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รายงานผลการวิจัย

บทบาทของอินเตอร์ลิวคิน 17 ในโรคปริทันต์อักเสบ

The role of IL-17 in periodontitis

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ชื่อโครงการวิจัย บทบาทของอินเตอร์ลิวคิน 17 ในโรคปริทันต์อักเสบ

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บทคัดย่อ

ความเป็นมาและวัตถุประสงค์ : อินเตอร์ลิวคิน-17 (IL-17) ซึ่งเป็นไซโตไคน์ของทีเซลล์ชนิดใหม่ที่กระตุ้นให้เกิดการตอบสนองทางการอักเสบได้ พบได้ทั้งในชิ้นเนื้อเหงือกและน้ำเหลืองเหงือกของผู้ป่วยที่เป็นโรคปริทันต์อักเสบ ในการวิจัยครั้งนี้ผู้วิจัยตรวจสอบผลของอินเตอร์ลิวคิน-17เพียงอย่างเดียว หรือเมื่อรวมกับอินเตอร์เฟอรอน-แกมมาเกี่ยวกับการปรับเปลี่ยนภูมิคุ้มกันของเซลล์ไฟโบรบลาสต์จากเหงือกของคนซึ่งจะนำมาซึ่งการทำให้เกิดโรคของอวัยวะปริทันต์

ระเบียบวิธีวิจัยและผลการทดลอง : อินเตอร์ลิวคิน-17 หรืออินเตอร์เฟอรอน-แกมมา (IFN-γ) ที่ความเข้มข้นต่างๆ หรือไซโตไคน์ทั้งสองชนิดร่วมกัน ใส่ลงในการเพาะเลี้ยงเซลล์ไฟโบรบลาสต์จากเหงือกของคน ผู้วิจัยทำการวัดการแสดงออกของไอแคม-1 (ICAM-1) เอชแอลเอ-ดีอาร์ (HLA-DR) และซีดี 40 (CD 40) ด้วยการวิเคราะห์โดยใช้โฟลไซโตเมทรี (flow cytometry) และวัดการผลิตอินเตอร์ลิวคิน-18 ด้วยวิธีอีไลซ่า (ELISA) ผลการวิจัยแสดงให้เห็นว่า อินเตอร์เฟียรอน-แกมมาสามารถส่งเสริมการแสดงออกของเอชแอลเอ-ดีอาร์ และไอแคม-1 บนเซลล์ไฟโบรบลาสต์ได้อย่างชัดเจน และมีผลต่อการแสดงออกของซีดี 40 เพียงเล็กน้อย ในทางตรงกันข้ามอินเตอร์ลิวคิน-17 ไม่มีผลต่อโมเลกุลทั้งสาม เมื่อร่วมกันอินเตอร์ลิวคิน-17 ไม่มีผลส่งเสริมอินเตอร์เฟียรอน-แกมมาในการกระตุ้นการแสดงออกของเอชแอลเอ-ดีอาร์ และซีดี 40 แต่มีผลส่งเสริมแสดงออกของไอแคม-1 อย่างมีนัยสำคัญ ($p<0.01$) ในทางตรงกันข้ามกับอินเตอร์เฟียรอน-แกมมา อินเตอร์ลิวคิน-17 สามารถกระตุ้นให้เซลล์ไฟโบรบลาสต์ผลิตอินเตอร์ลิวคิน-8 ได้ และเมื่อกระตุ้นด้วยไซโตไคน์ทั้งสองชนิดร่วมกัน อินเตอร์เฟียรอน-แกมมามีผลส่งเสริมอินเตอร์ลิวคิน-17ในการกระตุ้น การผลิตอินเตอร์ลิวคิน-8 จากเซลล์ไฟโบรบลาสต์อย่างมีนัยสำคัญ ($p<0.05$)

สรุป : การวิจัยนี้แสดงให้เห็นว่าอินเตอร์ลิวคิน-17 และอินเตอร์เฟียรอน-แกมมามีผลที่แตกต่างบนเซลล์ไฟโบรบลาสต์จากเหงือกของคนในเรื่องของการแสดงออกของโค-สติมมูลาโทรี โมเลกุล และโมเลกุลยึดเกาะเช่นเดียวกับสารสื่ออักเสบ โมเลกุลเหล่านี้ทั้งหมดจำเป็นในการควบคุมการตอบสนองทางภูมิคุ้มกัน จึงเป็นการบ่งชี้ถึงบทบาททางการควบคุมระบบภูมิคุ้มกันเฉพาะที่ของอินเตอร์ลิวคิน-17 และอินเตอร์เฟียรอน-แกมมาในเนื้อเยื่อปริทันต์ที่อักเสบ

ศูนย์วิทยทรัพยากร จุฬาลงกรณ์มหาวิทยาลัย

Project Title	The role of IL-17 in periodontitis
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Abstract

Background and objectives: IL-17 is a novel T cell-derived cytokine that promotes inflammatory responses. It is presence in inflamed gingival tissues and gingival crevicular fluid of periodontitis patients. In this study we investigated the effects of IL-17 alone or in combination with IFN-γ on the immune modulation of human gingival fibroblasts (HGFs) which would contribute to pathogenesis of periodontium.

Methods and results: Various concentrations of IL-17, IFN-γ, or the combination of these two cytokines were added to HGF cultures. The expression of ICAM-1, HLA-DR, and CD40 was assessed by flow cytometry and IL-8 production was determined by ELISA. Our results demonstrated that IFN-γ markedly induced expression of HLA-DR and ICAM-1 and slightly induced CD40 expression on HGFs. In contrast, IL-17 had no effect on these molecules. When combined, IL-17 did not enhance IFN-γ-induced HLA-DR and CD40 expression but significantly up-regulated ICAM-1 expression ($p<0.01$). Unlike IFN-γ, IL-17 induced IL-8 production from HGFs. When combined, IFN-γ synergistically enhanced IL-17-induced IL-8 production in all HGF lines ($p<0.05$).

Conclusions: These findings indicate that IL-17 and IFN-γ have different effects on HGFs regarding the expression of co-stimulatory molecule and adhesion molecule as well as immune mediator. All of these molecules are critical in controlling immune response, thus indicating the immunoregulatory role of locally produced IL-17 and IFN-γ in inflamed periodontal tissue.

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Report of the role of IL-17 in periodontitis

Introduction

Periodontitis is an immune mediated disease associated with chronic bacterial infection which results in progressive destruction of connective tissue and bone. The lesion is characterized by dense lymphoid infiltrates such as T and B cells, as well as their cell-derived cytokines.

IL-17, a CD4+ T cell-derived cytokine, has emerged as a crucial regulator of inflammatory responses. It does not seem to regulate T cell function but acts mostly on other cell types, for example fibroblasts, epithelial cells, endothelial cells and osteoblasts (Fossiez et al., 1996; Kotake et al., 1999; Laan et al., 1999; Yao et al., 1995). IL-17 treatment of these cells induces the expression of pro-inflammatory cytokines such as IL-6, IL-8, granulocyte colony-stimulating factor, prostaglandin E2 and matrix metalloproteinases (Chabaud et al., 2000; Fossiez et al., 1996; Laan et al., 1999; Rifas and Arackal, 2003). IL-17-producing CD4+ T cells have recently been proposed as a unique Th cell subset, called Th17, which seems to be distinct from traditional Th1 and Th2 in their cytokine-expression profile and immune function (Dong, 2006). Over-expression of IL-17 has been found to be associated with many inflammatory diseases in humans, such as rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, psoriasis, and allograft rejection (Albanesi et al., 2000; Antonysamy et al., 1999a; Antonysamy et al., 1999b; Chabaud et al., 1999; Homey et al., 2000; Hsieh et al., 2001; Kotake et al., 1999; Kurasawa et al., 2000; Loong et al., 2000; Teunissen et al., 1998; Wong et al., 2000). Very recent observations in periodontitis demonstrated the presence of IL-17 in the inflamed gingival tissue (Oda et al., 2003; Takahashi et al., 2005), solubilized gingival biopsies (Johnson et al., 2004), and gingival crevicular fluid taken from periodontally affected sites (Vernal et al., 2005). In addition, gingival T cell clones derived from periodontitis lesion expressed IL-17 mRNA (Ito et al., 2005). However, the relevant biological function of this cytokine, that affects cells of periodontium and may contribute to periodontal pathogenesis, is still lacking.

Accumulating data indicate that gingival fibroblasts, the most abundant cell in periodontal connective tissue, play active role not only in tissue homeostasis but also in immunoregulation of the periodontium. Our laboratory recently demonstrated that human gingival fibroblasts (HGFs) express a variety of Toll-like receptors (TLRs 2, 3, 4, 5, 6, and 9), key receptors of the innate immune system, and were able to recognize and respond to many pathogen associated molecular patterns (PAMPs) (Mahanonda et al., submit for publication). Upon stimulation with PAMPs as well as with cytokines, HGFs release a variety of pro-inflammatory mediators for example IFN-γ, PGE_2, IL-1, IL-6 and IL-8

(Daghigh et al., 2002; Sakuta et al., 1998; Takashiba et al., 1992; Wang et al., 2003), and up-regulate expression of HLA-DR and ICAM-1 (Shimabukuro et al., 1996). These fibroblast-derived mediators and surface antigens are known as essential molecules in immunoregulation of the cells in innate and adaptive inflammatory response.

Immunohistochemistry findings of periodontitis lesion have demonstrated that locally infiltrated T cells and gingival fibroblasts are in close proximity in (Murakami et al., 1999; Okada et al., 1983). It is, therefore, reasonable to speculate that these immune 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. In this study, we investigated the effects of IL-17, a regulatory T cell cytokine, alone or in combination with IFN-γ, a well known Th1 cytokine, on the immune modulation of HGFs. Upon cytokine (single or combined) stimulation, we analyzed HGFs in terms of expression of co-stimulatory molecules (HLA-DR and CD40), adhesion molecule (ICAM-1) and chemokine (IL-8).

Survey of Related Literature

1. Immunopathogenesis

1.1. 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, 2000) 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, 2000). The key periodontal pathogens in chronic periodontitis are *P. gingivalis, and T. forsythia* whereas those in localized aggressive periodontitis are *A. actinomycetemcomitans* (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 (Slots and Ting, 2000; Socransky and Haffajee, 1991). 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 et al., 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; Yamazaki 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 (Aarvak et al., 1999; Yao et al., 1995). 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.

1.2. 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 (Dongari-Bagtzoglou and Ebersole, 1996a; Dongari-Bagtzoglou and Ebersole, 1996b; Imatani et al., 2001; Ohmori et al., 1987; Takada et al., 1991; Tamura et al., 1992). 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 (Sakuta et al., 1998; Takashiba et al., 1992).

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 and Ebersole, 1998; Dongari-Bagtzoglou et al., 1997).

2. HLA-DR and CD40 on human gingival fibroblasts

2.1. 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).

2.2. 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-Bagtzoglou 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.

3. 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., 2000). They are usually produced transiently, are extremely potent, generally acting at picomolar concentrations and interact with specific receptors at the cell membrane, setting of a cascade that leads to induction, enhancement or inhibition of a number of cytokine-regulated genes in the nucleus (Balkwill and Burke, 1989).

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

Cytokines are known to be major participants in acute and chronic inflammation regardless of its location, and there is strong evidence for participation of these mediators in periodontitis. They are produced by activated resident gingival cells and infiltrating immune cells. In periodontitis lesion, high levels of inflammatory mediators/cytokines such as IL-1β, TNF-α, IFN-γ, IL-6, IL-10, IL-13, IL-4, IL-8, MMP and PGE_2 have been detected (Kornman et al., 2000; Okada and Murakami, 1998).

3.1. 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 (Baggiolini et al., 1994; Bickel, 1993). IL-8 attracts neutrophils by inducing neutrophil extravasation at the site of inflammation. It then activates the cells to undergo the metabolic burst and to degranulate on arrival at the site of the challenge (Kornman et al., 2000). This cytokine has been thought to play a significant role in various forms of periodontitis (Bickel, 1993; Fitzgerald and Kreutzer, 1995; Gainet et al., 1998; Takashiba et al., 1992).

IL-8 concentration was shown to be increased in gingival crevicular fluid from patients with periodontitis (Tsai et al., 1995), and remained elevated in patients who did not respond to treatment (Chung et al., 1997). High levels of IL-8 in plasma were detected in patients with various forms of periodontitis and the presence of mRNA for IL-

8 was observed in gingival neutrophils (Gainet et al., 1998). In inflamed gingival tissues, it was observed that IL-8 was produced in epithelial cells, macrophages and fibroblasts (Tonetti et al., 1993). As mentioned earlier, HGF IL-8 could be induced by stimulation with bacterial LPS or other cytokines (Sakuta et al., 1998; Steffen et al., 2000; Takashiba et al., 1992; Takigawa et al., 1994). The decisive role of IL-8 in periodontal disease is not clear. It is possible that at an early phase of periodontal inflammation, IL-8 may be required to attract neutrophils and leukocytes to eliminate the infection. On the other hand, at the chronic stage of periodontal inflammation, excessive IL-8 may be unwanted but inevitably present due to continual activation by etiologic bacterial plaque and the local cellular/cytokine network in the lesion. Therefore, additional work is required to determine the significance of this cytokine in periodontal disease.

3.2. 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.

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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.

จุฬาลงกรณ์มหาวิทยาลัย

3.3. 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 (Aarvak et al., 1999; Yao et al., 1995). IL-17 exhibits pleiotrophic biologic activities on various types of cells, such as fibroblasts, endothelial cells, and epithelial cells, mediating a wide range of responses, mostly proinflammatory and hematopoietic (Chabaud et al., 2001; Fossiez et al., 1996; Rouvier et al., 1993; Yao et al., 1995). Many IL-17 studies were done in area of rheumatoid arthritis (Yao et al., 1995) suggesting that IL-17 has the potential to be an important cytokine in the immune system, and associated with disease states. Interestingly, IL-17 was also detected in inflamed gingival tissues, both gingivitis and periodontitis (Oda et al., 2003), particularly, in 4 to 5 mm pockets (Johnson et al., 2004).

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

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

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cytokines IL-17 and IFN-γ on HGF with regard to co-stimulatory molecule expression (CD40 and HLA-DR) and cytokine production (IL-8)

Procedures

1. Reagents and monoclonal antibodies (mAbs)

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 systems Inc. (Minneapolis, MA, USA). MAbs against ICAM1 (PE) and HLA-DR (PerCP) were obtained from BD PharMingen (San Diego, CA, USA). MAbs against CD40 and mouse isotype control monoclonal antibodies (FITC, PE, and PerCP) were obtained from Becton Dickinson (San Jose, CA, USA).

2. HGFs

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

3. Stimulation of HGFs 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.

4. Flow cytometry analysis

HGFs were gently trypsinized and washed twice with phosphate buffered saline (PBS). The cells were then stained with mAbs anti-ICAM1 (PE), anti-CD40 (FITC) and HLA-DR (PerCP). Mouse isotype mAbs conjugated with FITC, PE, and PerCP were used as control. HGFs 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).

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.

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.

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

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).

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Results

IL-17 specifically and dose dependently augmented IFN-γ-induced ICAM-1 expression on HGF at the protein level whereas HLA-DR, CD40 levels were not modulated by IL-17. On the other hand, IL-17 alone did not affect ICAM-1. In addition, IL-17, both directly and in synergism with IFN-γ

stimulated the release of IL-8 by HGFs. The synthesis of IFN-γ-induced IDO was not changed by adding IL-17. Taken together, the results suggest that IL-17 is an important player of T cell-mediated periodontal immune response, with synergistic effects on IFN-γ-stimulated HGF activation.

1. The kinetic and dose response experiments of IL-17 and IFN-γ on HLA-DR, CD40 and ICAM-1 expression on HGFs.

A kinetic experiment of IL-17- and IFN-γ-induced HLA-DR, CD40 and ICAM-1 expression on HGFs was shown in Figure 1. HGFs were cultured with IFN-γ (5000 U/ml) and IL-17 (500 ng/ml) for 1, 2, 3, 4, and 5 days and the expression of surface molecules was analyzed by flow cytometry. As compared with un-stimulated HGFs, IFN-γ markedly up-regulated HLA-DR and ICAM-1 expression on HGFs. The IFN-γ–induced ICAM-1 and HLA-DR expression was early detected at day 1 (MFI = 109.18) and day 2 (MFI =17.83) after incubation, respectively. The expression of both molecules increased over time and peaked at day5 (MFI = 78.98 for HLA-DR, Figure 1A and MFI = 157.17 for ICAM-1, Figure 1C). In addition, IFN-γ also up-regulated CD40 expression on HGFs, but the induced effect was lower than HLA-DR and ICAM-1 expression (Figure 1B). IL-17 had no effect on the expression of all surface molecules on HGFs (Figure 1A, 1B, 1C).

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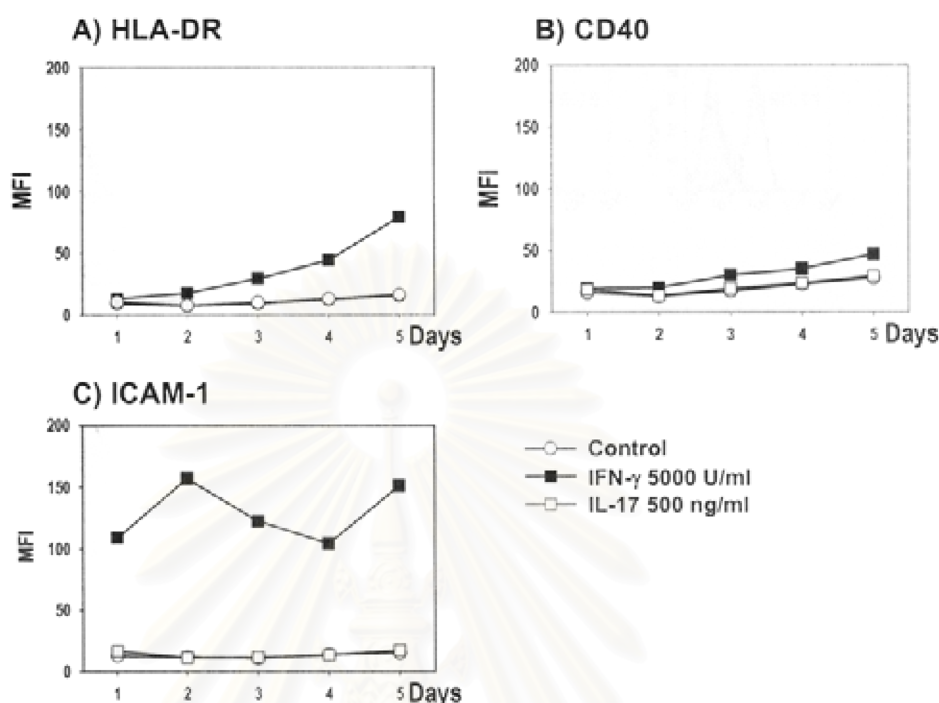

Figure 1. Kinetics of HLA-DR (A), CD40 (B), and ICAM-1 (C) expression on HGFs. HGFs (1×10^5 cells/ml) were stimulated with and without IFN-γ (5000 U/ml), and IL-17 (500 ng/ml) for various periods of time and the expression of HLA-DR, CD40, and ICAM-1 was determined by flow cytometry.

A dose response experiment using various concentrations of IFN-γ (50, 500, and 5000 ng/ml) was shown in Figure 2. Representative histograms of IFN-γ-induced HLA-DR, CD40 and ICAM-1 expression on HGFs (n=13) demonstrated a clear enhanced effect of HLA-DR expression at 500 U/ml of IFN-γ and 500 U/ml of ICAM-1 (Figure 2A, 2C), whereas, a higher concentration of IFN-γ (5000 U/ml) was required to induce CD40 expression (Figure 2B).

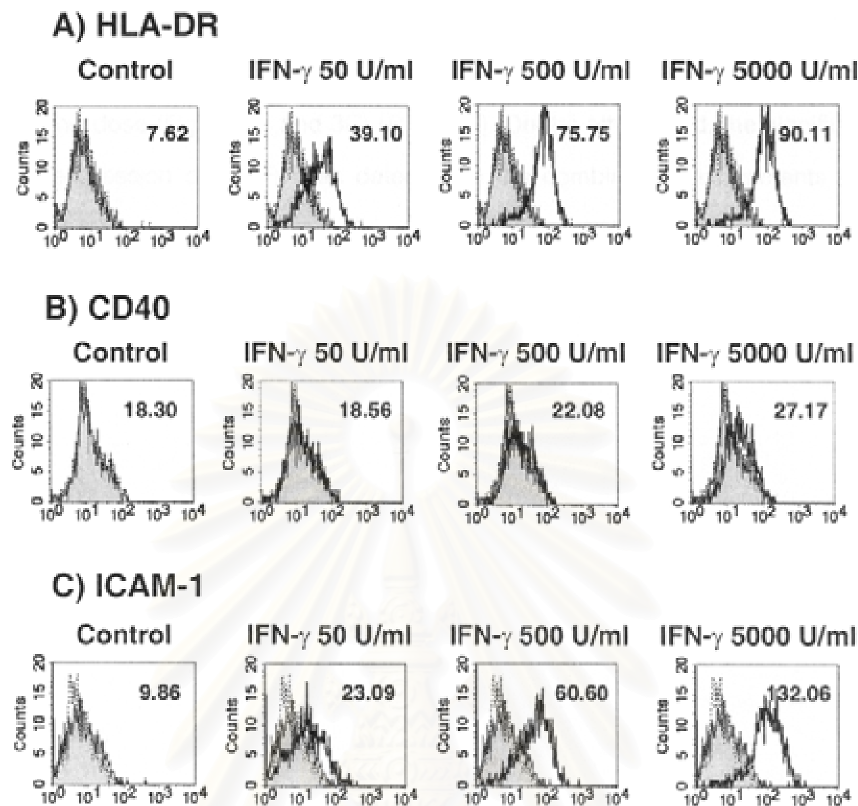

Figure 2. Representative histograms showing dose response analysis of HLA-DR (A), CD40(B), and ICAM-1 (C)expression on HGFs (n=13). HGF cultures were stimulated with various dose of IFN-γ (50, 500, 5000 U/ml) for 5 days. The expression of HLA-DR, CD40, and ICAM-1 was determined by flow cytometry. Dotted 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 mean fluorescence intensity (MFI).

2. The combination effects of IL-17 and IFN-γ on HLA-DR, CD40, and ICAM-1 expression on HGFs

We next evaluated the combination effects of IL-17 and IFN-γ on HLA-DR, CD40, and ICAM-1 expression on HGF cell lines (n = 11). HGFs were cultured with IFN-γ (50, 500, and 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 cell surface antigens were analyzed by flow cytometry. Our results revealed that

the levels expression of HLA-DR and CD40 on HGFs after stimulation with the combination of the two cytokines (Figure 3D) were not different from those stimulation with the single cytokine at each corresponding dose (Figure 3B and 3C) ($P > 0.05$). On the other hand, the significant enhancement of ICAM-1 expression on HGFs was detected in the combination experiments (n=11, $P < 0.01$) (Figure 3D).

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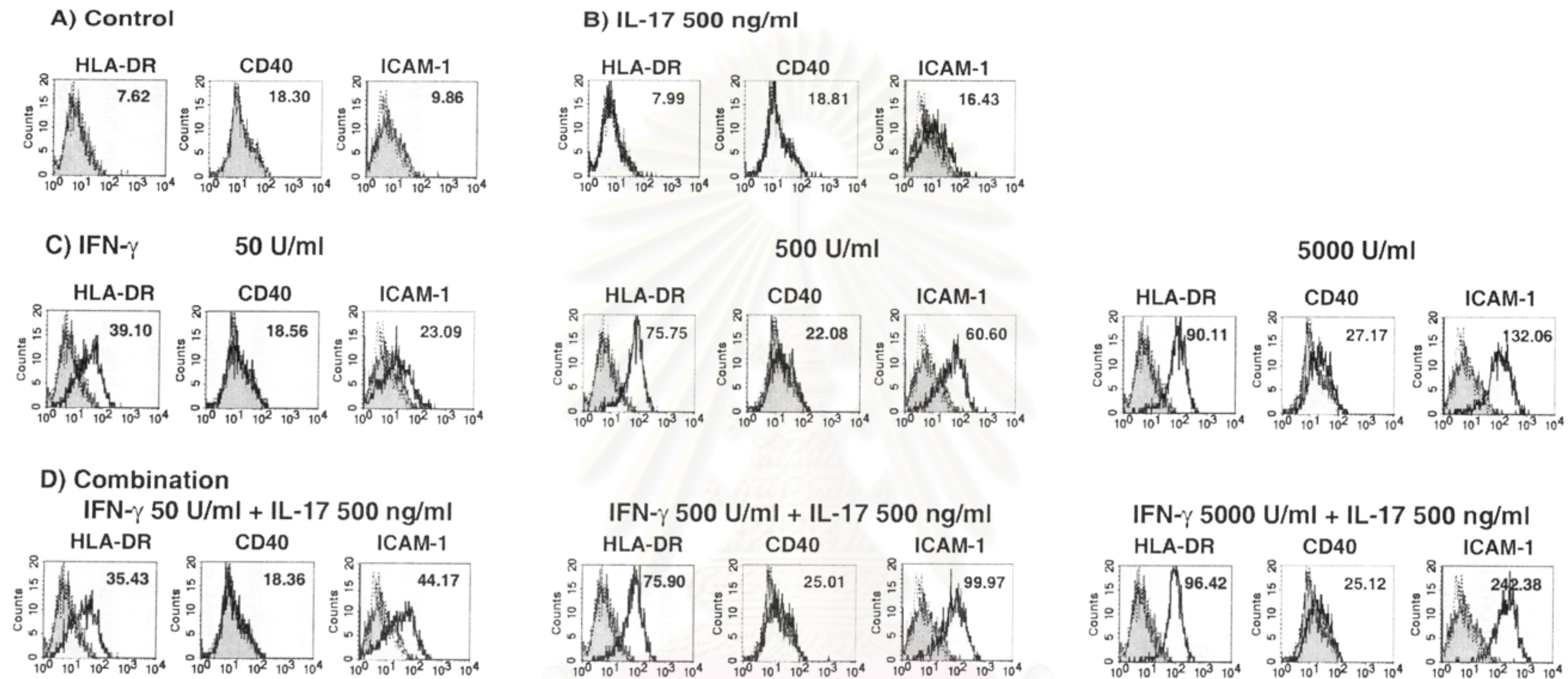


Figure 3. Representative histograms showing the combination effects of IFN-γ and IL-17 on of HLA-DR, CD40, and ICAM-1 expression on HGFs (n=11). HGFs were cultured with and without (A) IFN-γ (50, 500, 5000 U/ml) (C), IL-17 (500 ng/ml) (B), or the combination of 2 cytokines (50 U/ml IFN-γ + 500 ng/ml IL-17; 500 U/ml IFN-γ + 500 ng/ml IL-17; 5000 U/ml IFN-γ + 500 ng/ml IL-17) (D) for 5 days. The expression of HLA-DR, CD40, and ICAM-1 was determined by flow cytometry. 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 mean fluorescence intensity (MFI).

3. Dose response experiments of IL-17 and IFN-γ on IL-8 production

Various doses of IFN-γ (50, 500, and 5000 U/ml) and IL-17 (5, 50, and 500 ng/ml) were used to stimulate HGF cultures (n=4). After 2 days of incubation, culture supernatants were collected for analysis of IL-8 production by ELISA. The results demonstrated that IL-17 induced IL-8 production from HGFs in a dose dependent manner (Figure 4A), whereas IFN-γ had no effect (Figure 4B).

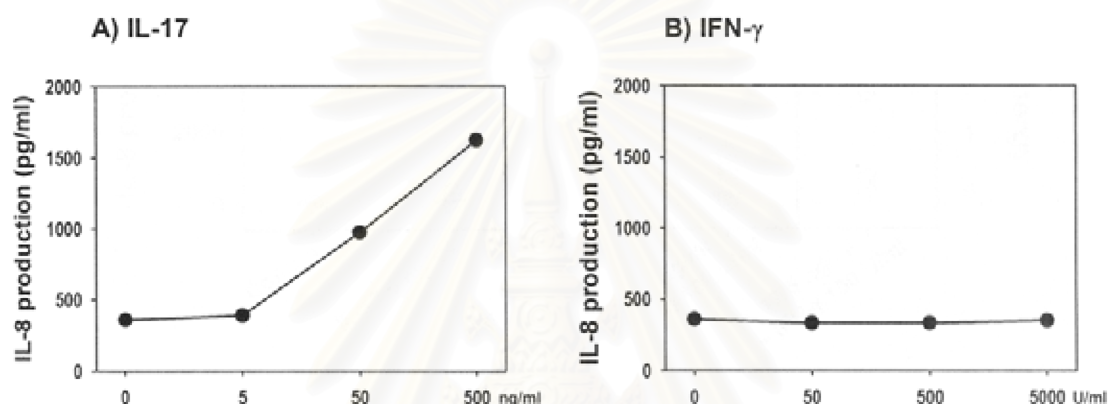
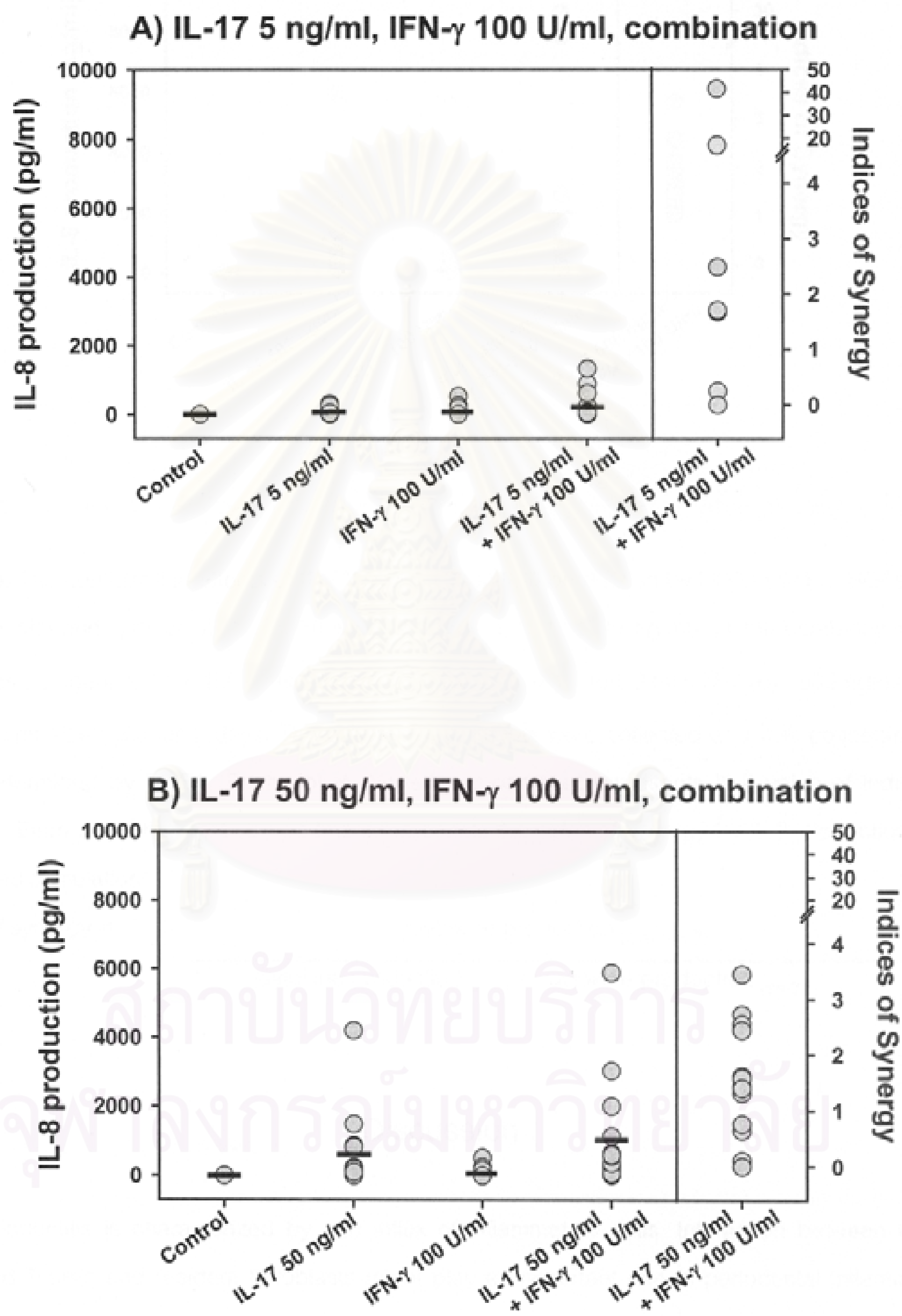


Figure 4. A representative of dose response of IL-8 production in HGF cultures (n=4). HGF ($1x10^5$ cells /ml) were stimulated with various concentrations of IL-17 (5, 50, and 500 ng/ml) (A) or IFN-γ (50, 500, and 5000 U/ml) (B) for 2 days. The culture supernatants were collected and IL-8 concentrations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

4. The combination effect of IL-17 and IFN-γ on IL-8 production by HGFs

We also evaluated the combination effect of IL-17 and IFN-γ on IL-8 production. HGF cultures were stimulated with 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) and IL-8 production was determined after 2 day incubation by ELISA. Interestingly, IFN-γ synergistically enhanced IL-17-induced IL-8 production in all HGF cell lines (n=15) when combined with a high dose of IL-17 (500ng/ml) (Figure 5C). However, when IFN-γ combined with lower doses of IL-17 at 5 and 50 ng/ml, synergy of IL-8 production was observed in 6 and 11 HGF cell lines respectively (Figures 5A, 5B), while the others showed no effect (Figures 5A, 5B).



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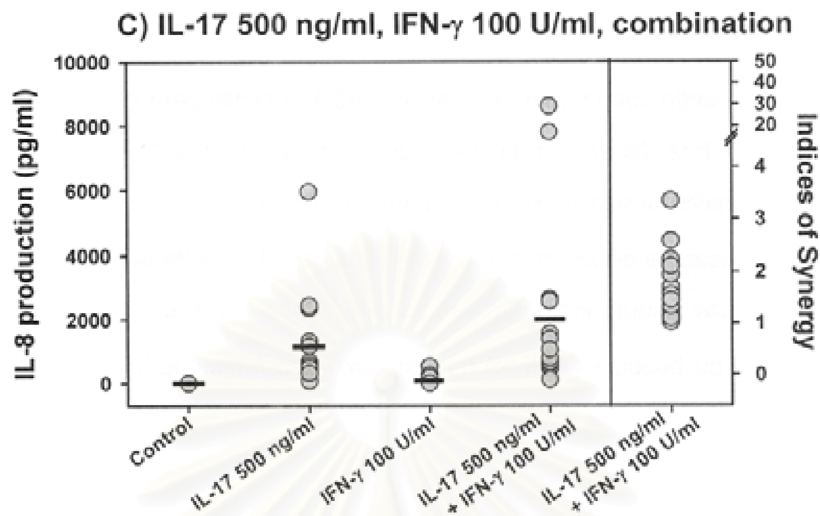

Figure 5. The combination effects of IFN-γ and IL-17 on IL-8 production by HGFs (n=11). HGFs were cultured with and without IL-17 (5, 50,and 5000 ng/ml), IL-17 (500 ng/ml), or the combination of 2 cytokines , 5 ng/ml IL-17 + 100 U/ml IFN-γ; 50 ng/ml IL-17 (A)+ 100 U/ml IFN-γ (B); 500 ng/ml IL-17 + 100 U/ml IFN-γ (C) for 2 days. The culture supernatants were collected and IL-8 concentrations were determined by ELISA. Each dot in the left compartment represents IL-8 value of individual subject. Each dot in the right compartment represents the index of synergy for IL-8 production after combined stimulation.

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

Discussion

Periodontitis is characterized by and influx of inflammatory cells. Interaction between locally infiltrated T cells and resident fibroblasts might play an important role in periodontal inflammatory response. Previous observations demonstrates that IL-17, a product of activated T cells, is locally expressed in periodontal inflamed lesion (Ito et al., 2005; Oda et al., 2003; Takahashi et al., 2005). To obtain more detailed insight in the consequences of T cell/gingival fibroblast interaction, we analyzed the expression of co-stimulatory molecules, adhesion molecule and chemokine on cultured HGFs in

response to activation with IL-17 or in combined with other T cell-derived cytokine , IFN-γ, *in vitro*. Our results show that IL-17 alone, IFN-γ alone, or combined T-cell derived cytokines, have differential immunoregulatory effects on HGFs.

Unlike IL-17, IFN-γ greatly induced up-regulation of HLA-DR and ICAM-1 expression on HGFs 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 HGFs 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 HGFs (Wassenaar et al., 1997). It is possible that endogenous mediator(s) induced from IFN-γ-treated HGFs may contribute to the up-regulation of HLA-DR expression. Inconsistent with other observations, our data indicated that IL-17 did not enhance IFN-γ- induced HLA-DR and CD40 expression on HGFs (Albanesi et al., 1999; Teunissen et al., 1998). Unlike dendritic cells, fibroblasts are recognized as non-professional antigen-presenting cells and cannot prime antigen-specific naïve T cells (Murakami and Okada, 1997). Previous reports indicated that the majority of CD4+ Tcells infiltrating in periodontitis lesions were antigen-specific memory T cells (CD45RO+ CD4+ T cells) (Gemmell et al., 1992; Yamazaki et al., 1993). Our findings that HGFs can be induced to markedly up-regulate HLA-DR and minimal CD40 expression by IFN-γ may imply that the activated HGFS might possibly be able to stimulated these antigen-specific memory T cells which requires low threshold of co-stimulatory molecule expression.

Migration and retention of inflammatory cells in periodontally diseased tissue may be mediated by a family of cell surface receptors known as the cell adhesion molecules, e.g. ICAM-1. IFN-γ enhances ICAM-1 expression on HGFs whereas IL-17 had no effect. When simultaneous activation of HGFs with IL-17 and IFN-γ synergistically enhanced ICAM-1 expression (at least 2 fold higher than sum of single stimulations) as demonstrated by statistical analysis ($p<0.01$). On the other hand, the combined cytokine stimulation did not have additive effect on HLA-DR and CD40 expression. Similar synergy on ICAM-1 expression due to combined cytokine stimulation could also be observed in human skin keratinocyte (breast skin and foreskin) cultures (Teunissen et al., 1998). Thus, it seems to be likely that the strong induction of ICAM-1 expression by these two cytokines may play crucial role in recruitment and retention of infiltrating T cells which is a hallmark of periodontitis lesion.

IL-8 represents a key chemokine involving PMN recruitment to the site of inflammation (Bickel, 1993). 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, 2001). Also such association has been reported in periodontitis (Chung et al., 1997;

Tsai et al., 1995). In this study, we evaluated the effect of IL-17 and IFN-γ on IL-8 production by HGFs. We showed that HGFs 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 HGFs 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 (Albanesi et al., 1999; Andoh et al., 2001; Teunissen et al., 1998). Interestingly, the synergy of IL-8 production could be detected in all HGFS 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 HGFs response from different healthy donors after stimulation of HGFs 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 subpopulations and other as yet unidentified mechanisms (Dongari-Bagtzoglou and Ebersole, 1998; Kornman et al., 2000). The issue of heterogeneous response in IL-8 production from HGFS is very interesting and may contribute to disease susceptibility and severity. Further studies are required to precisely understand the mechanisms

Conclusion

In summary, our results indicate that IL-17 has effect on IL-8 production from HGFs whereas IFN-γ has effect on HLA-DR, CD40 and ICAM-1 expression. The data imply that co-stimulatory molecule expression, adhesion molecule expression, and chemockine production of HGFs may be controlled by different mechanisms. Expression of co-stimulatory molecules, HLA-DR and CD40, adhesion molecule, ICAM-1, as well as IL-8 production are important in immune response. The combination of IL-17 and IFN-γ results in synergisms of expression of ICAM-1 and IL-8. We, therefore, suggest the critical role in immunoregulation of the locally produced T-cell cytokines, IL-17 and IFN-γ, that may exacerbate periodontal inflammatory response via gingival fibroblast activation.

Suggestion for Further Work

Our study highlights that IL-17, a T cell-derived cytokine, may play important role in the immunopathogenesis of periodontitis, the advanced from of disease. IL-17 together with IFN-γ, this combination synergized chemokine IL-8 and adhesion molecule expression, thus promothing inflammation. One of the main functions of IL-17 involves connective tissue and bone destruction as

can be seen in rheumatoid arthritis, a similar T cell-mediated disease. Further investigation into the effect of IL-17 on osteoblast cell differentiation would be of interest, and thus providing a better understanding of the mechanisms underlying periodontal tissue destruction which may lead to tooth loss. Our laboratory is now generating human osteoblasts derived from alveolar bone and if possible the preliminary study of such investigation may take place.

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Report of peripheral blood mononuclear cell (PBMC) study

Table 1. shows IL-17 production in PBMC cultures (n=10) after stimulation with *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* and *Actinomyces viscosus*. Inconsistent data of IL-17 production could be observed. There were 5 out of 10 PBMC cultures that responded to *P.gingivalis* and *A. viscosus* and IL-17 protein could be detected. Whereas none of *A. actinomycetemcomitans*-stimulated PBMC cultures produced IL-17. In our proposal, the method of this protein detection was carried out using ELISA. Therefore, we included RT-PCR technique, a sensitive method to detect IL-17 mRNA expression in *P. gingivalis*-stimulated PBMC. Again, mRNA expression of IL-17 results confirmed the inconsistent data.

The problems that we encountered may be because our study was originally designed based on the observation that IL-17 is produced from non-antigen specific T cells (via IL-23 from activated monocytes) (Aggarwal et al., 2003). However, accumulating data from recent studies suggest that IL-17 is produced from antigen-specific T cells (Th17) (Harrington et al., 2005; Park et al., 2005). The Th17 data support our observation that primary stimulation of PBMC with *Porphyromonas gingivalis, Actinobacillus actinomycetemcomitans* and *Actinomyces viscosus* could not induce IL-17 production. Due to these facts, we could not further investigate the cell source of IL-17 using PBMC model.

Subject	Control	Pg	Aa	Av
1	-	-	-	-
2	-	-	-	75.27
3	-	-	-	26.91
4	-	28.32	-	47.65
5	28.01	-	-	-
6	-	-	-	165.7
7	-	40.16	-	-
8	-	26.52	-	-
9	-	29.61	-	88.32
10	-	97.74	-	-

Table 1. IL-17 production by bacterial stimulated PBMCs (n=10). PBMC were cultured with 10 μg/ml of *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, or *Actinomyces viscosus* for 5 days. The supernatants were collected for analysis of IL-17 by ELISA.

Pg = *Porphyromonas gingivalis* , Av = *Actinomyces viscosus* , Aa = *Actinobacillus actnimycetemcomitans*

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