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IL-8 and IDO Expression by Human Gingival Fibroblasts via TLRs[1]

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Human gingival fibroblasts (HGFs), a predominant cell type in tooth-supporting structure, are presently recognized for their active role in the innate immune response. They produce a variety of inflammatory cytokines in response to microbial components such as LPS from the key periodontal pathogen, *Porphyromonas gingivalis*. In this study, we demonstrated that HGFs expressed mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9, but not TLRs 7, 8, and 10. Stimulation of HGFs with highly purified TLR2 ligand (*P. gingivalis* LPS), TLR3 ligand (poly(I:C)), TLR4 ligand (*Escherichia coli* LPS), and TLR5 ligand (*Salmonella typhimurium* flagellin) led to expression of IL-8 and IDO. A potent TLR 9 ligand, CpG oligodeoxynucleotide 2006 had no effect, although HGFs showed a detectable TLR9 mRNA expression. No significant enhancement on IL-8 or IDO expression was observed when HGFs were stimulated with various combinations of TLR ligands. Surprisingly, the TLR9 ligand CpG oligodeoxynucleotide 2006 was able to specifically inhibit poly(I:C)-induced IL-8 and IDO expression. TNF-α enhanced TLR ligand-induced IL-8 production in HGFs, whereas IFN-γ enhanced TLR ligand-induced IDO expression. HGF production of IDO in response to *P. gingivalis* LPS, IFN-γ, or the two in combination inhibited T cell proliferation in MLRs. The observed T cell inhibition could be reversed by addition of either 1-methyl-DL-tryptophan or L-tryptophan. Our results suggest an important role of HGFs not only in orchestrating the innate immune response, but also in dampening potentially harmful hyperactive inflammation in periodontal tissue. ***The Journal of Immunology,*** **2007, 178: 1151–1157.**

Periodontitis is a chronic bacterial infection of tooth-supporting structures. It causes destruction of periodontal connective tissue and bone and, in severe cases, tooth loss. Key oral plaque bacteria including *Porphyromonas gingivalis*, *Actinobacillus actinomycetemcomitans*, and *Tanerella forsythia* are recognized as etiologic agents in periodontitis. The disease initiation and progression results from the host response to plaque bacteria. Immunohistochemistry studies reveal dense cellular infiltration, including numerous T and B cells in periodontitis lesions. In addition, high levels of inflammatory mediators such as IL-1β, TNF-α, PGE_2, IFN-γ, and IL-8 can be detected in inflamed gingival tissues and gingival crevicular fluid (1, 2).

Gingival fibroblasts, the major cell type in periodontal connective tissues, provide a tissue framework for tooth anchorage. Until recently, they were presumed to be immunologically inert. Currently, however, researchers recognize their active role in host defense. Upon stimulation with bacterial pathogens and their products, as well as with cytokines, gingival fibroblasts secrete various soluble mediators of inflammation such as IFN-γ, PGE_2, IL-1, IL-6, and IL-8 (3–6) and up-regulate expression of HLA-DR and ICAM-1 (7). These fibroblast-derived mediators and surface Ags are thought to play an important role in periodontal inflammatory response. Recently, human gingival fibroblasts (HGFs)[3] have been demonstrated to express TLRs 2, 4, and 9 (8–10). TLRs are recognized as key pathogen recognition receptors that sense microbial invasion. TLR ligation triggers inflammatory innate immune response, which is critical for pathogen elimination (11). It is likely that the release of inflammatory mediators from HGFs in response to microbial stimuli occurs via TLR triggering.

Recent findings also suggest that fibroblasts play an important role in negative feedback inhibition of inflammatory T cell response. IFN-γ-treated dermal fibroblasts express IDO (12, 13). IDO is known as an enzyme that catabolizes tryptophan, an essential amino acid. Immune inhibitory effects by IDO is due to tryptophan depletion and/or cytotoxic effects by the tryptophan metabolites, such as kynurenine, 3-hydroxykynurenine, and 3-hydroxyanthranilic acid (14, 15). Recent observation showed that a synthetic derivative of the tryptophan metabolite anthranilic acid reversed paralysis in mice with experimental autoimmune encephalomyelitis by suppression of myelin-reactive T cell responses (16). It is becoming clear that IDO can act as a critical immune suppressive molecule responsible for the attenuation of T cell hyperactivity.

Gingiva, the outer layer of the oral cavity, is consistently exposed to ~500 bacterial species of both commensal and pathogenic bacteria (17). How the oral tissues orchestrate their response to bacterial stimuli via TLR signaling, and thereby either maintain homeostasis or mediate expression of disease, is thus a very important research topic. We investigated the local innate immune

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[3] Abbreviations used in this paper: HGF, human gingival fibroblast; PAMP, pathogen-associated molecular patterns; ODN, oligodeoxynucleotide.

response and immune regulation by focusing on TLR expression of HGFs and on their function after triggering by their specific ligands.

Materials and Methods

Reagents and Abs

Medium for HGF cultures was DMEM that was purchased from Invitrogen Life Technologies. The medium was supplemented with penicillin G (50 U/ml), streptomycin (50 μg/ml), Fungizone (2.5 μg/ml), and 10% heat-inactivated FCS. Highly purified TLR ligands including LPS from *Porphyromonas gingivalis* (TLR2 ligand), poly(I:C) (TLR3 ligand), LPS from *Escherichia coli* K12 strain (TLR4 ligand), flagellin from *Salmonella typhimurium* (TLR5 ligand), loxoribine (guanosine analog, TLR7 ligand), and single-stranded poly(U) oligonucleotide complexed with LyoVec (TLR8 ligand) were obtained from InvivoGen. CpG ODN 2006 (TLR9 ligand) was obtained from Coley Pharmaceutical Group. IFN-γ and TNF-α were purchased from R&D Systems. Anti-human TLR3 mAb (TLR3.7) was obtained from eBioscience. Both 1-methyl-DL-tryptophan and L-tryptophan were purchased from Sigma-Aldrich.

Human gingival fibroblasts

Gingival tissue samples were collected from subjects who had clinically healthy periodontium and no history of periodontitis. The gingival biopsies were obtained at the time of the 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 (18). In brief, 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 passages four to eight were used throughout the study.

Preparation of P. gingivalis *sonicates*

P. gingivalis FDC-381 was cultured in trypticase soy broth (Sigma-Aldrich) at 37°C under an anaerobic chamber (Thermo Electron). The bacteria were harvested by centrifugation (Beckman Coulter) at 2000 $\times$ *g* for 15 min and washed twice in PBS. The purity was assessed by Gram staining and colony morphology on trypticase soy agar. The microorganisms were subjected to sonication with high-density ultrasonication (High Intensity Ultrasonic Processor, microprocessor controlled 600-W model; Sonics and Material) at 4°C for 20 min elapsed time, with pulse on 2.5 s and pulse off 2 s. The sonicated extracts were examined microscopically for complete breakage of cells. Protein concentration of the sonicates was determined by using a Bio-Rad protein assay. The bacterial stock was aliquoted and stored at −20°C until use.

mRNA expression of TLRs in HGFs

Total RNA from HGFs was isolated by using a RNeasy Mini Kit from Qiagen. One microgram of DNase I-treated total RNA was reverse transcribed using ImProm-II Reverse Transcription System for RT-PCR (Promega). TLRs 1–10 and GAPDH were amplified using specific primers purchased from Sigma-Aldrich as described as follows and the PCR conditions were described as in previous studies (19, 20): *TLR1* (5′-CGTAAA ACTGGAAGCTTTGCAAGA-3′/5′-CCTTGGGCCATTCCAAATAAGT CC-3′); *TLR2* (5′-GGCCAGCAAATTACCTGTGTG-3′/5′-CCAGGTAG GTCTTGGTGTTCA-3′); *TLR3* (5′-ATTGGGTCTGGGAACATTTCTC TTC-3′/5′-GTGAGATTTAAACATTCCTCTTCGC-3′); *TLR4* (5′-CTG CAATGGATCAAGGACCA-3′/5′-TCCCACTCCAGGTAAGTGTT-3′); *TLR5* (5′-CCTCATGACCATCCTCACAGTCAC-3′/5′-GGCTTCAAGG CACCAGCCATCTC-3′); *TLR6* (5′-ACTGACCTTCCTGGATGTGG-3′/ 5′-TGGCACACCATCCTGAGATA-3′); *TLR7* (5′-ACAAGATGCCTT CCAGTTGC-3′/5′-ACATCTGTGGCCAGGTAAGG-3′); *TLR8* (5′-CA GAATAGCAGGCGTAACACATCA-3′/5′-AATGTCACAGGTGCATTC AAAGGG-3′); *TLR9* (5′-GCGAGATGAGGATGCCCTGCCCTACG-3′/ 5′-TTCGGCCGTGGGTCCCTGGCAGAAG-3′); *TLR10* (5′-GGCCAG AAACTGTGGTCAAT-3′/5′-AACTTCCTGGCAGCTCTGAA-3′); and *GAPDH* (5′-TCATCTCTGCCCCCTCTGCTG-3′/5′-GCCTGCTCACCACC TTCTTG-3′).

Flow cytometric analysis of TLR3 expression

The specific localization of TLR3 of the HGFs was investigated by flow cytometry. For surface TLR3 staining, HGFs were incubated with PE-conjugated anti-human TLR3 mAb (clone TLR3.7, 1 μg) for 30 min at 4°C. For intracellular staining, cells were pretreated with fixation/permeabilization solution (BD Pharmingen) for 20 min at 4°C, washed once with PBS, and then incubated with the same mAb for 1 h at 4°C. Mouse isotype mAbs conjugated with PE was used as control. The stained cells were then analyzed on a FACSCalibur (BD Biosciences).

TLR ligation on HGFs after stimulation with TLR ligand(s) and/or cytokine

HGFs (1.5 $\times$ 10^5 cells/ml) in 96-well plates or 24-well plates (Corning Glass) were treated with either 1) various single TLR ligands: *P. gingivalis* LPS (50 μg/ml), poly(I:C) (100 μg/ml), *E. coli* LPS (10 μg/ml), *Salmonella typhimurium* flagellin (5 μg/ml); loxoribine (100 μM), ssPolyU (5 μg/ml), and CpG ODN 2006 (10 μg/ml); 2) TLR ligand combinations: *P. gingivalis* LPS (50 μg/ml) plus poly(I:C) (100 μg/ml), *P. gingivalis* LPS (50 μg/ml) plus *E. coli* LPS (10 μg/ml), *P. gingivalis* LPS (50 μg/ml) plus *S. typhimurium* flagellin (5 μg/ml), *P. gingivalis* LPS (50 μg/ml) plus CpG ODN 2006 (10 μg/ml), poly(I:C) (100 μg/ml) plus *E. coli* LPS (10 μg/ml), poly(I:C) (100 μg/ml) plus *S. typhimurium* flagellin (5 μg/ml), poly(I:C) (100 μg/ml) plus CpG ODN 2006 (10 μg/ml), *E. coli* LPS (10 μg/ml) plus *S. typhimurium* flagellin (5 μg/ml), *E. coli* LPS (10 μg/ml) plus CpG ODN 2006 (10 μg/ml), or *S. typhimurium* flagellin (5 μg/ml) plus CpG ODN 2006 (10 μg/ml); 3) cytokines: IFN-γ (100 U/ml) and TNF-α (50 ng/ml); or 4) TLR ligand and cytokine combinations: *P. gingivalis* LPS (50 μg/ml) plus IFN-γ (5 U/ml), poly(I:C) (10 μg/ml) plus IFN-γ (5 U/ml), *E. coli* LPS (10 μg/ml) plus IFN-γ (5 U/ml), *S. typhimurium* flagellin (5 μg/ml) plus IFN-γ (5 U/ml), *P. gingivalis* LPS (50 μg/ml) plus TNF-α (1 ng/ml), poly(I:C) (10 μg/ml) plus TNF-α (1 ng/ml), *E. coli* LPS (10 μg/ml) plus TNF-α (1 ng/ml), and *S. typhimurium* flagellin (5 μg/ml) plus TNF-α (1 ng/ml).

After stimulation with TLR ligand(s) and/or cytokine for 12–24 h, the cells and culture supernatants were collected for measurement of IL-8 and IDO expression.

Determination of IL-8

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

mRNA expression of IDO

The kinetics study of IDO mRNA expression (6, 12, and 24 h) was conducted using IFN-γ- or TNF-α-treated HGFs. Twelve-hour-treated cells showed optimal mRNA expression of IDO. The total RNA of HGFs after 12 h of stimulation with TLR ligand(s) and/or cytokine was reverse transcribed and treated with DNase I as previously mentioned. IDO was amplified using specific primer (5′-CTTCCTGGTCTCTCTATTGG-3′/5′-GAAGTTCCTGTGAGCTGGT-3′; Sigma-Aldrich) (21). The expected size of the PCR product was 430 bp. For semiquantitative RT-PCR analysis, band intensities on scanned gels were analyzed (GeneTools; Synoptics) using specific bands of the housekeeping gene GAPDH as a reference.

IDO activity: kynurenine assay

IDO-dependent catabolism of tryptophan produces kynurenine. Hence, the biological activity of IDO was evaluated by measuring the level of kynurenine in HGF culture supernatants (22). One hundred microliters of culture supernatants of HGFs after stimulation with TLR ligand(s) and/or cytokine was mixed with 50 μl of 30% trichloroacetic acid, vortexed, and centrifuged at 8000 $\times$ *g* for 5 min. Then, 75 μl of the supernatant was added to an equal volume of Ehrlich reagent (100 mg of *p*-dimethylbenzaldehyde in 5 ml of glacial acetic acid) in a 96-well microtiter plate, and the absorbance was read at an OD of 492 nm. A standard curve of defined kynurenine concentration (0–100 μM) permitted analysis of unknowns.

Suppression of T cell response in MLR

To assess whether IDO-expressed HGFs inhibit allogeneic T cell responses, MLRs were performed on a layer of HGFs that had been treated for 2 days with either *P. gingivalis* LPS (50 μg/ml), IFN-γ (5 U/ml), or the combination of *P. gingivalis* (50 μg/ml) and IFN-γ (5 U/ml). PBMC were isolated from the blood of healthy human donors. MLCs were performed by mixing PBMC (each at 4 $\times$ 10^7 cells/ml in PBS) from two healthy donors. A total of 4 $\times$ 10^5 mixed PBMC in 10 μl of PBS was cocultured with a confluent layer of stimulated HGFs (200 μl/well) in 96-well plates. ^{3}[H]Thymidine (0.5 μCi/200 μl/well) was added on day 5 and cell cultures were incubated for another 18 h. Cells were harvested onto glass filter paper and radioactivity was measured (beta plate; PerkinElmer Wallac). To

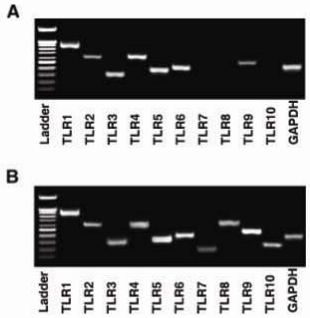

FIGURE 1. TLR expression in HGFs. *A*, TLRs 1–10 mRNA expression was measured in cultured HGFs by RT-PCR. *B*, PBMC mRNA was used as positive control. GAPDH mRNA was used as an internal control. Data are representative of four separate HGF lines and PBMC.

confirm the inhibitory effect of IDO, 1-methyl-DL-tryptophan (1000 μg/ml) or L-tryptophan (1000 μM) was added during the coculture of the stimulated HGFs with mixed PBMC.

Statistical analysis

Statistical comparisons among treatment conditions with respect to production of IL-8 and IDO and to inhibition of the T cell response were analyzed using SigmaStat (Jandel). The parametric Student's *t* test was used for normally distributed data, and the nonparametric Mann-Whitney *U* rank-sum test was used for nonnormally distributed data. A value of $p < 0.05$ was considered statistically significant.

Results

mRNA expression of TLRs on HGFs

TLRs have been found on many cell types and are known to play a central role in pathogen recognition in the innate immune system. To evaluate the expression of TLRs in HGFs, total RNA from HGFs was analyzed by RT-PCR using a panel of specific primers of TLRs 1–10. We found the mRNA expression of TLRs 1, 2, 3, 4, 5, 6, and 9 on HGFs but not TLRs 7, 8, and 10 (Fig. 1*A*). The results were reproducible in all four HGF lines. Human PBMC were used as a positive control and shown to express all mRNA of TLRs 1–10 (Fig. 1*B*).

TLR3 is generally recognized as an intracellular receptor, but a recent study showed a surface TLR3 on human skin and lung fibroblasts (23). Thus, we analyzed the specific location of TLR3 expression in HGFs. Using the same mAb specific to TLR3 (clone TLR 3.7), we demonstrated the detection of intracellular TLR3 on HGFs but not on the cell surface (Fig. 2).

We next evaluated whether TLR expression could be modulated by oral bacterial products. Coculture of HGFs with sonicated extracts of *P. gingivalis* for 24 h did not lead to a significant upregulation of any TLR mRNA expression (data not shown).

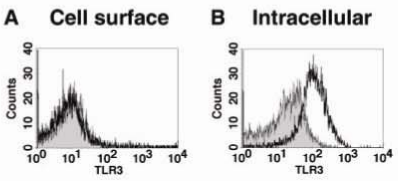

FIGURE 2. Flow cytometric analysis of cell surface and intracellular TLR3 expression in HGFs. HGFs were stained for cell surface (*A*) or intracellular TLR3 (*B*) with mAb against human TLR3 (clone TLR3.7, open histograms). Shaded histograms represent cells stained with isotype-matched control Abs.

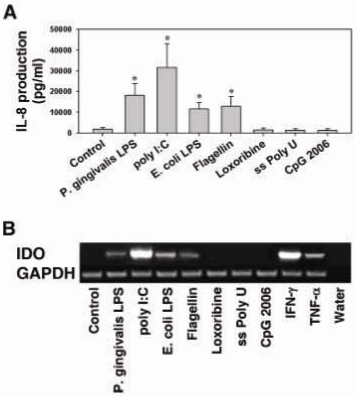

FIGURE 3. Expression of IL-8 and IDO in HGFs after stimulation with various TLR ligands. HGFs were cultured in 96-well plates or 24-well plates and stimulated with the following ligands: *P. gingivalis* LPS (TLR2 ligand), poly(I:C) (TLR3 ligand), *E. coli* LPS (TLR4 ligand), *S. typhimurium* (TLR5 ligand), loxoribine (TLR7 ligand), poly(U) (TLR8 ligand), and CpG ODN 2006 (TLR9 ligand). Culture medium was used as a control. For assessment of IL-8 production, culture supernatants of stimulated HGFs were harvested after 24 h and then assayed by ELISA. Data shown are mean ± SEM of four separate experiments (*, $p < 0.05$, compared with unstimulated control, *A*). To measure IDO expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. IFN-γ- and TNF-α-stimulated HGFs were used as positive controls. GAPDH mRNA was used as an internal control. Data are representative of four separate experiments (*B*).

TLR ligands stimulate expression of IL-8 and IDO

To characterize the functional relevance of TLRs in HGFs, expression of IL-8 and IDO was determined after stimulation with highly purified TLR ligand(s). IL-8 production coincided with mRNA expression of TLRs (i.e., TLRs 2, 3, 4, and 5; Fig. 3*A*). On the contrary, no IL-8 production was observed in HGFs stimulated with CpG ODN 2006, even though the cells expressed TLR9 mRNA (Fig. 3*A*).

Skin fibroblasts are known to express IDO when treated with IFN-γ (12, 13). In this study, the IDO expression of HGFs after TLR ligation was also evaluated. Similar to IL-8 production, *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin, respective ligands for TLRs 2, 3, 4, and 5, induced IDO mRNA expression. IFN-γ- and TNF-α-treated HGFs were used as positive controls (Fig. 3*B*).

TLR ligand combinations (*P. gingivalis* LPS plus poly(I:C), *P. gingivalis* LPS plus *E. coli* LPS, *P. gingivalis* LPS plus *S. typhimurium* flagellin, *P. gingivalis* LPS plus CpG ODN 2006, poly(I:C) plus *E. coli* LPS, poly(I:C) plus *S. typhimurium* flagellin, poly(I:C) p;us CpG ODN 2006, *E. coli* LPS plus *S. typhimurium* flagellin, *E. coli* LPS plus CpG ODN 2006, and *S. typhimurium* flagellin plus CpG ODN 2006) did not lead to a significant enhancement of IL-8 production (Fig. 4*A*) or IDO expression (Fig. 4*B*), as compared with the sum of individual ligands. Surprisingly, CpG ODN 2006 specifically inhibited poly(I:C)-induced IL-8 production ($p < 0.05$) and poly(I:C)-induced IDO expression ($p < 0.05$; Fig. 4).

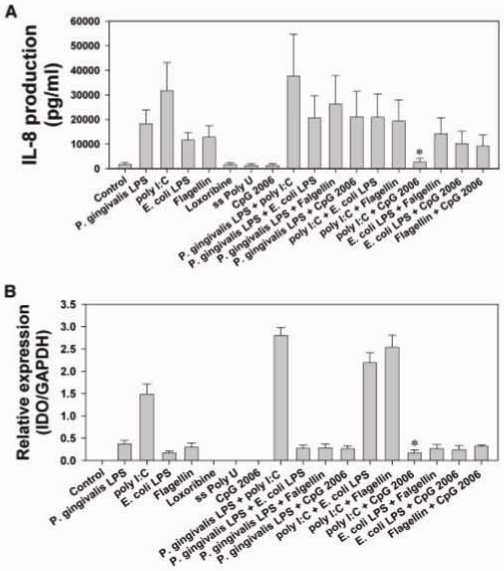

FIGURE 4. Expression of IL-8 and IDO in HGFs after stimulation with TLR ligand combination. HGFs were cultured in 96-well plates or 24-well plates and stimulated with the following ligand combinations: *P. gingivalis* LPS + poly(I:C), *P. gingivalis* LPS + *E. coli* LPS, *P. gingivalis* LPS + *S. typhimurium* flagellin, *P. gingivalis* LPS + CpG ODN 2006, poly(I:C) + *E. coli* LPS, poly(I:C) + *S. typhimurium* flagellin, poly(I:C) plus CpG ODN 2006, *E. coli* LPS + *S. typhimurium* flagellin, *E. coli* LPS + CpG ODN 2006, and *S. typhimurium* flagellin + CpG ODN 2006. Culture medium was used as a control. Culture supernatants of stimulated HGFs were harvested after 24 h and IL-8 production was determined by ELISA. Data shown are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with poly(I:C) treatment, *A*). For semiquantitative analysis of IDO mRNA expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. IFN-γ- and TNF-α-stimulated HGFs were used as positive controls. Data shown are the mean ratio ± SEM of IDO:GAPDH from four separate experiments (*, $p < 0.05$, compared with poly(I:C) treatment, *B*).

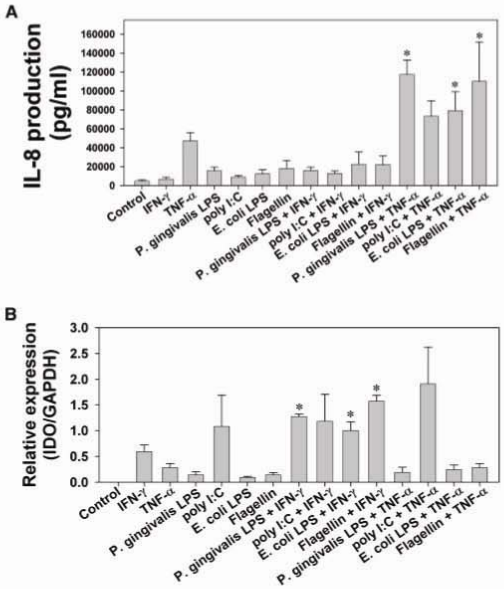

FIGURE 5. Expression of IL-8 and IDO in HGFs after stimulation with TLR ligand and cytokine combination. HGFs were cultured in 96-well plates or 24-well plates and stimulated with the following ligand and cytokine combinations: *P. gingivalis* LPS + IFN-γ, poly(I:C) + IFN-γ, *E. coli* LPS + IFN-γ, *S. typhimurium* flagellin + IFN-γ, *P. gingivalis* LPS + TNF-α, poly(I:C) + TNF-α, *E. coli* LPS + TNF-α, and *S. typhimurium* flagellin + TNF-α. Culture medium was used as a control. Culture supernatants of stimulated HGFs were harvested after 24 h and IL-8 production was determined by ELISA. Data shown are the mean ± SEM of four separate experiments (*, $p < 0.05$, compared with the sum of two individual stimulators, *A*). For semiquantitative analysis of IDO expression, stimulated HGFs were harvested after 12 h and mRNA expression of IDO was analyzed by RT-PCR. Data shown are the mean ratio ± SEM of IDO:GAPDH from four separate experiments (*, $p < 0.05$, compared with the sum of two individual stimulators, *B*).

Combination of TLR ligand and cytokine stimulates expression of IL-8 and IDO

Cytokines IFN-γ, and TNF-α have been consistently detected in periodontitis lesions (6, 7). We next investigated the effects on HGF production of IL-8 and IDO by either cytokine or by the combination of cytokine with different TLR ligands, specifically *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, or *S. typhimurium* flagellin. Fig. 5*A* demonstrates that unlike IFN-γ, TNF-α, when combined with *P. gingivalis* LPS, *E. coli* LPS, or *S. typhimurium* flagellin, significantly induced more IL-8 from HGFs than the additive ($p < 0.05$). Interestingly, the results of IDO mRNA expression were quite different. IFN-γ, but not TNF-α when combined with *P. gingivalis* LPS, *E. coli* LPS, or *S. typhimurium* flagellin significantly induced IDO mRNA expression greater than the sum of individual stimulators ($p < 0.05$; Fig. 5*B*).

P. gingivalis *LPS and/or IFN-γ-treated HGFs induce suppression of T response via IDO*

According to the enhancement of IDO mRNA expression on HGFs after stimulation with the combination of *P. gingivalis* LPS and IFN-γ, we next assessed the biological activity of IDO by measuring the kynurenine concentration in those cultured supernatants. Fig. 6*A* demonstrates that the kynurenine could be detected within 24-h culture supernatants of stimulated HGFs. The levels of kynurenine continued to increase up to 72 h in cultures.

We further evaluated whether HGFs, which were stimulated with *P. gingivalis* LPS, IFN-γ, or the two in combination could inhibit T cell response in MLRs. Cocultures of mixed PBMC with 2-day *P. gingivalis*- or IFN-γ-stimulated HGF showed 10–32% inhibition of the T cell proliferative response (*, $p < 0.05$) as compared with unstimulated HGFs. However, the combination of *P. gingivalis* and IFN-γ-stimulated cells markedly inhibited T cell proliferation by 62% (**, $p < 0.001$; Fig. 6*B*). The response of T cells was restored when 1-methyl-DL-tryptophan or L-tryptophan was added to the cultures (Fig. 6*B*).

Discussion

It is known that host immune response employs TLR and non-TLR pathways to recognize pathogens and commensal bacteria (24, 25). This recognition leads to expression of mediators that limit microbial invasion. When gingival epithelium is ruptured, HGFs can be

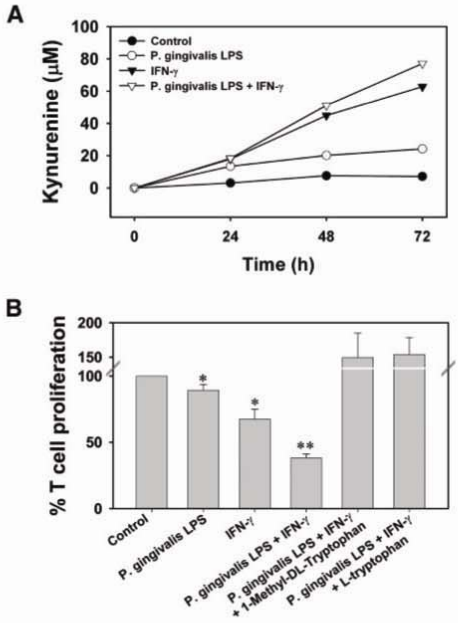

FIGURE 6. Kinetics of IDO activity and suppression of T cell proliferation in MLRs. HGFs were stimulated with either IFN-γ, *P. gingivalis* LPS, or the combination of IFN-γ and *P. gingivalis* LPS. Culture supernatants were harvested at different time points (0, 24, 48, and 72 h) and the concentration of kynurenine was determined (*A*). Suppression of T cell proliferation in MLR was assessed by coculturing mixed PBMC from two donors with *P. gingivalis* LPS, IFN-γ, or the combination of *P. gingivalis* LPS and IFN-γ-stimulated HGFs. Either 1-methyl-DL-tryptophan or L-tryptophan was added to the stimulated HGF cultures at the same time as mixed PBMC. After 6 days of incubation, T cell proliferative response was determined by tritiated thymidine uptake. T cell proliferation was calculated as a percentage of control. Data shown are mean ± SEM from four separate experiments (*, $p < 0.05$; **, $p < 0.001$, compared with unstimulated control, *B*).

exposed to many bacterial pathogen-associated molecular patterns (PAMPs). Clinical observations demonstrated the presence of periodontopathic bacteria in epithelial and connective tissue layers of periodontitis lesions (26–33). The pathogens such as *P. gingivalis, A. actinomycetemcomitans,* and *Fusobacterium nucleatum* were also shown to invade human gingival epithelial cells and fibroblasts *in vitro* as well as *in vivo* (34–37). The ability of HGFs to recognize and respond to such patterns renders them crucial in dealing with microbial invasion. In this study, we evaluated the expression of TLRs and their role in signaling by HGFs. Our results demonstrate that HGFs derived from healthy gingival tissues expressed mRNA of TLRs 1, 2, 3, 4, 5, 6, and 9, but not of TLRs 7, 8, and 10. This is similar to observations in nasal fibroblasts (38). A recent study showed that fibroblasts from human foreskin and lung expressed cell surface TLR3 (23). However, our study, using the same clone of mAb, demonstrated the presence of intracellular TLR3, but not cell surface TLR3, on HGFs. Thus, fibroblast TLR expression may differ across anatomic sites. Further study is needed to identify and compare the physiologic significance of intracellular and cell surface TLR3.

In line with the TLR mRNA expression, HGFs expressed IL-8 and IDO in response to *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin, respective ligands for TLRs 2, 3, 4, and 5. It has been shown that highly purified *P. gingivalis* LPS possess lipid A heterogeneity, which may contribute to their ability to interact with either TLR2 or TLR4 (39). *P. gingivalis* LPS at a concentration of 50 μg/ml used in our study predominantly stimulated HGFs via TLR2 and to a lesser extend via TLR4 (InvivoGen product information). Poly(I:C) appeared to induce higher IL-8 and IDO expression than did other TLR ligands. Surprisingly, CpG ODN 2006, a potent ligand for TLR9, did not induce IL-8 or IDO expression. Similarly, purified DNA derived from either *E. coli*, *P. gingivalis*, or *A. actinomycetemcomitans*, which is also recognized as a TLR9 ligand, did not induce either of these mediators (data not shown). Our findings may indicate a nonfunctional TLR9 in HGFs. (Gingival epithelial cells also express TLR9 mRNA, but do not respond to CpG ODN 2006; R. Mahanonda and S. Pichyangkul, unpublished observations.) In contrast, some recent studies showed that DNA preparation from periodontopathic bacteria activated HGFs via TLR9 to produce IL-6 or IL-8 (10, 40). This inconsistency requires further investigation.

The finding that HGFs expressed TLRs 2, 4, and 5 supports their role in the innate immune response against bacteria. Oral plaque bacteria are known to have PAMPs that are recognized by TLRs 2, 4, and 5. For example, *P. gingivalis* LPS and *P. gingivalis* fimbriae are recognized by TLR2 (41–43); LPS from *A. actinomycetemcomitans* and *Bacteroides fragilis* are recognized by TLR4 (44–46). Flagellin of *Treponema denticola* is most likely recognized by TLR5. The expression of TLR3 in HGFs is interesting because TLR3 recognizes dsRNA, a by-product of viral replication and transcription (47). A possible role of herpesviruses in etiology and severity of periodontal diseases has been reported (48–50). The presence of TLR3 thus suggests a role of HGFs in antiviral response.

The effects of TLR ligand combinations on IL-8 and IDO expression by HGFs were not significantly different from those of single ligands or the sum of individual ligands, except for the combination of CpG ODN 2006 with poly(I:C). Addition of CpG ODN 2006 markedly inhibited poly(I:C)-induced IL-8 and poly(I:C)-induced IDO expression. CpG ODN 2006, by itself, had no effect on the expression of either IL-8 or IDO. The inhibitory effect of CpG ODN 2006 on poly(I:C)-stimulated HGFs is unlikely to be limited to the early phase, because the addition of CpG ODN 2006 at 6 h after poly(I:C) treatment still completely suppressed IL-8 production (data not shown). Further studies will be needed to understand the inhibitory effect of CpG ODN on poly(I:C)-induced HGF activation.

Previous studies demonstrated that different cytokines have different effects on HGFs in IL-8 production (3, 51). TNF-α, but not IFN-γ, induced IL-8 production from HGFs; these observations agree with those of previous studies (3, 51, 52). Combinations of cytokines and bacterial PAMPs are known to modulate cytokine production from different cell types (53, 54). A high level of IL-8 as well as the increased presence of IL-8-secreting fibroblasts has been detected in periodontitis lesions (6, 55). Our data demonstrate that stimulation of HGFs with TNF-α, combined with TLR ligands 2, 4, or 5, synergistically enhanced IL-8 production. The IL-8 response in periodontal tissue could have both beneficial and deleterious effects. IL-8 is important in neutrophil activation and recruitment. On one hand, undue down-regulation of this function could compromise antimicrobial defense. On the other hand, unduly vigorous or sustained IL-8 response could cause chronic inflammatory tissue destruction.

It is reported that skin fibroblasts can dampen local immune cell responses via IDO. In this study, we demonstrated that HGFs were also able to induce IDO expression in response to *P. gingivalis* LPS, poly(I:C), *E. coli* LPS, and *S. typhimurium* flagellin. IDO