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γ-Interferon enhances expression of CD14/MyD88 and subsequent responsiveness to lipopolysaccharide from *Actinobacillus actinomycetemcomitans* in human gingival fibroblasts

S. Mochizuki[1], M. Kobayashi[1], T. Suzuki[1], A. Oikawa[1], T. Koseki[2], T. Nishihara[3], K. Hasegawa[1]
[1]Department of Periodontology, Showa University Dental School, Tokyo, [2]Department of Preventive Dentistry, Tohoku University, School of Dentistry, Hiyagi and [3]Department of Oral Microbiology, Kyushu Dental Collage, Fukuoka, Japan

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Objectives: CD14, toll-like receptor 4 (TLR4) and MyD88 have been shown to mediate responsiveness in host cells to lipopolysaccharide. We investigated here the regulatory effects of inflammatory cytokines on the expression of membrane CD14 (mCD14), TLR4 and MyD88, and on subsequent responsiveness to lipopolysaccharide from *Actinobacillus actinomycetemcomitans* in human gingival fibroblasts.

Materials and methods: Following treatment with either interleukin-1β, tumor necrosis factor-α (TNF-α) or γ-interferon (IFN-γ), expression of mCD14/TLR4 and MyD88 was determined by flow cytometry and western blotting, respectively. After pretreatment with IFN-γ, cells were pre-incubated with either anti-CD14 antibody MY4 or anti-TLR4 antibody HTA125 and subsequently treated with *A. actinomycetemcomitans* lipopolysaccharide. Then, phosphorylation of mitogen-activated protein (MAP) kinases and IκBα was examined by western blotting, and production of interleukin-6 and interleukin-8 was measured by their respective enzyme-linked immunosorbent assay (ELISA) kits.

Results: IFN-γ stimulated expression of mCD14, whereas -1β and TNF-α did not. Expression of MyD88 but not TLR4 was also enhanced by IFN-γ. The lipopolysaccharide activated MAP kinases, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38, and IκBα and stimulated production of interleukin-6 and interleukin-8. The lipopolysaccharide-stimulated interleukin-6 and interleukin-8 production was markedly inhibited by MY4 or HTA125. Pretreatment with IFN-γ augmented the following activation of MAP kinases and IκBα and production of interleukin-6 and interleukin-8 in response to the lipopolysaccharide.

Makoto Kobayashi, DDS, PhD, Department of Periodontology, Showa University Dental School, 2-1-1 Kitasenzoku, Ohta-ku, Tokyo 145-8515, Japan
Tel: +81 33787 1151 (ext. 354)
Fax: +81 33787 9290
e-mail: perikoba@senzoku.showa-u.ac.jp

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Conclusions: These results suggest that the augmentation by IFN-γ of the responsiveness to *A. actinomycetemcomitans* lipopolysaccharide, such as activation of MAP kinases and IκBα and terminal cytokine production in human gingival fibroblasts, may be partially mediated by up-regulation of CD14 and MyD88 expression.

Lipopolysaccharide from periodontopathic bacteria has been recognized as an important pathogenic component in the initiation and progression of periodontal disease, because the lipopolysaccharide has been shown to stimulate production of inflammatory cytokines in various kinds of host cells and to induce bone resorption, directly or indirectly. Human gingival fibroblasts, a major constituent of gingiva, may also act as a regulator of the cytokine network in periodontal tissues because they produce various inflammatory cytokines when stimulated with the periodontopathic bacterial lipopolysaccharide (1–5) as well as inflammatory cytokines.

CD14 is considered to play an essential role in the first event occurring in host infection by bacteria, as it acts as a receptor for lipopolysaccharide combined with lipopolysaccharide-binding protein (6–8). CD14 exists in two forms: membrane CD14 (mCD14), which is a glycosylphosphatidylinositol (GPI)-anchored protein on the cell surface (9), and the soluble form (sCD14), which lacks the GPI-anchor and is present in serum and urine (10). Recently, 10 human toll-like receptors (TLRs) have been identified, and the TLRs have been shown to play an essential role in intracellular signaling of host cells in response to bacterial cell surface components including lipopolysaccharide (11–14). TLR4 has been particularly well documented as an lipopolysaccharide-signaling receptor in previous *in vivo* and *in vitro* studies (15–20). MyD88, an adapter molecule for the TLR/interleukin-1 receptor family, is also essential for the cellular responsiveness to the bacterial cell surface components such as lipopolysaccharide (21). Human gingival fibroblasts constitutively express CD14 (22–26), TLR4 (27, 28) and MyD88 (28), and these molecules therefore may mediate cytokine production in lipopolysaccharide-stimulated human gingival fibroblasts. Actually, the findings of recent studies have demonstrated that CD14 (22–26) and TLR4 (27, 28) mediated production of inflammatory cytokines in response to lipopolysaccharide from *Porphyromonas gingivalis* in human gingival fibroblasts.

The downstream pathways mediated by the TLRs are starting to be clarified. The TLR-mediated signaling leads to phosphorylation of IκB and dissociation of IκB from nuclear factor-κB (NF-κB) (29). These events ultimately result in translocation of NF-κB to the nucleus and initiation of gene transcription. Mitogen-activated protein (MAP) kinases also have been shown to be rapidly phosphorylated by lipopolysaccharide-challenge in several cell types and to be involved in the following regulation of many intracellular signaling pathways. Actually, the involvement of MAP kinases, such as c-Jun N-terminal kinase (JNK), p38 and extracellular signal-regulated kinase (ERK), in lipopolysaccharide-induced cytokine production has been documented (30–36). Therefore, periodontopathic bacterial lipopolysaccharide may also activate IκB and/or MAP kinases via CD14 and TLR4 in human gingival fibroblasts.

Some *in vitro* and *in vivo* studies revealed the possible role of inflammatory cytokines on regulation of CD14 expression. interleukin-1β and/or tumor necrosis factor-α (TNF-α) stimulate CD14 mRNA expression in hepatocytes (37). The enhancement of CD14 expression by these cytokines also has been observed in liver and kidney (38–40). γ-Interferon (IFN-γ) markedly enhanced mCD14 expression in high-CD14-expressing human gingival fibroblasts (41), and release of sCD14 in the cells was also augmented by IFN-γ in accordance with the increase expression of mCD14 (24). There have been a few reports concerning the regulatory effects of the cytokines on expression of TLR4 and MyD88. In the inflamed gingiva, the expression of TLR4 and MyD88, as well as CD14 and the subsequent lipopolysaccharide-responsiveness in human gingival fibroblasts, may be modulated by various inflammatory cytokines, including interleukin-1β, TNF-α and IFN-γ. We therefore investigated here the regulatory effects of these inflammatory cytokines on expression of mCD14, TLR4 and MyD88 and on subsequent responsiveness to lipopolysaccharide from *Actinobacillus actinomycetemcomitans*, including activation of MAP kinases and IκBα and production of interleukin-6 and interleukin-8, in human gingival fibroblasts.

Materials and methods

Cell culture

Human gingival fibroblasts were isolated from attached gingiva. Attached gingiva was obtained from the hard palate at a 5 mm distance from the gingival margin in three adult (male, 18–20 years of age) volunteers with clinically healthy periodontium. All volunteers were duly informed of the nature and extent of the study, and their informed consent was obtained according to the Helsinki Declaration. The protocol for our human study was approved by the ethical committee of Showa University Dental School, Tokyo, Japan. The human gingiva collected was cut into small pieces, placed in 35-mm^2 tissue culture dishes (Corning, New York, NY, USA) and cultured in Dulbecco's modified Eagle's medium (Flow Laboratories, McLean, VA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA), 100 U/ml of penicillin G (Banyu Pharmaceutical Co., Tokyo, Japan) and 100 μg/ml of streptomycin (Meiji Kaisha Ltd, Tokyo, Japan). The cultures were maintained at 37°C in 95% air and 5% CO_2 until confluent cell monolayers were formed. The typical human gingival fibroblasts prepared from each of three volunteers were designated HGF-

1, -2 and -3, respectively. Each human gingival fibroblast sample (1×10^5 cells) was subcultured in 25-cm^2 culture flasks (Corning) and used at passages 4–6 in all experiments.

Cell treatment

Each human gingival fibroblast sample was seeded in either 24-well flat-bottomed tissue culture plates (Corning), six-well flat-bottomed tissue culture plates (Corning) or 100-mm diameter culture dishes at a density of 5×10^4, 1×10^5 or 1×10^6 cells/well or dish, respectively, and cultured for 3 days until confluence in 1 ml, 3 ml or 10 ml of Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. After being cultured for 12 h in Dulbecco's modified Eagle's medium containing 2% fetal bovine serum, each human gingival fibroblast sample was treated with either recombinant human (rh) interleukin-1β (0.025–25 ng/ml; Genzyme Co., Boston, MA, USA), rhTNF-α (0.025–25 ng/ml; Genzyme) or rhIFN-γ (10–10,000 U/ml; Genzyme) for 6–96 h in Dulbecco's modified Eagle's medium containing 2% fetal bovine serum. Each human gingival fibroblast sample was either pretreated with rhIFN-γ (1000 U/ml) or untreated for 72 h. All were then washed three times, preincubated for the last 30 min with either 10 μg/ml of anti-CD14 monoclonal antibody MY4 (mouse IgG2b; Coulter Co., Miami, FL, USA), isotype-matched mouse IgG2b (10 μg/ml, Coulter), 5 μg/ml of anti-TLR4 monoclonal antibody HTA125 (mouse IgG2a; provided by Dr K. Miyake, The Institute of Medical Science, The University of Tokyo, Tokyo, Japan) or isotype-matched mouse IgG2a (5 μg/ml, Coulter) and subsequently treated with *A. actinomycetemcomitans* strain Y4 lipopolysaccharide [10–10,000 ng/ml (42)] for 5–30 min or 24 h. The *A. actinomycetemcomitans* lipopolysaccharide was isolated from whole cells by the phenol–water procedure of Westphal and Jann (43). The crude extracts were treated with nuclease, washed extensively with pyrogen-free water by ultracentrifugation and lyophilized (44).

Flow cytometric analysis

Human gingival fibroblasts treated with each cytokine were analyzed for expression of mCD14 and TLR4 with flow cytometry. After each treatment, the cells were collected and washed once with 0.5% phosphate-buffered saline (Sigma Chemical Co., St. Louis, MO, USA) containing 0.5% bovine serum albumin (Sigma). For analysis of CD14 expression, the cells were stained with either fluorescein isothiocyanate (FITC)-conjugated MY4 or control FITC-conjugated mouse IgG2b at 4°C for 30 min. For analysis of TLR4 expression, the cells were stained with either HTA125 or control mouse IgG2a at 4°C for 30 min, followed by FITC-conjugated goat anti-mouse IgG (Biosource International Inc., Camerillo, CA, USA) at 4°C for a further 30 min. Flow cytometric analysis was performed by using a fluorescence-activated cell sorter (FACScan; Becton Dickinson, Mountain View, CA, USA).

Western blot analysis

After each treatment, the cells were washed with cold phosphate-buffered saline and lyzed by adding 1 × sodium dodecyl sulfate sample buffer [0.05 M Tris-HCl (pH 6.8)/2% w/v sodium dodecyl sulfate/6% β-mercaptoethanol/10% glycerol]. The lysates were immediately scraped, collected into microcentrifuge tubes and sonicated for 10–15 s on ice. The sonicated samples were thereafter centrifuged at 15,000 × *g* for 10 min at 4°C. Protein amounts were determined with a protein assay kit (Bio-Rad Laboratories Inc., Hercules, CA, USA). Protein samples (20 μg/line) were run on 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were then electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using a semi-dry blotter (MilliBlot-SDE system; Millipore Co., Bedford, MA, USA), according to the manufacturer's instructions. The membranes were washed once with 10 mM Tris-HCl (pH 7.2) containing 150 mM NaCl and 0.1% (v/v) Tween 20 (Tris-buffered saline with Tween 20) and then blocked for 1 h in Tris-buffered saline with Tween 20 containing 2% (w/v) skim milk. After washing the membranes with Tris-buffered saline with Tween 20, polyclonal antibodies against ERK1/2, phosphorylated ERK1/2, JNK1/2, phosphorylated JNK1/2, p38, phosphorylated p38, IκBα, phosphorylated IκBα (Cell Signaling Technology Inc., Beverly, MA, USA) and MyD88 (StressGen Biotechnologies Corp., Victoria, BC Canada) were added at a dilution of 1 : 1000 in Tris-buffered saline with Tween 20 and incubated for 8 h at 4°C. Following washing three times with Tris-buffered saline with Tween 20, the membrane were treated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG (Cell Signaling; diluted 1 : 2000) and horseradish peroxidase-conjugated anti-biotin IgG (Cell Signaling; diluted 1 : 1000) in Tris-buffered saline with Tween 20 at room temperature. After washing three times with Tris-buffered saline with Tween 20, the protein bands were visualized using an enhanced chemiluminescence western blot analysis system (Amersham Biosciences, Piscataway, NJ, USA). The quantitative analysis was performed using a densito graph software library laser analyzer (ATTO Corp., Tokyo, Japan).

Quantification of cytokines produced

At the end of each treatment, the culture supernatant was harvested and stored at −80°C until required. The amount of interleukin-6 or interleukin-8 secreted into the culture supernatant was determined by using enzyme-linked immunosorbent assay (ELISA) kits for human interleukin-6 or human interleukin-8 (Genzyme).

Statistical analysis

All experiments were performed three times for each cell population of human gingival fibroblasts. In the assay for cytokine production, experiments were also performed three times, with each experiment conducted in triplicate for each cell population of human gingival fibroblasts. Means and standard deviations (SD) were then calculated, and the statistical significance of

differences among each group was examined with a one-way ANOVA and *post hoc t*-test. The *post hoc t*-test was performed when the ANOVA test was significant at the $p < 0.05$ level.

Results

Effects of inflammatory cytokines on mCD14 expression in human gingival fibroblasts

Flow cytometric analysis demonstrated that all three human gingival fibroblasts, which were obtained from three different donors, expressed weak but similar levels of mCD14 (percentage of positive cells: HGF-1 cells, 16.2%; HGF-2 cells, 12.3%; HGF-3 cells, 13.8%) on the cell surfaces. The expression of mCD14 was not appreciably changed in any of the human gingival fibroblasts when treated with either rhinterleukin-1β or rhTNF-α at 0.025–25 ng/ml for 24–96 h (data not shown). On the other hand, rhIFN-γ at 1000 U/ml weakly stimulated expression of mCD14 in HGF-1 cells after treatment for 48 h, and its expression increased up until 96 h. The mean fluorescence intensity of mCD14 expression in the cells either treated for 72 h with rhIFN-γ or untreated was 11.8 or 4.1, respectively, with a negative control value of 3.6 (Fig. 1). When HGF-1 cells were treated with rhIFN-γ at 10–10,000 U/ml for 72 h, this cytokine also dose-dependently enhanced mCD14 expression (Fig. 2). The stimulatory effect of rhIFN-γ on mCD14 expression was also observed in HGF-2 and -3 cells. After the treatment with rhIFN-γ (1000 U/ml) for 72 h, the mean fluorescence intensity of mCD14 expression in HGF-2 and -3 cells was 3.1 and 4.5 for the untreated cells and 7.2 and 8.5 for the rhIFN-γ-treated cells, with a negative control value of 2.6 and 3.8, respectively (data not shown).

Effect of γ-interferon on expression of toll-like receptor 4 and MyD88 in human gingival fibroblasts

The three human gingival fibroblasts samples also expressed similar levels of TLR4 (percentage of positive cells: HGF-1 cells, 29.1%; HGF-2 cells, 22.3%; HGF-3 cells, 23.8%) on the cell surfaces. As shown in Fig. 3, the treatment with rhIFN-γ at 1000 U/ml for 24–96 h did not cause any appreciable changes in the expression of TLR4 in HGF-1 cells. The mean fluorescence intensity of TLR4 expression in the cells treated for 72 h with rhIFN-γ or not was 21.0 or 21.8, respectively, with a negative control value of 6.3. Similar results were obtained in HGF-2 and -3 cells. After the treatment for 72 h, the mean fluorescence intensity of TLR4 expression in HGF-2 and -3 cells was 18.4 and 20.6 for the untreated cells and 18.6 and 20.5 for the rhIFN-γ-treated cells, with a negative control value of 6.0 and 6.2, respectively (data not shown).

Expression of MyD88 protein in the three human gingival fibroblasts either treated for 6–72 h with 1000 U/ml of rhIFN-γ or untreated was determined by western blotting. As shown in Fig. 4, a 35-kDa MyD88 protein was constitutively expressed in untreated human gingival fibroblasts, and its expression was not appreciably changed throughout the culture periods. However, the expression of the MyD88 protein was stimulated by treatment

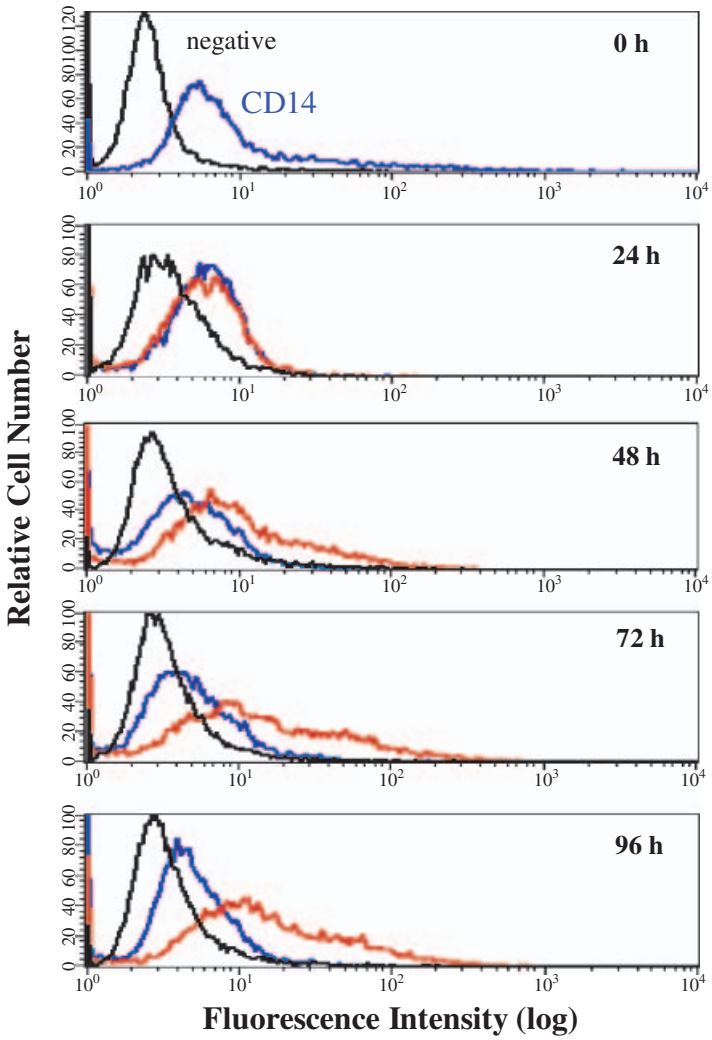


Fig. 1. Time-dependent effect of recombinant human γ-interferon (rhIFN-γ) on mCD14 expression in human gingival fibroblasts. HGF-1 cells at confluence were treated for 24–96 h in the presence (red line) or absence (blue line) of 1000 U/ml of rhIFN-γ. After each treatment, the cells were stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD14 monoclonal antibody MY4 and analyzed by a fluorescence-activated cell sorter (FACScan). The fluorescence of negative control cells was incubated with the FITC-conjugated isotype-matched mouse IgG2b (black line). The results shown are representative of those from the three separate experiments.

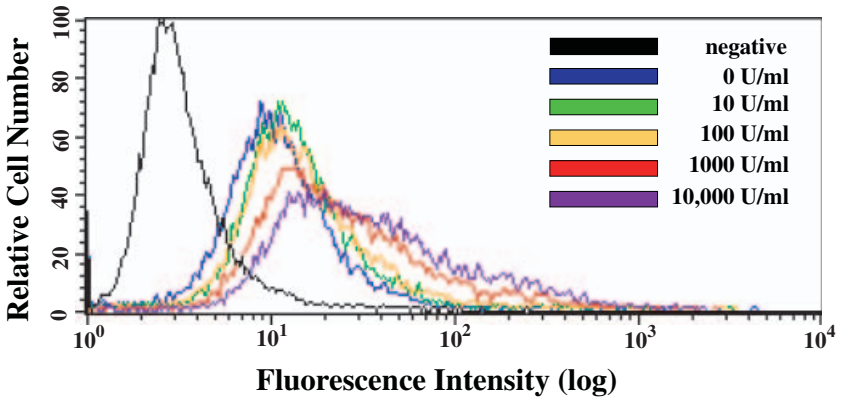


Fig. 2. Dose-dependent effect of recombinant human γ-interferon (rhIFN-γ) on mCD14 expression in human gingival fibroblasts. HGF-1 cells at confluence were either treated for 72 h with 10–10,000 U/ml of rhIFN-γ or untreated (0 U/ml: blue line; 10 U/ml: green line; 100 U/ml: orange line; 1000 U/ml: red line; 10,000 U/ml: purple line). After each treatment, the cells were stained with the fluorescein isothiocyanate (FITC)-conjugated MY4 and analyzed by the fluorescence-activated cell sorter (FACScan). The fluorescence of negative control cells was incubated with the FITC-conjugated mouse IgG2b (black line). The results shown are representative of those from the three separate experiments.

with rhIFN-γ for 12–48 h in the cells (a 1.8-fold increase at 12 h, a 1.5-fold increase at 24 h and a 1.4-fold increase at 48 h, as compared with untreated cells).

Priming effect of γ-interferon on *A. actinomycetemcomitans* lipopolysaccharide-induced activation of mitogen-activated protein kinases and IκBα in human gingival fibroblasts

Because rhIFN-γ enhanced expression of mCD14 and MyD88 in the human gingival fibroblasts, it may augment subsequent cellular responsiveness to periodontopathic bacterial lipopolysaccharide in the cells. As shown in Fig. 5, when HGF-2 cells were treated with *A. actinomycetemcomitans* 1000 ng/ml lipopolysaccharide alone, expression of the phosphorylated forms of ERK, p38 and JNK was stimulated at 5–10 min after the treatment. Pretreatment with rhIFN-γ at 1000 U/ml for 72 h did not cause any appreciable changes in the levels of the phosphorylated forms of MAP kinases at time 0, whereas the rhIFN-γ priming remarkably augmented the subsequent lipopolysaccharide-stimulated phosphorylation of JNK and p38 in the cells (Fig. 5). At the same time, the rhIFN-γ priming slightly enhanced the lipopolysaccharide-stimulated phosphorylation of ERK (Fig. 5). The lipopolysaccharide-stimulated activation of MAP kinases and the augmentation by rhIFN-γ pretreatment of the lipopolysaccharide-stimulated activation of MAP kinases were also observed in HGF-1 and HGF-3 cells (data not shown).

The treatment with 1000 ng/ml of *A. actinomycetemcomitans* lipopolysaccharide also stimulated phosphorylation of IκBα at 5–10 min after the treatment, and the degradation of IκBα was subsequently observed at 30 min in HGF-2 cells (Fig. 5). When the cells were pretreated with 1000 U/ml of rhIFN-γ for 72 h, the subsequent lipopolysaccharide-stimulated phosphorylation of IκBα was enhanced in the cells (Fig. 5). The augmentation by the rhIFN-γ priming of the lipopolysaccharide-stimulated activation of IκBα was also observed in HGF-1 and HGF-3 cells (data not shown).

Priming effect of γ-interferon on *A. actinomycetemcomitans* lipopolysaccharide-stimulated production of interleukin-6 and interleukin-8 in human gingival fibroblasts

We next investigated the priming effect of rhIFN-γ on terminal interleukin-6 and interleukin-8 production induced by *A. actinomycetemcomitans* lipopolysaccharide in the human gingival fibroblasts, because the pretreatment with hIFN-γ enhanced the subsequent lipopolysaccharide-induced activation of MAP kinases and IκBα in the cells. As shown in Figs 6(A) and (B), the treatment with *A. actinomycetemcomitans* lipopolysaccharide for 24 h significantly stimulated interleukin-6 and interleukin-8 production at concentrations above 1000 ng/ml in the three human gingival fibroblasts tested. Pretreatment with 1000 U/ml of rhIFN-γ for 72 h had no effect on production of interleukin-6 and interleukin-8 in the cells, whereas the rhIFN-γ-priming significantly enhanced interleukin-6 and interleukin-8 production induced by the subsequent treatment with the lipopolysaccharide at 1000 ng/ml for 24 h (Fig. 7).

Participation of CD14 and toll-like receptor 4 with interleukin-6 and interleukin-8 production by γ-interferon-primed and unprimed human gingival fibroblasts in response to *A. actinomycetemcomitans* lipopolysaccharide

We finally investigated whether CD14 and TLR4 mediate the *A. actinomycetemcomitans* lipopolysaccharide-stimulated interleukin-6 and interleukin-8 production in the rhIFN-γ-primed and unprimed human gingival fibroblasts. Pretreatment with either MY4 (10 μg/ml) or HTA125 (5 μg/ml) for 30 min significantly inhibited the interleukin-6 and interleukin-8 production stimulated with *A. actinomycetemcomitans* lipopolysaccharide to almost the control levels in the three human gingival fibroblasts tested; however, pretreatment with the control mouse IgG2b (10 μg/ml) or IgG2a (5 μg/ml) had no effect (Fig. 6C). The interleukin-6 and interleukin-8 production in response to the lipopolysaccharide in the rhIFN-γ-pretreated human gingival fibroblasts was also significantly inhibited to almost control levels by the pretreatment with MY4, whereas the HTA125-pretreatment significantly, but partially, inhibited it (Fig. 7).

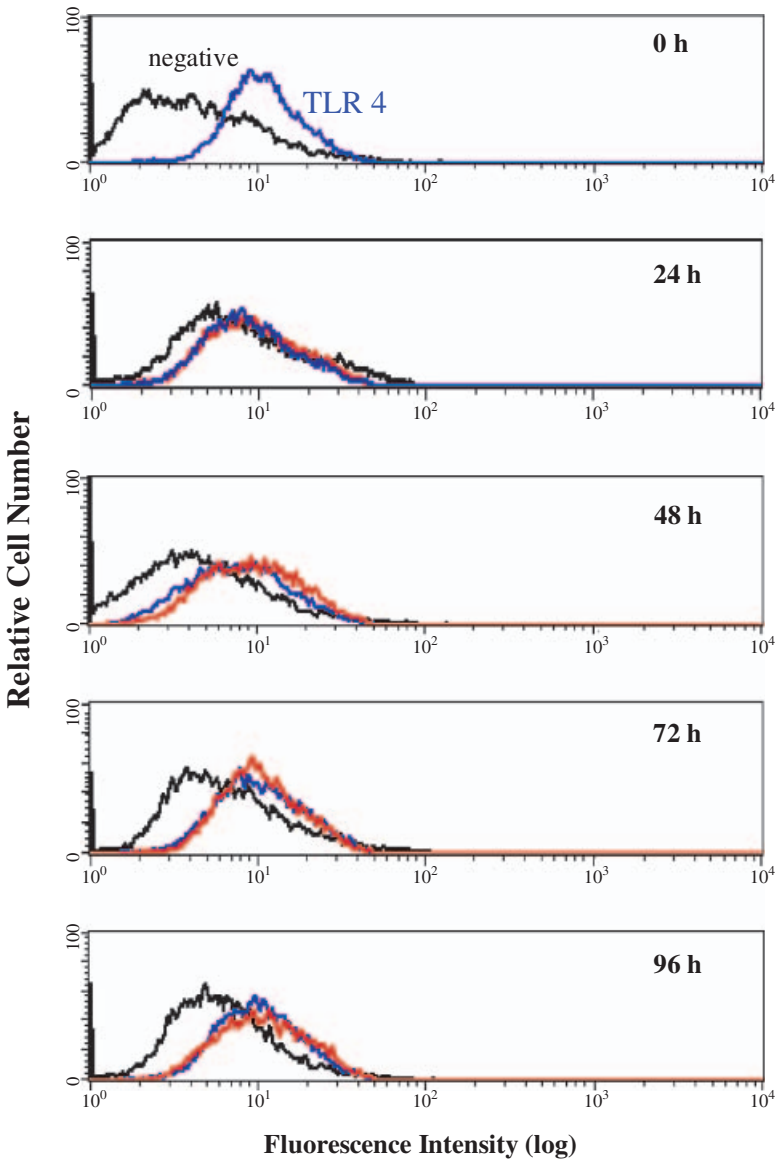


Fig. 3. Effect of recombinant human γ-interferon (rhIFN-γ) on toll-like receptor 4 (TLR4) expression in human gingival fibroblasts. HGF-1 cells at confluence were either untreated or treated for 24–96 h with 1000 U/ml of rhIFN-γ (untreated cells: blue line; rhIFN-γ-treated cells: red line). After each treatment, the cells were stained with either anti-TLR4 monoclonal antibody HTA125 or control isotype-matched mouse IgG2a (black line), followed by fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG and analyzed by the fluorescence-activated cell sorter (FACScan). The results shown are representative of those from the three separate experiments.

Discussion

The evidences for the expression of CD14 in human gingival fibroblasts are controversial. Some reports demonstrated that CD14 is expressed in human gingival fibroblasts (25–27), whereas other reports found that human gingival fibroblasts fail to express CD14 (23, 45). Sugawara *et al.* (24) showed that human gingival fibroblasts consist of populations that are heterogeneous on the basis of different levels of mCD14 expression, and that this explains the possible reasons for the heterogeneous expression of mCD14 in human gingival fibroblasts. Previous studies demonstrated that human gingival fibroblasts expressed TLR4 (27, 28, 41) and MyD88 (28, 41). The present study also showed that the three human gingival fibroblasts tested expressed mCD14, TLR4 and MyD88, all of which are important signaling molecules mediating lipopolysaccharide-responsiveness.

It is interesting to investigate the regulatory effects of inflammatory cytokines on CD14, TLR4 and MyD88 expression in human gingival fibroblasts, because the expression in the cells may be modulated by various inflammatory cytokines in inflamed gingiva. This study demonstrated that expression of mCD14 in the human gingival fibroblasts is enhanced by IFN-γ; however, interleukin-1β and TNF-α fail to stimulate it. The up-regulation by IFN-γ of mCD14 expression in human gingival fibroblasts is supported by previous reports of Sugawara *et al.* (24) and Tamai *et al.* (41). Conversely, regulatory effects of interleukin-1β and TNF-α on CD14 expression are controversial. Liu *et al.* (37) reported that CD14 mRNA expression of hepatocytes is up-regulated effectively by interleukin-1β and/or TNF-α *in vitro* and *in vivo*. The role of interleukin-1β and TNF-α in the enhancement of CD14 mRNA in liver and kidney also has been revealed by the studies of Fearns *et al.* (38, 39) and Takakuwa *et al.* (40). These data suggest that CD14 expression is differentially regulated by interleukin-1β and TNF-α in different cell types and organs. Bosisio *et al.* (45) demonstrated that IFN-γ augments expression of TLR4 and MyD88 in human mononuclear phagocytes, whereas our present study showed that IFN-γ stimulates expression of MyD88, but not TLR4, in the human gingival fibroblasts. Tamai *et al.* (41) also showed that expression of MyD88, but not TLR4, is markedly enhanced by IFN-γ in high-CD14-expressing human gingival fibroblasts, and that IFN-γ slightly increases MyD88 and scarcely affects TLR4 levels in low-CD14-expressing human gingival fibroblasts.

The augmentation by IFN-γ of CD14 and MyD88 expression in human gingival fibroblasts may cause the subsequent enhancement of lipopolysaccharide-responsiveness. In the present study, *A. actinomycetemcomi-*

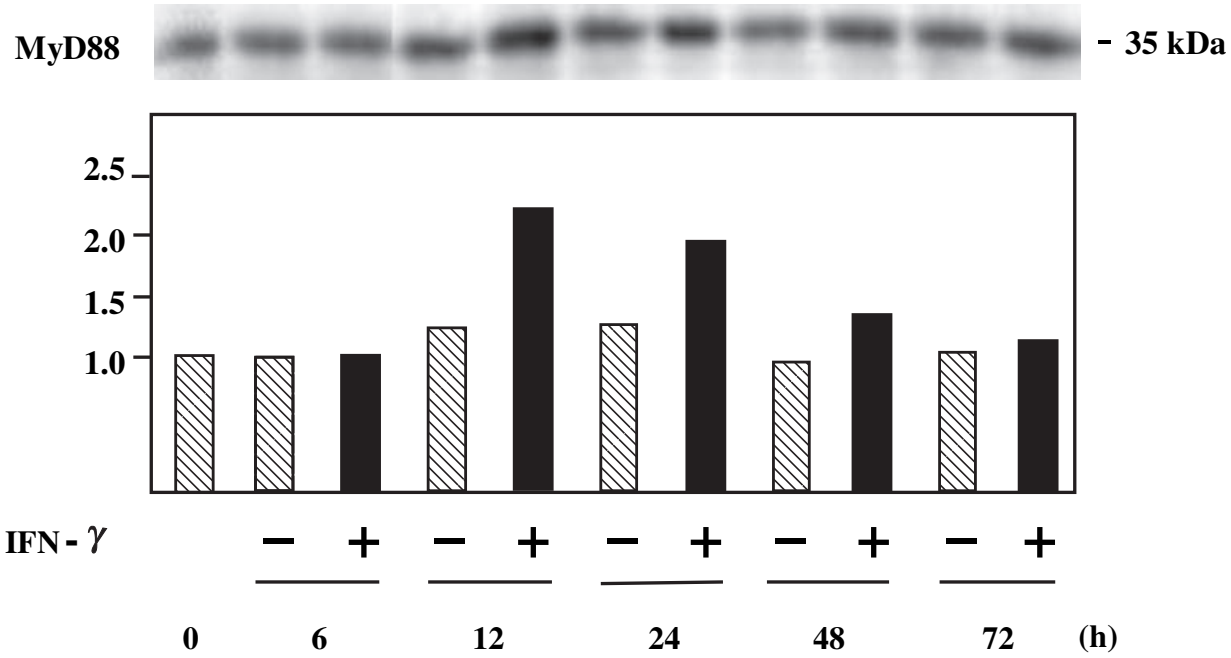


Fig. 4. Effect of recombinant human γ-interferon (rhIFN-γ) on MyD88 expression in human gingival fibroblasts. Three cell populations of human gingival fibroblasts at confluence were either treated for 6–72 h with 1000 U/ml of rhIFN-γ or untreated. Expression of MyD88 protein was assessed by western blotting. Protein levels of MyD88 are representative of the results from one of three separate experiments in HGF-1 cells. The graph demonstrates the MyD88 protein levels as a multiple of the value in time 0. The values represent the means of the values obtained in three separate experiments for each of the three cell populations of human gingival fibroblasts ($n = 9$).

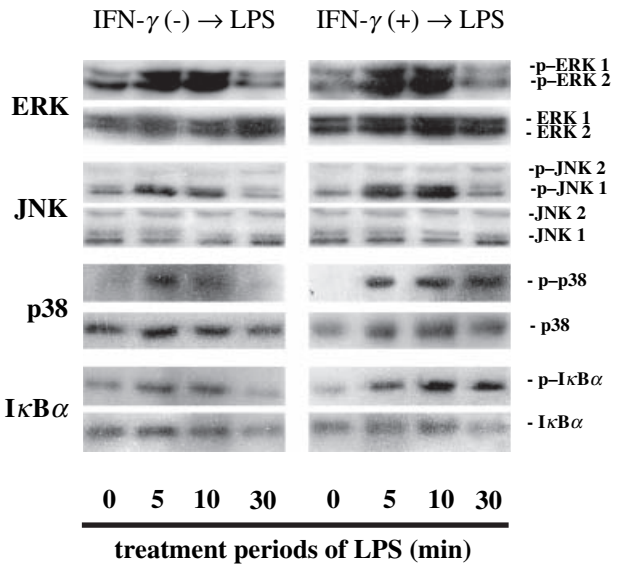


Fig. 5. Priming effects of recombinant human γ-interferon (rhIFN-γ) on activation of mitogen-activated protein (MAP) kinases and inhibitor of IκBα in human gingival fibroblasts treated with lipopolysaccharide (LPS) from *A. actinomycetemcomitans*. HGF-2 cells at confluence were pretreated for 72 h either with or without 1000 U/ml of rhIFN-γ, and subsequently treated for 5–30 min with 1000 ng/ml of the lipopolysaccharide (left panels: cells without rhIFN-γ pretreatment; right panels: rhIFN-γ-pretreated cells). After each treatment, the activation of extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase 1/2 (JNK1/2), p38 and IκBα were determined by western blotting. Upper panels show the phosphorylated form of each MAP kinase and IκBα detected. Lower panels show each protein of MAP kinases and IκBα detected. The results shown are representative of those from the three separate experiments.

tans lipopolysaccharide activated ERK, JNK, p38 and IκBα in the human gingival fibroblasts. This result supports a previous study (28), in part, which demonstrated that *P. gingivalis* lipopolysaccharide stimulated tyrosine phosphorylation of p42/p44 proteins and following DNA binding of two transcriptional factors, AP-1 and NF-κB via TLR4. However, the priming effect of IFN-γ on lipopolysaccharide-stimulated activation of MAP kinases and IκBα in human gingival fibroblasts has not been determined yet. The present study demonstrated that IFN-γ-pretreatment enhances the activation of MAP kinases and IκBα induced by the subsequent challenge of *A. actinomycetemcomitans* lipopolysaccharide in the human gingival fibroblasts.

In this study, production of interleukin-6 and interleukin-8 upon stimulation with *A. actinomycetemcomitans* lipopolysaccharide was significantly inhibited by pretreatment with the anti-CD14 antibody MY4 in the human gingival fibroblasts. Some studies using MY4 have also clarified that human gingival fibroblasts produced several inflammatory cytokines,

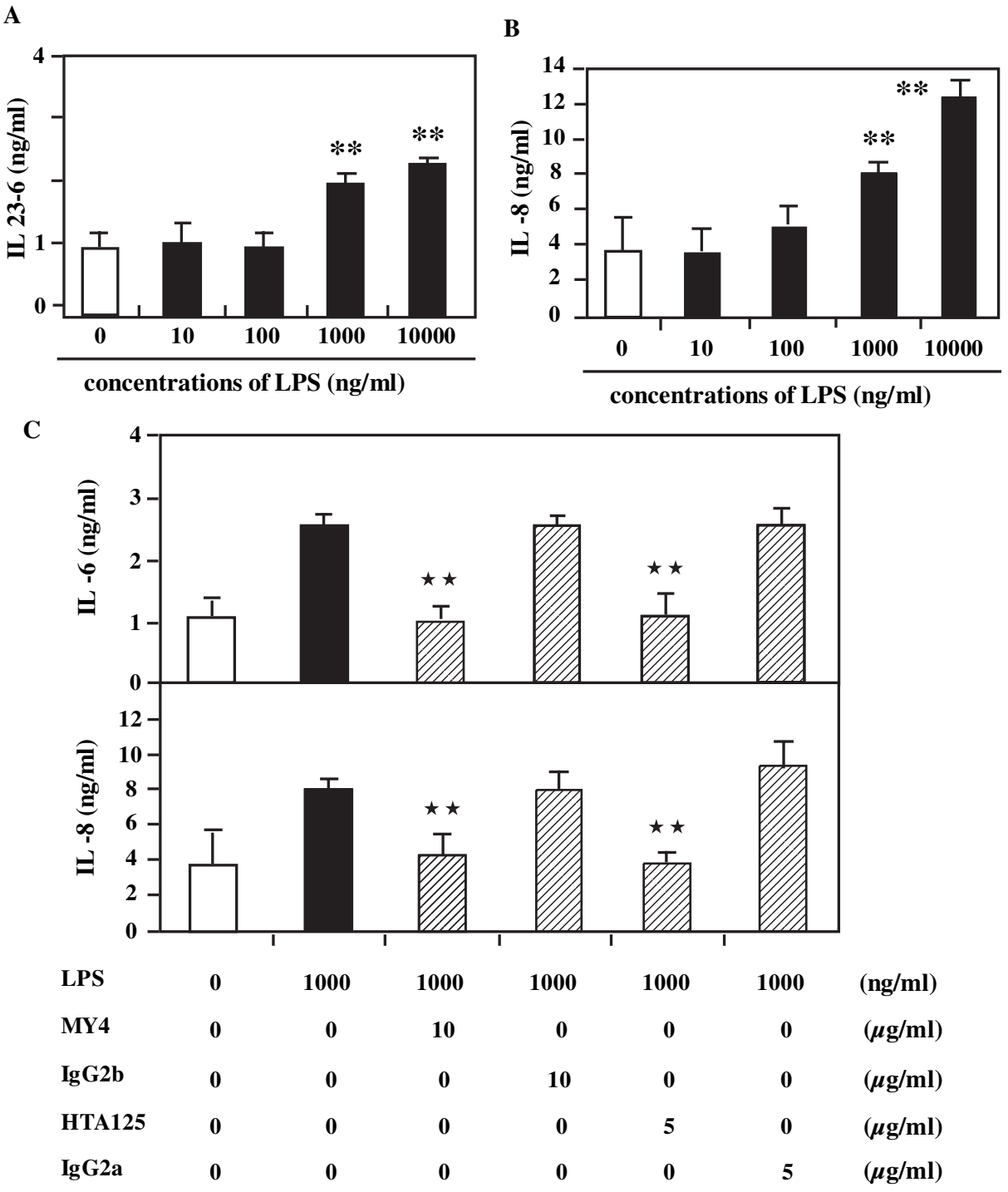
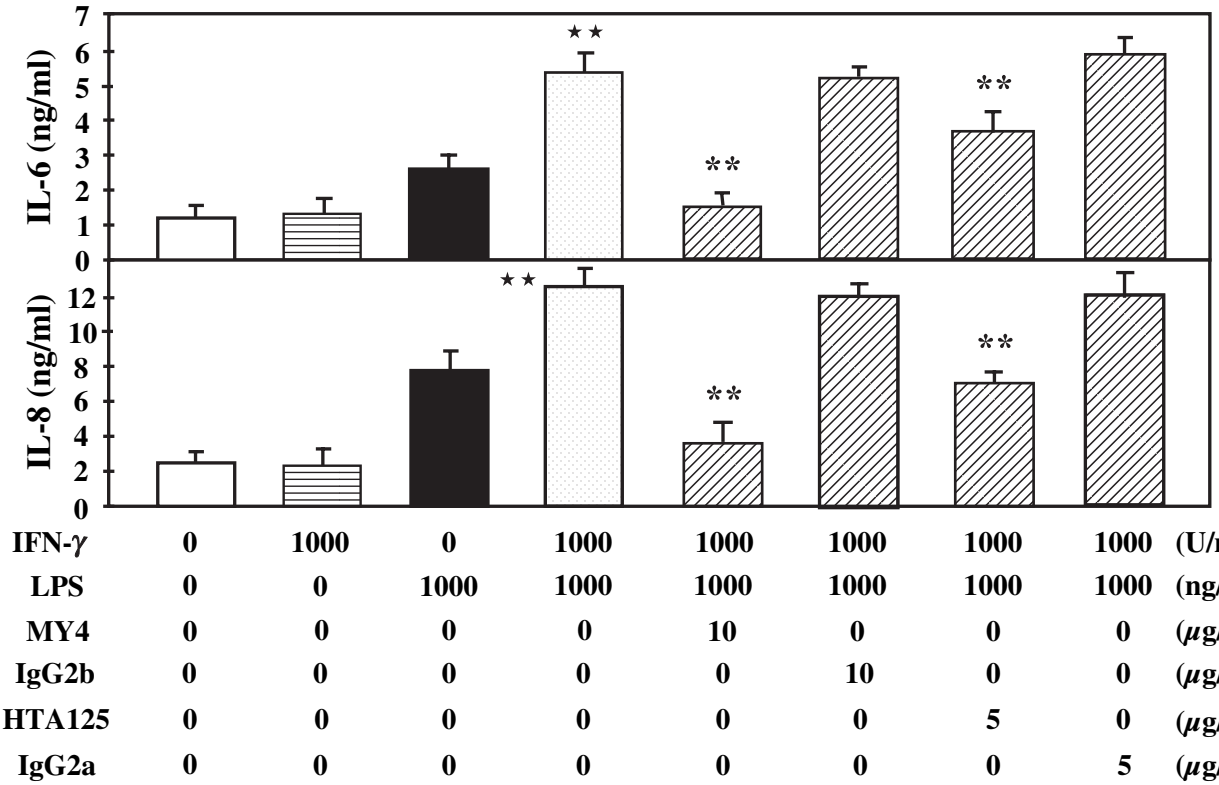


Fig. 6. Participation of CD14 and toll-like receptor 4 (TLR4) in interleukin (interleukin)-6 and interleukin-8 production stimulated with *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS) in human gingival fibroblasts. Dose-dependent effect of the lipopolysaccharide on interleukin-6 (A) and interleukin-8 (B) production in human gingival fibroblasts. Each of the human gingival fibroblast cultures, HGF-1, -2 and -3, at confluence was treated for 24 h with 0–1000 ng/ml of the lipopolysaccharide. (C) Participation of CD14 and TLR4 with the lipopolysaccharide-stimulated interleukin-6 and interleukin-8 production in human gingival fibroblasts. Each of the cell cultures at confluence was either untreated or pretreated with either 10 μg/ml of MY4, mouse IgG2b (10 μg/ml), 5 μg/ml of HTA125, or mouse IgG2a (5 μg/ml) for 30 min, and subsequently treated for 24 h with 1000 ng/ml of the lipopolysaccharide. After each treatment, the amount of interleukin-6 or interleukin-8 secreted into the culture supernatant was measured with enzyme-linked immunosorbent assay (ELISA) kits for human interleukin-6 or interleukin-8. The values (ng/ml) shown are the means ± SD of three separate experiments, each conducted in triplicate, for each of the three human gingival fibroblast cultures. The difference from the value in untreated cells or cells treated with the lipopolysaccharide alone was significant at $p < 0.01$ (**) or $p < 0.01$ (★★), respectively.



IFN-γ	**0**	**1000**	**0**	**1000**	**1000**	**1000**	**1000**	**1000**	**(U/ml)**
LPS	**0**	**0**	**1000**	**1000**	**1000**	**1000**	**1000**	**1000**	**(ng/ml)**
MY4	**0**	**0**	**0**	**0**	**10**	**0**	**0**	**0**	**(μg/ml)**
IgG2b	**0**	**0**	**0**	**0**	**0**	**10**	**0**	**0**	**(μg/ml)**
HTA125	**0**	**0**	**0**	**0**	**0**	**0**	**5**	**0**	**(μg/ml)**
IgG2a	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**5**	**(μg/ml)**

Fig. 7. Priming effect of γ-interferon (IFN-γ) on interleukin (interleukin)-6 and interleukin-8 production in response to *Actinobacillus actinomycetemcomitans* lipopolysaccharide (LPS) in human gingival fibroblasts. Each of the human gingival fibroblast cultures, HGF-1, -2 and -3, at confluence was either pretreated for 72 h with 1000 U/ml of recombinant human γ-interferon (rhIFN-γ) or untreated, then pre-incubated for the last 30 min either with MY4, mouse IgG2b, HTA125 or mouse IgG2a or without any additions, and subsequently treated for 24 h with 1000 ng/ml of the lipopolysaccharide. After each treatment, the amount of interleukin-6 or interleukin-8 secreted into the culture supernatant was measured with enzyme-linked immunosorbent assay (ELISA) kits for human interleukin-6 or interleukin-8. The values (ng/ml) shown are the means ± SD of three separate experiments, each conducted in triplicate, for each of the three human gingival fibroblast cultures. The difference from the value in the cells treated with the lipopolysaccharide alone or the cells treated with rhIFN-γ and the lipopolysaccharide was significant at $p < 0.01$ (★★) or $p < 0.01$ (**), respectively.

such as interleukin-6, interleukin-8 and MCP-1, in response to lipopolysaccharide from *P. gingivalis* and enterobacterial species *via* CD14 (22–26). Accordingly, these data emphasize the predominant role of CD14 in lipopolysaccharide-induced inflammatory cytokines in human gingival fibroblasts. Recent studies strongly suggest TLR4 as the primary lipopolysaccharide signaling receptor (15–20); however, TLR2 is also implicated in the lipopolysaccharide signaling. Through transfection studies and the use of anti-TLR4 and anti-TLR2 antibodies, it is suggested that the contribution of each TLR to lipopolysaccharide-induced inflammatory responses is dependent upon the cell type used and the source of the lipopolysaccharide. Actually, in contrast to the predominant role of TLR4 in signaling of enterobacterial lipopolysaccharide, some previous reports revealed that the relative contribution of TLR2 vs. TLR4 in lipopolysaccharide-responsiveness might be different among each periodontopathic bacterial lipopolysaccharide (46–52). In the present study, *A. actinomycetemcomitans* lipopolysaccharide-induced interleukin-6 and interleukin-8 productions are also significantly inhibited by pretreatment with the anti-TLR4 antibody HTA125 in the human gingival fibroblasts. This result suggests that the TLR4-mediated signal, at least, confers the production of inflammatory cytokines in response to *A. actinomycetemcomitans* lipopolysaccharide in human gingival fibroblasts. Recent studies using MyD88-deficient mice demonstrate the existence of MyD88-dependent and -independent pathways in lipopolysaccharide signaling (53, 54). Namely, lipopolysaccharide activates at least two signaling pathways to induce different subsets of genes; the MyD88-dependent pathway regulates expression of interleukin-6, interleukin-1β, TNF-α and cyclooxygenase-2, whereas the MyD88-independent pathway regulates expression of IFN-regulated genes, possibly through coordinate action of IFN regulatory factor-3 and NF-κB. Furthermore, Kawai *et al.* (54) revealed that TLR2 activates NF-κB and MAP kinases through only the MyD88-dependent pathway, whereas TLR4 activates these kinases through both the MyD88-dependent and MyD88-independent pathways. However, for the present, the nature and

role of the MyD88-independent pathway are not well understood. Accordingly, we think that MyD88 may also mediate the *A. actinomycetemcomitans* lipopolysaccharide-induced interleukin-6 and interleukin-8 production in human gingival fibroblasts.

As pretreatment with IFN-γ enhanced the subsequent *A. actinomycetemcomitans* lipopolysaccharide-induced activation of MAP kinases and IκBα in human gingival fibroblasts, the IFN-γ-priming might augment terminal interleukin-6 and interleukin-8 production in response to the lipopolysaccharide. As expected, the IFN-γ-pretreatment significantly enhanced the subsequent *A. actinomycetemcomitans* lipopolysaccharide-induced interleukin-6 and interleukin-8 productions in the human gingival fibroblasts. The present study also shows that the lipopolysaccharide-stimulated interleukin-6 and interleukin-8 production in the IFN-γ-pretreated human gingival fibroblasts are completely inhibited to almost control levels by the pretreatment with MY4, whereas the HTA125-pretreatment partially inhibits it. As mentioned above, IFN-γ stimulates expression of mCD14 and MyD88, but not TLR4, in the human gingival fibroblasts. However, participation of endogenous sCD14 with the enhancement by IFN-γ of the *A. actinomycetemcomitans* lipopolysaccharide-induced interleukin-6 and interleukin-8 production is deniable, because previous reports demonstrated that sCD14 and lipopolysaccharide binding protein might also participate with lipopolysaccharide-responsiveness in human gingival fibroblasts (45) and that the release of sCD14 in high-CD14-expressing human gingival fibroblasts was enhanced by IFN-γ in accordance with the increased expression of mCD14 (24). Therefore, the augmentation by IFN-γ-pretreatment of the lipopolysaccharide-stimulated interleukin-6 and interleukin-8 production may be partially due to the up-regulation by IFN-γ of CD14 and MyD88 expression in human gingival fibroblasts.

In conclusion, the present data propose that IFN-γ stimulates expression of mCD14 in human gingival fibroblasts, whereas interleukin-1β and TNF-α do not. IFN-γ also enhances the expression of MyD88, but not TLR4, in the cells. Furthermore, IFN-γ-pretreatment augments the following responsiveness to *A. actinomycetemcomitans* lipopolysaccharide, such as activation of MAP kinases and IκBα and terminal cytokine production in the cells. This suggests that the augmentation by IFN-γ of the lipopolysaccharide-responsiveness in human gingival fibroblasts may be partially mediated by up-regulation of CD14 and MyD88 expression. These results also lead to a possibility that, in inflamed gingiva, human gingival fibroblasts become more sensitive to periodontopathic bacterial lipopolysaccharide because the cells are stimulated to express lipopolysaccharide-signaling-related molecules such as CD14 and MyD88 by IFN-γ, which is mainly secreted from activated epithelial cells and Th1 cells.

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