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Porphyromonas gingivalis lipopolysaccharide induces tumor necrosis factor-α and interleukin-6 secretion, and CCL25 gene expression, in mouse primary gingival cell lines: interleukin-6-driven activation of CCL2

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Background and Objective: Porphyromonas gingivalis infection is strongly associated with periodontitis. Although *P. gingivalis* is known to elicit a strong inflammatory response, details of that remain fragmentary. To understand the local response to *P. gingivalis*, primary cell lines derived from mouse gingival tissues were exposed to *P. gingivalis* or *Escherichia coli* lipopolysaccharide, and the production of interleukin-6 and tumor necrosis factor-α was measured. CCL25 gene expression was measured by real-time polymerase chain reaction. Cells stimulated with combinations of interleukin-6, soluble interleukin-6 receptor and/or soluble gp130 were assayed for CCL2 and tumor necrosis factor-α secretion.

Material and Methods: Primary cell lines were generated from mouse gingival tissues. Enzyme-linked immunosorbent assays were used to determine cytokine levels, and real-time polymerase chain reaction was used to quantify CCL25 gene expression.

Results: Exposure to *P. gingivalis* lipopolysaccharide but not to *E. coli* lipopolysaccharide resulted in significantly elevated levels of both interleukin-6 and tumor necrosis factor-α, and stimulation with *P. gingivalis* lipopolysaccharide also upregulated CCL25 gene expression. In one of three experiments, interleukin-6 induced CCL2 secretion, whereas interleukin-6 plus soluble interleukin-6 receptor induced CCL2 secretion in all three experiments, suggesting that both direct interleukin-6 signaling and interleukin-6 trans-signaling may be involved. However, because soluble gp130 did not inhibit trans-signaling, and because direct stimulation of gingival cells with soluble gp130 resulted in CCL2 secretion, the

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possibility exists that soluble gp130 forms binary complexes with soluble interleukin-6 receptor that promote direct interleukin-6 stimulation.

Conclusion: These findings define a pathway in which exposure of gingival cells to *P. gingivalis* induces the release of interleukin-6 and tumor necrosis factor-α; interleukin-6, in turn, induces CCL2 secretion.

Porphyromonas gingivalis is a periodontopathic bacterium of humans. To date, most studies of the interactions between gingival tissues and *P. gingivalis* have been carried out using human gingival fibroblasts. Thus, when exposed to *P. gingivalis* or *P. gingivalis* lipopolysaccharide, human gingival tissue elaborates a number of immunoregulatory and pro-inflammatory analytes, including interleukin-1, interleukin-6, interleukin-8 (1–5), prostaglandin E2 (6), tumor necrosis factor-α (7), and CXCL12 and CXCR4 (8). The effects of interleukin-6 can be inhibited by interleukin-10 (9), a cytokine with strong immunosuppressive activities. These findings point to a complex pattern of cytokine synthesis by gingival tissues and cells following infection with *P. gingivalis* or exposure to *P. gingivalis* lipopolysaccharide.

Interleukin-6 is an inductive signal for the synthesis of several chemokines, including CCL2 (10–12), also referred to as monocyte chemoattractant protein-1. The induction of CCL2 secretion can occur through several mechanisms. Direct stimulation of interleukin-6 receptor-bearing cells results in the incorporation of the interleukin-6/interleukin-6 receptor complex into the membrane homodimeric gp130 unit, which then delivers an intracellular signal for activation (13,14). Alternatively, free interleukin-6 combined with soluble interleukin-6 receptor can bind directly to membrane gp130, thereby stimulating an interleukin-6-mediated response. The latter mechanism, referred to as trans-signaling, is important for interleukin-6 activation of nonhematopoietic cells that do not express surface interleukin-6 receptor.

CCL25, a CC chemokine with strong chemotactic activity, is selectively expressed in the thymus, the intestinal epithelium and the oral mucosa (15–17). CCL25 may also be used in the development of pockets of T cells in the intestine (18). Additionally, studies in our laboratory have demonstrated that the introduction of replicating antigen (influenza virus) or nonreplicating antigen (hen-egg lysozyme) induces high expression of CCL25 in the oral mucosa of mice (15). The extent to which CCL25 is associated with the host response to *P. gingivalis* in gingival tissues has yet to be explored.

The goal of the present study was twofold. First, we wished to determine whether *P. gingivalis* lipopolysaccharide would elicit CCL25 synthesis in primary mouse gingival cells. If so, this would provide evidence that the host response to immunogenic *P. gingivalis* lipopolysaccharide in gingival tissues may contribute to the early, and possibly also to the sustained, inflammatory immune response. Second, given that interleukin-6 is known for its strong pro-inflammatory activity, we were interested in determining what effects interleukin-6 has on CCL2 secretion from gingival cells, and to do so in the context of direct interleukin-6 signaling and interleukin-6 trans-signaling.

As described here, within 24 h of exposure of mouse gingival cell lines to *P. gingivalis* lipopolysaccharide, but not to *E. coli* lipopolysaccharide, there was a significant increase in two important pro-inflammatory cytokines, namely interleukin-6 and tumor necrosis factor-α. Stimulation with *Porphyromonas gingivalis* lipopolysaccharide increased CCL25 gene expression. Gingival cells exposed to interleukin-6 plus soluble interleukin-6 receptor secreted CCL2 but not tumor necrosis factor-α. Interestingly, in the presence of soluble gp130 alone, mouse gingival cells also induced CCL2 secretion. These findings define an inflammatory response cascade initiated by the exposure of gingival cells to *P. gingivalis* lipopolysaccharide, involving interleukin-6, tumor necrosis factor-α, CCL2 and CCL25.

Material and methods

Animals and reagents

Adult female 6–16-wk-old C57BL/6 mice were purchased from Harlan (Indianapolis, IN, USA). Animals were used according to protocols approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at Houston (TX, USA). Dulbecco's modified Eagle's medium and Dulbecco's phosphate-buffered saline were purchased from Sigma-Aldrich (St Louis, MO, USA). Dulbecco's modified Eagle's medium was supplemented with fetal bovine serum (10%, v/v) (Invitrogen, Carlsbad, CA, USA), 100 U/mL of penicillin–streptomycin, 2 mM L-glutamine and 5×10^{-5} M 2-mercaptoethanol (2-ME) (Sigma-Aldrich). The reagents used in this study included ultrapure *P. gingivalis* lipopolysaccharide (Invitrogen) and *E. coli* lipopolysaccharide (Sigma); recombinant interleukin-6 (< 0.01 ng of endotoxin per μg of cytokine) (eBioscience, San Diego, CA, USA); soluble interleukin-6 receptor (< 0.0 endotoxin per 1 μg of cytokine receptor) (R&D Systems, Minneapolis, MN, USA); and two preparations of soluble gp130: recombinant human soluble gp130 (< 1.0 endotoxin per 1 μg of receptor) (R&D Systems) and recombinant mouse soluble gp130/Fc chimera (< 1.0 ng of endotoxin per 1 μg of receptor) (R&D systems).

The G8.8 hybridoma cell line (19), which secretes a monoclonal antibody reactive with mouse epithelial cells, was

purchased from the Developmental Studies Hybridoma Bank of the University of Iowa (Iowa City, IA, USA). Other antibodies used included: biotinylated mouse IgG anti-rat immunoglobulin (RG7/1.30) (BD-PharMingen, San Diego, CA, USA); alexa Fluor 488-labeled anti-vimentin (S-20) serum reactive with cells of mesenchymal origin (Santa Cruz, Santa Cruz, CA, USA); anti-mouse CD16/32 Fc blocking IgG2b immunoglobulin (2.4G2), streptavidin–fluorescein isothiocyanate conjugate and streptavidin–phycoerythrin conjugate (BD-PharMingen); purified anti-mouse TLR4 (e-Bioscience); purified goat anti-gp130 (R&D Systems), biotin-labeled anti-goat serum (Vector Laboratories, Burlingame, CA, USA); and streptavidin-alaphycocyanin (e-Bioscience).

Gingival tissue recovery and culture

6–16-wk-old mice were killed by cervical dislocation and gingival tissues were dissected from below the mandibular incisors (Fig. 1) using a #15C scalpel. Gingival tissues were washed in Dulbecco's phosphate-buffered saline, minced and placed into 96-well or 24-well Costar tissue culture plates (Fisher Scientific, Pittsburg, PA, USA) in supplemented Dulbecco's modified Eagle's medium and allowed to adhere for 2–3 h in an incubator at 37°C in a 10% CO_2 environment. Dulbecco's modified Eagle's medium, supplemented as described in the previous paragraph, was added and tissues were monitored daily for growth and were fed by replacing 25% of the medium with fresh Dulbecco's modified Eagle's medium, supplemented as described above, every 3–4 d.

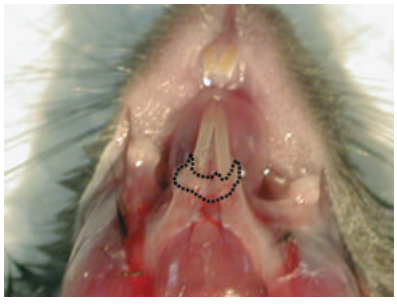

Fig. 1. Mouse gingival region used for explant to generate gingival cell lines.

Immunocytochemical and immunofluorescence staining

Gingival cells were grown in Lab-Tek chambered cover glass slides (Nalge Nunc, Naperville, IL, USA). Cell monolayers were fixed in methanol for 5 min, air dried and washed and rehydrated in phosphate-buffered saline. A 1:50 dilution of Fc blocking antibody was added for 20 min at room temperature. Primary antibodies (a 1:50 dilution of Alexa Fluor 488-labeled anti-vimentin serum, or undiluted tissue culture supernatant of G8.8 antibody) were then added and allowed to react overnight at 4°C. Biotin anti-rat IgG (at a dilution of 1:25) was added to cells stained with G8.8 antibody; after 45 min the cells were washed with phosphate-buffered saline, reacted with streptavidin–phycoerythrin conjugate for 15 min in the dark and then washed with phosphate-buffered saline. Cells were examined using a Nikon 80I microscope (Nikon, Dallas, TX, USA). Control tissues were stained with secondary antibody alone (for G8.8 staining), or with streptavidin–fluorescein isothiocyanate-488 conjugate for vimentin control staining.

Cell stimulation and enzyme-linked immunosorbent assays

Gingival monolayers were exposed for 24 h to the following: 1 μg/mL of *P. gingivalis* lipopolysaccharide; 10 ng/mL of recombinant interleukin-6; 10 ng/mL of recombinant interleukin-6 plus 50 ng/mL of soluble interleukin-6 receptor; 10 ng/mL of recombinant interleukin-6 plus 50 ng/mL of soluble interleukin-6 receptor plus 50 ng/mL of soluble gp130; 50 ng/mL of soluble gp130; or phosphate-buffered saline. The amounts of interleukin-6, tumor necrosis factor-α and CCL2 secreted were measured in cell-free supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience) and a Tricontinent Multi-Wash Advantage automated ELISA reader and washer (Molecular Devices, Berkshire, UK).

Real-time polymerase chain reaction

Gingival cell lines were rinsed with Dulbecco's phosphate-buffered saline, trypsinized, washed in supplemented Dulbecco's modified Eagle's medium and the number of cells was determined. RNA was isolated using a Qiagen (Valencia, CA, USA) RNA easy kit. RNA concentrations were estimated spectrophotometrically. One-hundred nanograms of total RNA was amplified in a MiniOpticon Real-Time polymerase chain reaction (PCR) instrument (Bio-Rad, Hercules, CA, USA) with CCL25 forward (5′-GGAAAAGGC TAGTCCACTGGAA-3′) and reverse (5′-CCTTGGACTTCATATGGTTT GACTT-3′) primers (Integrated DNA Technologies, Coralville, IA, USA), and 18s primers (Mouse 18s primer set; cat. no. SP-10003; Maxim Biotech, Inc., Rockville, MD, USA.), with a SYBR green master mix (Bio-Rad). Analysis of gene expression was performed using the $2^{-\Delta\Delta Ct}$ method of Livak & Schmittgen with Bio-Rad software (20).

Statistical analysis

Cytokine production was compared using the Student's *t*-test for unpaired observations. A *p*-value of ≤ 0.05 was considered statistically significant.

Results

Growth and maintenance of gingival cell lines

Gingival tissues were recovered from the mandibular region as shown in Fig. 1. A total of 23 lines were derived from C57BL/6 mice (Table 1). All 23 lines were maintained in culture for 1–8 wk, and twelve (MGC-3, MGC-6, MGC-7, MGC-9, MGC-10, MGC-11, MGC-15, MGC-18, MGC-19, MGC 20, MGC-21, MGC-23) were used in assays to study *P. gingivalis*-induced secretion of interleukin-6 tumor necrosis factor-α and CCL2, or CCL25 gene expression (Table 1).

Table 1. Culture and use of mouse gingival cell (MGC) lines[a]

Line	Weeks of culture	Use of line
MGC-3	6	IL-6 and TNF-α ELISA
MGC-6	3	IL-6 and TNF-α ELISA; CCL25 PCR
MGC-7	4	IL-6 and TNF-α ELISA; CCL25 PCR
MGC-9	5	CCL2 and TNF-α ELISA
MGC-10	8	CCL2; and TNF-α ELISA
MGC-11	6	TNF-α ELISA
MGC-15	6	IL-6 ELISA
MGC-18	3	CCL2 and IL-6 ELISA
MGC-19	6	immunocytochemical staining, CCL2, IL-6 and TNF-α ELISA
MGC-20	6	IL-6 and TNF-α ELISA, CCL25 PCR
MGC-21	3	Immunocytochemical staining
MGC-23	7	IL-6, TNFα and CCL2 ELISA; sp130 and TLR4 block; gp130 immunofluorescence staining

[a]Twelve of the 23 gingival cell lines were used for functional studies. The remaining 11 lines were isolated and grown, but were not employed functionally.
ELISA, enzyme-linked immunosorbent assay; IL-6, interleukin-6; PCR, polymerase chain reaction; TLR4, Toll-like receptor 4; TNF-α, tumor necrosis factor-α.

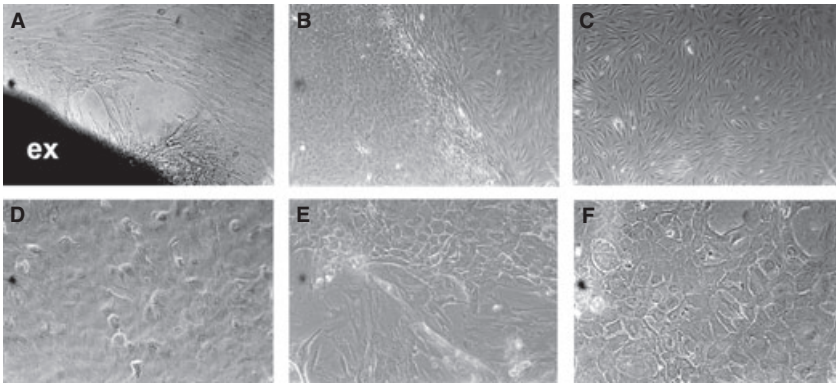

Fig. 2. Gingival cells surrounding explants (A–D) in culture plates. By 2–3 wk the cells have grown to become mostly confluent (E, F). ex, explant.

The growth of gingival cells with a spindle shape is shown in a 7-d culture near the gingival explant (Fig. 2A). By day 13 of culture, cells had spread across the tissue culture plate and consisted of spindle-shaped cells (Fig. 2B,C) that eventually gathered into rounded or cuboidal cells (Fig 2B,C). Gingival cells could be maintained for many weeks, as shown in Fig. 2E,F, which are day-24 cultures. Note the combination of spindle-shaped cells and rounded cells at that time (Fig. 2E).

To evaluate the cellular composition of the gingival lines, cells were stained with rat IgG2a G8.8 immunoglobulin that is reactive with mouse epithelial cells, or with anti-vimentin serum that is reactive with cells of mesenchymal origin, including fibroblasts. Most cell lines were heterogeneous with regard to the types of cells present. This is shown in Fig. 3, which demonstrates reactivity with both G8.8 (Fig. 3A,B) and anti-vimentin (Fig. 3D,E) serum.

P. gingivalis but not *E. coli* lipopolysaccharide stimulation of gingival cell lines promotes interleukin-6 and tumor necrosis factor-α secretion

After a minimum of 3 wk in culture, gingival cells were stimulated by the addition of *P. gingivalis* or *E. coli* lipopolysaccharide at a final concentration of 1 μg/mL. Cell-free supernatants were collected after 24 h and assayed for interleukin-6 and tumor necrosis factor-α production. As shown in Fig. 4A, low levels of interleukin-6 were produced by gingival cell lines in the absence of *P. gingivalis* lipopolysaccharide stimulation; however, there was a significant increase in interleukin-6 secretion in cultures following exposure to *P. gingivalis* lipopolysaccharide. A similar pattern also was observed for tumor necrosis factor-α (Fig. 4A), indicating that the levels of both cytokines were elevated following exposure to *P. gingivalis* lipopolysaccharide.

In contrast to the findings from *P. gingivalis* lipopolysaccharide-stimulated cells, gingival cells stimulated with *E. coli* lipopolysaccharide did not show significantly increased levels of either interleukin-6 or tumor necrosis factor-α (Fig. 4B). It is important to note that because cytokine secretion was studied in supernatants from gingival cells that had been cultured for a minimum of 3 wk in Dulbecco's modified Eagle's medium, which does not sustain the long-term growth of lymphocytes *in vitro*, the source of cytokines would be from gingival epithelial cells or fibroblasts and not from contaminating lymphocytes.

To understand the differences in stimulation by *P. gingivalis* lipopolysaccharide vs. *E. coli* lipopolysaccharide, rat IgG2a anti-Toll-like receptor 4 immunoglobulin was added to lipopolysaccharide-stimulated cultures. Because *E. coli*, but not *P. gingivalis*, lipopolysaccharide signals through Toll-like receptor 4, it was anticipated that rat IgG2a anti-Toll-like receptor 4 immunoglobulin would suppress the effect of *E. coli* stimulation of interleukin-6 but would have no such effect upon stimulation with *P. gingivalis* lipopolysaccharide. These anticipated results were observed and are presented in Fig. 4C. Note the statistically significant suppressed level of interleukin-6 secretion in *E. coli* lipopolysaccharide-stimulated cultures and the statistically significant increase in *P. gingivalis*-stimulated cultures containing rat IgG2a anti-Toll-like recep-

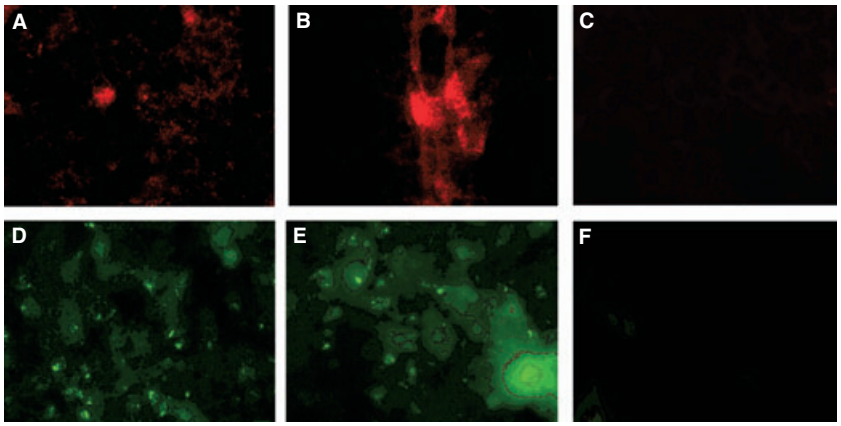

Fig. 3. Immunofluorescence staining of gingival cell lines with (A, B) anti-mouse epithelial cell rat IgG2a immunoglobulin G8.8 plus phycoerythrin-labeled anti-rat immunoglobulin, and (D, E) fluorescein isothiocyanate-labeled anti-mouse vimentin serum. In general, cell cultures were heterogeneous populations of both fibroblasts and epithelial cells. (C) Control staining consisting of phycoerythrin-labeled anti-rat serum, and (F) streptavidin–fluorescein isothiocyanate conjugate. Magnification: panels A, C, D and F, 20×; and panels B and E, 40×.

tor 4 immunoglobulin. The enhancing effect of the latter might be caused by some contaminating *E. coli* lipopolysaccharide in *P. gingivalis* lipopolysaccharide preparations such that blocking of Toll-like receptor 4 increased the availability of stimulation by *P. gingivalis* lipopolysaccharide. Regardless, these studies support the overall pattern of stimulation exerted by *P. gingivalis* lipopolysaccharide.

P. gingivalis lipopolysaccharide activates CCL25 gene expression

CCL25 is an important chemokine of leukocyte cell trafficking, the expression of which has been shown to be highly restricted to the thymus, the intestine and the oral epithelium (15–17,21). Because of the key role played by CCL25 in orchestrating the inflammatory response, experiments were carried out to determine whether gingival cells participate in the activation of leukocytes through CCL25 synthesis and to evaluate the capacity of *P. gingivalis* lipopolysaccharide to enhance CCL25 secretion. Real-time PCR analysis was used with CCL25-specific primers. Gingival cells from three cell lines were exposed to 1 μg/mL of *P. gingivalis* lipopolysaccharide or to phosphate-buffered saline for control cultures. Twenty-four hours later, cells were recovered, RNA was extracted and real-time PCR was carried out for CCL25 and the 18s housekeeping gene. Analysis of CCL25 gene expression for nonstimulated and lipopolysaccharide-stimulated cells was carried out using the $2^{-\Delta\Delta Ct}$ method of Livak & Schmittgen (20). As shown in Fig. 4D, there was a 2.3–8.4-fold increase in CCL25 RNA expression in the three cell lines following stimulation with *P. gingivalis* lipopolysaccharide compared with nonstimulated cells. These findings strongly imply that, following exposure to the lipopolysaccharide component of *P. gingivalis*, gingival tissues respond by production of the chemotactic cytokine, CCL25.

Interleukin-6 induces CCL2 secretion, but not tumor necrosis factor-α secretion, from gingival cells

Interleukin-6 has been shown to be an inductive signal for CCL2 by direct interleukin-6 receptor stimulation or interleukin-6 trans-signaling (22,23). Based on this, gingival cells were cultured for 24 h with the following: (i) recombinant interleukin-6; (ii) recombinant interleukin-6 plus soluble interleukin-6 receptor; (iii) recombinant interleukin-6 plus soluble interleukin-6 receptor plus soluble gp130; (iv) soluble gp130; or (v) phosphate-buffered saline, as described in the *Material and methods*, and CCL2 and tumor necrosis factor-α production were measured in cell-free supernatants after 24 h of culture. The results from three independent experiments are shown in Fig. 5A–C and summarized in Table 2. CCL2 secretion was elevated significantly in one of three cell cultures stimulated with interleukin-6 compared with unstimulated cultures. When cells were cultured with interleukin-6 plus soluble interleukin-6 receptor, the secreted levels of CCL2 were elevated in all three experimental groups relative to unstimulated cultures (Fig. 5A–C, Table 2). Although this might imply interleukin-6 trans-signaling, CCL2 secretion from none of these cultures was inhibited by soluble gp130 in the presence of interleukin-6 plus soluble interleukin-6 receptor compared to cultures stimulated with interleukin-6 plus soluble interleukin-6 receptor alone. Moreover, soluble gp130 alone induced CCL2 secretion from all experimental groups relative to unstimulated cells (Fig. 5A–C). The addition of a gp130 inhibitory antibody to cultures stimulated with soluble gp130 resulted in a level of CCL2 secretion that was higher than found in cultures stimulated with soluble gp130 alone, which was not a result of the stimulatory effects of the antibody (Fig. 5D).

To understand the basis of these results, some of the cells used in Fig. 5D were stained with goat IgG anti-gp130 immunoglobulin. As shown in Fig. 5E, only about one-fifth of the cells expressed surface gp130. This is consistent with the fact that the cell lines were phenotypically heterogeneous, as would be expected of primary cell cultures and as confirmed by the staining shown in Fig. 3. Hence, it is perhaps not surprising that the effects of soluble gp130 do not replicate what occurs with homogeneous cell populations.

When tumor necrosis factor-α production was assayed in the same cell-free supernatants using those same combinations of stimulation, no tumor

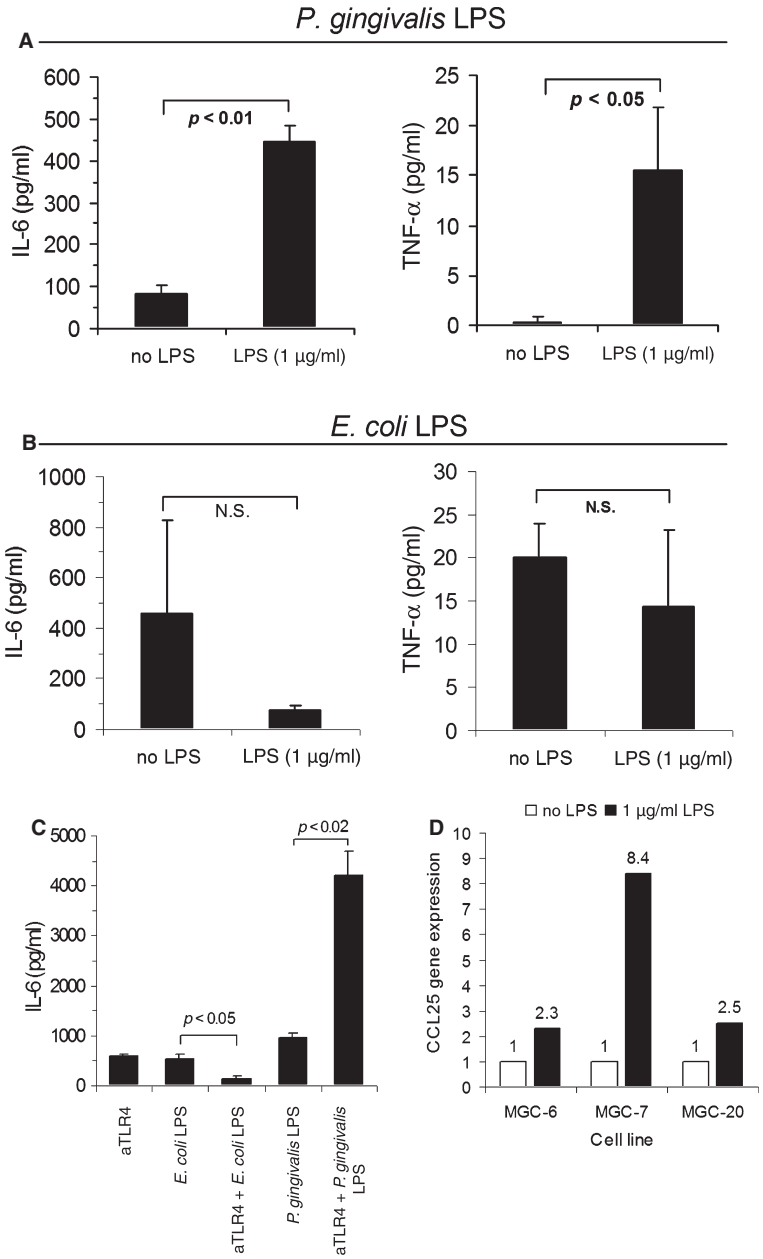

Fig. 4. (A) Interleukin-6 and tumor necrosis factor-α levels in tissue culture supernatants from unstimulated (no lipopolysaccharide) and *Porphyromonas gingivalis* lipopolysaccharide-stimulated cell lines, and (B) interleukin-6 and tumor necrosis factor-α levels in tissue culture supernatants from unstimulated (no lipopolysaccharide) and *Escherichia coli* lipopolysaccharide-stimulated cell lines. (C) Rat IgG2a anti-Toll-like receptor 4 immunoglobulin suppressed the secretion of interleukin-6 when stimulated by *E. coli* lipopolysaccharide but not by *P. gingivalis* lipopolysaccharide, implying a different signaling mechanism mediated by lipopolysaccharide from those organisms. Data are the mean values ± standard error of the mean of two to three experiments. (D) *P. gingivalis* lipopolysaccharide stimulation of three separate gingival cell lines, as indicated, resulted in a 2.3–8.4-fold increase in CCL25 RNA levels compared with unstimulated (phosphate-buffered saline) cells. Gene expression levels were determined for each sample relative to the 18s gene expression of that sample, as described in the *Material and methods*. Because all *P. gingivalis* lipopolysaccharide-stimulated cultures had greater CCL25 gene expression than unstimulated cultures, gene expression levels for unstimulated cultures were assigned a value of 1.0, and gene expression levels of stimulated cultures were expressed as fold increase over unstimulated cultures. aTLR4, anti-Toll-like receptor 4 immunoglobulin; IL-6, interleukin-6; LPS, lipopolysaccharide; MGC, mouse gingival cell; NS, not statistically significant; TNF-α, tumor necrosis factor-α.

necrosis factor-α secretion was detected (Fig. 5F). These findings indicate that interleukin-6 is a stimulatory signal for the release of CCL2 from gingival cells, which occurs through a direct pathway, rather than a trans-signaling pathway.

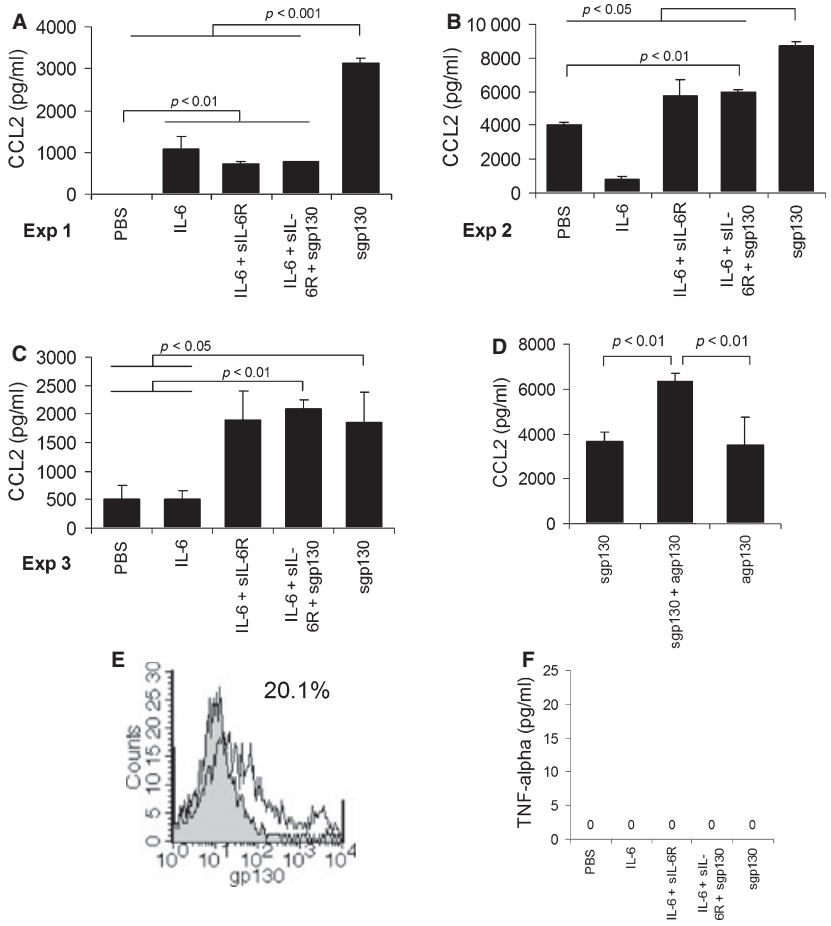

Fig. 5. (A–C) CCL2 and tumor necrosis factor-α levels in cell-free supernatants 24 h after stimulation with recombinant interleukin-6, recombinant interleukin-6 plus soluble interleukin-6 receptor, recombinant interleukin-6 plus soluble interleukin-6 receptor plus soluble gp130, soluble gp130, or phosphate-buffered saline; data were obtained from three independent sets of experiments and show an increase in CCL2 secretion from gingival cells stimulated with recombinant interleukin-6 plus soluble interleukin-6 receptor, recombinant interleukin-6 plus soluble interleukin-6 receptor plus soluble gp130 or soluble gp130; all compared with phosphate-buffered saline control cultures. (D) Neutralization of soluble gp130 by goat IgG anti-gp130 immunoglobulin caused an increase in CCL2 secretion. (E) A gingival cell line stained with soluble gp130 indicated that about one-fifth of the cells express gp130. (F) Cell supernatants used for CCL2 assays had no detectable tumor necrosis factor-α production. PBS, phosphate-buffered saline; rIL-6, recombinant interleukin-(IL-6); sIL-6r, soluble interleukin-6 receptor; sgp130, soluble gp130

Table 2. Stimulatory effects on CCL2 secretion from gingival cell lines

	Type of stimulation			
Experiment	Direct IL-6	IL-6 + sIL-6R	sgp130 blockade of IL-6 + sIL-6R	sgp130 alone
1	+[a]	+[b]	–[c]	+[d]
2	–	+	–	+
3	–	+	–	+

[a]Increase in CCL2 level above that of phosphate-buffered saline cultures.
[b]Increase in CCL2 level above that of phosphate-buffered saline cultures; possible indicator of trans-signaling.
[c]Failure to block trans-signaling, implying a lack of trans-signaling activation.
[d]Increase in CCL2 level above that of phosphate-buffered saline cultures.
IL-6, interleukin-6; sgp130, soluble gp130; sIL-6R, soluble interleukin-6 receptor.

They also demonstrate that there is specificity in this response for CCL2 because interleukin-6 stimulation did not induce tumor necrosis factor-α secretion.

Discussion

We have demonstrated that the exposure of primary cell lines, derived from mouse gingival tissues, to *P. gingivalis* lipopolysaccharide results in the synthesis of both interleukin-6 and tumor necrosis factor-α, and that recombinant interleukin-6, with or with-

out soluble interleukin-6 receptor, serves as an inductive signal for the secretion of CCL2. Because the combination of recombinant interleukin-6 plus soluble interleukin-6 receptor increased the secretion of CCL2, it appears that activation may occur by interleukin-6 trans-signaling. This, however, is contradicted by the fact that the addition of soluble gp130 to cultures with recombinant interleukin-6 plus soluble interleukin-6 receptor did not inhibit CCL2, and by experiments showing that CCL2 secretion was elevated in gingival cell cultures following direct exposure to soluble gp130. This occurred in three groups of experiments, each with multiple data entries using two different sources of soluble gp130, both of which had endotoxin levels that meet industry standards for *in vitro* tissue culture systems.

There are several potential explanations for the positive stimulatory effect of soluble gp130. Previous studies have demonstrated that secreted soluble interleukin-6 receptor can form binary complexes with soluble gp130, which, once formed, are unable to bind interleukin-6 (24). Accordingly, this would leave interleukin-6 available to bind to membrane interleukin-6 receptor, resulting in enhanced CCL2 secretion as a consequence of direct signaling. Note that even in the absence of stimulation by lipopolysaccharide, gingival cells spontaneously produced some interleukin-6 (Fig. 4), indicating the availability of interleukin-6 for direct stimulation in cultures bearing interleukin-6 receptor.

Similarly, the addition of goat IgG anti-gp130 immunoglobulin could have enhanced CCL2 secretion by blocking soluble gp130, thereby promoting interleukin-6 stimulation directly via the surface interleukin-6 receptor. Future experiments are planned to measure and quantify the levels of interleukin-6 receptor produced in gingival cell cultures. Clearly, the heterogeneity associated with primary cell lines, which is evident by the immunofluorescence staining in Fig. 3 and by the flow cytometric analysis of surface gp130 expression in Fig. 5E, adds a level of complexity to the present study. However, it was the intention of this work to use primary cells because they exhibit biological properties that more closely reflect cells as they exist under natural conditions than transformed cell lines. Nonetheless, experiments are currently underway to generate transformed cell lines of gingival cells that can be cloned to homogeneity for *in vitro* use.

An important aspect of this study was the finding that CCL25 gene expression was upregulated in gingival cells following exposure to *P. gingivalis* lipopolysaccharide. Previous work from our laboratory provided the first report of CCL25 expression in oral tissues of mice, which occurred following the introduction of foreign antigen (hen-egg lysozyme) (15) as well as in tissues undergoing repair (21). CCL25 is particularly relevant because of its restricted tissue expression that is limited to the thymus (25), the intestinal epithelium (26,27) and the oral mucosa (15,21). The findings here, that there is an increase in CCL25 expression in gingival tissues following exposure to *P. gingivalis* lipopolysaccharide, suggest that CCL25 is used as a means of leukocyte recruitment into the gingiva following *P. gingivalis* infection. Predictably, this would involve the accumulation of polymorphonuclear leukocytes and monocytes within inflamed tissues; however, a role for γδ T cells in the leukocyte recruitment process also must be considered. In the skin epidermis, for example, γδ T cells traffic into wound areas where they participate in the process of tissue healing through the elaboration of keratinocyte growth factors (28–30). Whether this also holds true for the oral mucosa remains to be determined but is feasible given that γδ T cells are present in the oral epithelia of humans and mice (31–35). Moreover, γδ T cells that utilize the Vγ3 gene (i.e. the T-cell receptor element used by γδ T cells in tissue healing in the skin epidermis) (36) are present in the oral mucosa of mice, and expression of those cells is modulated following antigen exposure (33). This follows a pattern in which there is an initial decrease in the number of $V\gamma3^+$ cells in the oral mucosa during the early phase of antigen exposure, which subsequently results in the recovery of those cells later in the antigen-driven response (33). Those observations, coupled with the findings described in the present study, open the way for a closer examination of the potential role of γδ T cells in oral tissues following *P. gingivalis* infection, and to the involvement of CCL25 in the regulation of those cells.

These findings predict a model in which the exposure of gingival cells to *P. gingivalis* lipopolysaccharide results in the release of the pro-inflammatory cytokines tumor necrosis factor-α and interleukin-6, and induces the synthesis of CCL25. Interleukin-6, in combination with soluble interleukin-6 receptor, then serves as a signal for the local production of CCL2. The presence of CCL25 and CCL2 would promote an influx of polymorphonuclear and mononuclear cells into the inflammatory site. If infection of gingival tissues with *P. gingivalis* is effectively resolved, tissue healing will occur and the inflammatory response will subside. However, the continual presence of *P. gingivalis* may contribute to a chronic inflammatory response, thereby increasing the level of tissue destruction in oral mucosa. These findings facilitate future studies aimed at understanding local chemokine responses through the control of molecular signaling pathways associated with interleukin-6 and CCL2.

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