Revised MS - Cellular Microbiology

Interplay between *Porphyromonas gingivalis* and EGF signaling in the regulation of CXCL14

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Running title: Regulation of CXCL14 by *P. gingivalis*

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Abbreviations: ActD, actinomycin D; BHI, brain heart infusion; CCL, chemokine (C-C motif) ligand; CXCL, chemokine (C-X-C motif) ligand; hBD, human β -defensin; HPRT, hypoxanthine guanine phosphoribosyl transferase; IL, interleukin; Kgp, Lysine-specific gingipain; PAR, protease-activated receptor; PRR, pattern recognition receptor; Rgp, Arginine-specific gingipain; TBP, TATA-box binding protein; TLCK, N- α -toysl-L-lysine chloromethyl ketone hydrochloride; TLR, Toll-like receptor.

25 **SUMMARY (200 word limit – currently 188 words)**

26 *Porphyromonas gingivalis* is a keystone pathogen in chronic periodontitis. Its
27 expression of gingipain proteases (Kgp and RgpA/B) is central to the stimulation of chronic
28 inflammation. In this study, we investigated the inflammatory response of oral epithelial cells
29 to *P. gingivalis*. The cells responded by upregulating the expression of the orphan chemokine
30 CXCL14. The stimulation of CXCL14 expression was largely triggered by the gingipain
31 proteases, and was dependent on the host protease-activated receptor PAR-3. Significantly,
32 CXCL14 expression was transcriptionally repressed in response to EGF-induced activation of
33 the MEK-ERK1/2 pathway. *P. gingivalis* overcomes the repression of CXCL14 via the
34 gingipain protease-mediated degradation of EGF. Therefore, *P. gingivalis* not only directly
35 stimulates CXCL14 expression via PAR-3, but also promotes its expression by antagonizing
36 EGF signaling. In addition to chemotactic activity, some chemokines also have antimicrobial
37 activities. CXCL14 was demonstrated to have bactericidal activity, against commensal oral
38 streptococci associated with health. Notably though, *P. gingivalis* was not susceptible to
39 killing by CXCL14, potentially because the gingipain proteases can degrade CXCL14. This
40 suggests that the stimulation of dysregulated CXCL14 expression by *P. gingivalis* may help
41 promote dysbiosis and the development of chronic periodontitis.

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43 **1. INTRODUCTION**

44 The oral epithelium is a critical protective barrier to infection by microbial pathogens.
45 The barrier function of the epithelium is maintained through continuous cycles of cell
46 proliferation and differentiation whereby basal epithelial cells periodically withdraw from the
47 cell cycle and terminally differentiate as they migrate towards the epithelium surface
48 (Presland et al., 2000). Signaling by growth factor receptors, including the EGF receptor, is
49 important for maintaining the homeostatic balance between epithelial cell proliferation and
50 differentiation (Parkar et al., 2001). Oral epithelial cells also contribute to barrier function by
51 producing antimicrobial peptides (Dale et al., 2005). For example, the human β -defensins,
52 hBD1 and hBD2, which are constitutively expressed by differentiating epithelial cells in the
53 suprabasal layers of the gingival epithelium, are active against a range of bacteria and fungi
54 (Dale et al., 2001; Ouhara et al., 2005).

55 Oral epithelial cells also mediate host defense by triggering inflammation in response
56 to microbial pathogens. This is achieved through the activation of pathogen recognition
57 receptors (PRRs), including Toll-like receptors (TLRs) and protease-activated receptors
58 (PARs) (Kinane et al., 2008; Uehara et al., 2008). Chemokines are important mediators of the
59 inflammatory responses activated by PRRs. Interleukin-8 (IL-8) and chemokine (C-X-C
60 motif) ligand 1 (CXCL1) stimulate neutrophil recruitment, while chemokine (C-C motif)
61 ligand 2 (CCL2) stimulates the recruitment of monocytes and macrophages (Graves et al.,
62 1995; Kobayashi, 2006). These innate immune cells can amplify the immune response by
63 producing additional chemokines, including CCL5 and CXCL10, which recruit adaptive
64 immune cells, such as T-lymphocytes (Appay et al., 2001; Groom et al., 2011). Importantly,
65 the expression of chemokines by epithelial cells is also regulated by other receptors,
66 including the EGF receptor (Liu et al., 2008; Mascia et al., 2003). Accordingly, multiple
67 receptor signaling pathways contribute to the coordinated expression of chemokines by

epithelial cells.

In addition to stimulating the recruitment of immune cells, some chemokines also have direct antimicrobial activities (Yang et al., 2003). CCL20 and CCL28, for example, have been shown to have antimicrobial activities against a broad range of pathogens, including Gram-negative and Gram-positive bacteria, as well as fungi (Hieshima et al., 2003; Yang et al., 2003). A common feature of chemokines with antimicrobial activities appears to be the presence of a large, positively charged electrostatic surface-patch, which induces the lysis of cell membranes, resulting in microbial cell death (Yang et al., 2003; Yung et al., 2012).

Porphyromonas gingivalis is a keystone pathogen in chronic periodontitis, an inflammatory disease where the dysregulation of host inflammation causes the breakdown of periodontal tissues (Darveau, 2010; Hajishengallis, 2015). *P. gingivalis*, which colonizes the outer layers of a polymicrobial subgingival biofilm, expresses virulence factors that enable it to subvert the host immune response. In particular, the gingipain proteases (Kgp and RgpA/B) can degrade cytokines and chemokines (e.g. IL-6 and IL-8), as well as antimicrobial peptides (e.g. β -defensins) (Carlisle et al., 2009; Darveau et al., 1998; Stathopoulou et al., 2009). The gingipain proteases can also dysregulate the immune response by stimulating signaling crosstalk between TLRs, chemokine receptors, and complement receptors (Hajishengallis et al., 2008; Maekawa et al., 2014; Wang et al., 2010). Importantly, the dysregulation of the immune response also disrupts the homeostasis that normally exists between the host and the subgingival biofilm, resulting in biofilm dysbiosis and the stimulation of chronic periodontitis (Darveau, 2010; Hajishengallis, 2015).

Here, we demonstrate that *P. gingivalis* stimulates the expression of the orphan chemokine CXCL14 by oral epithelial cells. CXCL14 expression was primarily stimulated by the gingipain proteases, and was dependent on the host receptor PAR-3. Although, CXCL14

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93 expression is suppressed by EGF-induced activation of the MEK-ERK1/2 pathway,
94 *P. gingivalis* can degrade EGF. Therefore, the stimulation of CXCL14 expression by
95 *P. gingivalis* involves the activation of PAR-3-dependent signaling as well as the inhibition
96 of EGF signaling. Significantly, CXCL14 was shown to have bactericidal activity against oral
97 *Streptococcus* species associated with health, whereas *P. gingivalis* was resistant, most likely
98 because it can proteolytically degrade CXCL14. This study therefore not only extends our
99 understanding of the host response to *P. gingivalis*, but also identifies CXCL14 as a host
100 factor that when dysregulated may promote dysbiosis and chronic periodontitis.

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2. MATERIALS AND METHODS

2.1 Reagents

Keratinocyte serum-free medium and supplements (human EGF, bovine pituitary extract, and GlutaMax-1), Lipofectamine RNAiMAX transfection reagent, Opti-MEM I reduced serum medium, and precast 10% and 4-12% gradient NuPAGE gels were from Life Technologies. The ON-TARGETplus PAR-1, PAR-2, and PAR-3 siRNA were from GE Healthcare. The anti-phospho-ERK1/2, anti-ERK1/2, anti-phospho-p38 MAP kinase, and anti-p38 MAP kinase antibodies were from Cell Signaling Technology. U0126 and SB203580 were from Merck Millipore, and recombinant human CXCL14 was from R&D Systems. Brain heart infusion (BHI) medium was from BD Biosciences, and defibrinated horse blood was from Equicell. Hemin, menadione, N- α -toysl-L-lysine chloromethyl ketone hydrochloride (TLCK), actinomycin D, and IGEPAL CA-630 detergent were from Sigma-Aldrich.

2.2 Bacterial cell culture

P. gingivalis (ATCC 33277), *Streptococcus gordonii* (ATCC 35105), and *Streptococcus* sp. OT058 were obtained from the culture collection of the Melbourne Dental School (University of Melbourne). The isogenic Kgp and RgpA/B deficient *P. gingivalis* mutant KDP136 ($\Delta kgp \Delta rgpA \Delta rgpB$) was as previously described (Shi et al., 1999). The bacteria were maintained on horse-blood agar plates at 37 °C in an anaerobic atmosphere of 5% H₂, 80% N₂, and 15% CO₂. Bacterial colonies were used to inoculate BHI medium supplemented with 0.5 mg/mL cysteine, 5 µg/mL hemin, and 5 µg/mL menadione (Huynh et al., 2016). *Escherichia coli* was maintained on Luria Bertani agar plates, and grown in Luria Bertani broth at 37 °C.

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127 **2.3 Mammalian cell culture**

128 Human OKF6/TERT-2 oral epithelial cells (Dickson et al., 2000) (hereafter referred
129 to as OKF6 cells) were cultured in keratinocyte serum-free medium supplemented with 0.4
130 ng/mL human EGF, 25 µg/mL bovine pituitary extract, 0.4 mM CaCl₂, and 2 mM GlutaMax-
131 1. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂.

133 **2.4 Infection and treatment of oral epithelial cells with *P. gingivalis* and culture**
134 **supernatants**

135 *P. gingivalis* cell concentrations were determined spectrophotometrically and
136 confirmed retrospectively by counting viable cell colonies on horse-blood agar plates. Late
137 logarithmic growth phase bacteria were harvested by centrifugation at 7,000 x g for 20 min at
138 4 °C. The supernatants were passed through a 0.22 µm filter to obtain cell-free culture
139 supernatants, and the bacteria were suspended in keratinocyte growth medium. The cell-free
140 culture supernatants were assayed for Kgp and RgpA/B gingipain protease activities using the
141 chromogenic substrates, *N*-*p*-tosyl-Gly-Pro-Lys *p*-nitroanilide and *N*α-benzoyl-L-arginine *p*-
142 nitroanilide, respectively. Protease activity was measured in 50 mM Tris-HCl (pH 7.4), 5 mM
143 CaCl₂, 150 mM NaCl, and 20 mM cysteine at 37 °C, using a VICTOR3™ plate reader
144 (PerkinElmer). For the infection of OKF6 cells, approximately 3.5 x 10⁵ cells (per well) were
145 infected with 3.5 x 10⁷ *P. gingivalis*, for a multiplicity-of-infection (MOI) of 100:1 (Huynh et
146 al., 2016). For the treatment of OKF6 cells with *P. gingivalis* culture supernatants, 0.1 mL
147 cell-free culture supernatant (from a 10 mL *P. gingivalis* culture) was added to approximately
148 3.5 x 10⁵ cells (per well) in 1.0 mL keratinocyte growth medium.

150 **2.5 RNA purification and reverse transcription**

151 Total RNA was purified using the ReliaPrep RNA Cell miniprep system (Promega),

which included an on-column DNase-treatment step. RNA was reverse-transcribed using random primers and GoScript Reverse Transcriptase (Promega) according to the manufacturer's instructions.

2.6 Inflammatory gene expression profiling

Two µg total RNA was reverse-transcribed as described above. The cDNA (100 ng/µL) was mixed with TaqMan OpenArray Real-Time master mix (Life Technologies), and then loaded onto an OpenArray Human Inflammation plate (Life Technologies) using the OpenArray AccuFill System. PCR was performed on the QuantStudio™ 12K Flex Real-Time PCR System, and the data were normalized against HPRT gene expression using Expression Suite Software (version 1.0.1).

2.7 Quantitative real-time PCR

Quantitative real-time PCR (qPCR) was performed in duplicate using GoTaq qPCR Master Mix (Promega) and pre-developed TaqMan assays (Life Technologies) for the following genes: CXCL1 (Hs00236937_m1), CXCL14 (Hs01557413_m1), IL-8 (Hs00174103_m1), PAR1 (Hs00169258_m1), PAR2 (Hs00608346_m1), PAR3 (Hs00187982_m1), and PAR4 (Hs01006385_g1). PCR was performed on a QuantStudio 7 Flex Real-Time PCR system (Life Technologies). The data were normalized against the hypoxanthine guanine phosphoribosyl transferase (HPRT) or TATA-box binding protein (TBP) gene, and changes in gene expression were calculated using the ΔC_t method.

2.8 Enzyme-linked immunosorbent assay

ELISA assays were performed by incubating cell-free culture supernatants and standards in capture antibody-coated 96-well microplates for 90 min at 37 °C, per the

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3 177 manufacturer's instructions (Abcam). The contents of the wells were discarded, followed by
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5 178 the addition of a biotinylated anti-CXCL14 antibody and incubation for 60 min at 37 °C. The
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7 179 plate was washed, and then incubated with an avidin-biotin-peroxidase complex for 30 min at
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9 180 37 °C. Following washing, 3,3',5,5'-tetramethylbenzidine substrate was added and the plate
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11 181 incubated in the dark for 20-25 min at 37 °C. Color development was stopped and then
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13 182 measured at 450 nm using a VICTOR3™ plate reader (PerkinElmer).
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18 184 **2.9 RNA interference-mediated gene-silencing**

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20 185 A reverse transfection protocol was used for siRNA transfection of OKF6 cells (Kwa
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22 186 et al., 2014). Briefly, the siRNA were diluted to 120 nM with 100 µL Opti-MEM I reduced
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24 187 serum medium, mixed with 100 µL Opti-MEM medium containing 1 µL Lipofectamine
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26 188 RNAiMAX transfection reagent, and incubated at room-temperature for 15-20 min at room
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28 189 temperature. OKF6 cells (1 x 10⁵ cells in 1 mL keratinocyte serum-free medium
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30 190 supplemented with 0.4 ng/mL human EGF, 25 µg/mL bovine pituitary extract, 0.4 mM CaCl₂,
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32 191 and 2 mM GlutaMax-1) were seeded in 12-well plates and cultured with the transfection
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34 192 cocktail overnight. Thereafter, the medium was replaced and the cells infected with *P.*
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36 193 *gingivalis* 24 h later (i.e. 48 h post-transfection).
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42 195 **2.10 Cell lysis, SDS-PAGE, and Western blotting**

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44 196 OKF6 cells were washed twice with ice-cold PBS and then lysed on ice with IGEPAL
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46 197 lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630,
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48 198 10% glycerol, 20 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 10 mM NaF, and
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50 199 protease inhibitors) for 60 min. The lysates were clarified by centrifugation (13,000 x g for
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52 200 10 min at 4°C) and the protein concentrations measured using a protein assay kit (Bio-Rad).
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55 201 Cell lysates and recombinant proteins were incubated with NuPAGE LDS sample buffer for
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10 min at 70 °C, and then subjected to electrophoresis on a 10% (cell lysates) or 4-12% gradient (recombinant proteins) NuPAGE gels (Life Technologies). The silver staining of gels was performed as previously described (Heukeshoven et al., 1988). For Western blotting, proteins were transferred onto a PVDF membrane, which was then blocked with 3% BSA in TBST (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.02% Tween-20) for 60 min at room temperature. The membrane was then incubated with the primary antibody (diluted in 1% BSA in TBST) overnight at 4 °C. The membrane was washed with TBST, followed by incubation with a HRP-conjugated secondary antibody (diluted in 1% BSA in TBST) for 60 min at room temperature. Following washing with TBST, immunoreactive proteins were visualized using ECL reagents (Millipore) and a Fujifilm Las-3000 Imager (Fujifilm, Japan).

2.11 *In vitro* degradation of recombinant proteins by *P. gingivalis* and purified Kgp

Recombinant human EGF and CXCL14 (500 ng) were incubated at 37 °C in 200 mM HEPES (pH 7.2), 5 mM CaCl₂, and 10 mM cysteine with 1×10^8 *P. gingivalis*. TLCK was then added (2 mM final concentration), and the bacteria removed by centrifugation at 12,000 x g for 5 min at 4 °C. The supernatant was recovered and subjected to SDS-PAGE. *P. gingivalis* Kgp was purified as described previously (Huq et al., 2013). The Kgp was activated at 37 °C for 5 min in 200 mM HEPES (pH 7.2), 5 mM CaCl₂, and 10 mM cysteine, and then incubated (at a Kgp to substrate ratio of 1:25) with recombinant EGF or CXCL14 (500 ng). Aliquots of the digestion reaction were removed and Kgp activity inhibited by the addition of TLCK (2 mM final concentration). The aliquots were then subjected to SDS-PAGE or mass spectrometry.

2.12 Antimicrobial assay

Mid-logarithmic phase bacteria were harvested by centrifugation and washed with

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3 227 PBS. The bacteria (5×10^4) were resuspended in incubation buffer (10 mM Tris-HCl (pH 7.4),
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5 228 5 mM glucose) and incubated with serial-dilutions of carrier-free recombinant CXCL14 for
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7 229 60 min at 37 °C. Serial dilutions of the incubation mixtures were then plated on horse-blood
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9 230 agar plates (for *P. gingivalis*, *S. gordonii*, and *S. sp.* OT058) or LB agar plates (for *E. coli*).
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11 231 Bacterial cell numbers were enumerated and % killing expressed as: $(1 - [\text{colonies after}$
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13 232 CXCL14 incubation]/[colonies after control incubation]) x 100.
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18 234 **2.13 Mass spectrometry**

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20 235 Kgp digests of recombinant CXCL14 were desalted using C₁₈ zip-tips, and then
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22 236 analyzed on an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific) interfaced with an
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24 237 Ultimate 3000 nano-HPLC system (Thermo Scientific). The mass spectrometer was operated
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26 238 with a nano-ESI spray voltage of 2.0 kV, capillary temperature of 250 °C, and an S-lens RF
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28 239 value of 55%. All spectra were acquired in positive mode, with full scan MS spectra scanning
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30 240 from *m/z* 300–1650 in Fourier Transform mode at >240,000 full width at half maximum
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32 241 resolution. The ten most intense precursor ions were subjected to high energy collision
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34 242 induced dissociation. Dynamic exclusion with two repeat counts over 30 seconds and
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36 243 exclusion for 70 seconds was applied. Mascot MS/MS ion searches were performed with the
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38 244 following settings: *Swissprot* database, Lys-C, two missed cleavages, 10 ppm peptide
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40 245 tolerance, and 0.2 Da MS/MS tolerance.
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46 247 **2.14 Statistical analysis**

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48 248 Data combined from three or more independent biological replicate experiments are
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50 249 presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 7.
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52 250 Differences between two groups were evaluated using the Students' *t*-test. For multiple
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54 251 comparisons, statistical analysis was performed by ANOVA with Dunnett's (time course
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experiments) or Sidak's (gene silencing experiments) post-hoc test. A p value < 0.05 was considered to be statistically significant.

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3. RESULTS

3.1 Gingipain protease-dependent stimulation of CXCL14 expression in oral epithelial cells by *P. gingivalis*

The inflammatory response initiated at the oral epithelium is important for establishing host defense against infection by *P. gingivalis*. To identify mediators of the inflammatory response, an OpenArray inflammation gene panel was used to detect genes whose expression changed when oral epithelial cells (e.g. OKF6 cells) were infected with *P. gingivalis*. The gene encoding the orphan chemokine CXCL14 was found to be one of the most strongly stimulated, and which was confirmed by real-time PCR (qPCR) (Fig. 1A). Next, we measured the levels of CXCL14 in cell culture supernatants by ELISA. Although CXCL14 was detected in cell culture supernatants from uninfected OKF6 cells, the levels of CXCL14 in the supernatants from *P. gingivalis*-infected cells appeared to be lower (Fig. 1B), despite the increase in CXCL14 mRNA levels (Fig. 1A). Similar studies have reported that measuring the levels of cytokines and chemokines in cell culture supernatants is often compromised due to the proteolysis of the cytokines and chemokines by *P. gingivalis* proteases (e.g. Kgp and RgpA/B) (Darveau et al., 1998; Stathopoulou et al., 2009; Zhang et al., 1999). Notably, Kgp and RgpA/B have been shown to stimulate the expression of inflammatory cytokines and chemokines by oral epithelial cells (Chung et al., 2004; Dommisch et al., 2007; Loubakos et al., 2001). Therefore, we used *P. gingivalis* KDP136, an isogenic mutant that does not express Kgp and RgpA/B, to determine whether the gingipain proteases are important for the stimulation of CXCL14 expression. In contrast to wildtype *P. gingivalis*, the gingipain-deficient mutant did not stimulate CXCL14 expression (Fig. 1C). A complementary approach was also taken to confirm the importance of the gingipain proteases for the stimulation of CXCL14 expression; specifically, wildtype *P. gingivalis* was pretreated with the irreversible serine protease inhibitor, TLCK. Consistent with the data

presented in Fig. 1C, the inactivation of Kgp and RgpA/B activity by TLCK inhibited the stimulation of CXCL14 expression (Fig. 1D). In addition to being retained on the outer membrane, *P. gingivalis* also releases the gingipain proteases into the culture supernatants as components of outer membrane vesicles (O'Brien-Simpson et al., 2003). Therefore, to determine whether CXCL14 expression can also be stimulated by outer membrane vesicle-associated gingipain proteases, OKF6 cells were treated with cell-free culture supernatants from wildtype *P. gingivalis* and *P. gingivalis* KDP136. First, the presence of high levels of Kgp and RgpA/B activity in culture supernatants from wildtype *P. gingivalis*, and the absence of Kgp and RgpA/B activity in those from *P. gingivalis* KDP136, was confirmed (Fig. 1E-F). As seen in Figure 1G, CXCL14 expression was significantly stimulated by culture supernatants from wildtype *P. gingivalis*. By contrast, the stimulation of CXCL14 expression was significantly lower when the cells were treated with culture supernatants from *P. gingivalis* KDP136 (Fig. 1G). Taken together, these data indicate that the stimulation of CXCL14 expression by *P. gingivalis* is primarily mediated by the gingipain proteases.

3.2 Stimulation of CXCL14 expression by *P. gingivalis* is PAR-3-dependent

Protease-activated receptors (PARs) have previously been shown to be important for the stimulation of cytokine and chemokine expression by the gingipain proteases (Dommisch et al., 2007; Giacaman et al., 2009; Loubakos et al., 2001). Therefore, the role of PARs in regulating the stimulation of CXCL14 expression by *P. gingivalis* was investigated. *P. gingivalis* was found to stimulate increased PAR-2 expression in OKF6 cells (Fig. 2A). In contrast, PAR-1 and PAR-3 expression were not affected (Fig. 2A), and, consistent with an earlier report (Giacaman et al., 2009), PAR-4 expression was not detected (data not shown). We next determined the effects of the siRNA-mediated gene-silencing of PAR-1, PAR-2, and PAR-3 on the stimulation of CXCL14 expression by *P. gingivalis*. The transfection of OKF6

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3 305 cells with PAR-1, PAR-2 or PAR-3 siRNA reduced their respective expression levels by >80%
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5 306 (Fig. 2B, D and F). The silencing of either PAR-1 or PAR-2 did not affect the stimulation of
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7 307 CXCL14 expression by *P. gingivalis* (Fig. 2C and 2E). In contrast, the stimulation of
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9 308 CXCL14 expression was significantly inhibited by the silencing of PAR-3 (Fig. 2G). This
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11 309 indicates that the gingipain protease-mediated stimulation of CXCL14 expression by
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13 310 *P. gingivalis* is PAR-3-dependent.
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18 312 **3.3 CXCL14 expression in oral epithelial cells is negatively regulated by EGF**
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20 313 **signaling**

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22 314 CXCL14 gene expression in epidermal keratinocytes was recently reported to be
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24 315 inhibited by EGF signaling (Lichtenberger et al., 2013). Furthermore, *P. gingivalis* has been
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26 316 shown to antagonize EGF signaling in epidermal fibroblasts (Pyrce et al., 2013). This raised
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28 317 the possibility that, in addition to directly stimulating the expression of CXCL14,
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30 318 *P. gingivalis* might also promote CXCL14 expression by interfering with EGF signaling.
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32 319 Therefore, we first sought to establish whether EGF regulates CXCL14 expression in OKF6
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34 320 cells. EGF was found to strongly inhibit the expression of CXCL14 (Fig. 3A). Time-course
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36 321 experiments revealed that CXCL14 mRNA levels were rapidly reduced in response to EGF
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38 322 stimulation (Fig. 3B). To determine whether this resulted in a corresponding decrease in
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40 323 CXCL14 protein, the levels of CXCL14 in cell culture supernatants were measured. As
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42 324 shown in Fig. 3C, CXCL14 levels were lower in culture supernatants from EGF-treated
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44 325 OKF6 cells. EGF signaling in epidermal keratinocytes has also been shown to regulate the
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46 326 expression of the neutrophil chemokine CXCL1 (Lichtenberger et al., 2013). Thus, we also
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48 327 investigated the effect of EGF on CXCL1 expression by OKF6 cells for comparison. In
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50 328 contrast to CXCL14, CXCL1 expression was stimulated by EGF (Fig. 3D). Interestingly, IL-
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52 329 8 expression was not similarly stimulated by EGF (Fig. 3E). EGF activates several signaling
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pathways, including the ERK1/2 and p38 MAP kinase pathways. Indeed, Western blotting of cell lysates with phospho-specific antibodies demonstrated the activation of ERK1/2 and p38 MAP kinase in OKF6 cells by EGF (Fig. 3F-G). Therefore, we investigated whether the inhibition of CXCL14 expression in OKF6 cells by EGF was mediated by the ERK1/2 or p38 MAP kinase pathway. Inhibition of ERK1/2 signaling with the pharmacologic MEK inhibitor, U0126, resulted in a significant increase in CXCL14 mRNA levels (Fig. 3H). In contrast, CXCL14 expression was not increased when the cells were treated with the p38 MAP kinase inhibitor, SB203580 (Fig. 3H). Therefore, EGF-mediated suppression of CXCL14 is ERK1/2-dependent. The increased CXCL14 mRNA levels in cells treated with U0126 could be attributed to increased gene transcription or increased mRNA stability. To determine the mechanism of regulation, gene transcription in U0126-treated OKF6 cells was inhibited with actinomycin D (ActD). Significantly, ActD inhibited the increase in CXCL14 mRNA levels caused by U0126 (Fig. 3I). Consistent with EGF reducing CXCL14 mRNA levels by inhibiting CXCL14 transcription, EGF did not affect the half-life of CXCL14 mRNA transcripts in OKF6 cells (data not shown). Collectively, these data indicate that EGF-induced activation of the MEK-ERK1/2 pathway inhibits CXCL14 transcription in oral epithelial cells.

3.4 *P. gingivalis* antagonizes the negative regulation of CXCL14 expression by EGF

We next investigated whether EGF affects the stimulation of CXCL14 expression by *P. gingivalis*. Specifically, OKF6 cells were cultured with 20 ng/mL EGF prior to being infected with *P. gingivalis*. EGF reduced *P. gingivalis*-stimulated CXCL14 expression by approximately 50% (Fig. 4A). The stimulation of CXCL14 expression by *P. gingivalis* was not significantly reduced when the cells were treated with 2 ng/mL EGF (data not shown). Notably, in addition to not stimulating CXCL14 expression, the gingipain protease-deficient

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3 355 *P. gingivalis* mutant (*P. gingivalis* KDP136) did not relieve the CXCL14 gene from EGF-
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5 356 induced transcriptional repression (Fig. 4A). For comparison, we also investigated the effect
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7 357 of EGF on the stimulation of CXCL1 expression by *P. gingivalis*. Although *P. gingivalis* and
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9 358 EGF both stimulated CXCL1 expression, the effects were not additive (Fig. 4B), thus
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11 359 suggesting that *P. gingivalis* inhibits the stimulation of CXCL1 expression by EGF.
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13 360 *P. gingivalis* has previously been reported to inhibit the biological activity of EGF via its
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15 361 expression of a peptidyl arginine deiminase (PPAD), which can catalyze the citrullination of
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17 362 the arginine residue at the C-terminus of EGF (Pyrce et al., 2013). Here, we directly tested the
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19 363 ability of *P. gingivalis* to proteolytically degrade EGF. Specifically, recombinant EGF was
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21 364 incubated with *P. gingivalis* for up to 3 h, and its proteolysis was then assessed. For reference,
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23 365 the relative ratio of *P. gingivalis* to EGF in Fig. 4A-B was approximately 2×10^6
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25 366 *P. gingivalis* per ng EGF. The incubation of 500 ng EGF with 1×10^6 *P. gingivalis* (i.e.
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27 367 2×10^3 *P. gingivalis* per ng EGF) resulted in the rapid proteolysis of EGF, and complete
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29 368 degradation when incubated with 1×10^8 *P. gingivalis* (Fig. 4C). In contrast, significant
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31 369 degradation by the gingipain protease-deficient mutant was only evident when EGF was
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33 370 incubated with at least 1×10^8 *P. gingivalis* KDP136 (Fig. 4C), and presumably mediated by
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35 371 other *P. gingivalis*-expressed proteases (e.g. PrtT and Tpr) (Otogoto et al., 1993; Park et al.,
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37 372 1993). Importantly, EGF was also degraded when incubated with highly purified Kgp (Fig.
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39 373 4D). Taken together, these data indicate that the gingipain protease-mediated degradation of
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41 374 EGF is likely to be important for the stimulation of CXCL14 expression by *P. gingivalis*.
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48 376 **3.5 CXCL14 exhibits antimicrobial activity against oral bacteria but not *P. gingivalis***

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50 377 In addition to chemotactic activity (Cao et al., 2000; Kurth et al., 2001; Schaerli et al.,
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52 378 2005), CXCL14 has recently been demonstrated to also have antimicrobial activity, including
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54 379 against *Escherichia coli* (Dai et al., 2015; Maerki et al., 2009). Consistent with those reports,
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we likewise found that CXCL14 could kill *E. coli* (Fig. 5A). Therefore, we tested the ability of CXCL14 to kill *P. gingivalis*. As determined by colony-forming assay, *P. gingivalis* was resistant to killing by CXCL14 (Fig. 5B). Consequently, we tested the ability of CXCL14 to kill other oral bacteria; specifically, commensal oral streptococci associated with periodontal health, *Streptococcus gordonii* and *Streptococcus* sp. OT058 (Hong et al., 2015). As shown in Fig. 5C, *S. gordonii* was highly susceptible to killing by CXCL14. For example, >95% killing of *S. gordonii* was achieved at 1 μ M CXCL14 (Fig. 5C). *S. sp.* OT058 was also highly susceptible to killing by CXCL14 (Fig. 5D). These data demonstrate significant differences in the ability of CXCL14 to kill oral bacteria; moreover, they show that *P. gingivalis* is resistant to killing by CXCL14.

3.6 Gingipain protease-dependent degradation of CXCL14 by *P. gingivalis*

The secreted (i.e. mature) form of CXCL14 contains fourteen lysine and seven arginine residues (Fig. 6A). This would potentially make CXCL14 highly susceptible to degradation by the lysine-specific gingipain protease, Kgp, and the arginine-specific gingipain proteases, RgpA/B, expressed by *P. gingivalis*. Moreover, degradation of CXCL14 by the gingipain proteases could explain, at least in part, the resistance of *P. gingivalis* to killing by CXCL14. Therefore, we directly tested the ability of *P. gingivalis* to degrade CXCL14. Specifically, recombinant CXCL14 was incubated with *P. gingivalis* for up to 1 h, and its proteolysis was then assessed. The incubation of 500 ng CXCL14 with 1×10^4 *P. gingivalis*, which was equivalent to the ratio of CXCL14 to *P. gingivalis* in the antimicrobial assays in Figure 5, resulted in the rapid proteolysis of CXCL14 (Fig. 6B). CXCL14 was completely degraded when incubated with greater numbers of *P. gingivalis* (e.g. 1×10^6 *P. gingivalis*). In contrast, significant degradation by the gingipain protease-deficient mutant was only evident when CXCL14 was incubated with at least 1×10^8 *P. gingivalis*

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405 KDP136 (Fig. 6B). Consistently, the incubation of CXCL14 with purified Kgp also resulted
406 in its rapid degradation (Fig. 6C). The first thirteen amino acids of the mature CXCL14
407 protein (i.e. Ser1–Arg13) have been shown to largely mediate its antimicrobial activity (Dai
408 et al., 2015). Therefore, we were interested in determining whether any peptides containing
409 this sequence might be produced when CXCL14 was degraded by *P. gingivalis*. Therefore,
410 aliquots of the digestion reactions shown in Fig. 6C were subjected to analysis by mass
411 spectrometry to identify the CXCL14-derived peptides generated by Kgp digestion. Although
412 several peptides from the N-terminal half of CXCL14 were identified, none contained Ser1–
413 Arg13 (Table 1). This was not particularly surprising given that the amino acid sequence
414 spanning Ser1–Arg13 contains four lysine residues (Fig. 6A). Several relatively short
415 peptides from the C-terminal half of CXCL14 were also identified (Table 1). Collectively,
416 these data indicate that the gingipain proteases of *P. gingivalis* can degrade CXCL14.
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DISCUSSION

Chemokines are key mediators of host defense against infection by microbial pathogens. In this study, oral epithelial cells were shown to express the orphan chemokine CXCL14 in response to the keystone periodontal pathogen *P. gingivalis*. The stimulation of CXCL14 expression was primarily mediated by the gingipain proteases of *P. gingivalis*, and was dependent on the host protease-activated receptor PAR-3. EGF-induced signaling was found to transcriptionally repress CXCL14 expression, and thus the gingipain protease-mediated degradation of EGF is likely to be important for the stimulation of CXCL14 expression by *P. gingivalis*. Furthermore, our data indicate that CXCL14 can directly kill oral *Streptococcus* species. *P. gingivalis*, however, was not susceptible to killing by CXCL14, most likely because it can proteolytically degrade CXCL14. Consequently, the stimulation of dysregulated CXCL14 expression by *P. gingivalis* might promote dysbiosis.

The gingipain proteases (Kgp and RgpA/B) are critical virulence factors of *P. gingivalis*. Our data indicate that they play an important role in mediating the stimulation of CXCL14 expression in oral epithelial cells (e.g. OKF6 cells) by *P. gingivalis*. Consistently, *P. gingivalis* has also been shown to stimulate CXCL14 expression in human gingival epithelial cells (Chung et al., 2008). Our conclusion that the stimulation of CXCL14 expression is primarily mediated by the gingipain proteases is based on two lines of evidence. First, CXCL14 expression was not stimulated by *P. gingivalis* KDP136, an isogenic mutant that does not express Kgp and RgpA/B. Second, treating *P. gingivalis* with the serine protease inhibitor TLCK blocked the stimulation of CXCL14 expression. In addition to degrading host proteins, the gingipain proteases are also important for the processing of *P. gingivalis* fimbriae (Kadowaki et al., 1998; Nakayama et al., 1996), which mediate its attachment and invasion of epithelial cells (Njoroge et al., 1997; Weinberg et al., 1997; Yilmaz et al., 2002). However, the fact that treating wildtype *P. gingivalis* with TLCK

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3 443 inhibited the stimulation of CXCL14 expression largely excludes the possibility that impaired
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5 444 fimbriae processing might have been responsible for the lack of CXCL14 stimulation by the
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7 445 gingipain protease-deficient mutant.
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9 446 Interestingly, despite the transcriptional upregulation of CXCL14, the protein levels
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11 447 of CXCL14 in culture supernatants from *P. gingivalis*-infected OKF6 cells appeared to be
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13 448 lower than in those from uninfected cells. This suggested that CXCL14 might be degraded by
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15 449 *P. gingivalis* proteases (e.g. Kgp and RgpA/B) following its secretion. Indeed, similar *in vitro*
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17 450 studies have found that secreted cytokines and chemokines (e.g. IL-8) are degraded by
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19 451 *P. gingivalis* proteases (Darveau et al., 1998; Stathopoulou et al., 2009; Zhang et al., 1999).
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21 452 As well as being retained on the outer membrane, *P. gingivalis* also releases the gingipain
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23 453 proteases as components of outer membrane vesicles (O'Brien-Simpson et al., 2003). We
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25 454 found that CXCL14 expression was also stimulated by the outer membrane vesicle-associated
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27 455 gingipain proteases. This is consistent with other studies that have demonstrated the
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29 456 stimulation of chemokine expression (e.g. CCL20) by cell-free *P. gingivalis* culture
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31 457 supernatants (Dommisch et al., 2007). Interestingly, although primarily mediated by the
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33 458 gingipain proteases, other factors that are released by *P. gingivalis* also appear to be capable
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35 459 of weakly stimulating CXCL14 expression. Further studies will be required to identify these
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37 460 factors.
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41 461 The gingipain proteases have been shown to stimulate the expression of cytokines and
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43 462 chemokines by activating PARs (Dommisch et al., 2007; Lourbakos et al., 2001). This
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45 463 prompted us to investigate a role for PARs in the stimulation of CXCL14 expression by
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47 464 *P. gingivalis*. Gene silencing experiments revealed that PAR-3 was important for the
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49 465 stimulation of CXCL14 expression. Other studies, using cell-free culture supernatants, have
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51 466 suggested that PAR-1 and PAR-2 are important for the gingipain protease-mediated
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53 467 stimulation of cytokine and chemokine expression (e.g. IL-6 and CCL20) by *P. gingivalis*
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(Dommisch et al., 2007; Loubakos et al., 2001). Therefore, this is the first report to ascribe a role for PAR-3 in the stimulation of cytokine or chemokine expression by *P. gingivalis*. Although PAR-3 is generally considered to function as a co-receptor with PAR-1 (McLaughlin et al., 2007), studies have suggest that PAR-3 may also have autonomous functions (Bretschneider et al., 2003; Ostrowska et al., 2008; Segal et al., 2014). Furthermore, PARs have been shown to act in synergy with other PRRs, including TLRs, to mediate the stimulation of cytokine expression in monocytes by *P. gingivalis* (Uehara et al., 2008). Additional studies will therefore be required to establish whether PAR-3 functions together with other PRRs to regulate CXCL14 expression.

CXCL14 expression in OKF6 cells was inhibited in response to the EGF-induced activation of the MEK-ERK1/2 pathway. In contrast, EGF stimulated the expression of the neutrophil chemokine CXCL1. These findings are similar to those reported for the regulation of CXCL14 and CXCL1 in epidermal keratinocytes (Lichtenberger et al., 2013; Mascia et al., 2003). Importantly, our data indicate that *P. gingivalis* can largely overcome the transcriptional repression of CXCL14 via the gingipain protease-mediated degradation of EGF. A prior study demonstrated that *P. gingivalis* can also antagonize EGF-induced signaling via its expression of a peptidyl arginine deiminase, which catalyzes the citrullination and inactivation of EGF (Pyrc et al., 2013). Thus, *P. gingivalis* can not only directly stimulate CXCL14 expression via PAR-3, but the gingipain protease-mediated degradation of EGF is also likely to be important for the stimulation of CXCL14 expression (Fig. 7). In addition to EGF, other growth factors, including keratinocyte growth factor and insulin-like growth factor 1, which likewise activate MEK-ERK1/2, are also upregulated during inflammation and wound healing (Chang et al., 1996; Li et al., 2005; Werner et al., 2004). Therefore, *P. gingivalis* gingipain protease-mediated degradation of various host factors that can activate the MEK-ERK1/2 pathway potentially contribute to the optimal

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493 stimulation of CXCL14 expression. Interestingly, the ability of *P. gingivalis* to antagonize
494 EGF function may also reduce CXCL1-elicited neutrophil recruitment. Although EGF did not
495 similarly regulate the expression of IL-8, *P. gingivalis* can suppress IL-8 transcription by
496 inhibiting NF- κ B activation (Takeuchi et al., 2013).

497 The function of CXCL14 has not been fully elucidated, including establishing its
498 receptor specificity. The expression of CXCL14 in mucosal tissues, including those of the
499 oral cavity (Frederick et al., 2000; Shellenberger et al., 2004; Shurin et al., 2005), and its
500 ability to stimulate the migration of immune cells (e.g. monocytes) suggests that CXCL14
501 plays a role in mucosal homeostasis and inflammation (Cao et al., 2000; Kurth et al., 2001;
502 Shurin et al., 2005). Therapeutic blockade of EGF signaling, which results in the elevated
503 expression of CXCL14, as well as other chemokines (e.g. CCL5), has been shown to
504 stimulate the excessive infiltration of immune cells into the epidermis, resulting in skin
505 inflammation (Lichtenberger et al., 2013). Although inflammation normally protects the host
506 from infection by microbial pathogens, *P. gingivalis* relies on inflammation to obtain
507 nutrients from tissue breakdown products. Importantly, the expression of the gingipain
508 proteases by *P. gingivalis* facilitates the subversion of host-mediated bacterial killing
509 mechanisms whilst stimulating dysregulated inflammation (Hajishengallis, 2015; Lamont et
510 al., 2015). For instance, *P. gingivalis* has been shown to impair host defense by stimulating
511 signaling crosstalk between TLR2 and the chemokine receptor, CXCR4, in macrophages
512 (Hajishengallis et al., 2008). Notably, CXCL14 was recently shown to function as a positive
513 allosteric modulator of CXCR4 signaling (Collins et al., 2017), and thus CXCL14 might
514 potentiate the signaling crosstalk that *P. gingivalis* instigates between CXCR4 and TLR2 to
515 impair host defense.

516 Similar to several other chemokines (e.g. CCL20 and CCL28) (Yang et al., 2003),
517 CXCL14 has recently been shown to have antimicrobial activity, and to kill bacteria that

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3 518 cause respiratory tract and skin infections (e.g. *Pseudomonas aeruginosa* and *Staphylococcus*
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5 519 *aureus*) (Dai et al., 2015). We have shown here that CXCL14 can also kill the commensal
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7 520 oral *Streptococcus* species, *S. gordonii* and *S. sp.* OT058. This is consistent with the report of
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9 521 CXCL14 also killing another commensal oral *Streptococcus*, *S. mitis* (Dai et al., 2015), which
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11 522 is one of the most abundant species of oral microbiomes associated with health (Aas et al.,
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13 523 2005). Notably though, *P. gingivalis* was not susceptible to killing by CXCL14, most likely
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15 524 because it can degrade CXCL14. The degradation of CXCL14 is largely mediated by the
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17 525 gingipain proteases, although our data indicate that other *P. gingivalis* proteases (e.g. PrtT
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19 526 and Tpr) may also contribute to its degradation. A similar protease-mediated protective
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21 527 mechanism has been proposed for the resistance of *Finnegoldia magna*, an opportunistic
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23 528 pathogen, to CXCL14 (Frick et al., 2011).

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26 529 The antimicrobial activity of CXCL14 is largely associated with a short peptide at its
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28 530 N-terminus (e.g. CXCL14₁₋₁₃) (Dai et al., 2015). Interestingly, neutrophil protease 3 can
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30 531 cleave CXCL14 and produce the antimicrobial active peptide CXCL14₁₋₁₇ (Dai et al., 2015).
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32 532 Although we identified peptides from the N-terminal half of CXCL14 following its *in vitro*
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34 533 digestion by Kgp, no peptides that contained Ser1–Arg13 were identified, which was likely
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36 534 due to the presence of multiple lysine residues in this region of CXCL14. Importantly,
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38 535 *P. gingivalis* forms mutualistic interactions with a number of bacteria in the oral biofilm
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40 536 (Kolenbrander et al., 2010). Therefore, degradation of CXCL14 *in vivo* by *P. gingivalis* may
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42 537 also protect otherwise susceptible bacteria, including accessory pathogens. A peptide
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44 538 spanning Tyr14–Lys54 of CXCL14 (CXCL14₁₄₋₅₄) was shown to stimulate the chemotaxis of
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46 539 human monocytes (e.g. THP-1 cells) (Dai et al., 2015). However, no such peptide was
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48 540 identified following the digestion of CXCL14 by Kgp, and thus *P. gingivalis* can likely also
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50 541 abolish CXCL14 chemotactic activity.

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53 542 In summary, our findings provide important mechanistic insights into the regulation
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543 of CXCL14 by *P. gingivalis*. Although the expression of CXCL14 by oral epithelial cells is a
544 host-initiated response, its role in host defense might need to be reconsidered in the context of
545 infection by pathogens that rely on dysbiosis and host inflammation to fulfil their nutritional
546 requirements. Specifically, the ability of CXCL14 to kill commensal oral species but not
547 *P. gingivalis* suggests that the dysregulation of CXCL14 by *P. gingivalis* may help promote
548 dysbiosis and chronic periodontitis.

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554
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775 **Table 1. Peptides identified following Kgp digestion of CXCL14**

Peptide identified	Ion score
¹² IRYSDVKK ¹⁹	10
¹⁹ KLEMKPK ²⁵	34
²⁰ LEMKPKYPHCEEK ³²	57
²⁶ YPHCEEK ³²	17
³³ MVIITTK ³⁹	53
⁶⁴ WYNAWNEK ⁷¹	36
⁶⁴ WYNAWNEKRRVYEE ⁷⁷	9

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FIGURE LEGENDS

Figure 1. Gingipain protease-mediated stimulation of CXCL14 expression in oral epithelial cells by *P. gingivalis*. (A) OKF6 cells were cultured with *P. gingivalis* for the time indicated, and CXCL14 mRNA levels then measured ($n = 3$). (B) OKF6 cells were cultured with *P. gingivalis* for 24 h, and CXCL14 protein levels in the culture supernatants then measured ($n = 3$). (C) OKF6 cells were cultured with *P. gingivalis* or *P. gingivalis* KDP136 for 24 h, and CXCL14 mRNA levels then measured ($n = 3$). (D) OKF6 cells were cultured for 24 h with *P. gingivalis* that had been pretreated with TLCK, and CXCL14 mRNA levels then measured ($n = 3$). (E-F) Proteolytic activity of (E) Kgp and (F) RgpA/B in cell-free culture supernatants from *P. gingivalis* and *P. gingivalis* KDP136 were measured ($n = 3$) (G) OKF6 cells were stimulated for 24 h with BHI medium or cell-free culture supernatants from *P. gingivalis* and *P. gingivalis* KDP136, and CXCL14 mRNA levels then measured ($n = 3$). *** = $p < 0.001$, ** = $p < 0.01$.

Figure 2. PAR-3-dependent stimulation of CXCL14 expression by *P. gingivalis*. (A) OKF6 cells were cultured with *P. gingivalis* for 24 h, and PAR-1, PAR-2, and PAR-3 mRNA levels then measured ($n = 3$). (B-G) OKF6 cells were transfected with (B-C) PAR-1, (D-E) PAR-2, (F-G) PAR-3 siRNA, or control (-) siRNA. Forty-eight h later, (B) PAR-1, (D) PAR-2, and (F) PAR-3 mRNA levels were measured ($n = 3$). (C, E, and G) The cells were cultured with *P. gingivalis* for 24 h, and CXCL14 mRNA levels then measured ($n = 3$). *** = $p < 0.001$, * = $p < 0.05$.

Figure 3. Regulation of CXCL14 expression by EGF. (A) OKF6 cells were stimulated with the indicated concentration of EGF for 24 h, and CXCL14 mRNA levels then measured

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3 803 ($n = 3$). **(B)** OKF6 cells were stimulated with 5 ng/mL EGF for the time indicated, and
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5 804 CXCL14 mRNA levels then measured ($n = 3$). **(C)** OKF6 cells were stimulated with 20
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7 805 ng/mL EGF for 24 h, and CXCL14 protein levels in the culture supernatants then measured
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9 806 ($n = 3$). **(D-E)** OKF6 cells were stimulated with the indicated concentration of EGF for 24 h,
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11 807 and **(D)** CXCL1 and **(E)** IL-8 mRNA levels then measured ($n = 3$). **(F-G)** OKF6 cells were
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13 808 stimulated with 20 ng/mL EGF for the time indicated, and cell lysates then subjected to
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15 809 Western blotting with **(F)** anti-phospho-ERK1/2 and anti-ERK2 antibodies, and **(G)** anti-
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17 810 phospho-p38 MAP kinase and anti-p38 MAP kinase antibodies. The data are representative
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19 811 of two independent experiments. **(H)** OKF6 cells were stimulated with 20 ng/mL EGF for 24
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21 812 h, and then treated with 10 μ M U0126 or 5 μ M SB203580 for 8 h. CXCL14 mRNA levels
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23 813 were then measured ($n = 3$). **(I)** OKF6 cells were stimulated with 20 ng/mL EGF for 24 h,
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25 814 and then treated with 10 μ M U0126 and 1 μ g/mL actinomycin D (ActD) for 8 h. CXCL14
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27 815 mRNA levels were then measured ($n = 3$). *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.
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33 817 **Figure 4. *P. gingivalis* antagonizes the regulation of CXCL14 by EGF.** **(A-B)** OKF6 cells
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35 818 were stimulated with 20 ng/mL EGF for 24 h, and then cultured with *P. gingivalis* or *P.*
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37 819 *gingivalis* KDP136 for a further 24 h. **(A)** CXCL14 and **(B)** CXCL1 mRNA levels were then
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39 820 measured ($n = 3$). **(C)** EGF (500 ng) was incubated with 1×10^6 , 1×10^7 , or 1×10^8
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41 821 *P. gingivalis* or *P. gingivalis* KDP136 for the time indicated, and then subjected to SDS-
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43 822 PAGE and silver staining. The data are representative of three independent experiments. **(D)**
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45 823 EGF was incubated with purified Kgp for the time indicated, and aliquots of the incubation
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47 824 mixtures then subjected to SDS-PAGE and silver staining. The data are representative of two
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49 825 independent experiments. ** = $p < 0.01$, * = $p < 0.05$.
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55 827 **Figure 5. Bactericidal activity of CXCL14.** **(A)** *Escherichia coli*, **(B)** *P. gingivalis*, **(C)**

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3 828 *Streptococcus gordonii*, and (D) *Streptococcus* sp. OT058 were incubated with the indicated
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5 829 concentration of recombinant CXCL14 for 1 h, and killing then measured by CFU assay ($n =$
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7 830 3). *** = $p < 0.001$, ** = $p < 0.01$, * = $p < 0.05$.
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11 832 **Figure 6. Gingipain protease-mediated degradation of CXCL14. (A)** Amino acid
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13 833 sequence of mature human CXCL14. **(B)** CXCL14 (500 ng) was incubated with 1×10^4 ,
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15 834 1×10^6 , or 1×10^8 *P. gingivalis* or *P. gingivalis* KDP136 for the time indicated, and then
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17 835 subjected to SDS-PAGE and silver staining. **(C)** CXCL14 was incubated with purified Kgp
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19 836 for the time indicated, and then subjected to SDS-PAGE and silver staining. The data in **(B)**
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21 837 and **(C)** are representative of two independent experiments.
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26 839 **Figure 7. A proposed model for the regulation of CXCL14 expression in oral epithelial**
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28 840 **cells by *P. gingivalis*.** *P. gingivalis* stimulates the expression of CXCL14 via PAR-3. The
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30 841 stimulation of CXCL14 expression by *P. gingivalis* also involves the gingipain protease-
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32 842 mediated degradation of EGF, which relieves the transcriptional repression of CXCL14 by
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34 843 the MEK-ERK1/2 pathway.
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Figure 1. Gingipain protease-mediated stimulation of CXCL14 expression in oral epithelial cells by *P. gingivalis*. (A) OKF6 cells were cultured with *P. gingivalis* for the time indicated, and CXCL14 mRNA levels then measured (n = 3). (B) OKF6 cells were cultured with *P. gingivalis* for 24 h, and CXCL14 protein levels in the culture supernatants then measured (n = 3). (C) OKF6 cells were cultured with *P. gingivalis* or *P. gingivalis* KDP136 for 24 h, and CXCL14 mRNA levels then measured (n = 3). (D) OKF6 cells were cultured for 24 h with *P. gingivalis* that had been pretreated with TLCK, and CXCL14 mRNA levels then measured (n = 3). (E-F) Proteolytic activity of (E) Kgp and (F) RgpA/B in cell-free culture supernatants from *P. gingivalis* and *P. gingivalis* KDP136 were measured (n = 3) (G) OKF6 cells were stimulated for 24 h with BHI medium or cell-free culture supernatants from *P. gingivalis* and *P. gingivalis* KDP136, and CXCL14 mRNA levels then measured (n = 3). *** = p<0.001, ** = p <0.01.

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Figure 2. PAR-3-dependent stimulation of CXCL14 expression by *P. gingivalis*. (A) OKF6 cells were cultured with *P. gingivalis* for 24 h, and PAR-1, PAR-2, and PAR-3 mRNA levels then measured (n = 3). (B-G) OKF6 cells were transfected with (B-C) PAR-1, (D-E) PAR-2, (F-G) PAR-3 siRNA, or control (-) siRNA. Forty-eight h later, (B) PAR-1, (D) PAR-2, and (F) PAR-3 mRNA levels were measured (n = 3). (C, E, and G) The cells were cultured with *P. gingivalis* for 24 h, and CXCL14 mRNA levels then measured (n = 3). *** = p < 0.001, * = p < 0.05.

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Figure 3. Regulation of CXCL14 expression by EGF. (A) OKF6 cells were stimulated with the indicated concentration of EGF for 24 h, and CXCL14 mRNA levels then measured (n = 3). (B) OKF6 cells were stimulated with 5 ng/mL EGF for the time indicated, and CXCL14 mRNA levels then measured (n = 3). (C) OKF6 cells were stimulated with 20 ng/mL EGF for 24 h, and CXCL14 protein levels in the culture supernatants then measured (n = 3). (D-E) OKF6 cells were stimulated with the indicated concentration of EGF for 24 h, and (D) CXCL1 and (E) IL-8 mRNA levels then measured (n = 3). (F-G) OKF6 cells were stimulated with 20 ng/mL EGF for the time indicated, and cell lysates then subjected to Western blotting with (F) anti-phospho-ERK1/2 and anti-ERK2 antibodies, and (G) anti-phospho-p38 MAP kinase and anti-p38 MAP kinase antibodies. The data are representative of two independent experiments. (H) OKF6 cells were stimulated with 20 ng/mL EGF for 24 h, and then treated with 10 μ M U0126 or 5 μ M SB203580 for 8 h. CXCL14 mRNA levels were then measured (n = 3). (I) OKF6 cells were stimulated with 20 ng/mL EGF for 24 h, and then treated with 10 μ M U0126 and 1 μ g/mL actinomycin D (ActD) for 8 h. CXCL14 mRNA levels were then measured (n = 3). *** = p < 0.001, ** = p < 0.01, * = p < 0.05.

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Figure 4. *P. gingivalis* antagonizes the regulation of CXCL14 by EGF. (A-B) OKF6 cells were stimulated with 20 ng/mL EGF for 24 h, and then cultured with *P. gingivalis* or *P. gingivalis* KDP136 for a further 24 h. (A) CXCL14 and (B) CXCL1 mRNA levels were then measured (n = 3). (C) EGF (500 ng) was incubated with 1 x 10⁶, 1 x 10⁷, or 1 x 10⁸ *P. gingivalis* or *P. gingivalis* KDP136 for the time indicated, and then subjected to SDS-PAGE and silver staining. The data are representative of three independent experiments. (D) EGF was incubated with purified Kgp for the time indicated, and aliquots of the incubation mixtures then subjected to SDS-PAGE and silver staining. The data are representative of two independent experiments. ** = p < 0.01, * = p < 0.05.

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Figure 5. Bactericidal activity of CXCL14. (A) *Escherichia coli*, (B) *P. gingivalis*, (C) *Streptococcus gordonii*, and (D) *Streptococcus sp. OT058* were incubated with the indicated concentration of recombinant CXCL14 for 1 h, and killing then measured by CFU assay (n = 3). *** = p < 0.001, ** = p < 0.01, * = p < 0.05.

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Figure 6. Gingipain protease-mediated degradation of CXCL14. (A) Amino acid sequence of mature human CXCL14. (B) CXCL14 (500 ng) was incubated with 1 x 10⁴, 1 x 10⁶, or 1 x 10⁸ *P. gingivalis* or *P. gingivalis* KDP136 for the time indicated, and then subjected to SDS-PAGE and silver staining. (C) CXCL14 was incubated with purified Kgp for the time indicated, and then subjected to SDS-PAGE and silver staining. The data in (B) and (C) are representative of two independent experiments.

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Figure 7. A proposed model for the regulation of CXCL14 expression in oral epithelial cells by *P. gingivalis*. *P. gingivalis* stimulates the expression of CXCL14 via PAR-3. The stimulation of CXCL14 expression by *P. gingivalis* also involves the gingipain protease-mediated degradation of EGF, which relieves the transcriptional repression of CXCL14 by the MEK-ERK1/2 pathway.

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