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

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Abstract

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 *E. coli* lipopolysaccharide (LPS), and cytokine production for interleukin-6 (IL-6) and tumor necrosis factor- α (TNF α) were measured. CCL25 gene expression was measured by real-time PCR. Cells stimulated with combinations of IL-6, soluble IL-6 receptor (sIL-6R), and/or soluble gp130 (sgp130) were assayed for CCL2 and TNF α secretion.

Methods—Primary cell lines were generated from mouse gingival tissues. Enzyme-linked assays were used to determine cytokine levels, and real-time PCR was used to quantify CCL25 gene expression.

Results—Exposure to *P. gingivalis* but not *E. coli* LPS resulted in significantly elevated levels of both IL-6 and TNF α , and *P. gingivalis* LPS-stimulation also upregulated CCL25 gene expression. In one of three experiments, IL-6 induced CCL2 secretion, whereas IL-6 with sIL-6R induced CCL2 secretion in all three experiments, suggesting that both direct IL-6 signaling and IL-6 trans-signaling may be involved. However, because sgp130 did not inhibit trans-signaling, and because direct stimulation of gingival cells with sgp130 resulted in CCL2 secretion, the possibility exists that sgp130 forms binary complexes with soluble IL-6R that promote direct IL-6 stimulation.

Conclusion—These findings define a pathway in which exposure of gingival cells to *P. gingivalis* induces the release of IL-6 and TNF α ; IL-6 in turn induces CCL2 secretion.

Keywords

inflammation; oral; mucosa; periodontium; *P. gingivalis*

Introduction

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

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

CCL25, a CC chemokine with strong chemotactic activity, is selectively expressed in the thymus, the intestinal epithelium, and the oral mucosa (15-17). CCL25 also may be used in the development of pockets of T cells in the intestine (18). Additionally, studies in our laboratory demonstrate that the introduction of replicating antigen (influenza virus) or non-replicating 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 this study was two-fold. First, we wished to determine whether *P. gingivalis* LPS would elicit CCL25 synthesis in primary mouse gingival cells. If so, this would provide evidence that the host response to immunogenic *P. gingivalis* LPS in gingival tissues may contribute to the early, and possibly also to the sustained, inflammatory immune response. Second, given that IL-6 is known for its strong proinflammatory activity, we were interested in determining what effects IL-6 has on CCL2 secretion from gingival cells, and to do so in the context of direct IL-6 signaling and IL-6 trans-signaling.

As described here, within 24 hrs of exposure of mouse gingival cell lines to *P. gingivalis* but not *E. coli* LPS there was a significant increase in two important proinflammatory cytokine, IL-6 and TNF α . *P. gingivalis* LPS stimulation increased CCL25 gene expression. Gingival cells exposed to IL-6 plus sIL-6R resulted in CCL2 but not TNF α secretion. Interestingly, in the presence of sgp130 alone, mouse gingival cells also induced CCL2 secretion. These findings define an inflammatory response cascade initiated by exposure of gingival cells to *P. gingivalis* LPS, involving IL-6, TNF α , CCL2, and CCL25.

Materials and methods

Animals and reagents

Adult female 6-8 week old C57BL/6 mice were purchased from Harlan (Indianapolis, IN). 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. Dulbecco's modified Eagle's medium (DMEM) and Dulbecco's PBS (DPBS) were purchased from Sigma-Aldrich (St. Louis, MO). DMEM was supplemented with FBS (10% v/v) (Invitrogen; Carlsbad, CA),

100 U/ml penicillin-streptomycin, 2 mM L-glutamine, and 5×10^{-5} M 2-ME (Sigma-Aldrich). Reagents used in this study included ultrapure *P. gingivalis* LPS (Invitrogen; San Diego, CA) and *E. coli* LPS (Sigma); recombinant IL-6 (rIL-6) (<0.01 ng endotoxin per μ g cytokine (eBioscience; San Diego, CA); sIL-6R (<0.0 EU endotoxin per 1 μ g cytokine receptor) (R&D Systems; Minneapolis, MN); two preparations of sgp130: recombinant human sgp130 (<1.0 EU endotoxin per 1 μ g receptor) (R&D Systems), and recombinant mouse sgp130/Fc chimera (<1.0 ng endotoxin per 1 μ g 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). Other antibodies include: biotinylated mouse anti-rat antibody (RG7/1.30) (BD-PharMingen; San Diego, CA); alexa Fluor 488-labeled anti-vimentin (S-20) antibody reactive with cells of mesenchymal origin (Santa Cruz; Santa Cruz, CA); anti-mouse CD16/32 Fc blocking antibody (2.4G2), streptavidin FITC, and streptavidin PE (BD-PharMingen); purified anti-mouse TLR4 (e-Bioscience); purified goat anti-gp130 (R&D Systems; Minneapolis, MN), biotin-labeled anti-goat antibody (Vector Laboratories; Burlingame, CA); and SA-APC (e-Bioscience).

Gingival tissue recovery and culture

Following euthanization, gingival tissues were dissected from below the mandibular incisors (Fig. 1) using a #15C scalpel. Gingival tissues were washed in DPBS, minced, and placed into 96-well or 24-well Costar tissue culture plates (Fisher Scientific; Pittsburg, PA) in supplemented DMEM and allowed to adhere for 2-3 hrs in a 37°C incubator in a 10% CO₂ environment. Supplemented DMEM was added and tissues were monitored daily for growth and were fed by replacing 25% of the medium with fresh supplemented DMEM every 3-4 days.

Immunocytochemical and Immunofluorescence staining

Gingival cells were grown in Lab-Tek chambered cover glass slides (Nalge Nunc; Naperville, IL). Monolayers were fixed in methanol for 5 min, air dried, and washed and rehydrated in PBS. A 1:50 dilution of Fc blocking antibody was added for 20 min at room temperature. Primary antibodies, 1:50 dilution of Alexa Fluor 488-labeled anti-vimentin antibody, or undiluted tissue culture supernatant of G8.8 antibody, was reacted overnight at 4°C. Biotin anti-rat IgG was added for 45 min at a dilution of 1:25 to cells stained with G8.8 antibody, washed with PBS, reacted with streptavidin-PE for 15 min in dark followed by PBS washing. Cells were examined using a Nikon 80I microscope (Dallas, TX). Control tissues were stained with secondary antibody alone (for G8.8 staining), or streptavidin FITC-488 for vimentin control staining.

Cell stimulation and ELISA assays

Gingival monolayers were exposed for 24 hr to 1 μ g/ml *P. gingivalis* LPS; 10 ng/ml rIL-6; 10 ng/ml rIL-6 plus 50 ng/ml sIL-6R; 10 ng/ml rIL-6 plus 50 ng/ml sIL-6R plus 50 ng/ml sgp130; 50 ng/ml sgp130, or PBS. IL-6, TNF α , and CCL2 secretion were measured in cell-free supernatants using commercial enzyme-linked immunosorbent assay (ELISA) kits (eBioscience; San Diego, CA) and a Tricontinent Multi-Wash Advantage automated ELISA reader and washer (Molecular Devices; Berkshire, England).

Real-time PCR

Gingival cell lines were rinsed with DPBS, trypsinized, washed in supplemented DMEM, and the number of cells was determined. RNAs were isolated using a Qiagen (Valencia, CA) RNA easy kit. RNA concentrations were estimated spectrophotometrically. 100 ng of total RNA

were amplified in a MiniOpticon Real-Time PCR instrument (Bio-Rad; Hercules, CA) with CCL25 forward (5'-GGAAAAGGCTAGTCCACTGGAA-3') and reverse (5'-CCTTGGACTTCATATGGTTTGACTT-3') primers (Integrated DNA Technologies; Coralville, IA), and 18s primers (Mouse 18s primer set; Maxim Biotech, Inc., cat. no. SP-10003), with a SYBR green master mix (Bio-Rad). Analysis of gene expression was done using the $2^{-\Delta\Delta C_t}$ method of Livak and Schmittgen with Bio-Rad software (20).

Statistical analysis

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

Results

Growth and maintenance of gingival cell lines

Gingival tissues were recovered from mandibular region as shown in Fig. 1. A total of 23 lines were derived from C57BL/6 mice (Table 1). Lines were maintained in culture for 1-8 weeks, of which 8 lines (MGC-3, MGC-6, MGC-7, MGC-9, MGC-10, MGC-11, MGC-15, and MGC-18) were used for *P. gingivalis*-induced IL-6, TNF α , CCL2 assays, or CCL25 gene expression (Table 1).

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

To evaluate the cellular composition of the gingival lines, cells were stained with G8.8 antibody that is reactive with mouse epithelial cells, or with anti-vimentin antibody 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 and B) and anti-vimentin (Fig. 3 D and E) antibodies.

P. gingivalis but not *E. coli* LPS stimulation of gingival cell lines promotes IL-6 and TNF α secretion

After a minimum of 3 weeks of culture, gingival cells were stimulated by the addition of *P. gingivalis* or *E. coli* LPS at a final concentration of 1 μ g/ml. Cell-free supernatants were collected after 24 hrs and assayed for IL-6 and TNF α production. As shown in Fig. 4A, in this cell line low levels of IL-6 were produced by gingival cell lines in the absence of *P. gingivalis* LPS stimulation; however, there was a significant increase in IL-6 secretion in cultures following exposure to *P. gingivalis* LPS. A similar pattern also was observed for TNF α (Fig. 4A), indicating that both cytokine levels were elevated following *P. gingivalis* LPS exposure.

In contrast to the findings from *P. gingivalis* LPS-stimulated cells, gingival cells stimulated with *E. coli* LPS did not result in significantly increased levels of either IL-6 or TNF 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 weeks in DMEM, which does not sustain 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* LPS vs. *E. coli* LPS, anti-TLR4 antibody was added to LPS-stimulated cultures. Since *E. coli* but not *P. gingivalis* LPS signals through TLR4, it was anticipated that anti-TLR4 antibody would suppress the effect of *E. coli* stimulation of IL-6 but would have no negative effect on *P. gingivalis* LPS stimulation. These anticipated results were observed as shown in Fig. 4C. Note the statistically-significant suppressed level of IL-6 secretion in *E. coli* LPS-stimulated cultures and the statistically-significant increase in *P. gingivalis*-stimulated cultures containing anti-TLR4 antibody. The enhancing effect of the latter could have to do with some contaminating *E. coli* LPS in *P. gingivalis* LPS preparations such that blocking of TLR4 increased the availability of stimulation by *P. gingivalis* LPS. Regardless, these studies support the overall pattern of stimulation exerted by *P. gingivalis* LPS.

***P. gingivalis* LPS 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 done to determine whether gingival cells participate in the activation of leukocytes through CCL25 synthesis, and to evaluate the capacity of *P. gingivalis* LPS 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 *P. gingivalis* LPS or to PBS for control cultures. 24 hrs later, cells were recovered, RNA was extracted, and real-time PCR was done for CCL25 and the 18s housekeeping gene. Analysis of CCL25 gene expression for non-stimulated and LPS-stimulated cells was done using the 2-ΔΔCt method of Livak and 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* LPS compared to non-stimulated 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.

IL-6 induces CCL2 but not TNFα secretion from gingival cells

IL-6 has been shown to be an inductive signal for CCL2 by direct IL-6R stimulation or IL-6 trans-signaling (22,23). Based on that, gingival cells were cultured for 24 hr with: (i) rIL-6; (ii) rIL-6 plus sIL-6R; (iii) rIL-6 plus sIL-6R plus sgp130; (iv) sgp130; or (v) PBS as described in the Materials and Methods, and CCL2 and TNFα production were measured in cell-free supernatants after 24 hrs of culture. Results from three independent experiments are shown in Fig. 5A-C and summarized in Table 2. CCL2 secretion was elevated significantly in 1 of 3 cell cultures stimulated with IL-6 compared to non-stimulated cultures. When cells were cultured with IL-6 plus sIL-6R, secreted levels of CCL2 were elevated over non-stimulated cultures in all three experimental groups relative to non-stimulated cultures (Fig. 5A-C and Table 2). Although this might imply IL-6 trans-signaling, CCL2 secretion from none of those was inhibited by sgp130 in the presence of IL-6 plus sIL-6R compared to cultures stimulated with IL-6 plus sIL-6R stimulation alone. Moreover, sgp130 by itself induced CCL2 secretion from all experimental groups relative to non-stimulated cells (Fig. 5A-C). Addition of a gp130 inhibitory antibody to cultures stimulated with sgp130 resulted in CCL2 secretion that was elevated above that of cultures stimulated with sgp130 alone, which was not due to 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 anti-gp130 antibody. 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 sgp130 do not replicate what occurs with homogeneous cell populations.

When TNF α production was assayed from the same cell-free supernatants using those same combinations of stimulation, no TNF α secretion was detected (Fig. 5F). These findings indicate that IL-6 is a stimulatory signal for the release of CCL2 from gingival cells, which occurs through a direct rather than a trans-signaling pathway. They also demonstrate that there is specificity in this response for CCL2 since IL-6 stimulation did not induce TNF α secretion.

Discussion

We have demonstrated that exposure of primary cell lines derived from mouse gingival tissues to *P. gingivalis* LPS results in the synthesis of both IL-6 and TNF α , and that rIL-6 with or without sIL-6R serves as an inductive signal for the secretion of CCL2. Since the combination of rIL-6 plus sIL-6R increased CCL2 secretion, it would appear that activation may occur by IL-6 trans-signaling. This, however, is contradicted by the fact that the addition of sgp130 to cultures with rIL-6 plus sIL-6R did not inhibit CCL2, and by experiments showing that CCL2 secretion was elevated in gingival cell cultures following direct exposure to sgp130. This occurred in three groups of experiments each with multiple data entries using two different sources of sgp130, 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 sgp130. Previous studies have demonstrated that secreted soluble IL-6R can form binary complexes with sgp130 which, once formed, are unable to bind IL-6 (24). Accordingly, this would leave IL-6 available to bind to membrane IL-6R, resulting in enhanced CCL2 secretion as a consequence of direct signaling. Note that even in the absence of stimulation by LPS, gingival cells spontaneously produced some IL-6 (Fig. 4), indicating the availability of IL-6 for direct stimulation in cultures bearing IL-6R.

Similarly, the addition of anti-gp130 antibody could have enhanced CCL2 secretion by blocking soluble gp130, thereby promoting direct IL-6 stimulation via surface IL-6R. Future experiments are planned to measure and quantify secreted IL-6R levels in gingival cell cultures. Clearly, the heterogeneity associated with primary cell lines, which is evident by the immunofluorescence staining in Fig. 3 and 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 since they exhibit biological properties that more closely reflect cells as they exist under natural conditions than transformed cell lines. Nonetheless, experiments are currently under way 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* LPS. 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 tissue repair (21). CCL25 is particularly relevant due to 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* LPS would suggest that CCL25 is used as a means of leukocyte recruitment into the gingiva following *P. gingivalis* infection. Predictably, this would involve polymorphonuclear leukocytes and monocytes accumulation within inflamed tissues; however, a role for $\gamma\delta$ T cells in the leukocyte recruitment process also must be considered. In the skin epidermis, for example, $\gamma\delta$ 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 $\gamma\delta$ T cells are present in oral epithelia of humans and mice (31-35). Moreover, $\gamma\delta$ T cells that utilize the V γ 3 gene, i.e., the TCR element used by $\gamma\delta$ 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 V γ 3+ cells in the oral mucosa during the early phase of antigen exposure, which subsequently results in recovery of those cells later in the antigen-driven response (33). Those observations coupled with the findings described here open the way for a closer examination of the potential role for $\gamma\delta$ 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 exposure of gingival cells to *P. gingivalis* LPS results in the release of the proinflammatory cytokines, TNF α and IL-6, and induces the synthesis of CCL25. IL-6 in combination with sIL-6R 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 by *P. gingivalis* is effectively resolved, tissues 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 IL-6 and CCL2.

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

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

Fig. 3. Immunofluorescence staining of gingival cell lines with (A-B) anti-mouse epithelial cell antibody G8.8 plus PE-labeled anti-rat antibody, and (D-E) FITC-labeled anti-mouse vimentin antibody. In general, cell cultures were heterogeneous populations of both fibroblast and epithelial cells. (C) Control staining consisting of PE-labeled anti-rat antibody, and (F) streptavidin FITC. Magnification: A, C, D, and F, 20x; B and E, 40x.

Fig. 4.

(A) IL-6 and TNF α levels in tissue culture supernatants from unstimulated (no LPS) and *P. gingivalis* LPS-stimulated cell lines, and (B) unstimulated (no LPS) and *E. coli* LPS-stimulated cell lines. (C) Anti-TLR4 antibody suppressed the secretion of IL-6 when stimulated by *E. coli* but not *P. gingivalis* LPS, implying different signaling mechanism mediated by LPS from those organisms. Data are mean values $\pm$ SEM of 2-3 experiments. (D) *P. gingivalis* LPS stimulation of three separate gingival cell lines, as indicated, resulted in a 2.3 – 8.4 fold increase in CCL25 RNA levels compared to non-stimulated (PBS) cells. Gene expression levels were determined for each sample relative to the 18s gene expression of that sample as described in the Materials and methods. Since all *P. gingivalis* LPS stimulated cultures had greater CCL25

gene expression than non-stimulated cultures, gene expression levels for non-stimulated cultures were assigned a value of 1.0 and gene expression levels of stimulated cultures were expressed as fold increase over non-stimulated cultures.

Fig. 5.

(A-C) CCL2 and TNF α levels in cell-free supernatants 24 hr after stimulation with rIL-6, rIL-6 plus sIL-6R, rIL-6 plus sIL-6R plus sgp130, sgp130, or PBS; data from 3 independent sets of experiments showing an increase in CCL2 secretion from gingival cells stimulated with rIL-6 plus sIL-6R; rIL-6 plus sIL-6R plus sgp130; or sgp130 compared to PBS control cultures. (D) Neutralization of sgp130 by anti-gp130 antibody causes an increase in CCL2 secretion. (E) A gingival cell line stained with sgp130 indicated that about one-fifth of the cells express gp130. (F) Cell supernatants used for CCL2 assays had no detectable TNF α production.

Table 1Culture 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, TNF α , and CCL2 ELISA; sp130 and TLR4 block; gp130 immunofluorescence staining

^a 11 of a total of the 23 gingival cell lines that were used for functional studies. An additional 12 lines were isolated and grown, but were not employed functionally.

Table 2

Stimulatory effects on CCL2 secretion from gingival cell lines

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

^a Increase in CCL2 level above PBS cultures.^b Increase in CCL2 level above PBS 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 PBS cultures.