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Porphyromonas gingivalis Selectively Up-Regulates the HIV-1 Coreceptor CCR5 in Oral Keratinocytes

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Primary infection of oral epithelial cells by HIV-1, if it occurs, could promote systemic infection. Most primary systemic infections are associated with R5-type HIV-1 targeting the R5-specific coreceptor CCR5, which is not usually expressed on oral keratinocytes. Because coinfection with other microbes has been suggested to modulate cellular infection by HIV-1, we hypothesized that oral keratinocytes may up-regulate CCR5 in response to the oral endogenous pathogen Porphyromonas gingivalis by cysteine-protease (gingipains) activation of the protease-activated receptors (PARs) or LPS signaling through the TLRs. The OKF6/TERT-2-immortalized normal human oral keratinocyte line expressed CXCR4, whereas CCR5 was not detectable. When exposed to P. gingivalis ATCC 33277, TERT-2 cells induced greater time-dependent expression of CCR5-specific mRNA and surface coreceptors than CXCR4. By comparing arg- (Rgp) and lys-gingipain (Kgp) mutants, a mutant deficient in both proteases, and the action of trypsin, P. gingivalis Rgp was strongly suggested to cleave PAR-1 and PAR-2 to up-regulate CCR5. CCR5 was also slightly up-regulated by an isogenic gingipain-deficient mutant, suggesting the presence of a nongingipain-mediated mechanism. Purified P. gingivalis LPS also up-regulated CCR5. Blocking TLR2 and TLR4 receptors with Abs attenuated induction of CCR5, suggesting LPS signaling through TLRs. P. gingivalis, therefore, selectively up-regulated CCR5 by two independent signaling pathways, Rgp acting on PAR-1 and PAR-2, and LPS on TLR2 and TLR4. By inducing CCR5 expression, P. gingivalis coinfection could promote selective R5-type HIV-1 infection of oral keratinocytes. The Journal of Immunology, 2007, 179: 2542–2550.
Genes involved in the inflammatory response, such as cytokines and chemokines (30), are regulated through TLR activation. TLR signaling results from ligation of bacterial pathogen-associated molecular patterns (31, 32), structural groups of macromolecules including LPS. *P. gingivalis* LPS binds TLRs on oral keratinocytes and could represent another mechanism to enhance chemokine receptor expression. By signaling through PARs and TLRs, therefore, *P. gingivalis* was hypothesized to up-regulate the expression of CCR5 by oral keratinocytes. If this hypothesis is valid, *P. gingivalis* in biofilms proximal to mucosal epithelium could regulate the chemokine receptor gatekeeper to enable oral keratinocytes to selectively capture and transcytose R5-type HIV-1. We now show that *P. gingivalis* selectively up-regulates CCR5 and, albeit weakly, CXCR4. Furthermore, *P. gingivalis* up-regulation of CCR5 is mediated by the action of *P. gingivalis* gingipains on oral keratinocyte PARs and of LPS on TLRs.

**Materials and Methods**

**Cells**

Immortalized human oral keratinocytes OKF6/TERT-2 (TERT-2) were provided by J. Rheinwald (Harvard Medical School, Cambridge, MA) (33). Cells were grown in T-75 flasks in keratinocyte serum-free and calcium-free medium (Invitrogen) supplemented with 0.4 mM CaCl₂, 25 μg/ml bovine pituitary extract, and 0.2 mg/ml epidermal growth factor in 5% CO₂ at 37°C. Cells were grown to 70–80% confluence, changing medium every 3 days. Control cell lines were HeLa (CXCR4 positive, CCR5 negative; obtained from the American Type Culture Collection) and TZM-bl (HeLa transfected to express surface CD4 and CCR5; obtained from the National Institutes of Health AIDS Research and Reference Reagent Program) (34). Both cell lines were cultured in DMEM (Mediatech) supplemented with 10% (v/v) FBS (Mediatech).

**Bacteria**

*P. gingivalis* strain ATCC 33277 and a panel of isogenic mutants that fail to express Kgp (KDP 129; Δkgp), Rgp (KDP 133; ΔrgpA ΔrgpB), or both (KDP 136; Δkgp ΔrgpA ΔrgpB) (gifts from K. Nakayama, Nagasaki University, Nagasaki, Japan) were grown under anaerobic conditions in a Coy anaerobic chamber (85% N₂, 5% CO₂, and 10% H₂) at 37°C on Todd-Hewitt agar plates (Difco) or Todd-Hewitt broth. Agar and broth were supplemented with 5 μg/ml hemin (Sigma-Aldrich) and 1 μg/ml menadione (Sigma-Aldrich). Agar plates were also supplemented with 5% (v/v) defibrinated sheep blood. Bacteria were grown to early stationary phase (OD₆₀₀nm of 0.9–1.1). *Streptococcus gordonii* DL1 (Challis) was grown in Todd-Hewitt broth under aerobic conditions at 37°C in 5% CO₂. When cultured TERT-2 cells were 70–80% confluent, the medium was removed, replaced with prewarmed serum-free MEM (Mediatech), and freshly harvested bacteria were added at 37°C in spent culture medium to a final volume of 10 ml at a multiplicity of infection (MOI) of 1:1, 10:1, 100:1, and 10,000:1. After *P. gingivalis* inoculation, cells were maintained at 37°C in 5% CO₂ for various times. Cells not exposed to bacteria or treated with other compounds were kept in identical medium and conditions.

**Immunofluorescence**

Cells (2 × 10⁴) were seeded on coverslips in 12-well plates and cultured to 70% confluency at 37°C in 5% CO₂. After inoculating with *P. gingivalis*, cells were washed three times with PBS and fixed in 4% (v/v) paraformaldehyde. Fixed cells were blocked with 3% (v/v) BSA for 30 min at room temperature, incubated with 80 μl of a 1/100 dilution of CD184 (CXCR4/Fusin) or CD195 (CCR5) murine anti-human IgG2a mAb (BD Biosciences) for 1 h, washed five times, and then incubated in the dark with Alexa Fluor 568-conjugated goat anti-mouse IgG Ab (Molecular Probes) diluted 1/2000 and 4′,6-diamidino-2-phenylindole (DAPI) (Molecular Probes) at 4°C overnight. Cells were washed three times with PBS and detached from the vessel using 0.2% (v/v) EDTA. No trypsin was used. Cells were counted, fixed in 4% (v/v) paraformaldehyde for 10 min at 4°C, and incubated with murine mAbs against CD184 (CXCR4/Fusin) or CD195 (CCR5) (BD Biosciences) for 30 min at 4°C. Cells were washed three times with PBS supplemented with 2% (v/v) FBS and then incubated in the dark for 30 min with goat anti-mouse IgG secondary Ab conjugated with PE (Jackson ImmunoResearch Laboratories). Murine IgG2a (BD Biosciences) was used as an isotype control. HeLa and TZM-bl cells were used as positive cell controls for coreceptor expression. Coreceptor expression was analyzed using a FACScan (BD Biosciences) with CellQuest software. All experiments were performed in triplicate.

**RT-PCR**

Total RNA was extracted from oral keratinocytes using TRIzol (Invitrogen) according to the manufacturer’s instructions. RNA (1 μg) was reverse transcribed using the iScript cDNA Synthesis Kit (Bio-Rad) at 25°C for 5 min, followed by 30 min at 42°C and then 5 min at 85°C. Primers for human CXCR4 mRNA were: 5′-CCACGGGTATACCTGAGG-3′ (forward) and 5′-TGTGGTGCCGCTGGACGATG-3′ (reverse) (44); human CCR5, 5′-CTCCGCCTCTACTACCTGGTGT-3′ (forward) (36) and 5′-CCTGTCGCTCTTCCTCTCTCGGAG-3′ (reverse) (37) (51 bp); human GAPDH, 5′-GACCCCCCTTACACTTCCAAC-3′ (forward) (38) and 5′-AGGCTCCTTCATGGTTGAAC-3′ (reverse) (220 bp). RT-PCR was used to amplify each primer, 10 mM dNTP mix, 1.5 mM MgCl₂, and 0.25 μl of Taq DNA polymerase (Promega). Conditions for RT-PCR were as follows: initial denaturation at 95°C for 2 min, followed by 30 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s. RNA extraction and each RT-PCR were performed twice. For each sample, a control reaction was performed without reverse transcriptase and products, if any, were resolved on a 2% (w/v) agarose gel.

**Western blotting**

TERT-2 cells growing in monolayers were lysed using a buffer containing 5 M NaCl, 0.5% (v/v) Triton X-100, 0.6% (v/v) SDS, 1 M Tris-HCl (pH 8), 1% (v/v) sodium deoxycholate, 1 M NaF, 10 mM NaVO₃, 100 mM PMSF, 1 mg/ml pepstatin, 0.1 mg/ml aprotinin, and 0.25 mg/ml leupeptin, and were clarified by centrifugation. Proteins were quantified by BCA protein assays (Pierce). Proteins (35 μg) were then resolved by 10% SDS-PAGE, transferred to nitrocellulose membranes at 120 V for 90 min, and blocked with 10% (v/v) dry milk in 10 mM Tris-HCl, 100 mM NaCl (pH 7.6)-Tween 20. Membranes were then incubated with rabbit polyclonal anti-human CCR5 (CD195; 1/250 dilution) Abs (eBioscience) at 4°C overnight plus 1 h at room temperature. To detect primary Abs bound to specific Ags, membranes were incubated with goat anti-rabbit IgG conjugated with HRP (1/2500 dilution; Santa Cruz Biotechnology). Proteins were visualized by ECL (Amersham Biosciences).

**LPS and trypsin treatment**

TERT-2 cells were incubated for 30 min in serum-free MEM (Mediatech) at 37°C in 5% CO₂ with purified *P. gingivalis* LPS (10 μg/ml final concentration; gift from R. Darveau, University of Washington, Seattle, WA), purified *Escherichia coli* 055:B5 LPS (10 μg/ml; Sigma-Aldrich), or N-tosyl-l-phenylalanine chloromethyl ketone/trypsin (100 nM; Sigma-Aldrich) for 1 h. After incubation, cells were washed three times and processed for the corresponding assay. Using TERT-2 cells, we confirmed the report of Lourbakos et al. (26) showing that thrombin cleaves PAR-1, whereas trypsin cleaves PAR-1 and PAR-2 (data not shown).

**TLR and PAR blocking**

TERT-2 cells were incubated in serum-free MEM for 1 h at 37°C in 5% CO₂ with 0.2–2 μg/ml rabbit polyclonal anti-human THR2 (H-175), rabbit polyclonal anti-human TLR4 (H-80), mouse monoclonal anti-thrombin receptor (ATAP2), or mouse monoclonal anti-PAR-2 (SAM11) Abs (all from Santa Cruz Biotechnology). Cells were then washed five times with PBS and then inoculated with *P. gingivalis* ATCC 33277 at a MOI of 100:1. Total RNA was extracted at the indicated times of incubation and used as template for RT-PCR.

**Statistical analyses**

Statistical analyses were performed by the Student’s t test for paired values using GraphPad software. Data were considered significant at a p < 0.05.
Results

Human oral keratinocytes express CXCR4 but not CCR5

TERT-2 cells under normal tissue culture conditions expressed CXCR4 as shown by flow cytometry (Fig. 1A) and immunofluorescence (Fig. 1D). TERT-2 cells expressed less CXCR4 than HeLa (Fig. 1, B and F) or TZM-bl cells (Fig. 1, C and H). TERT-2 (Fig. 1, A and E) and HeLa cells (Fig. 1, C and H) appeared to express less CCR5 than TZM-bl cells (Fig. 1, B and G).

FIGURE 1. CXCR4 and CCR5 expression in TERT-2 cells under normal tissue culture conditions. Cells were grown as described in Materials and Methods. CXCR4 and CCR5 expression was analyzed by flow cytometry in TERT-2 (A), HeLa (B), and TZM-bl (C) cells. Using immunofluorescence microscopy, TERT-2 cells were analyzed for expression of CXCR4 (D) and CCR5 (E), HeLa cells for expression of CXCR4 (F) and CCR5 (G) and TZM-bl cells for CXCR4 (H) and CCR5 (I). For both assays, the isotype Ab control, IgG2a, was used at the same concentration used to detect the receptors. Scale bars, 20 μm. Blue, DAPI; red, Alexa Fluor 568-conjugated IgG. The key for flow cytometry is below the panels. Images are representative of three independent experiments.

FIGURE 2. Expression of CXCR4- and CCR5-specific mRNA in TERT-2 cells after exposure to P. gingivalis wild-type strain. A, TERT-2 cells were exposed to the wild-type strain (33277) at various MOIs for 1 h. B, Cells were incubated with P. gingivalis ATCC 33277 for 1, 2, 3, and 6 h at a MOI of 100:1. A and B. After cell exposure to P. gingivalis ATCC 33277, total RNA was extracted, reversed transcribed, and the resulting cDNA was used as template for RT-PCR. HeLa and TZM-bl cells were used as positive controls for detection of CXCR4 and CCR5, respectively. The 220-bp product at the bottom is GAPDH-specific mRNA in the same cDNA sample obtained from the cells. NT, No treatment, cells without P. gingivalis. Images are representative of three independent experiments. Quantitative analysis was conducted by densitometry using Kodak 1D 3.6.1 software. Expression of CXCR4 and CCR5 was represented as the ratio of their densitometric values to their corresponding GAPDH levels. Graphs show the relative expression of the molecules in each condition as a percentage of the ratio of the positive control, HeLa or TZM-bl cells, to GAPDH. Data in B reported as the mean ± SEM of three independent experiments.
P. gingivalis selectively up-regulates the expression of CCR5-specific mRNA

To determine whether P. gingivalis increases expression of CXCR4 and CCR5, TERT-2 cells were incubated with P. gingivalis ATCC 33277 at MOIs of 1:1–10,000:1 for 1 h at 37°C. Total RNA was extracted, reverse transcribed, and amplified by PCR. P. gingivalis optimally up-regulated CCR5-specific mRNA by 2- to 4-fold (lower panel) at a MOI of 100:1 (Fig. 2A). In the absence of P. gingivalis, expression of CCR5-specific mRNA was barely detectable (Fig. 2B, lane 1). Cells were then incubated with P. gingivalis at a MOI of 100:1 for up to 6 h. In the presence of P. gingivalis, CCR5-specific mRNA (591 bp) was up-regulated in a time-dependent manner for up to 6 h (Fig. 2B). Differences in CCR5 up-regulation were statistically significant (p < 0.01). In contrast, CXCR4-specific mRNA (441 bp) was unaffected for up to 6 h (Fig. 2B). Expression of specific mRNAs for CXCR4 and CCR5 in TERT-2 cells (Fig. 5A, lane 1), wild-type cells (Fig. 5A, lane 2), KDP 133 (Fig. 5A, lane 3), and KDP 136 (Fig. 5A, lane 4) was reduced about 2- to 4-fold more than KDP 133 or KDP 136 (Fig. 5A, lower panel). The expression of specific mRNAs for CXCR4 and CCR5 in TERT-2 cells was also shown by (G) flow cytometry. Cells were incubated with P. gingivalis for 3 h (solid line), 6 h (dotted line), or untreated for up to 6 h (gray histogram). Images are representative of three independent experiments.

P. gingivalis ATCC 33277 selectively up-regulates expression of CCR5 protein

By immunofluorescence, TERT-2 cells expressed CXCR4 (Fig. 3A), but levels of CCR5 were barely detectable (Fig. 3C) when compared with isotype control Ab staining (Fig. 3E). After incubation with P. gingivalis ATCC 33277 at a MOI of 100:1 for 3 h, cells showed a greater increase in expression of CCR5 (Fig. 3D) than CXCR4 (Fig. 3B). Expression of both chemokine receptors was greater at 3 h of incubation with P. gingivalis than at earlier times (data not shown). Consistent with immunofluorescence observations, CCR5 expression was maximal from 3 to 6 h of incubation with P. gingivalis when analyzed by flow cytometry (Fig. 3G).

P. gingivalis proteases up-regulate chemokine coreceptors through the PAR pathway

TERT-2 cells were incubated with P. gingivalis KDP 136, an isogenic mutant of P. gingivalis ATCC 33277 that lacks the Kgp and Rgp proteases (Δkgp ΔrgpA ΔrgpB) (34), at a MOI of 100:1 for 1–6 h. Total RNA was extracted and CXCR4-specific and CCR5-specific mRNAs were detected by RT-PCR. At each time point, CXCR4- and CCR5-specific mRNA expression was less in response to stain KDP 136 than the wild-type strain (Fig. 4A, upper panels). Up-regulation of CCR5 appeared to exceed CXCR4 at all times up to 6 h (Fig. 4A, lower panels). These findings suggest that P. gingivalis arg- and lys-gingipains could be required to induce chemokine receptor expression.

To determine which of the proteases induced CCR5, cells were incubated separately with specific gingipain mutants of P. gingivalis, KDP 129 (Δkgp), KDP 133 (ΔrgpA ΔrgpB), and KDP 136 (Δkgp ΔrgpA ΔrgpB) (34). In comparison to wild-type strain 33277 (Fig. 4B, upper panel, lane 2), KDP 133 (lane 4) and KDP 136 (lane 5) induced CCR5 slightly. ATCC 33277 (Fig. 4B, lane 2) and KDP 129 (lane 3), however, up-regulated CCR5 similarly, about 3- to 4-fold more than KDP 133 or KDP 136 (Fig. 4B, lower panel).

If the gingipains induce up-regulation of CCR5, the PARs are likely signaling receptors. To test this possibility, cells were pre-incubated for 1 h with mAbs against PAR-1 or PAR-2 and then exposed to the wild-type strain of P. gingivalis. The up-regulation of CCR5-specific mRNA (Fig. 4C, lane 2) was reduced about 2- to 3-fold (Fig. 4C, lower panel) when either PAR receptor was blocked (lanes 3 and 4). When cells were exposed to trypsin, which activates cells through PAR-1 and PAR-2 cleavage, CCR5 was also induced (data not shown). Taken together, these data strongly suggest that the P. gingivalis protease, arg-gingipain, regulates CCR5 through PAR-1 and PAR-2 signaling. At the protein level, P. gingivalis 33277 increased the expression of CCR5 for up to 24 h (Fig. 4D). Although the response was weaker, strain KDP 136 also induced CCR5 protein expression. Since strain KDP 136 is an arg- and lys-gingipain double mutant, other signaling pathways may also up-regulate CCR5.

P. gingivalis LPS up-regulates CCR5 through the TLR2 and TLR4 pathway

Since the arg- and lys-gingipain mutant, KDP 136, also up-regulated CCR5, but less effectively than the wild-type cells (Fig. 4A), we sought to learn whether LPS could contribute. TERT-2 cells were treated with purified LPS from P. gingivalis ATCC 33277 or E. coli or whole cells of Streptococcus gordonii. The expression of specific mRNAs for CXCR4 and CCR5 was assessed by RT-PCR. When compared with untreated TERT-2 cells (Fig. 5A, lane 1), wild-type cells (lane 2), purified LPS from P. gingivalis (lane 3) or E. coli (lane 4), each up-regulated CCR5, but not CXCR4 (data not shown). In contrast, S. gordonii DL1 (Challis), a LPS-negative oral commensal, was apparently unable to up-regulate CCR5-specific mRNA in TERT-2 cells (Fig. 5A, lane 5).
Since \textit{P. gingivalis} LPS up-regulated CCR5, we sought to learn whether signaling occurred through TLR2 or TLR4 (38). Cells were incubated with dilutions of anti-TLR2 and TLR4 Abs, washed, and then incubated with the \textit{P. gingivalis} wild-type strain. When compared with CCR5 expression in untreated TERT-2 cells (Fig. 5, B and C, lane 1), \textit{P. gingivalis} cells (Fig. 5, B and C, lane 2) and purified LPS (Fig. 5, B and C, lane 3) increased expression of CCR5-specific mRNA. The increase in chemokine receptor expression was inhibited in a dose-response manner by either anti-TLR2 (Fig. 5B, lanes 4 – 6) or anti-TLR4 Abs (Fig. 5C, lanes 4 – 6). The arg- and lys-gingipain-deletion mutant KDP 136 induced a small increase in CCR5 expression relative to the no treatment control (Fig. 4B), which was reduced with anti-TLR4 (data not shown). Anti-TLR4 appeared to block the weak LPS-mediated responses to strain KDP 136. In contrast, trypsin, which mimics PAR activation by the arg-gingipains, up-regulated CCR5-specific mRNA in the presence of anti-TLR4 (data not shown). Collectively, these data suggest strongly that up-regulation of CCR5 in response to

![Graph showing CCR5 expression](http://www.jimmunol.org/)

**FIGURE 4.** \textit{P. gingivalis} gingipains up-regulate HIV-1 coreceptors through the PAR signaling pathway. A, CXCR4- and CCR5-specific mRNA expression in TERT-2 cells after exposure to the wild-type strain and the \textit{P. gingivalis} protease-deletion mutant. TERT-2 cells were either untreated (NT; lane 1) or incubated with the strains at a MOI of 100:1 for 1–6 h. Total RNA was extracted, reversed transcribed, and the resulting cDNA was used to perform RT-PCR. Upper panel, CXCR4 mRNA expression; middle panel, CCR5; and lower panel, GAPDH, the housekeeping gene from the respective samples. Image is representative of three independent experiments. B, CCR5 up-regulation by selective protease activities. Cells were untreated (lane 1), treated with \textit{P. gingivalis} ATCC 33277 (lane 2), or exposed to specific gingipain-mutants of \textit{P. gingivalis}, KDP 129 (Δkgp; lane 3), KDP 133 (ΔrgpA ΔrgpB; lane 4), and KDP 136 (Δkgp ΔrgpA ΔrgpB; lane 5) at a MOI of 100:1 for 3 h. For each condition, total RNA was extracted, reversed transcribed, and the resulting cDNA was used as template for RT-PCR. Images are representative of three independent experiments. C, Effect of PAR-1 and PAR-2 signaling on CCR5 expression. RT-PCR products of extracted RNA from untreated cells (lane 1), cells exposed to \textit{P. gingivalis} ATCC 33277 (MOI = 100:1; lane 2), and cells preincubated for 1 h with 2 μg/ml (1/100 dilution) of monoclonal anti-PAR-1 and anti-PAR-2 (lane 4) Abs and then exposed to \textit{P. gingivalis} wild-type strain for 3 h (MOI = 100:1). Images are representative of two independent experiments. D, Induction of CCR5 protein by \textit{P. gingivalis} proteases. TERT-2 cells were incubated at a MOI of 100:1 with \textit{P. gingivalis} ATCC 33277 (lanes 1 and 3) or strain KDP 136 (lanes 2 and 4) for 3 h. TERT-2 cells were then washed to remove extracellular bacteria and fresh medium was added. Cultures were maintained for an additional 21 h (lanes 3 and 4). Cell lysates were resolved by 10% SDS-PAGE and Western blotting as described in Materials and Methods. Cell lysates taken at oh (lanes 1 and 2) were used as untreated controls. β-actin was visualized as a protein loading control. For all experiments, TZM-bl cells were used as positive controls for detection of CXCR4 (A) and CCR5 (A–D). Expression of the chemokine receptors was obtained as the ratio of their densitometric values to their corresponding GAPDH levels (A–C). Graphs show the relative expression of the molecules in each condition as a percentage of the ratio of the positive control, TZM-bl cells, to GAPDH.
P. gingivalis cells was mediated independently by LPS signaling through TLR4 (or TLR2) and arg-gingipain activation of PAR-1 and PAR-2.

**Discussion**

If direct primary HIV-1 infection of the CXCR4-positive, CCR5-negative oral mucosal epithelial cells (4, 38) is a plausible event in the pathogenesis of AIDS, the predominance of CCR5-dependent R5-type primary HIV-1 infections must be explained (13, 14). Intraepithelial Langerhans cells are CCR5 positive, but unlike the vaginal mucosa, there is little evidence that these immature dendritic cells are present in the upper third of the epithelium and can sample Ag on the surface of the oral (39, 40) or oropharyngeal (tonsil) (41) mucosa. Since the oral pathogen, *Actinobacillus actinomycetemcomitans*, up-regulates the expression of CXCR4 and CCR5 in a human monocyteic leukemia cell line (36), we considered the possibility that another precedent or coinfection could up-regulate expression of CCR5 on oral keratinocytes.

P. gingivalis gingipains are proteases that are strongly associated with the host innate immune response (42), signaling through oral keratinocyte PARs (23) to up-regulate expression of cytokines, chemokines (27), and defensins (43). In addition, *P. gingivalis* LPS signals through oral keratinocyte TLR2 and 4 to regulate expression of innate immune molecules (44, 45). In this study, we show evidence that both signaling pathways functioned to selectively induce expression of CCR5 when compared with CXCR4. The *P. gingivalis* proteases activated oral keratinocytes through PAR-1 and PAR-2 and, independently, LPS ligated TLR2 and TLR4 to signal selective up-regulation of CCR5.

*P. gingivalis* up-regulated the expression of both HIV chemokine coreceptors, albeit CXCR4 less than CCR5, in a time-dependent manner (Fig. 2B). Although expression peaked at a MOI of 100:1 (*P. gingivalis* cells:keratinocytes), neither CXCR4 nor CCR5 induction was dose dependent (Fig. 2A). At higher MOIs, the decline in expression may be attributable to cytotoxicity, as higher doses present higher concentrations of proteases and LPS in the medium and promote cell detachment (46).

The *P. gingivalis* protease activity accounted for substantial induction of CCR5, since a mutant in both proteases showed reduced induction of the receptor (Fig. 4, A and D). Indeed, the Rgp-deficient mutant induced less CCR5 expression than *P. gingivalis* wild-type or the Kgp-deficient mutant, strongly suggesting the involvement of Rgp (Fig. 4B). Rgp cleaves PAR-2 (26, 47). Thus, to learn whether PARs signaled for induction of CCR5, cells were blocked with mAbs against PAR-1 or PAR-2 and exposed to *P. gingivalis*. CCR5 induction appeared to be slightly more attenuated in the presence of anti-PAR-2 than cells in response to *P. gingivalis*. Cells were untreated (lane 1), incubated with *P. gingivalis* as above (MOI = 100:1; lane 2), incubated with purified *P. gingivalis* LPS at a final concentration of 10 μg/ml for 30 min (lane 3), or preincubated with dilutions of anti-human TLR2 (B) or TLR4 (C), followed by washing and incubation with *P. gingivalis* ATCC 33277 (MOI = 100:1) for an additional hour (lanes 4–6). After cell treatments, total RNA was extracted, as described in Materials and Methods, and RT-PCR was performed. The 591-bp product is CCR5 mRNA and the 220-bp product is GAPDH-specific mRNA obtained from the same cDNA sample. TZM-bl cells were used as positive controls for detection of CCR5. Images are representative of three independent experiments. A–C, A densitometric analysis was performed to compare CCR5 mRNA expression in the different conditions as described in the legend of Fig. 2.
anti-PAR-1 (Fig. 4C). These data strongly suggest that *P. gingivalis* Rgp signals through both PAR-1 and PAR-2 to induce expression of CCR5 on oral keratinocytes.

*P. gingivalis* also induced expression of CCR5 in the absence of the major arg- and lys-gingipains (Fig. 4A). *P. gingivalis* LPS was shown to induce keratinocyte expression of CCR5 through an independent and complementary pathway, which was simulated by highly purified LPS from *P. gingivalis* and *E. coli* (Fig. 5A). Since TLR4 is the prototype receptor for LPS on eukaryotic cells (31, 48), including keratinocytes (49), the role of these receptors was studied. Although *P. gingivalis* LPS activates TLR4 and TLR2 (45), the apparent involvement of TLR2 had been attributed to contamination with small traces of lipopolysaccharides, lipopeptides, or other unknown components (44). More recently, *P. gingivalis* 1828-LP, a lipoprotein present in a *P. gingivalis* LPS preparation, was shown to activate TLR2 (50). To specifically inhibit downstream TLR signaling (51), blocking Abs were used to segregate the TLR2 and TLR4 signaling responses to *P. gingivalis*. In response to wild-type *P. gingivalis*, CCR5 induction was reduced in a dose-dependent manner by anti-TLR2 (Fig. 5B) and anti-TLR4 (Fig. 5C). Hence, *P. gingivalis* LPS appears to induce expression of CCR5 by interactions with TLR2 and TLR4.

To determine whether CCR5 induction by PAR and TLR signaling were independent, trypsin was used as a probe. Trypsin is a PAR-1, PAR-2, and PAR-4 agonist (52–54) and induces expression of CCR5 in oral keratinocytes within 30 min (data not shown). Since keratinocytes that were preincubated with TLR4 Ab and then exposed to trypsin induced CCR5, signaling through PARs appeared to function independently of the TLR pathway. When TLR4 was blocked with Abs and keratinocytes were exposed to the double-protease deletion mutant of *P. gingivalis* (KDP 136), no CCR5 signal was seen (data not shown). Since mice express PAR-2-activating proteases in tissue fluids in response to experimental infections (55, 56), other mechanisms could contribute to CCR5 up-regulation by keratinocytes in vivo secondary to incubation with *P. gingivalis*. Nonetheless, signaling through PARs and TLRs appears to account for induction of CCR5 in response to *P. gingivalis* in this system.

Although the arg- and lys-proteases are characteristic of *P. gingivalis*, the LPS molecules are ubiquitous among Gram-negative bacteria. Hence, other Gram-negative bacteria could also induce CCR5 expression on oral keratinocytes. For example, *E. coli* LPS induced CCR5 in oral keratinocytes but signaling may differ from the LPS from *P. gingivalis* (KDP 136), no CCR5 signal was seen (data not shown). Since mice express PAR-2-activating proteases in tissue fluids in response to experimental infections (55, 56), other mechanisms could contribute to CCR5 up-regulation by keratinocytes in vivo secondary to incubation with *P. gingivalis*. Nonetheless, signaling through PARs and TLRs appears to account for induction of CCR5 in response to *P. gingivalis* in this system.

Generally, the oral cells that approximate *P. gingivalis* and other biofilm-associated bacteria are keratinocytes. Upon infection and with inflammation, oral bacteria can encounter other cells as the epithelium is breached. Responses to *P. gingivalis* LPS appear to vary with target cell type. For example, monocytes, but not endothelial cells, activate p38 MAPK in response to *P. gingivalis* LPS (57). Hence, induction or up-regulation of CCR5 could be a target cell-specific response. Yet, CCR5 can be expressed in cell lineages including neurons, astrocytes, capillary endothelial cells, epithelium, vascular smooth muscle, and fibroblasts (58). Although its function is still unclear (58), CCR5 appears to serve as a receptor for the chemotactic chemokine CCL5 (RANTES) (59). In response to LPS and arg-gingipain, oral keratinocytes up-regulate chemokine receptors, including CCR5, to participate in the network of chemokine-responsive cells in innate mucosal immunity.

Since LPS from other species of Gram-negative bacteria might also induce CCR5, *P. gingivalis* arg-gingipain may activate keratinocytes to be chemokine-responsive in a uniquely effective manner. CCR5 and other chemokine receptors are expressed at higher levels by inflamed gingival cells than normal cells in situ (38), suggesting that a low cytokine receptor keratinocyte population was replaced with a higher expressing population. During inflammation, these keratinocyte receptors may contribute to CCR5-dependent autocrine and paracrine signaling pathways. For example, keratinocytes in inflammatory disorders such as oral lichen planus express RANTES/CCL5, a ligand specific for CCR5 (60), and a potential ligand in a CCR5-dependent autocrine loop. Inflammatory cells in inflamed gingiva also express the chemokines MCP-2, MIP-1α, and MIP-1β, which are also ligands for CCR5 (61) and suggestive of a potential paracrine CCR5-dependent pathway. Although the function could be speculated, oral keratinocytes respond to proximal infecting bacteria by expressing receptors that could enable participation in autocrine- and paracrine-regulated local immunity.

HIV-positive individuals are at risk for severe periodontitis (62), but a few isolated cases suggest that past history of periodontal disease could be a risk factor for acquiring HIV during primary exposure (12). If our data are supported by careful ex vivo analysis of tissues, *P. gingivalis* infection of the periodontal tissues, including the gingiva approximating the teeth, may represent sites where R5-type HIV-1 could be selectively captured by oral keratinocytes. HIV-1 infection with other pathogens (63, 64) and the presence of inflammation (65) are associated with increased viral replication and propagation. Likewise, *P. gingivalis* infection and increased CCR5 expression could promote selective R5-type HIV-1 entry and infection of oral epithelial cells. Whether up-regulated CCR5 receptors in oral keratinocytes enhance the specific uptake of R5-type HIV-1 remains to be determined.

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**References**


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