFN-γ and IL-17 on HLA-DR and CD40 expression. HGF cell lines (n = 12) were cultured with IFN-γ (50, 500, 5000 U/ml), IL-17 (500 ng/ml), or the combination of the two cytokines (50 U/ml IFN-γ + 500 ng/ml IL-17; 500 U/ml IFN-γ + 500 ng/ml IL-17; 5000 U/ml IFN-γ + 500 ng/ml IL-17). After 5 days of incubation, HGF cultures were harvested and phenotypic analysis was determined by flow cytometry. Our results revealed that the levels expression of HLA-DR and CD40 on HGF after stimulation with the combination of the two cytokines (Figure 3D) was not different from those stimulation with the single cytokine at each corresponding dose (Figure 3B and 3C) ($P > 0.05$).

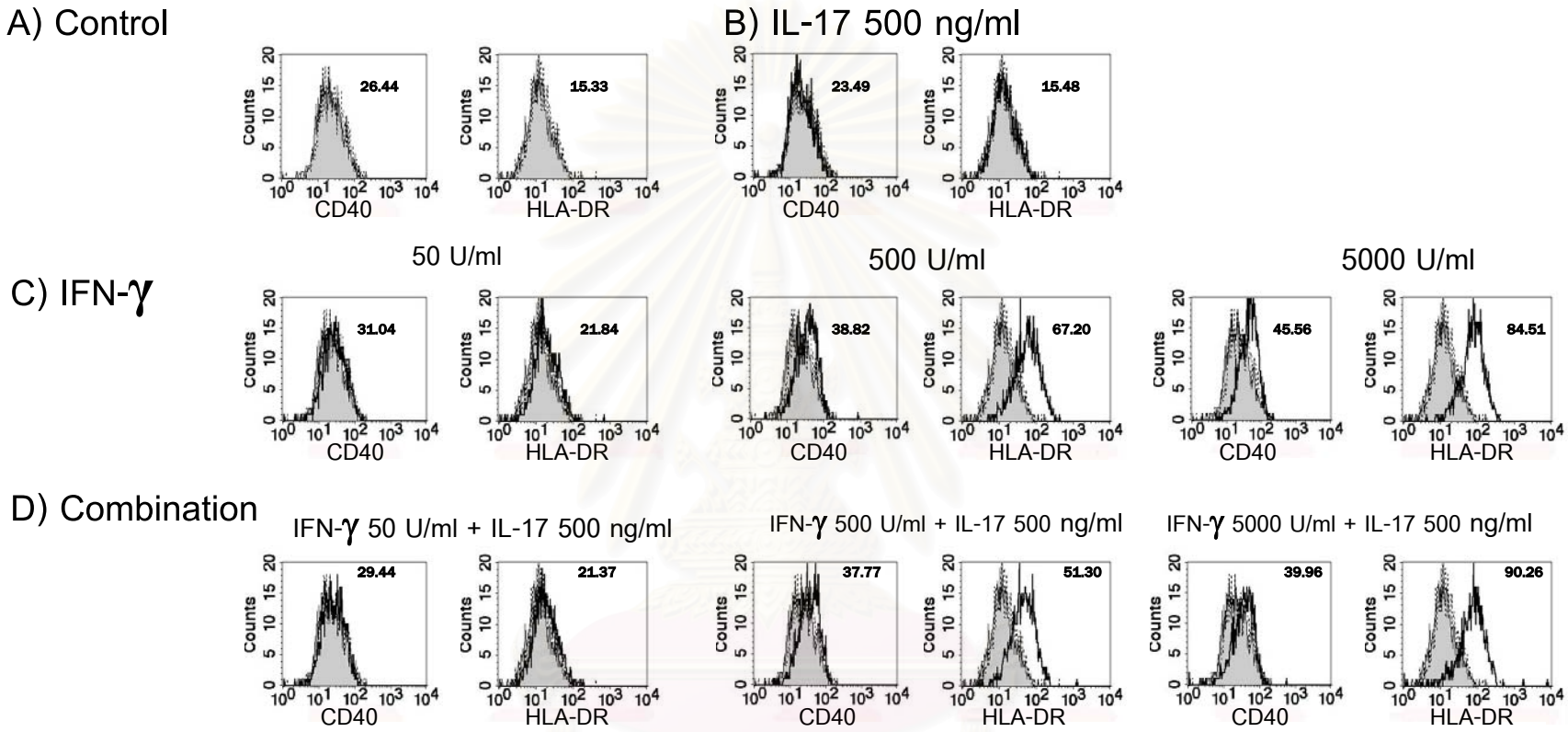

Figure 3. Representative histograms showing the effects of the combination of IFN-γ and IL-17 on CD40 and HLA-DR expression on HGF (n=12). HGF were cultured with 50, 500, 5000 U/ml IFN-γ (C), 500 ng/ml IL-17 (B) or the combination of 50 U/ml IFN-γ + 500 ng/ml IL-17; 500 U/ml IFN-γ + 500 ng/ml IL-17; 5000 U/ml IFN-γ + 500 ng/ml IL-17 (D). The cells were harvested and stained with 1) FITC-conjugated anti-CD40, 2) PerCP-conjugated anti-HLA-DR antibodies. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each histogram indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

4.3 The effect of IL-17 and IFN-γ on IL-8 production

Various doses of IFN-γ (50, 500, 5000 U/ml) and IL-17 (5, 50, 500 ng/ml) were used to stimulate HGF cultures. After 48 hours of incubation, culture supernatants were collected for analysis of IL-8 production using ELISA. The results in Figure 4 A, demonstrated that IL-17 induced IL-8 production in a dose dependent manner, whereas IFN-γ had no effect (Figure 4B).

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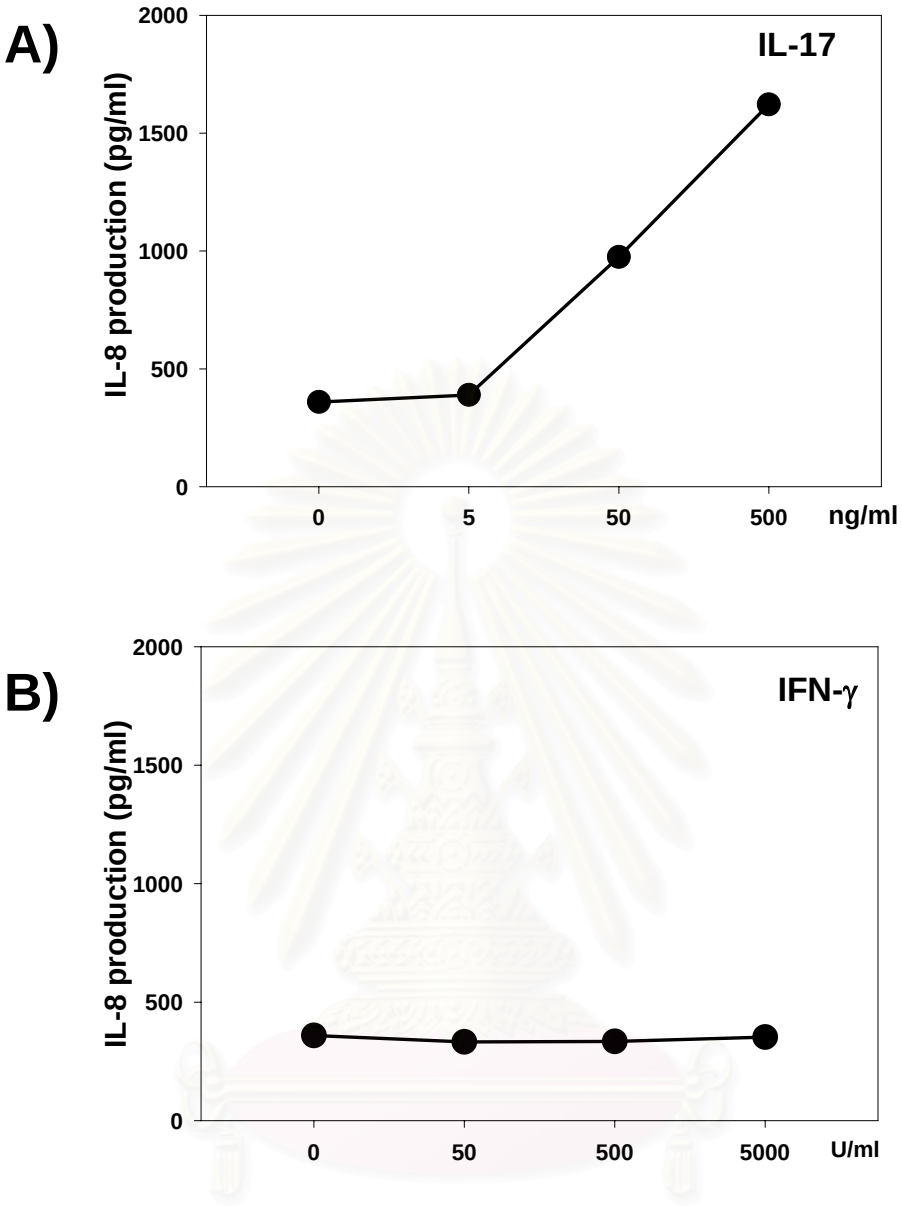

Figure 4. A representative of dose response of IL-8 production in HGF cultures (n=3). HGF ($1x10^5$ cells /ml) were incubated for 2 days with various concentrations of IL-17 (5, 50, 500 ng/ml) (A) or IFN-γ (50, 500, 5000 U/ml) (B). The culture supernatants were collected and IL-8 concentrations were determined by ELISA.

4.4 The combination effect of IL-17 and IFN-γ on IL-8 production by HGF

We also evaluated the combination effect of IL-17 and IFN-γ on IL-8 production. HGF cultures were stimulated with IL-17 (5, 50, 500 ng/ml), IFN-γ (100 U/ml) or the combination of the two cytokines (5 ng/ml IL-17 + 100 U/ml IFN-γ; 50 ng/ml IL-17+100 U/ml IFN-γ; 500 ng/ml IL-17+100 U/ml IFN-γ). After 2 days of incubation, culture supernatants were collected for analysis of IL-8 production using ELISA. Interestingly, IFN-γ synergistically enhanced IL-17-induced - IL-8 production in all HGF cell lines when combined with a high dose of IL-17 (500ng/ml) (Table2, Figures 5A). However, when IFN-γ combined with lower doses of IL-17, 5 and 50 ng/ml, synergy of IL-8 production was observed in six (PL, SP, PP, JP, TT, CK) and eleven HGF cell lines (PL, PR, TL, VK, PM, SP, PP, JP, NR, TT, CK) respectively (Table2), while the others showed no effect (Figure 5B). It should be noted that a high synergistic effect at lower doses of IL-17 (5 and 50 ng/ml) was detected in two HGF cell lines (TT and CK) (Table 2).

Table 2: Indices of synergy for the production of IL-8 after combined stimulation with IL-17 and IFN-γ

conditions	Donors															*P* value
	NH	PL	PR	TL	VK	PM	SP	SK	PS	SS	PP	JP	NR	TT	CK	
IL-17 5 ng/ml + IFN-γ 100 U/ml	0	1.67	0	0	0.25	0.25	1.69	0	0	0	1.71	2.49	0	17.26	41.65	0.59
IL-17 50 ng/ml + IFN-γ 100 U/ml	0.11	1.57	2.73	1.31	1.62	2.55	3.45	0	0.64	0.76	1.44	2.44	1.58	6.07	1.40	0.12
IL-17 500 ng/ml + IFN-γ 100 U/ml	1.62	1.14	1.06	1.01	1.70	1.93	2.58	1.55	1.24	1.09	1.32	2.22	2.10	3.34	1.45	0.001

The indices of synergy were calculated as described in the Materials and Methods section.

$$\text{Index of synergy} = \frac{\text{Cytokine production}_{(\text{IL-17+IFN-}\gamma)}}{\text{Cytokine production}_{(\text{IL-17})} + \text{Cytokine production}_{(\text{IFN-}\gamma)}}$$

Considered significantly synergistic with regards to index of synergy > 1 and $P < 0.05$

P values derived from the Wilcoxon matched pairs signed-ranks test.

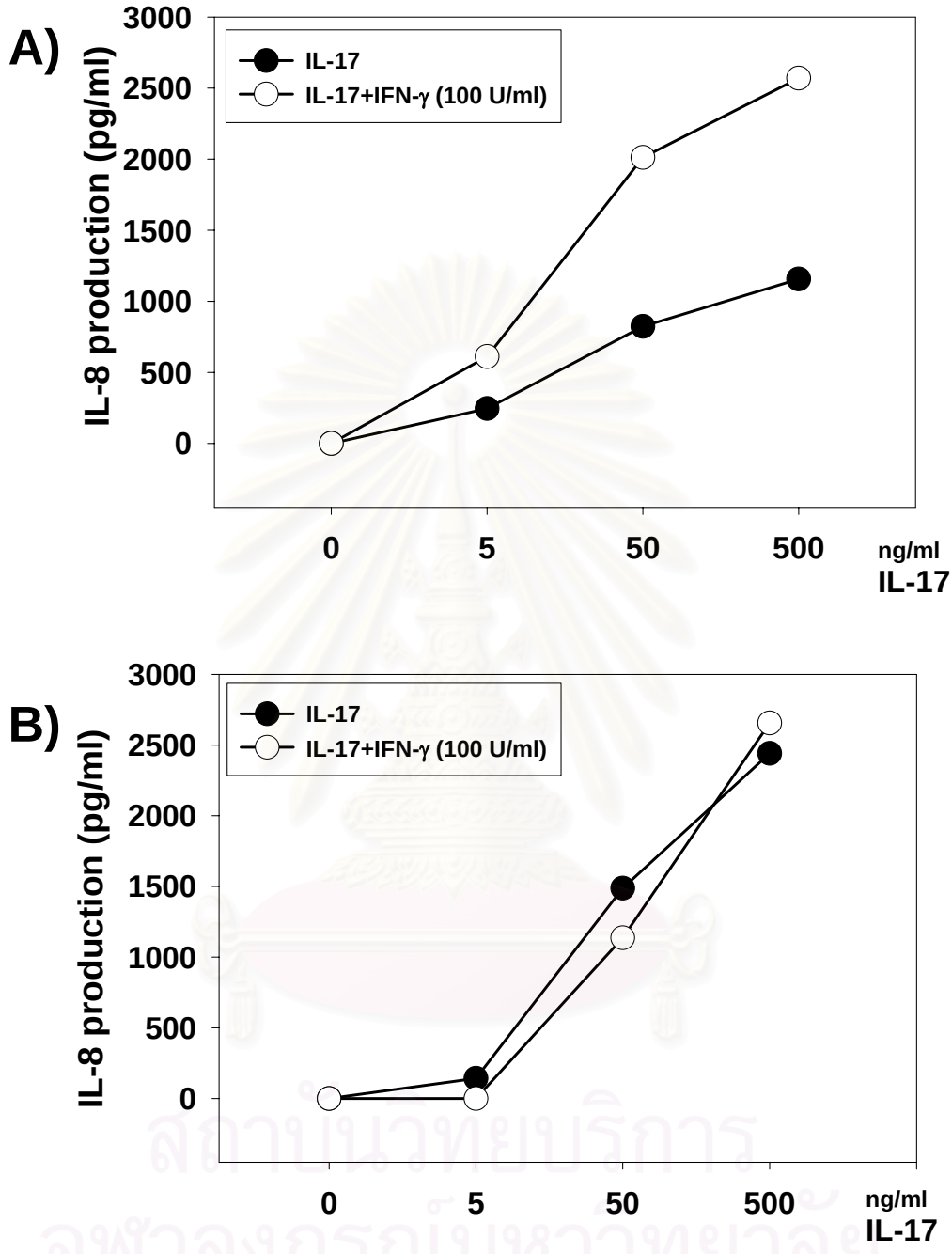

Figure 5. Heterogeneity of IL-8 production by HGF after stimulation with the combination of IL-17 and IFN-γ. A) Representative data of synergistic effect of IL-17 + IFN-γ on IL-8 production. B) Representative data of the absence of synergistic or additive effect of IL-17 (at lower doses of 5 and 50 ng/ml) + IFN-γ on IL-8 production.

CHAPTER 5

DISCUSSION AND CONCLUSION

Major characteristics of periodontitis lesions are increased numbers of activated T cells as well as over-production of cytokines to include IL-1, TNF-α, IFN-γ and IL-6 etc. (Roberts et al., 1997; Salvi et al., 1998; Takeichi et al., 2000). Immunohistochemistry data have demonstrated that T cells and gingival fibroblasts are in close proximity in the lesions, implying that fibroblasts could play an active role in immunoregulation and immunopathogenesis of the disease (Murakami et al., 1999). Several recent studies clearly indicated that fibroblasts actively participate in immune regulation and immunopathgenesis (Lekic et al., 1997; Murakimi and Okada, 1997; Takashiba et al., 2003). We demonstrated in this study that T cell-derived cytokines, IL-17 and IFN-γ, have immunostimulatory effects on human gingival fibroblasts. Unlike IL-17, IFN-γ greatly induced up-regulation of HLA-DR expression on HGF and minimally affected up-regulation of CD40 expression. In contrast, other studies demonstrated that IL-17 enhanced HLA-DR expression (Teunissen et al., 1998) and CD40 expression on keratinocytes (Woltman et al., 2000). This discrepancy could be due to different cell types used in the experiments. The clear effects of IFN-γ on co-stimulatory molecule expression on HGF required long incubation exposure (5 days). The finding agrees with other studies which demonstrated that the long culture period of at least 3 days was needed for IFN-γ-induced up-regulation of HLA-DR expression on HGF (Wassenaar et al., 1997). It is possible that endogenous mediator(s) induced from IFN-γ-treated HGF may contribute to the up-regulation of HLA-DR expression. In consistent with other observations, our data also indicated that IL-17 did not enhance IFN-γ- induced HLA-DR and CD40 expression on HGF (Teunissen et al., 1998; Albanesi et al., 1999).

Unlike dendritic cells, fibroblasts are recognized as non-professional antigen-presenting cells and cannot prime antigen-specific naïve T cells (Murakami and Okada, 1997). Our observation that HGF can be induced to markedly up-regulate HLA-DR and minimal CD40 expression by IFN-γ may imply that the activated HGF might possibly be able to stimulated antigen-specific memory T cells which requires low threshold of co-stimulatory molecule expression.

IL-8 represents a key chemokine involving PMN recruitment to the site of inflammation (Bickel, 1993; Witowski et al., 2004). High levels of IL-8 production have been shown to be associated with diseases such as rheumatoid arthritis (Furuzawa-Carballeda and Alcocer-Varela, 1999; Gerard et al., 2002) and severe asthma (Linden, et al 2001). Also such association has been reported in periodontitis (Tsai et al., 1995; Chung et al., 1997). In this study, we evaluated the effect of IL-17 and IFN-γ on IL-8 production by HGF. We showed that HGF produced IL-8 in response to IL-17 but not to IFN-γ. Although IFN-γ alone has no effect, significantly increased levels of IL-8 production from HGF could be observed when this cytokine was combined with IL-17. The synergistic effect on IL-8 production from keratinocytes induced by these two cytokines was also reported by other investigators (Teunissen et al., 1998; Albanesi et al., 1999; Andoh et al., 2001). Interestingly, the synergy of IL-8 production could be detected in all HGF cell lines at a higher dose of IL-17 (500 ng/ml) whereas at lower doses (5 and 50 ng/ml), heterogeneous response was observed in our study. The heterogeneity of fibroblasts is not new. Previous observations showed the heterogeneous HGF response from different healthy donors after stimulation of HGF either with bacterial products (lipopolysaccharide or lipoteichoic acid) or cytokine (IL-1) (Dongari-Bagtzoglou et al., 1997; Sugawara et al., 1998). These heterogeneity findings regarding cytokine production by fibroblasts may be dependent on many factors such as different kind of stimulants, genetic factors, selective expansion of certain

clonal fibroblast suppopulations and other as yet unidentified mechanisms (Kornman et al., 1997; Dongari-Bagtzoglou and Ebersole, 1998).

In summary, our results indicate that IFN-γ has effect on HLA-DR and CD40 expression on HGF whereas IL-17 has effect on IL-8 production. The data imply that co-stimulatory molecule expression and cytokine production of HGF are controlled by different mechanisms. Expression of co-stimulatory molecules, HLA-DR and CD40, as well as IL-8 production is important in immune response. We speculate that the locally produced IL-17 and IFN-γ could play role in local immunoregulation in inflamed periodontal tissue. Moreover, heterogeneous response in IL-8 production from HGF is a very interesting issue which could contribute to disease susceptibility and severity. Further studies are required to precisely understand the mechanisms.

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APPENDICES

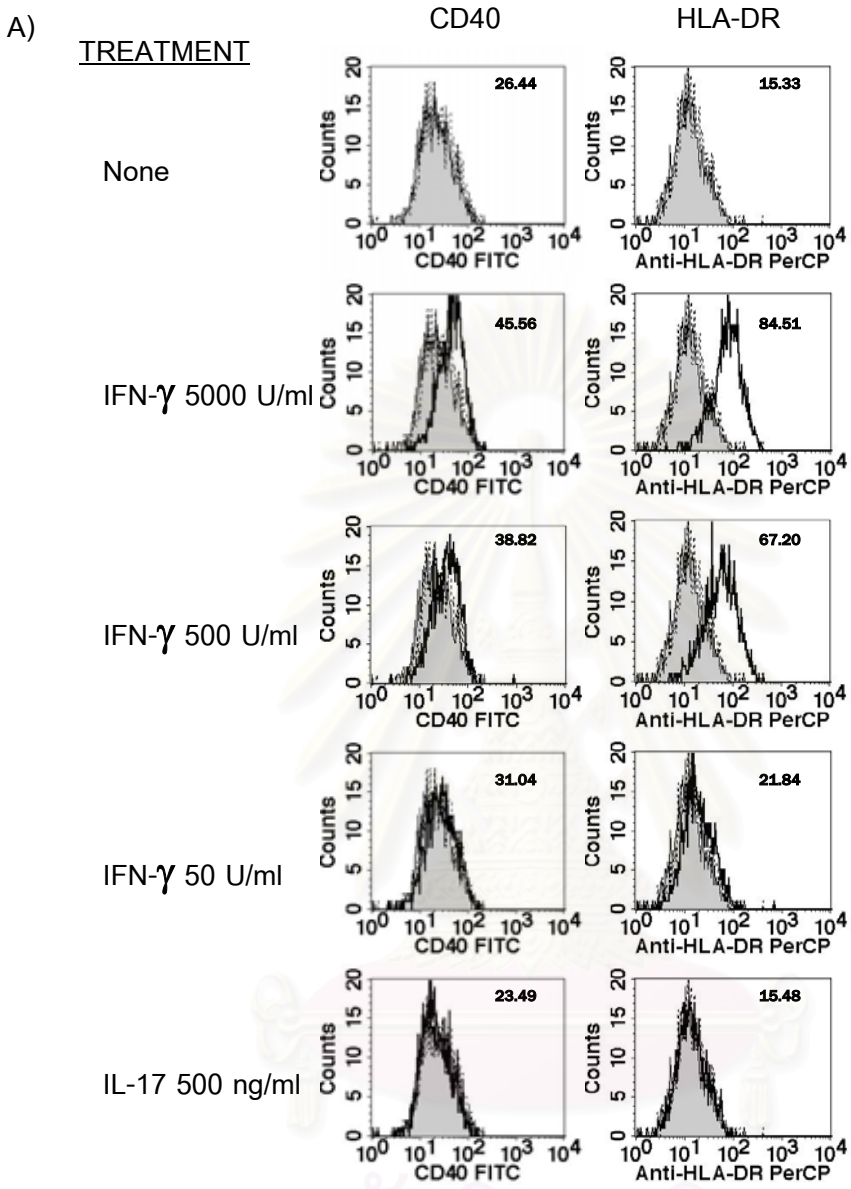

Figure 6A: NH-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

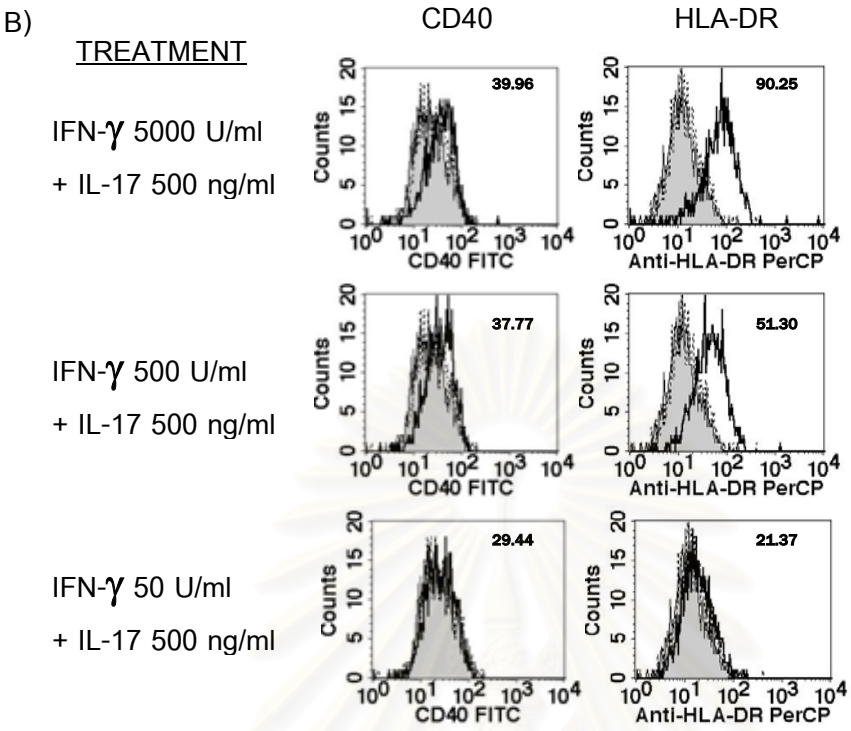

Figure 6B: NH-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

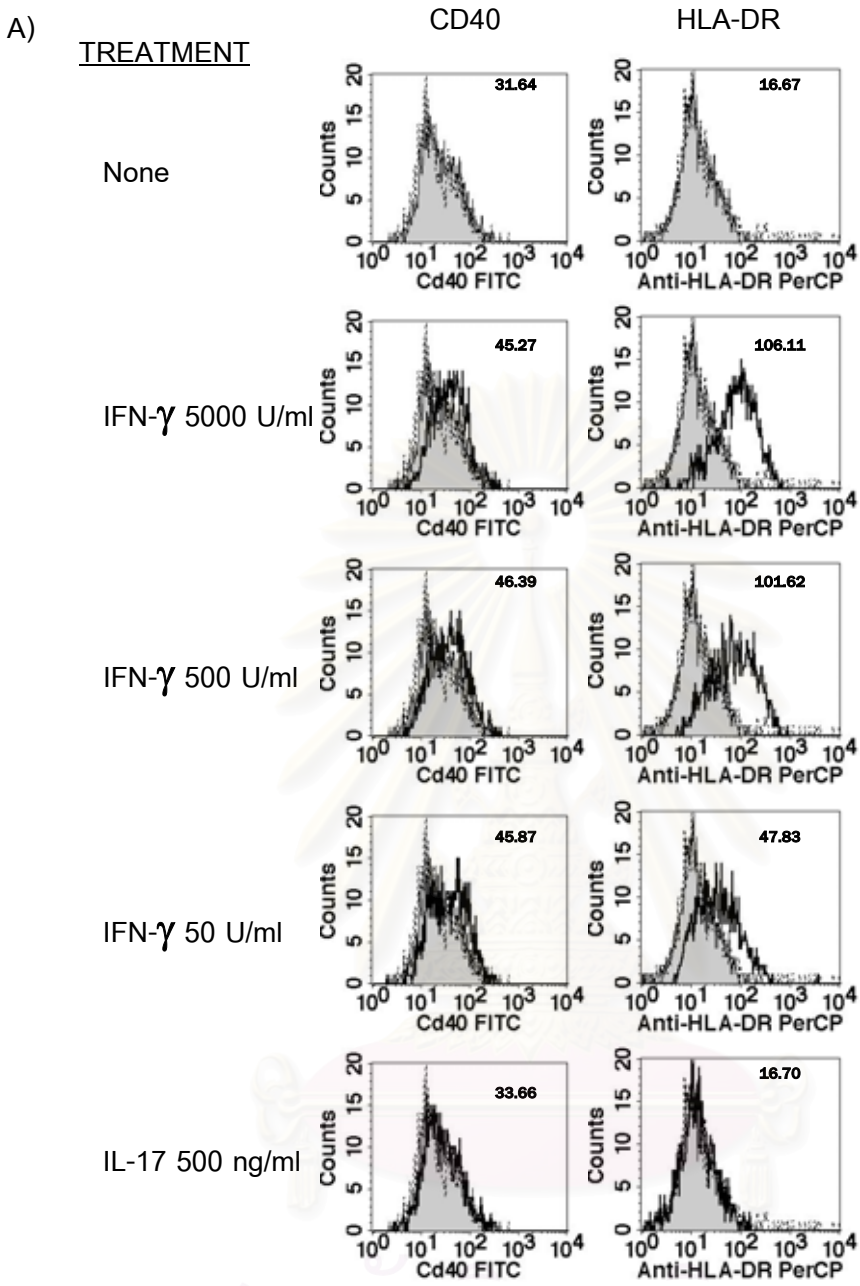

Figure 7A: PL-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

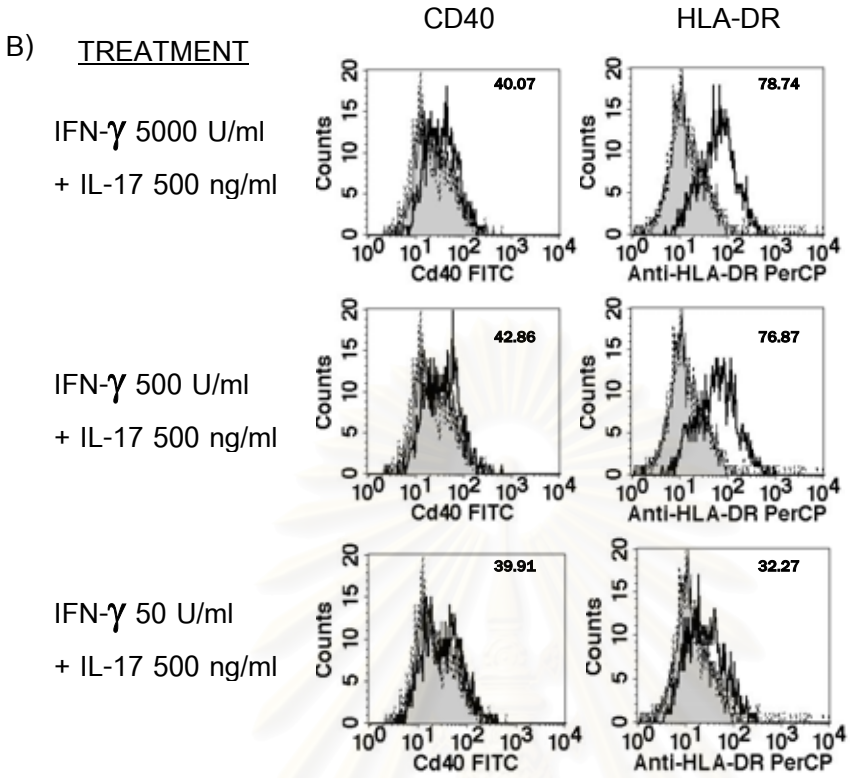

Figure 7B: PL-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

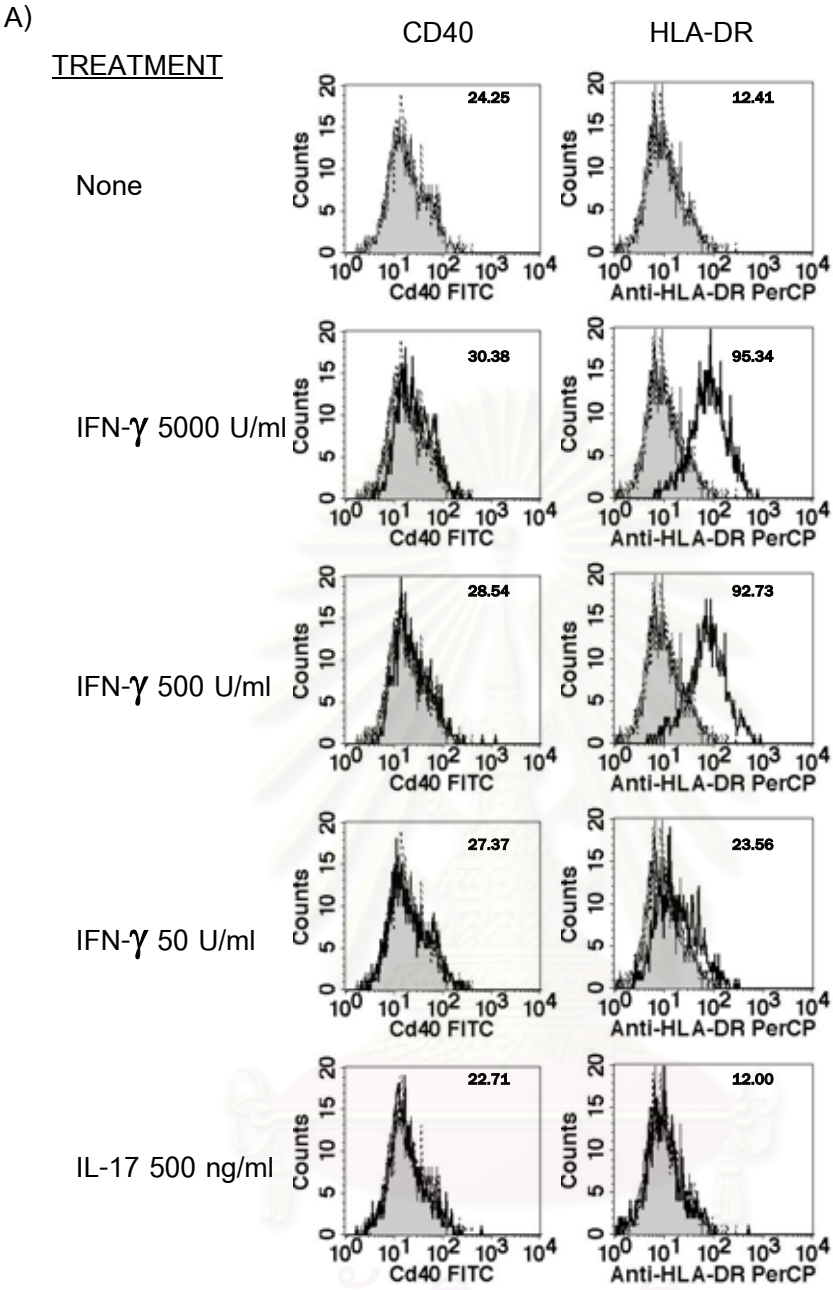

Figure 8A: PR-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

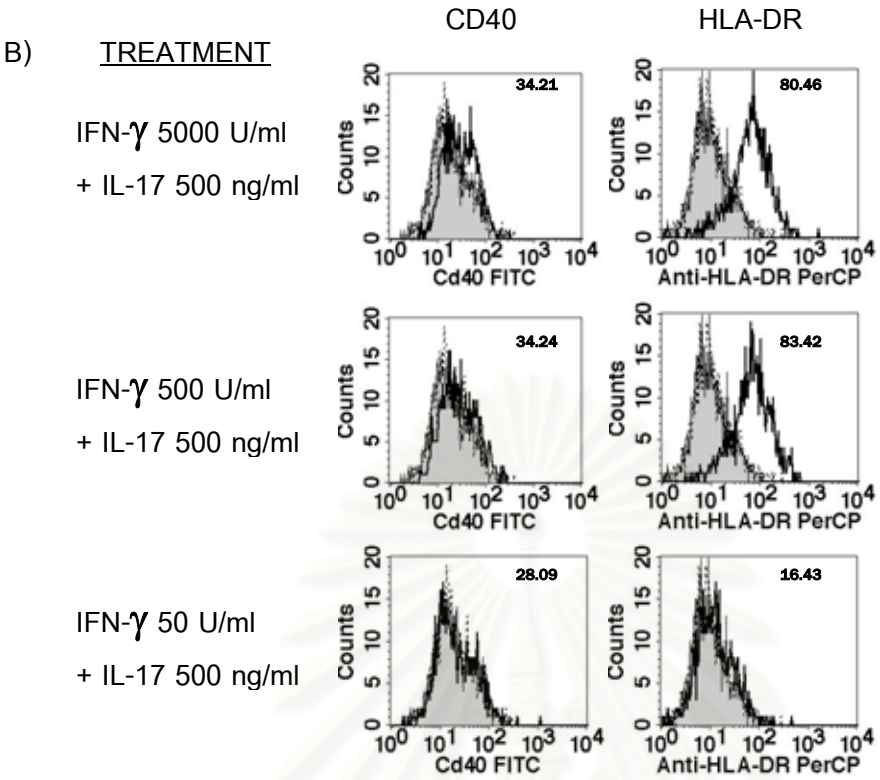

Figure 8B: PR-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

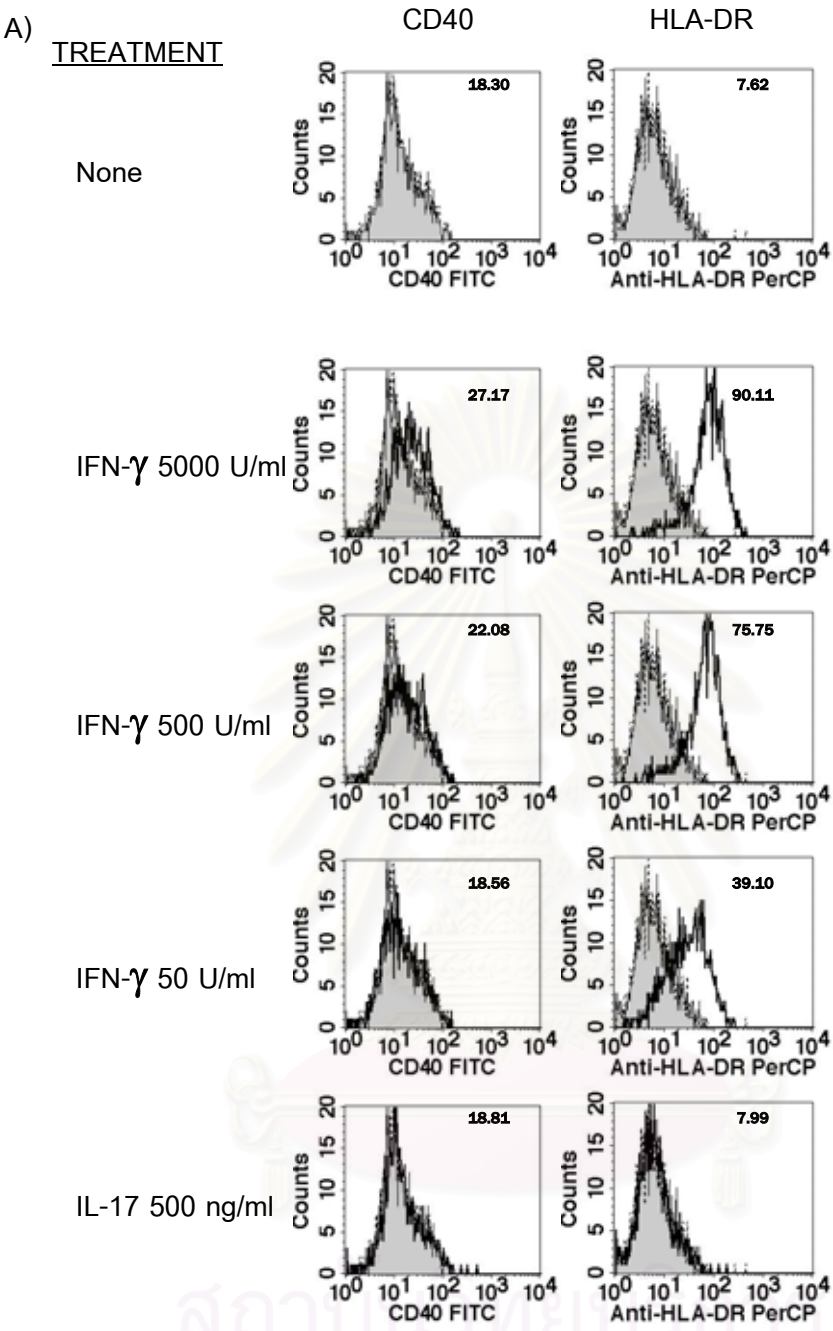

Figure 9A: TL-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

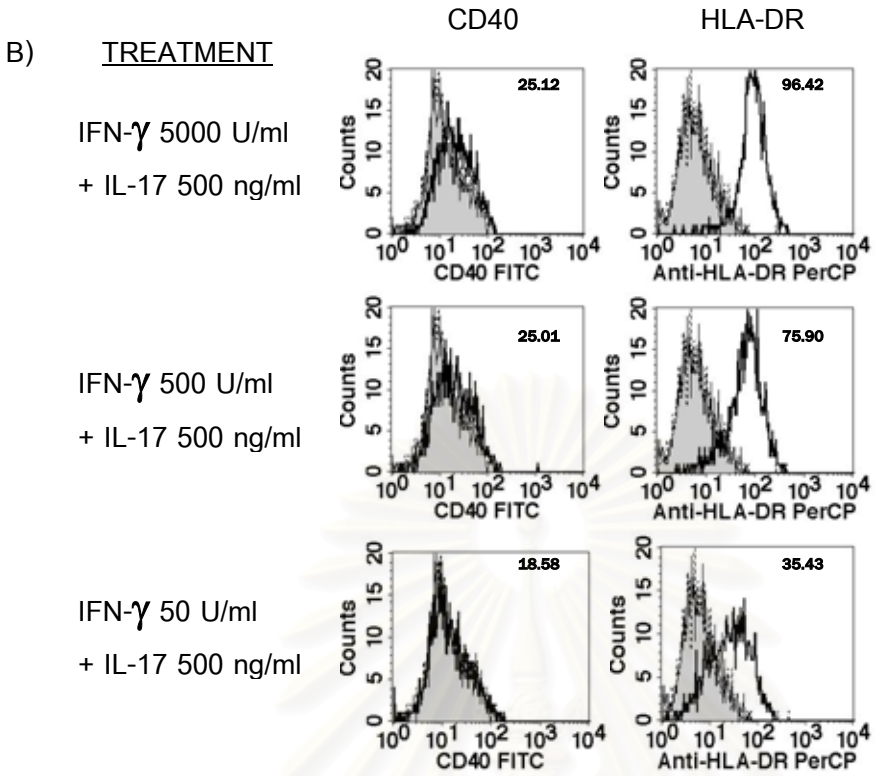

Figure 9B: TL-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

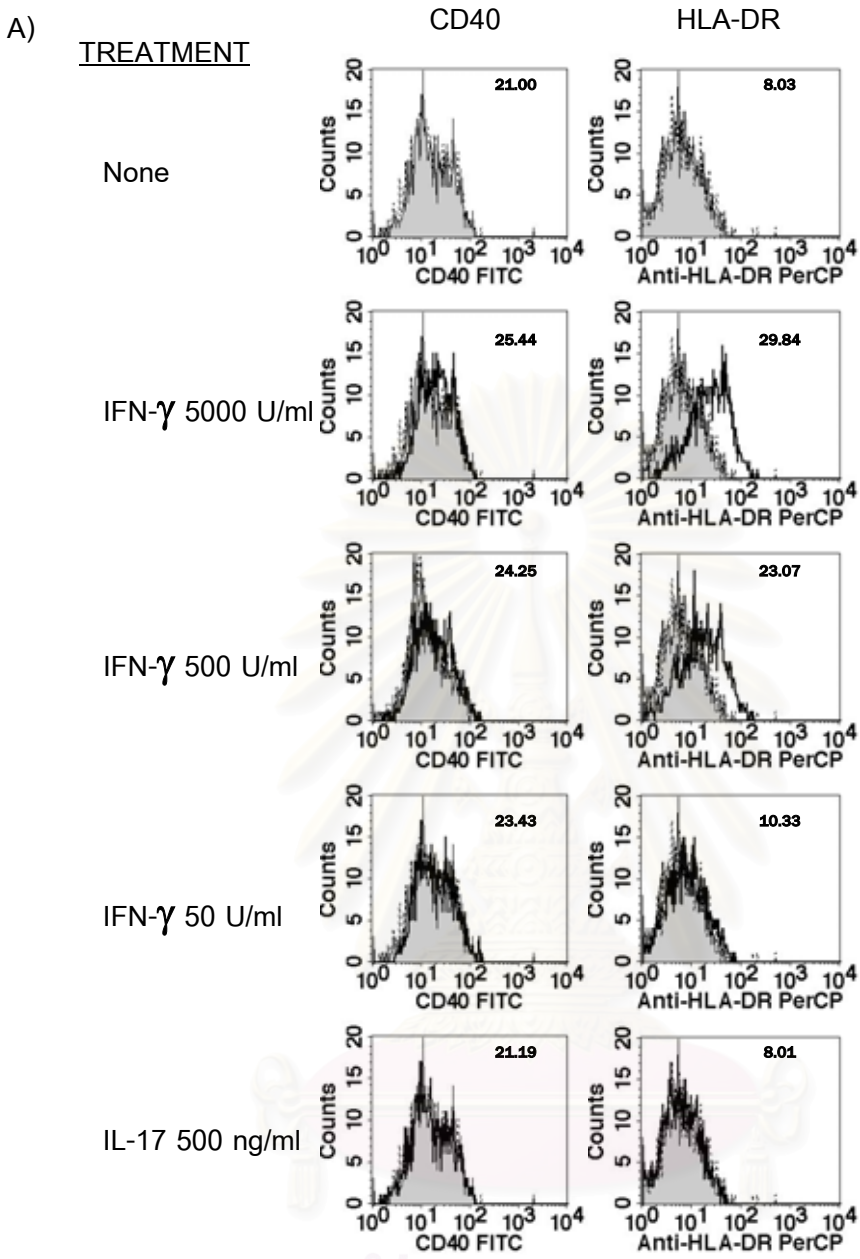

Figure 10A: VK-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

B)

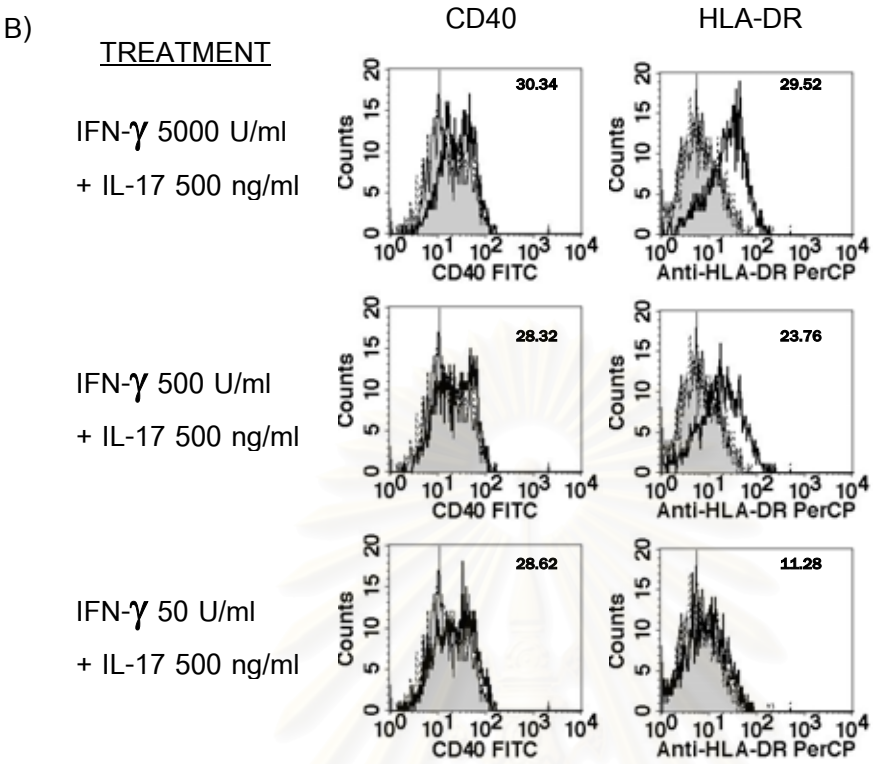

Figure 10B: VK-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

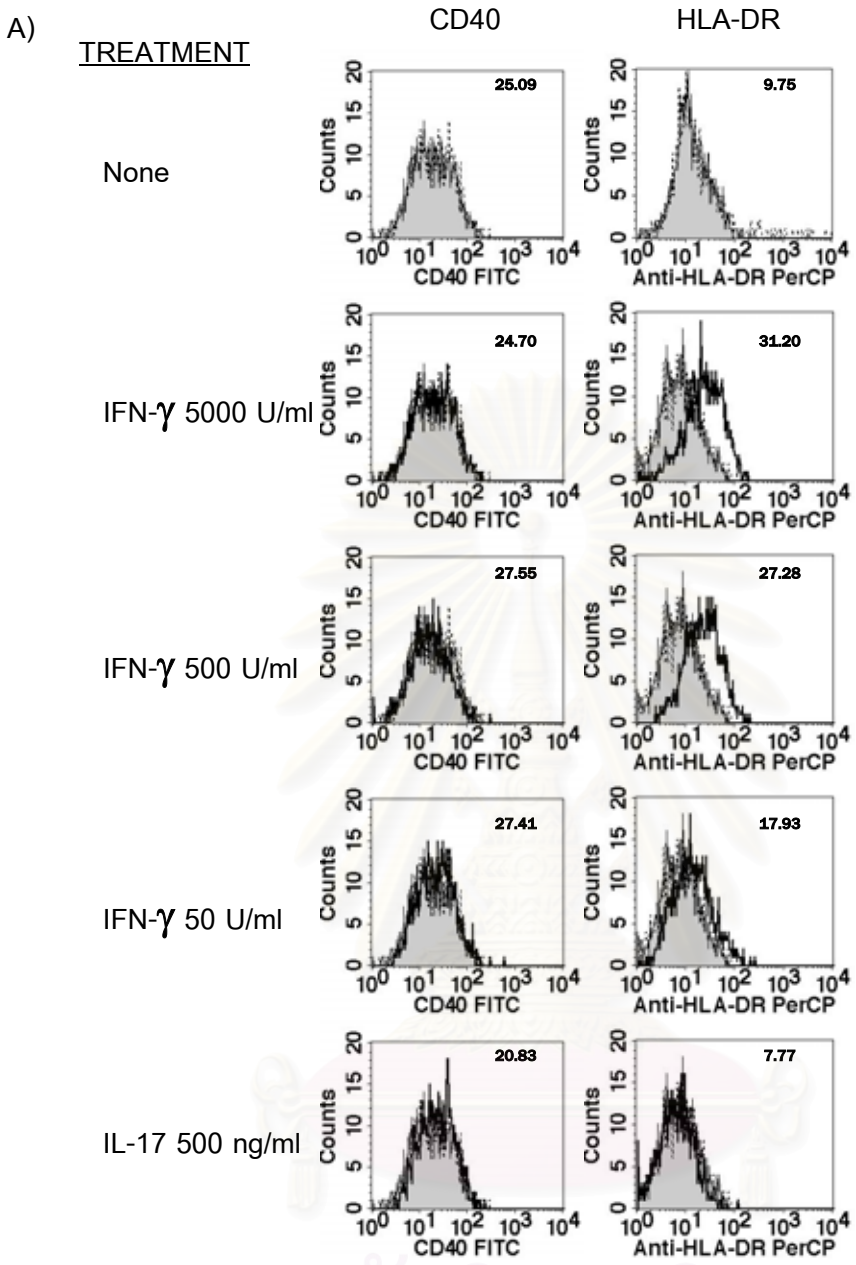

Figure 11A: PM-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

B)

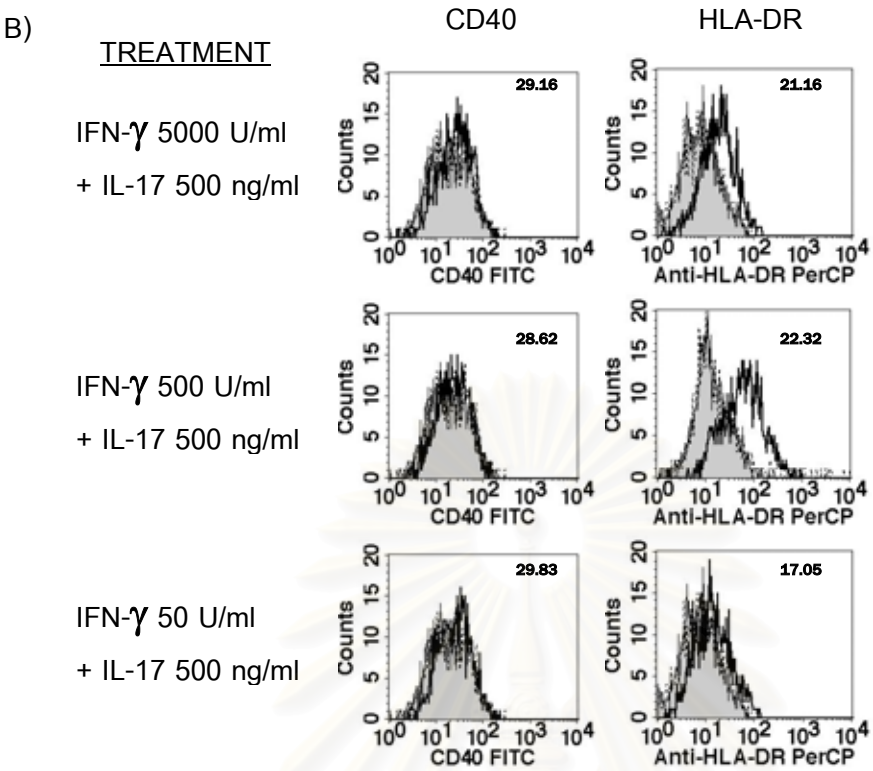

Figure 11B: PM-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

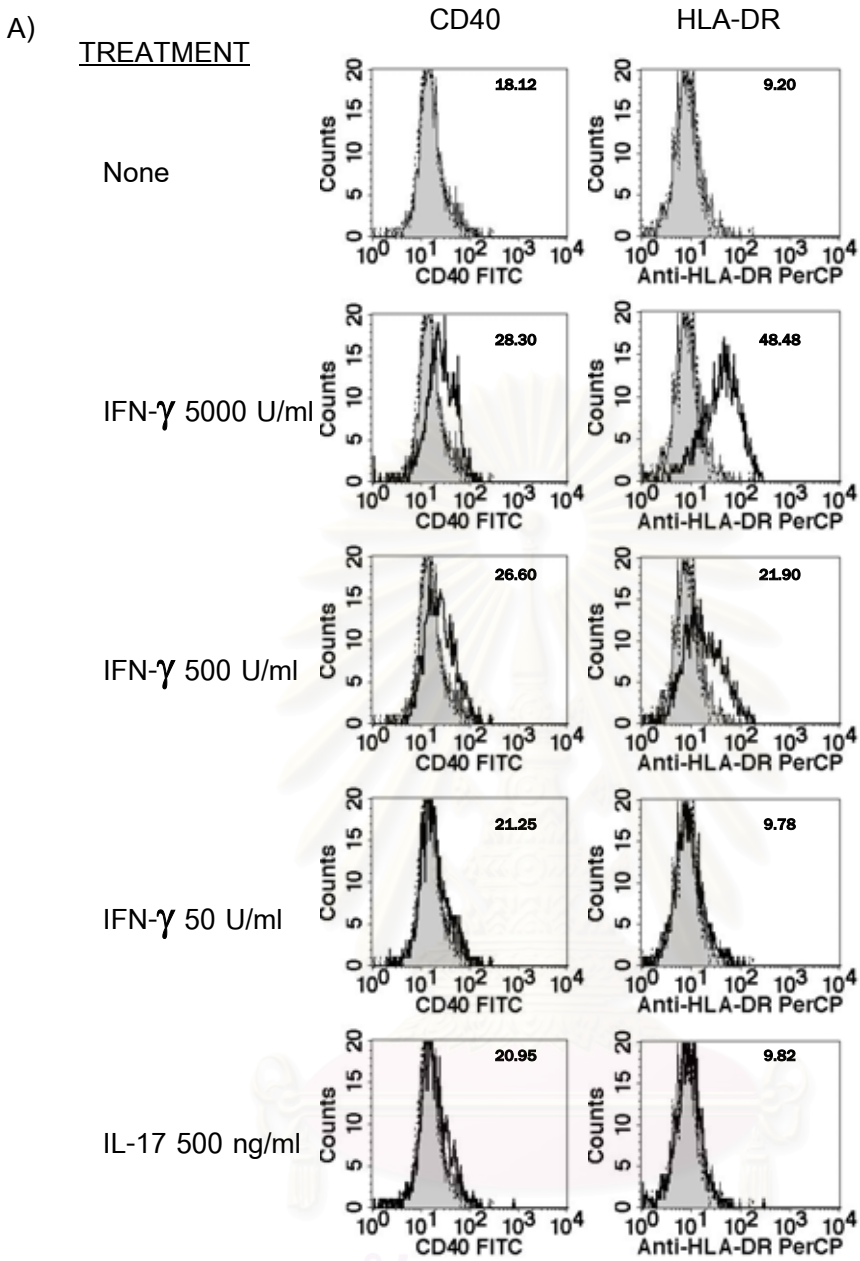

Figure 12A: SP-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

B)

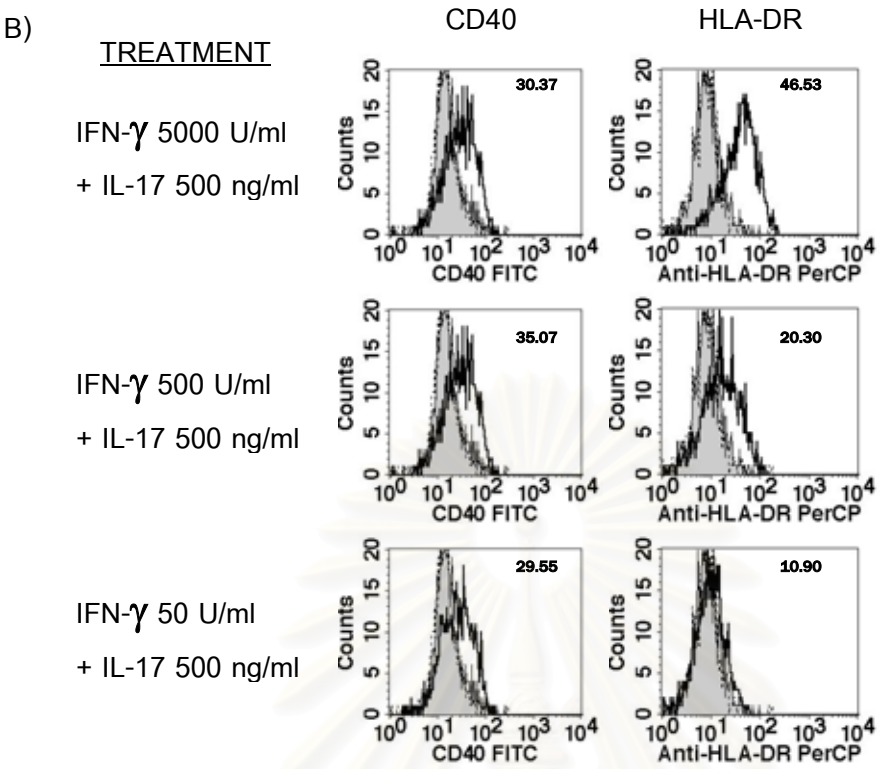

Figure 12B: SP-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

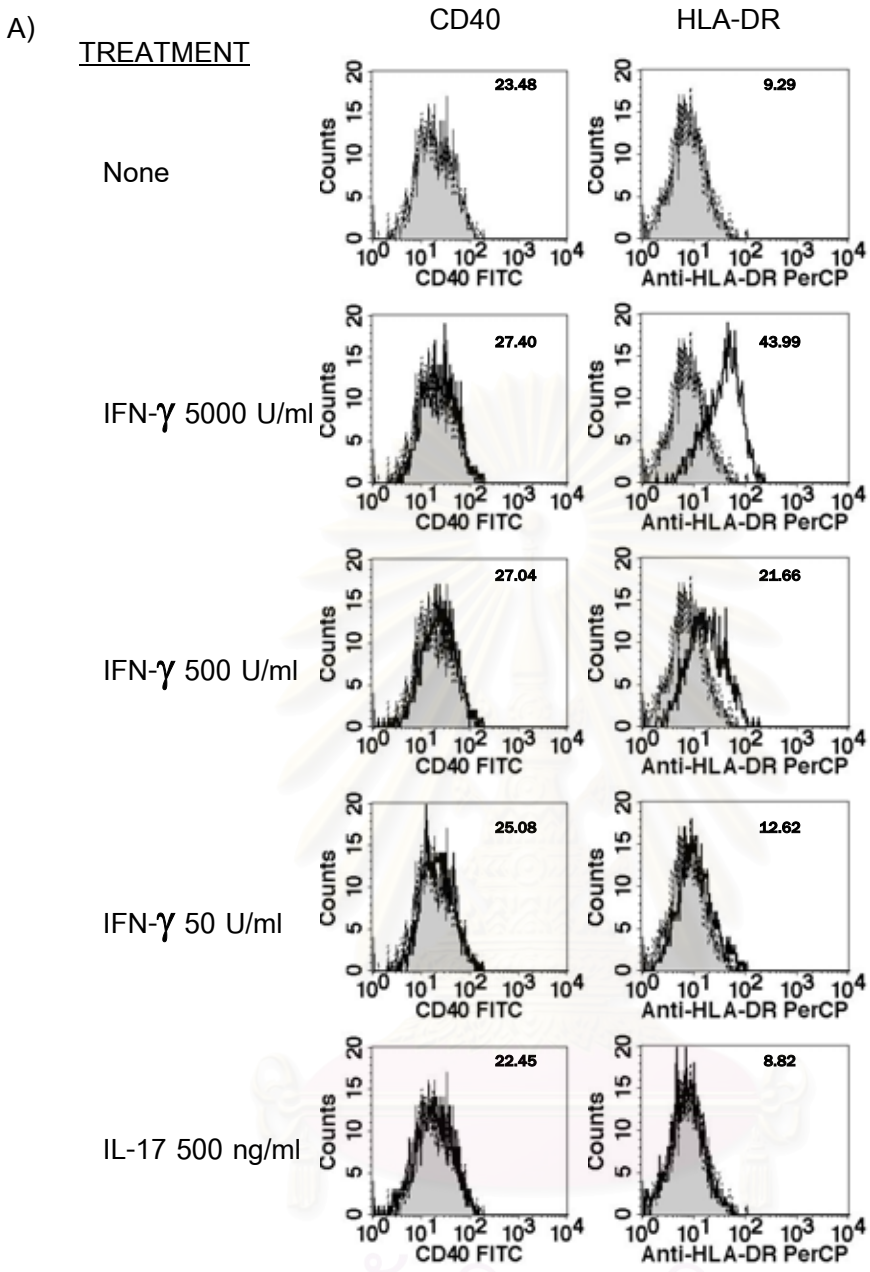

Figure 13A: PP-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

B)

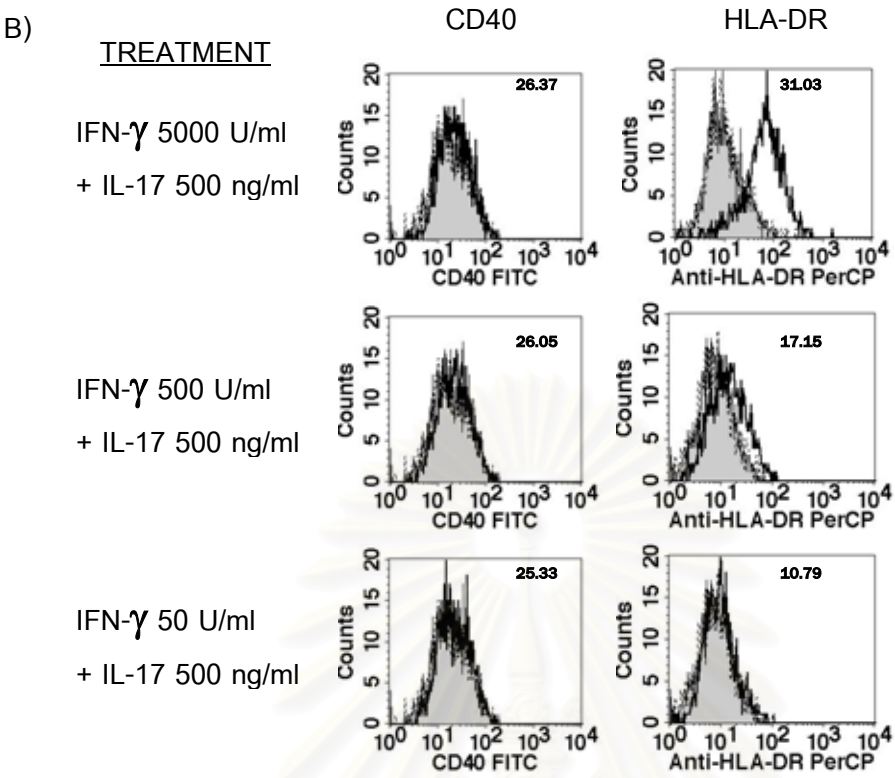

Figure 13B: PP-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

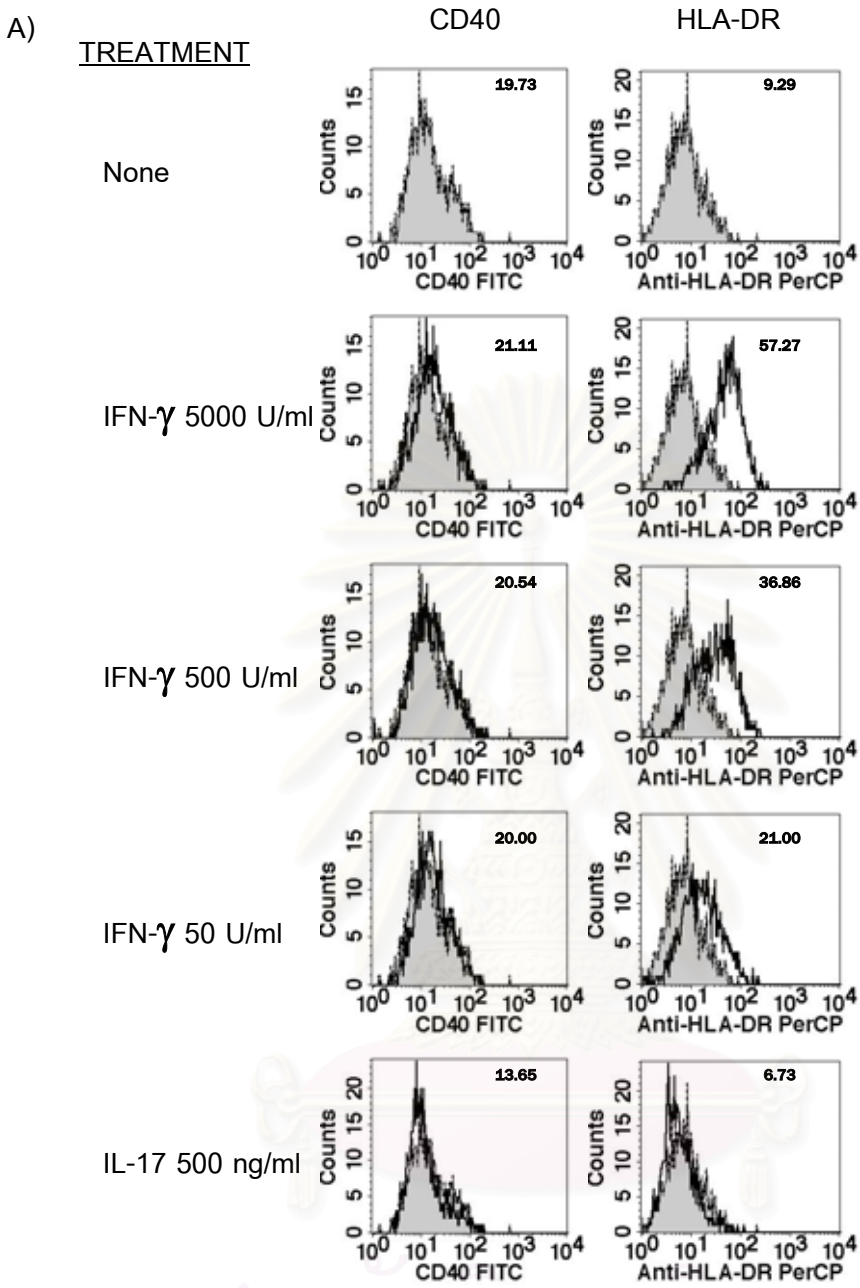

Figure 14A: CK-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

B)

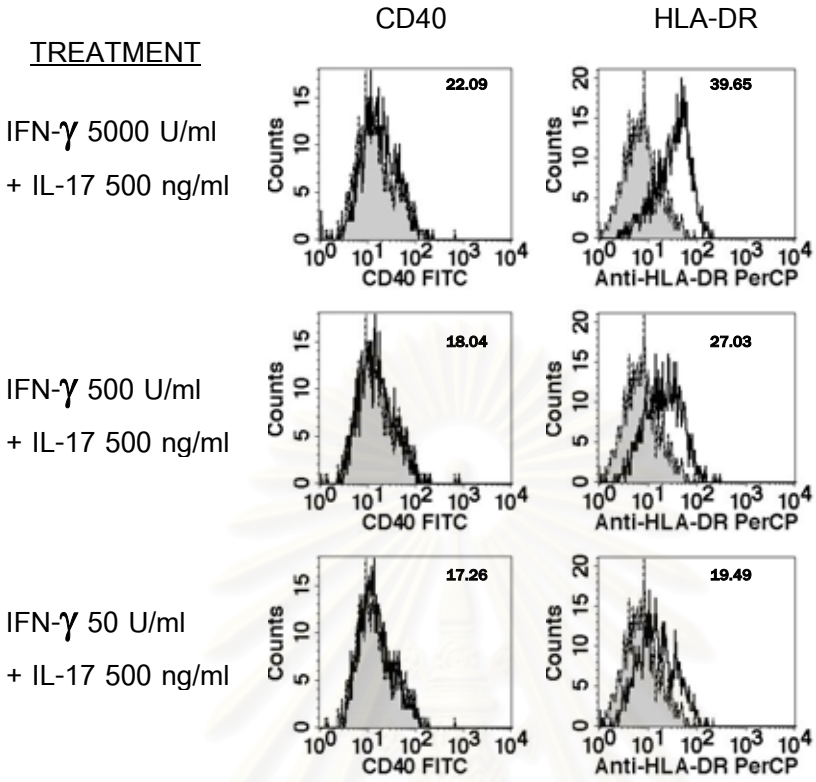

Figure 14B: CK-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

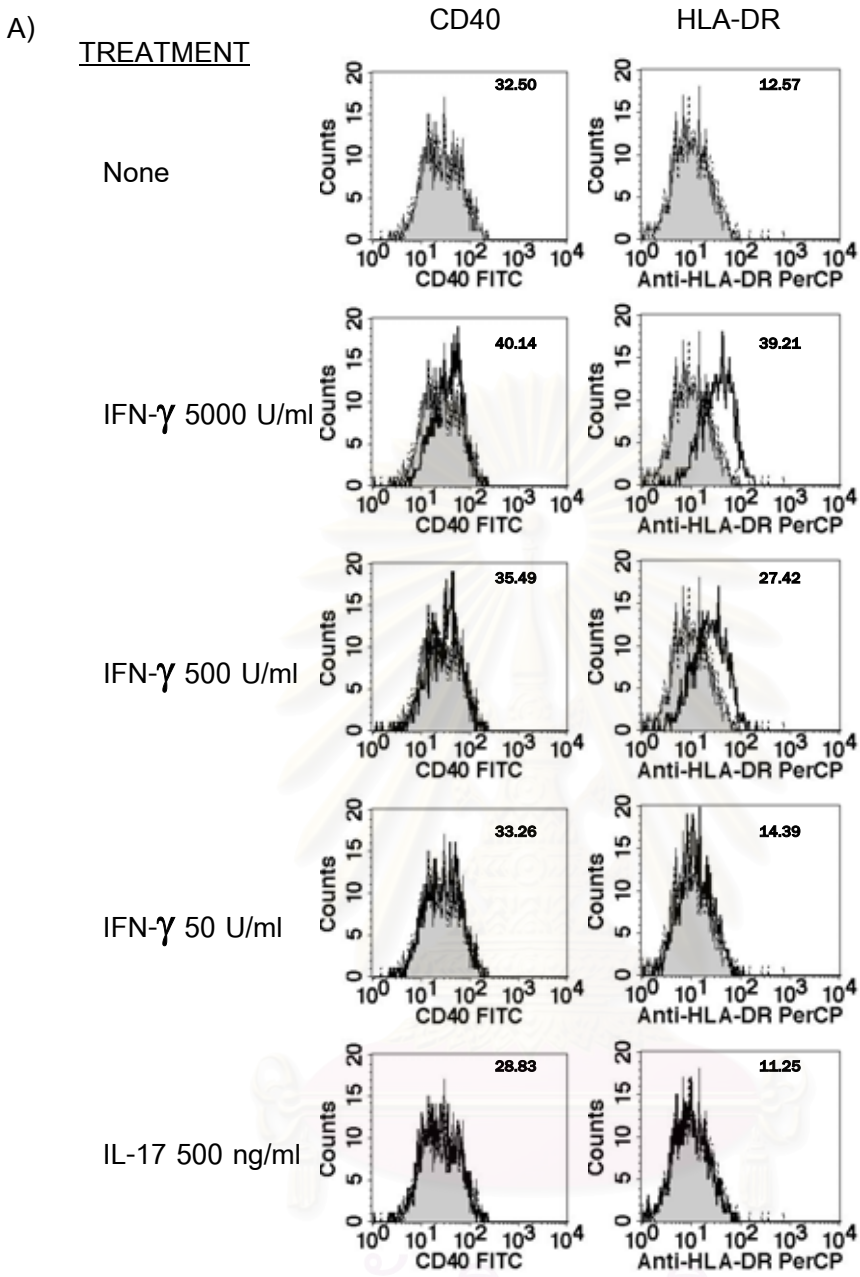

Figure 15A: JP-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

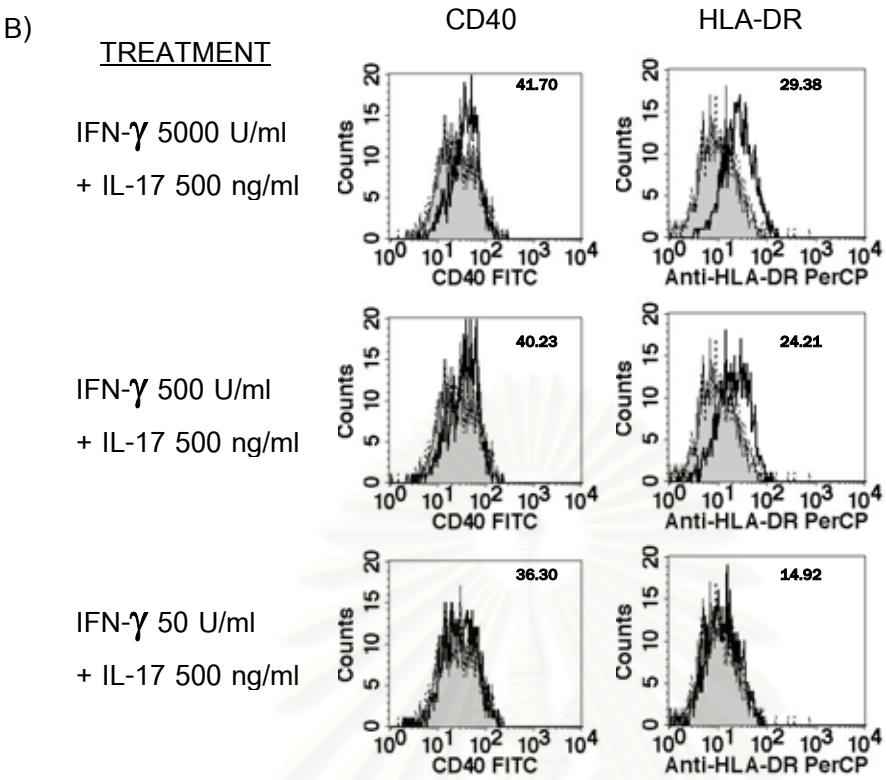

Figure 15B: JP-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

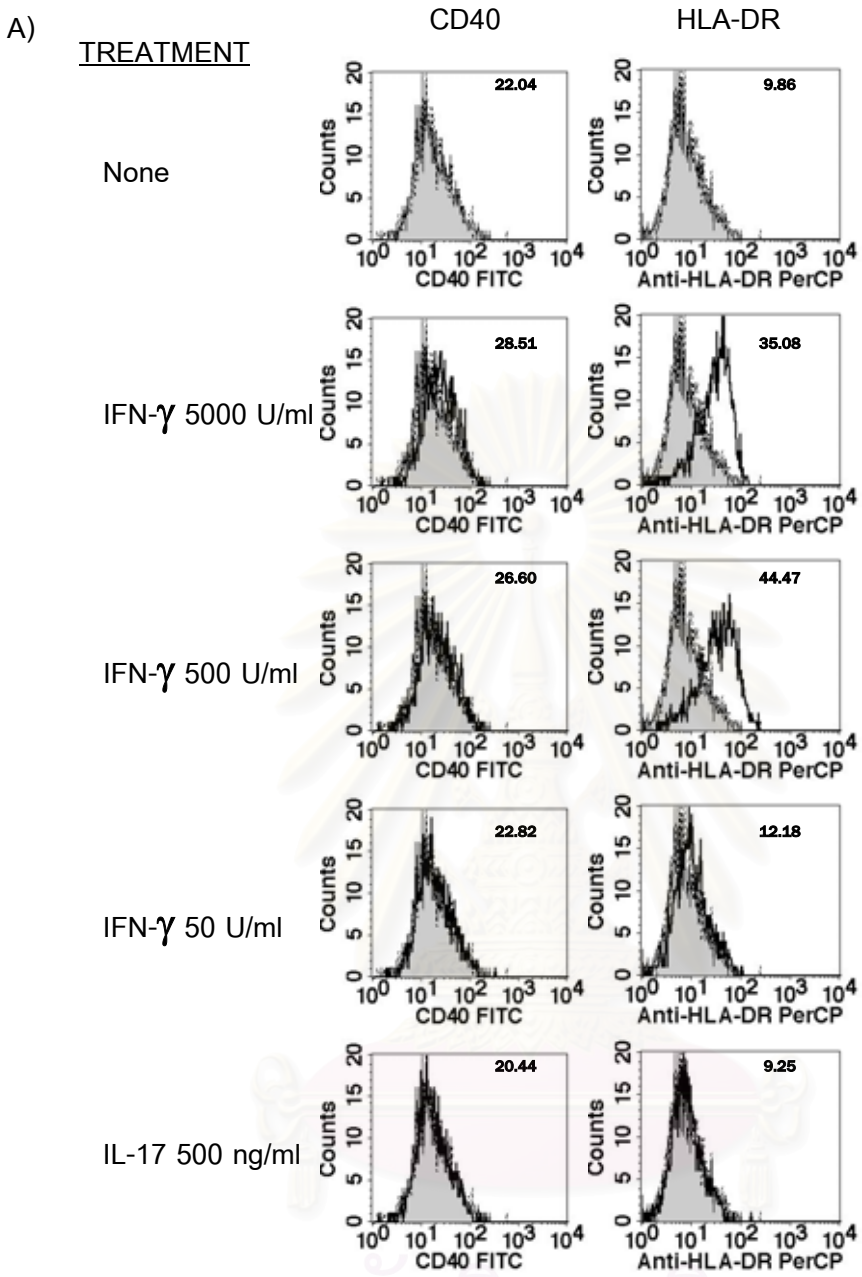

Figure 16A: NR-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

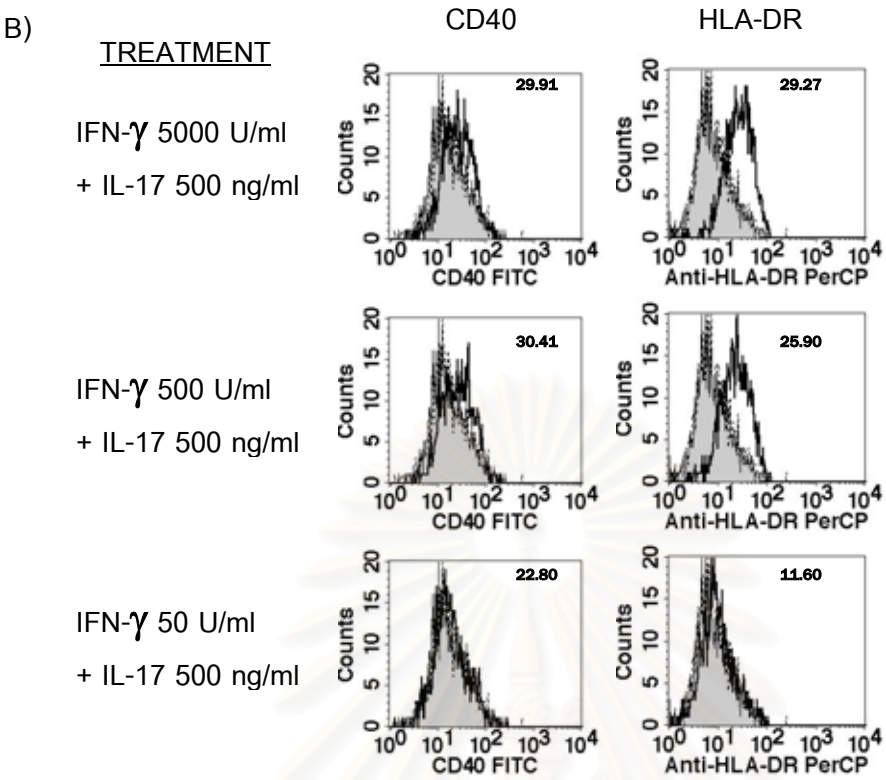

Figure 16B: NR-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

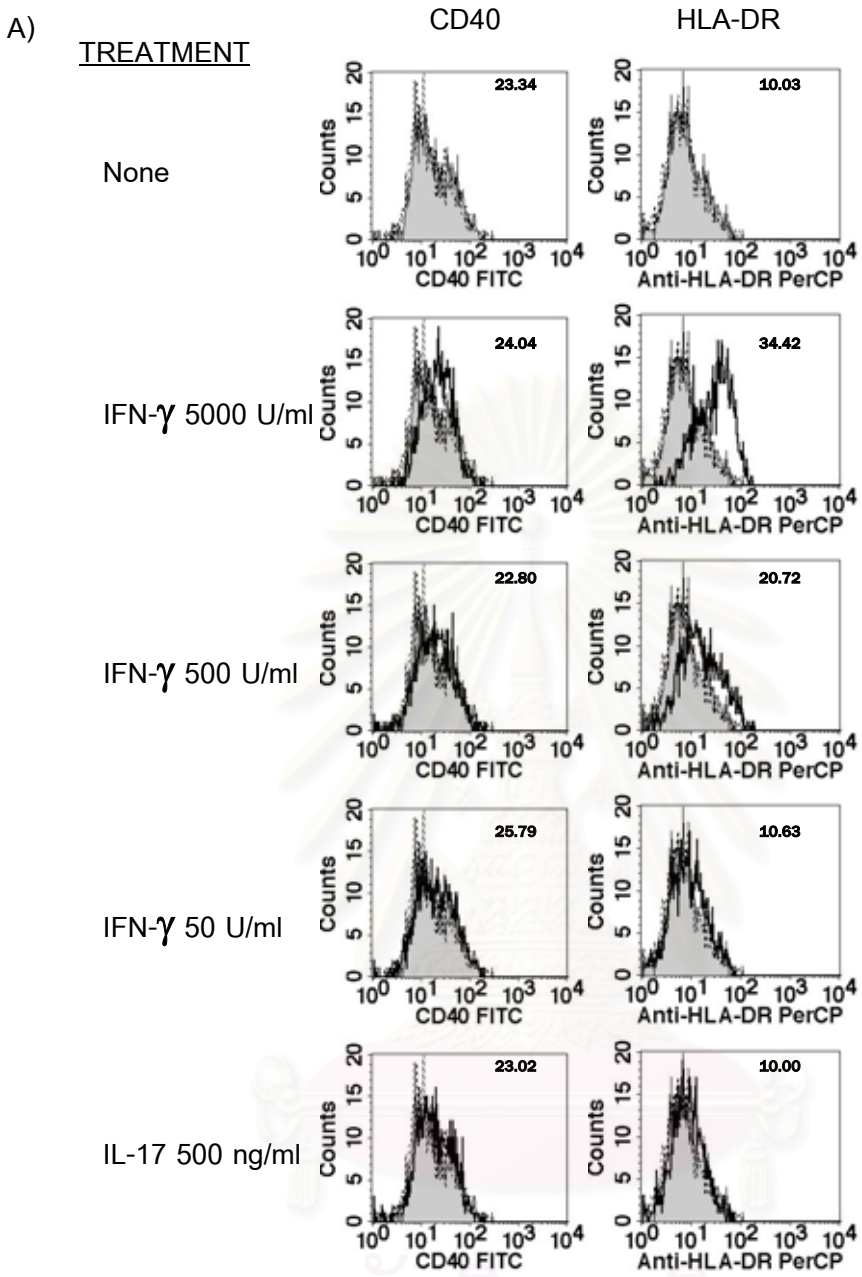

Figure 17A: TT-HGF: The effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts, shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

B)

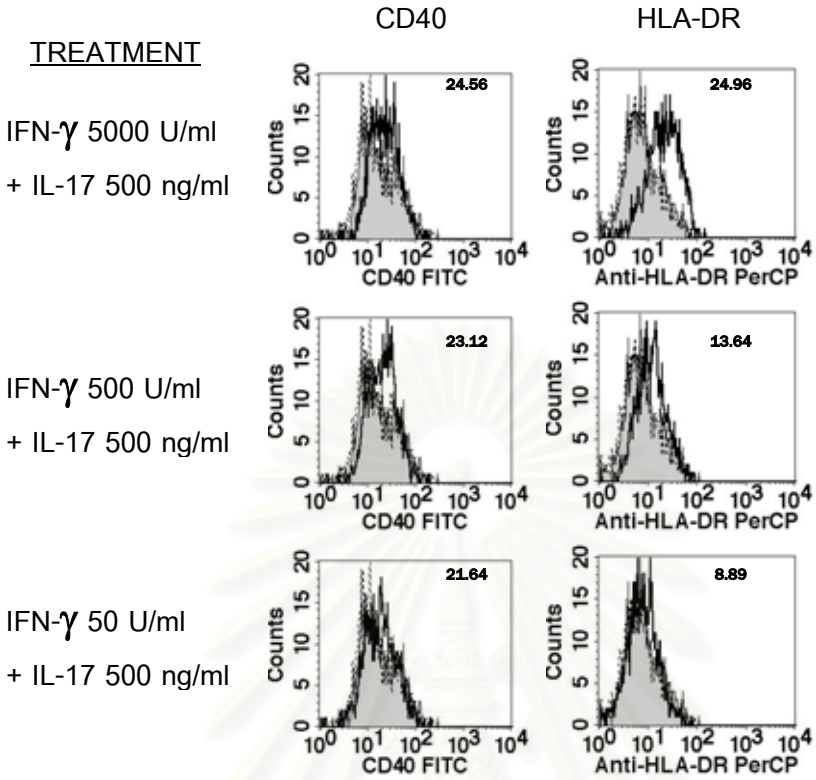

Figure 17B: TT-HGF: The combination effects of IFN-γ and IL-17 on CD40 and HLA-DR expression on human gingival fibroblasts shown as FACS profiles. Dotted lines are isotype controls, shaded areas are un-stimulated HGF and solid lines represent cytokine stimulation. The *x*-axis and the *y*-axis indicate the relative fluorescence intensity and cell number, respectively, and the number appearing in the upper right corner of each graph indicates CD40 or HLA-DR mean fluorescence intensity (MFI).

Table 3: The effect of IL-17 and IFN-γ on IL-8 production from fifteen independent donors.

conditions	IL-8 production (pg/ml)														
	NH	PL	PR	TL	VK	PM	SP	SK	PS	SS	PP	JP	NR	TT	CK
Control	967.74	112.87	193.57	115.04	168.39	173.01	451.74	170.78	261.46	734.72	375.69	235.77	718.96	16.57	218.65
IL-17 5 ng/ml	916.08	123.62	180.34	123.33	176.75	244.91	303.63	152.22	305.87	877.52	403.80	481.29	700.20	19.37	250.45
IL-17 50 ng/ml	1142.22	249.87	209.17	329.54	212.82	308.32	1271.07	159.33	869.52	2222.60	618.54	1058.76	909.99	115.96	4420.20
IL-17 500 ng/ml	1281.24	504.74	671.62	782.85	518.57	496.11	2812.62	240.24	1305.22	3175.18	947.25	1392.54	1201.89	328.21	6168.80
IFN-γ 100 U/ml	994.83	92.25	186.64	98.86	177.05	183.64	971.70	155.63	255.02	627.96	387.00	144.84	891.08	6.91	197.5
IL-17 5 ng/ml + IFN-γ 100 U/ml	863.01	130.77	221.37	110.99	172.57	194.02	1330.80	177.05	241.04	488.55	443.22	847.20	712.06	64.89	1543.00
IL-17 50 ng/ml + IFN-γ 100 U/ml	989.16	327.77	236.11	395.30	254.38	544.52	3482.07	236.09	647.65	1870.86	740.58	2247.15	1292.85	619.39	6103.05
IL-17 500 ng/ml + IFN-γ 100 U/ml	1517.94	560.07	699.80	789.13	778.61	816.27	8260.14	278.48	1556.12	3390.06	1142.49	2805.87	2096.83	1056.59	8827.45

Table 4: The effect of IL-17 and IFN-γ on IL-8 production from fifteen independent donors (minus control).

conditions	IL-8 production (pg/ml)														
	NH	PL	PR	TL	VK	PM	SP	SK	PS	SS	PP	JP	NR	TT	CK
IL-17 5 ng/ml	**0.00**	**10.75**	**0.00**	**8.29**	**8.36**	**71.90**	**0.00**	**0.00**	**44.41**	**142.80**	**28.11**	**245.52**	**0.00**	**2.80**	**31.80**
IL-17 50 ng/ml	**174.48**	**137.00**	**15.60**	**214.50**	**44.43**	**135.31**	**819.33**	**0.00**	**608.06**	**1487.88**	**242.85**	**822.99**	**191.03**	**99.39**	**4201.55**
IL-17 500 ng/ml	**313.50**	**391.87**	**478.05**	**667.81**	**350.18**	**323.10**	**2360.88**	**69.47**	**1043.76**	**2440.46**	**571.56**	**1156.77**	**482.93**	**311.64**	**5950.15**
IFN-γ 100 U/ml	**27.09**	**0.00**	**0.00**	**0.00**	**8.66**	**10.63**	**519.96**	**0.00**	**0.00**	**0.00**	**11.31**	**0.00**	**172.12**	**0.00**	**0.00**
IL-17 5 ng/ml + IFN-γ 100 U/ml	**0.00**	**17.90**	**27.80**	**0.00**	**4.18**	**21.01**	**879.06**	**6.27**	**0.00**	**0.00**	**67.53**	**611.43**	**0.00**	**48.32**	**1324.35**
IL-17 50 ng/ml + IFN-γ 100 U/ml	**21.42**	**214.90**	**42.54**	**280.26**	**85.99**	**371.51**	**3030.33**	**65.31**	**386.19**	**1136.14**	**364.89**	**2011.38**	**573.89**	**602.82**	**5884.40**
IL-17 500 ng/ml + IFN-γ 100 U/ml	**550.20**	**447.20**	**506.23**	**674.09**	**610.22**	**643.26**	**7808.40**	**107.70**	**1294.66**	**2655.34**	**766.80**	**2570.10**	**1377.87**	**1040.02**	**8608.80**

Table 5: Statistical analysis

A) Paired Samples Test CD40

Test	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
IFN-γ5000 vs IFN-γ 5000 U/ml + IL-17 500 ng/ml	-.4883	3.4285	.9897	-2.6667	1.6900	-.493	11	.631
IFN-γ500 vs IFN-γ 500 U/ml + IL-17 500 ng/ml	-1.9200	3.6203	1.0451	-4.2202	.3802	-1.837	11	.093
IFN-γ 50 vs IFN-γ50 U/ml + IL-17 500 ng/ml	-.4375	3.9550	1.1417	-2.9504	2.0754	-.383	11	.709

B) Paired Samples Test HLA-DR

Test	Paired Differences					t	df	Sig. (2-tailed)
	Mean	Std. Deviation	Std. Error Mean	95% Confidence Interval of the Difference				
				Lower	Upper			
IFN-γ 5000 vs IFN-γ 5000 U/ml + IL-17 500 ng/ml	8.1825	9.7368	2.8108	1.9960	14.3690	1.811	11	.055
IFN-γ500 vs IFN-γ 500 U/ml + IL-17 500 ng/ml	8.2400	7.9039	2.2816	3.2181	13.2619	1.795	11	.049
IFN-γ 50 vs IFN-γ 50 U/ml + IL-17 500 ng/ml	2.5642	4.6705	1.3482	-.4033	5.5316	1.902	11	.084

C) Test Statistics IL-8[b]

	IL-17 5 ng/ml + IFN-γ 100 U/ml vs IL-17 5 ng/ml	IL-17 50 ng/ml + IFN-γ 100 U/ml vs IL-17 50 ng/ml	IL-17 500 ng/ml + IFN-γ 100 U/ml vs IL-17 500 ng/ml
Z	-.534[a]	-1.538[a]	-3.296[a]
Asymp. Sig. (2-tailed)	.594	.124	.001

a Based on negative ranks.

b Wilcoxon Signed Ranks Test

BIOGRAPHY

Mr. Paiboon Jitprasertwong was born 3rd of February 1977 in Surin province. He graduated with D.D.S. (Doctor of Dental Surgery) from the Faculty of Dentistry, Khon Kaen University in 2000, and became a staff member of the Faculty of Dentistry, Naresuan University, Phitsanulok. He studied in a Master degree programs in Periodontology at Graduate School, Chulalongkorn University in 2002.