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

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

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Disclosures

The authors have no financial conflict of interest.

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RESEARCH REPORTS
Biological

Effects of IL-17 on Human Gingival Fibroblasts

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ABSTRACT

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

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

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INTRODUCTION

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

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

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

MATERIALS & METHODS

Reagents and Monoclonal Antibodies (mAbs)

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

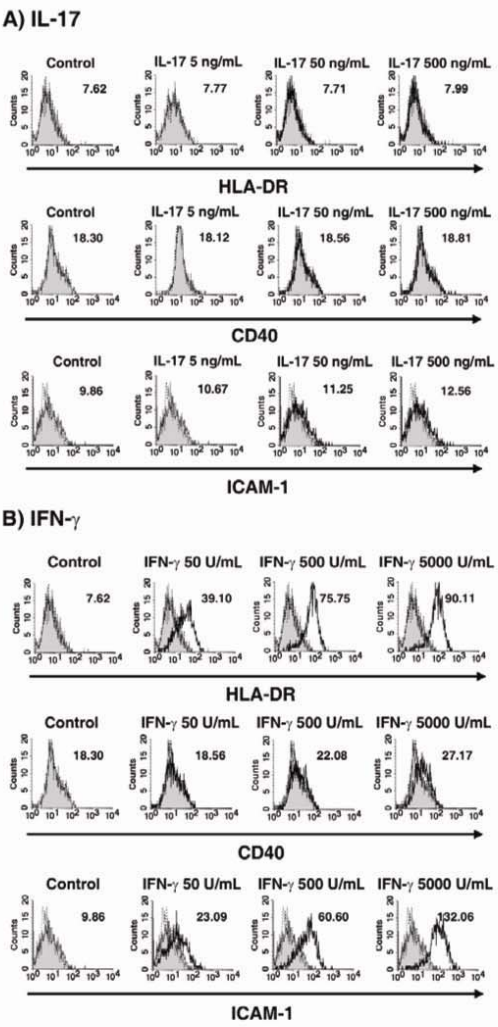

Figure 1. Flow cytometric analysis of HLA-DR, CD40, and ICAM-1 expression by HGFs after single stimulation with IL-17 or IFN-γ. HGF cultures were stimulated with indicated concentrations of IL-17 **(A)** and IFN-γ **(B)** for 5 days. Culture medium was used as a control. The surface molecule expression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

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

Human Gingival Fibroblasts

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

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

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

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

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

Determination of IL-8

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

Detection of IDO Expression and Activity

mRNA Expression of IDO

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

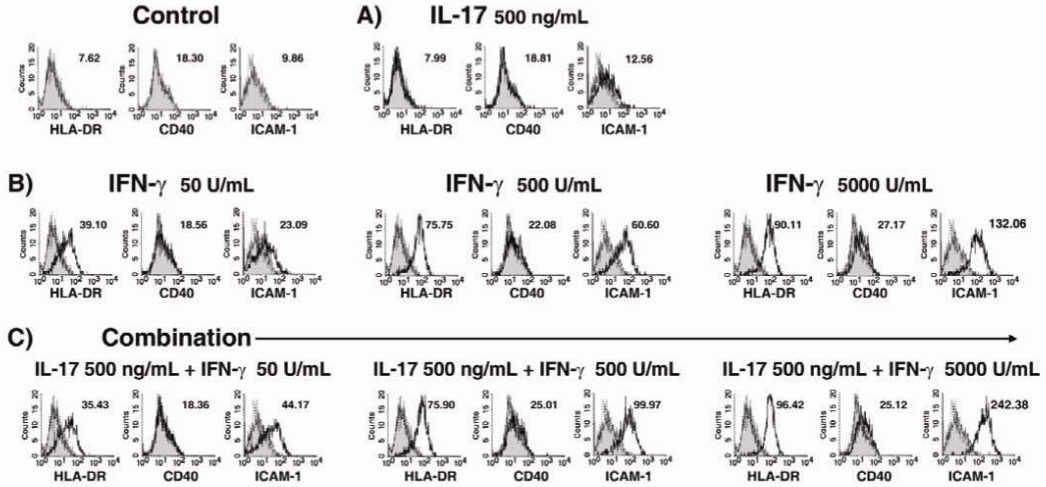

Figure 2. Flow cytometric analysis of HLA-DR, CD40, and ICAM-1 expression by HGFs after combined stimulation with IL-17 and IFN-γ. HGF cultures were stimulated with indicated concentrations of IL-17 **(A)**, IFN-γ **(B)**, or combined cytokines **(C)** for 5 days. Culture medium was used as a control.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

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

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

IDO Activity: Kynurenine Assay

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

Statistical Analysis

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

RESULTS

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

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

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

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

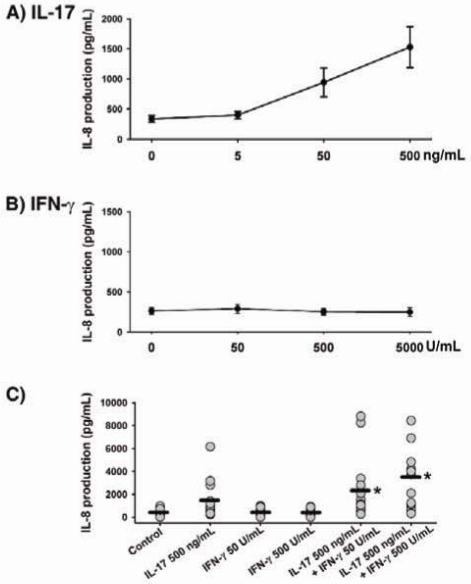

Figure 3. IL-8 production by HGFs after single stimulation with IL-17, IFN-γ, and combined cytokine stimulation. HGF cultures were stimulated with indicated concentrations of IL-17 **(A)**, IFN-γ **(B)**, and the combined cytokines **(C)** for 2 days. Culture medium was used as a control. The culture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

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

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

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

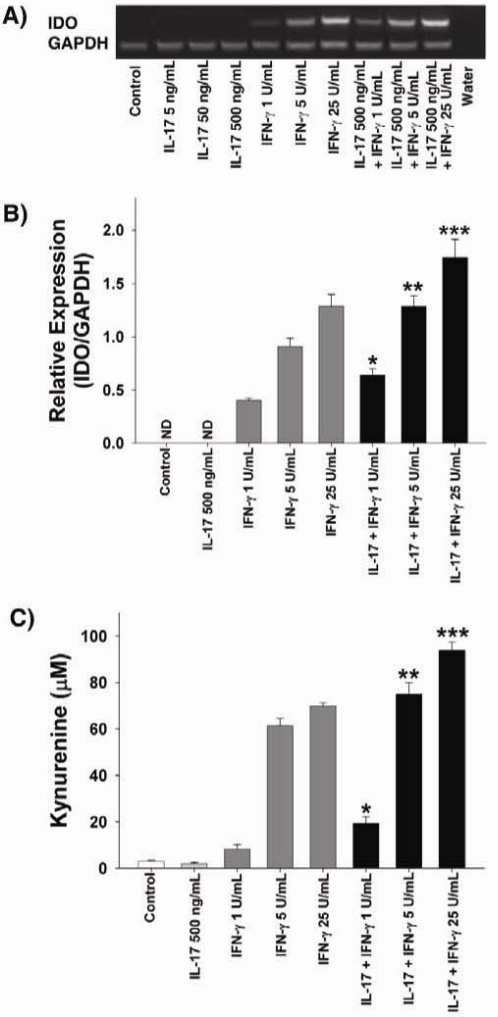

Figure 4. Expression of IDO in HGFs after stimulation with IL-17, IFN-γ, or combined cytokines. HGF cultures were stimulated with indicated concentrations of IL-17, IFN-γ, and combined cytokines. Culture medium was used as a control. Stimulated HGFs were harvested, and mRNA expression of IDO was analyzed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

DISCUSSION

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

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

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

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

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

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