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.
SUMMARY (200 word limit – currently 188 words)

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

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

Oral epithelial cells also mediate host defense by triggering inflammation in response to microbial pathogens. This is achieved through the activation of pathogen recognition receptors (PRRs), including Toll-like receptors (TLRs) and protease-activated receptors (PARs) (Kinane et al., 2008; Uehara et al., 2008). Chemokines are important mediators of the inflammatory responses activated by PRRs. Interleukin-8 (IL-8) and chemokine (C-X-C motif) ligand 1 (CXCL1) stimulate neutrophil recruitment, while chemokine (C-C motif) ligand 2 (CCL2) stimulates the recruitment of monocytes and macrophages (Graves et al., 1995; Kobayashi, 2006). These innate immune cells can amplify the immune response by producing additional chemokines, including CCL5 and CXCL10, which recruit adaptive immune cells, such as T-lymphocytes (Appay et al., 2001; Groom et al., 2011). Importantly, the expression of chemokines by epithelial cells is also regulated by other receptors, including the EGF receptor (Liu et al., 2008; Mascia et al., 2003). Accordingly, multiple receptor signaling pathways contribute to the coordinated expression of chemokines by...
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
expression is suppressed by EGF-induced activation of the MEK-ERK1/2 pathway, 
P. gingivalis can degrade EGF. Therefore, the stimulation of CXCL14 expression by 
P. gingivalis involves the activation of PAR-3-dependent signaling as well as the inhibition 
of EGF signaling. Significantly, CXCL14 was shown to have bactericidal activity against oral 
Streptococcus species associated with health, whereas P. gingivalis was resistant, most likely 
because it can proteolytically degrade CXCL14. This study therefore not only extends our 
understanding of the host response to P. gingivalis, but also identifies CXCL14 as a host 
factor that when dysregulated may promote dysbiosis and chronic periodontitis.
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-a-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 (ΔkgpΔrgpAΔrgpB) 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.
2.3 Mammalian cell culture

Human OKF6/TERT-2 oral epithelial cells (Dickson et al., 2000) (hereafter referred to as OKF6 cells) were cultured in keratinocyte serum-free medium supplemented with 0.4 ng/mL human EGF, 25 µg/mL bovine pituitary extract, 0.4 mM CaCl$_2$, and 2 mM GlutaMax-1. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO$_2$.

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

*P. gingivalis* cell concentrations were determined spectrophotometrically and confirmed retrospectively by counting viable cell colonies on horse-blood agar plates. Late logarithmic growth phase bacteria were harvested by centrifugation at 7,000 x g for 20 min at 4 °C. The supernatants were passed through a 0.22 µm filter to obtain cell-free culture supernatants, and the bacteria were suspended in keratinocyte growth medium. The cell-free culture supernatants were assayed for Kgp and RgpA/B gingipain protease activities using the chromogenic substrates, N-p-tosyl-Gly-Pro-Lys p-nitroanilide and Nα-benzoyl-L-arginine p-nitroanilide, respectively. Protease activity was measured in 50 mM Tris-HCl (pH 7.4), 5 mM CaCl$_2$, 150 mM NaCl, and 20 mM cysteine at 37°C, using a VICTOR3™ plate reader (PerkinElmer). For the infection of OKF6 cells, approximately 3.5 x 10$^5$ cells (per well) were infected with 3.5 x 10$^7$ *P. gingivalis*, for a multiplicity-of-infection (MOI) of 100:1 (Huynh et al., 2016). For the treatment of OKF6 cells with *P. gingivalis* culture supernatants, 0.1 mL cell-free culture supernatant (from a 10 mL *P. gingivalis* culture) was added to approximately 3.5 x 10$^5$ cells (per well) in 1.0 mL keratinocyte growth medium.

2.5 RNA purification and reverse transcription

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 ∆Ct 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
manufacturer’s instructions (Abcam). The contents of the wells were discarded, followed by
the addition of a biotinylated anti-CXCL14 antibody and incubation for 60 min at 37 °C. The
plate was washed, and then incubated with an avidin-biotin-peroxidase complex for 30 min at
37 °C. Following washing, 3,3',5,5'-tetramethylbenzidine substrate was added and the plate
incubated in the dark for 20-25 min at 37 °C. Color development was stopped and then
measured at 450 nm using a VICTOR3™ plate reader (PerkinElmer).

2.9 RNA interference-mediated gene-silencing

A reverse transfection protocol was used for siRNA transfection of OKF6 cells (Kwa
et al., 2014). Briefly, the siRNA were diluted to 120 nM with 100 µL Opti-MEM I reduced
serum medium, mixed with 100 µL Opti-MEM medium containing 1 µL Lipofectamine
RNAiMAX transfection reagent, and incubated at room-temperature for 15-20 min at room
temperature. OKF6 cells (1 x 10^5 cells in 1 mL keratinocyte serum-free medium
supplemented with 0.4 ng/mL human EGF, 25 µg/mL bovine pituitary extract, 0.4 mM CaCl₂,
and 2 mM GlutaMax-1) were seeded in 12-well plates and cultured with the transfection
cocktail overnight. Thereafter, the medium was replaced and the cells infected with P.
gingivalis 24 h later (i.e. 48 h post-transfection).

2.10 Cell lysis, SDS-PAGE, and Western blotting

OKF6 cells were washed twice with ice-cold PBS and then lysed on ice with IGEPAL
lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% IGEPAL CA-630,
10% glycerol, 20 mM β-glycerol phosphate, 1 mM sodium orthovanadate, 10 mM NaF, and
protease inhibitors) for 60 min. The lysates were clarified by centrifugation (13,000 x g for
10 min at 4°C) and the protein concentrations measured using a protein assay kit (Bio-Rad).
Cell lysates and recombinant proteins were incubated with NuPAGE LDS sample buffer for
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 CaCl$_2$, and 10 mM cysteine with $1 \times 10^8$ *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 CaCl$_2$, 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
PBS. The bacteria ($5 \times 10^4$) were resuspended in incubation buffer (10 mM Tris-HCl (pH 7.4), 5 mM glucose) and incubated with serial-dilutions of carrier-free recombinant CXCL14 for 60 min at 37 °C. Serial dilutions of the incubation mixtures were then plated on horse-blood agar plates (for *P. gingivalis, S. gordonii*, and *S. sp. OT058*) or LB agar plates (for *E. coli*). Bacterial cell numbers were enumerated and % killing expressed as: (1-[colonies after CXCL14 incubation]/[colonies after control incubation]) x 100.

### 2.13 Mass spectrometry

Kgp digests of recombinant CXCL14 were desalted using C\textsubscript{18} zip-tips, and then analyzed on an LTQ Orbitrap Elite mass spectrometer (Thermo Scientific) interfaced with an Ultimate 3000 nano-HPLC system (Thermo Scientific). The mass spectrometer was operated with a nano-ESI spray voltage of 2.0 kV, capillary temperature of 250 °C, and an S-lens RF value of 55%. All spectra were acquired in positive mode, with full scan MS spectra scanning from $m/z$ 300–1650 in Fourier Transform mode at >240,000 full width at half maximum resolution. The ten most intense precursor ions were subjected to high energy collision induced dissociation. Dynamic exclusion with two repeat counts over 30 seconds and exclusion for 70 seconds was applied. Mascot MS/MS ion searches were performed with the following settings: *Swissprot* database, Lys-C, two missed cleavages, 10 ppm peptide tolerance, and 0.2 Da MS/MS tolerance.

### 2.14 Statistical analysis

Data combined from three or more independent biological replicate experiments are presented as the mean ± SEM. Statistical analyses were performed using GraphPad Prism 7. Differences between two groups were evaluated using the Students’ *t*-test. For multiple comparisons, statistical analysis was performed by ANOVA with Dunnett’s (time course
experiments) or Sidak’s (gene silencing experiments) post-hoc test. A $p$ value < 0.05 was considered to be statistically significant.
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; Lourbakos 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; Lourbakos 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
cells with PAR-1, PAR-2 or PAR-3 siRNA reduced their respective expression levels by >80%
(Fig. 2B, D and F). The silencing of either PAR-1 or PAR-2 did not affect the stimulation of
CXCL14 expression by P. gingivalis (Fig. 2C and 2E). In contrast, the stimulation of
CXCL14 expression was significantly inhibited by the silencing of PAR-3 (Fig. 2G). This
indicates that the gingipain protease-mediated stimulation of CXCL14 expression by
P. gingivalis is PAR-3-dependent.

3.3 CXCL14 expression in oral epithelial cells is negatively regulated by EGF
signaling

CXCL14 gene expression in epidermal keratinocytes was recently reported to be
inhibited by EGF signaling (Lichtenberger et al., 2013). Furthermore, P. gingivalis has been
shown to antagonize EGF signaling in epidermal fibroblasts (Pyrc et al., 2013). This raised
the possibility that, in addition to directly stimulating the expression of CXCL14,
P. gingivalis might also promote CXCL14 expression by interfering with EGF signaling.

Therefore, we first sought to establish whether EGF regulates CXCL14 expression in OKF6
cells. EGF was found to strongly inhibit the expression of CXCL14 (Fig. 3A). Time-course
experiments revealed that CXCL14 mRNA levels were rapidly reduced in response to EGF
stimulation (Fig. 3B). To determine whether this resulted in a corresponding decrease in
CXCL14 protein, the levels of CXCL14 in cell culture supernatants were measured. As
shown in Fig. 3C, CXCL14 levels were lower in culture supernatants from EGF-treated
OKF6 cells. EGF signaling in epidermal keratinocytes has also been shown to regulate the
expression of the neutrophil chemokine CXCL1 (Lichtenberger et al., 2013). Thus, we also
investigated the effect of EGF on CXCL1 expression by OKF6 cells for comparison. In
contrast to CXCL14, CXCL1 expression was stimulated by EGF (Fig. 3D). Interestingly, IL-8
expression was not similarly stimulated by EGF (Fig. 3E). EGF activates several signaling
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
P. gingivalis mutant (P. gingivalis KDP136) did not relieve the CXCL14 gene from EGF-induced transcriptional repression (Fig. 4A). For comparison, we also investigated the effect of EGF on the stimulation of CXCL1 expression by P. gingivalis. Although P. gingivalis and EGF both stimulated CXCL1 expression, the effects were not additive (Fig. 4B), thus suggesting that P. gingivalis inhibits the stimulation of CXCL1 expression by EGF.

P. gingivalis has previously been reported to inhibit the biological activity of EGF via its expression of a peptidyl arginine deiminase (PPAD), which can catalyze the citrullination of the arginine residue at the C-terminus of EGF (Pyrc et al., 2013). Here, we directly tested the ability of P. gingivalis to proteolytically degrade EGF. Specifically, recombinant EGF was incubated with P. gingivalis for up to 3 h, and its proteolysis was then assessed. For reference, the relative ratio of P. gingivalis to EGF in Fig. 4A-B was approximately 2 x 10^6 P. gingivalis per ng EGF. The incubation of 500 ng EGF with 1 x 10^6 P. gingivalis (i.e. 2 x 10^3 P. gingivalis per ng EGF) resulted in the rapid proteolysis of EGF, and complete degradation when incubated with 1 x 10^8 P. gingivalis (Fig. 4C). In contrast, significant degradation by the gingipain protease-deficient mutant was only evident when EGF was incubated with at least 1 x 10^8 P. gingivalis KDP136 (Fig. 4C), and presumably mediated by other P. gingivalis-expressed proteases (e.g. PrtT and Tpr) (Otogoto et al., 1993; Park et al., 1993). Importantly, EGF was also degraded when incubated with highly purified Kgp (Fig. 4D). Taken together, these data indicate that the gingipain protease-mediated degradation of EGF is likely to be important for the stimulation of CXCL14 expression by P. gingivalis.

3.5 CXCL14 exhibits antimicrobial activity against oral bacteria but not P. gingivalis

In addition to chemotactic activity (Cao et al., 2000; Kurth et al., 2001; Schaarli et al., 2005), CXCL14 has recently been demonstrated to also have antimicrobial activity, including against Escherichia coli (Dai et al., 2015; Maerki et al., 2009). Consistent with those reports,
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 µM 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 $1 \times 10^4$ *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. $1 \times 10^6$ *P. gingivalis*). In contrast, significant degradation by the gingipain protease-deficient mutant was only evident when CXCL14 was incubated with at least $1 \times 10^8$ *P. gingivalis*.
KDP136 (Fig. 6B). Consistently, the incubation of CXCL14 with purified Kgp also resulted in its rapid degradation (Fig. 6C). The first thirteen amino acids of the mature CXCL14 protein (i.e. Ser1–Arg13) have been shown to largely mediate its antimicrobial activity (Dai et al., 2015). Therefore, we were interested in determining whether any peptides containing this sequence might be produced when CXCL14 was degraded by *P. gingivalis*. Therefore, aliquots of the digestion reactions shown in Fig. 6C were subjected to analysis by mass spectrometry to identify the CXCL14-derived peptides generated by Kgp digestion. Although several peptides from the N-terminal half of CXCL14 were identified, none contained Ser1–Arg13 (Table 1). This was not particularly surprising given that the amino acid sequence spanning Ser1–Arg13 contains four lysine residues (Fig. 6A). Several relatively short peptides from the C-terminal half of CXCL14 were also identified (Table 1). Collectively, these data indicate that the gingipain proteases of *P. gingivalis* can degrade CXCL14.
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...
inhibited the stimulation of CXCL14 expression largely excludes the possibility that impaired fimbriae processing might have been responsible for the lack of CXCL14 stimulation by the gingipain protease-deficient mutant.

Interestingly, despite the transcriptional upregulation of CXCL14, the protein levels of CXCL14 in culture supernatants from *P. gingivalis*-infected OKF6 cells appeared to be lower than in those from uninfected cells. This suggested that CXCL14 might be degraded by *P. gingivalis* proteases (e.g. Kgp and RgpA/B) following its secretion. Indeed, similar *in vitro* studies have found that secreted cytokines and chemokines (e.g. IL-8) are degraded by *P. gingivalis* proteases (Darveau et al., 1998; Stathopoulou et al., 2009; Zhang et al., 1999).

As well as being retained on the outer membrane, *P. gingivalis* also releases the gingipain proteases as components of outer membrane vesicles (O'Brien-Simpson et al., 2003). We found that CXCL14 expression was also stimulated by the outer membrane vesicle-associated gingipain proteases. This is consistent with other studies that have demonstrated the stimulation of chemokine expression (e.g. CCL20) by cell-free *P. gingivalis* culture supernatants (Domisch et al., 2007). Interestingly, although primarily mediated by the gingipain proteases, other factors that are released by *P. gingivalis* also appear to be capable of weakly stimulating CXCL14 expression. Further studies will be required to identify these factors.

The gingipain proteases have been shown to stimulate the expression of cytokines and chemokines by activating PARs (Domisch et al., 2007; Lourbakos et al., 2001). This prompted us to investigate a role for PARs in the stimulation of CXCL14 expression by *P. gingivalis*. Gene silencing experiments revealed that PAR-3 was important for the stimulation of CXCL14 expression. Other studies, using cell-free culture supernatants, have suggested that PAR-1 and PAR-2 are important for the gingipain protease-mediated stimulation of cytokine and chemokine expression (e.g. IL-6 and CCL20) by *P. gingivalis*.
(Dommisch et al., 2007; Lourbakos 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
stimulation of CXCL14 expression. Interestingly, the ability of *P. gingivalis* to antagonize
EGF function may also reduce CXCL1-elicited neutrophil recruitment. Although EGF did not
similarly regulate the expression of IL-8, *P. gingivalis* can suppress IL-8 transcription by
inhibiting NF-κB activation (Takeuchi et al., 2013).

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

Similar to several other chemokines (e.g. CCL20 and CCL28) (Yang et al., 2003),
CXCL14 has recently been shown to have antimicrobial activity, and to kill bacteria that
cause respiratory tract and skin infections (e.g. *Pseudomonas aeruginosa* and *Staphylococcus aureus*) (Dai et al., 2015). We have shown here that CXCL14 can also kill the commensal oral *Streptococcus* species, *S. gordonii* and *S. sp. OT058*. This is consistent with the report of CXCL14 also killing another commensal oral *Streptococcus, S. mitis* (Dai et al., 2015), which is one of the most abundant species of oral microbiomes associated with health (Aas et al., 2005). Notably though, *P. gingivalis* was not susceptible to killing by CXCL14, most likely because it can degrade CXCL14. The degradation of CXCL14 is largely mediated by the gingipain proteases, although our data indicate that other *P. gingivalis* proteases (e.g. PrtT and Tpr) may also contribute to its degradation. A similar protease-mediated protective mechanism has been proposed for the resistance of *Finegoldia magna*, an opportunistic pathogen, to CXCL14 (Frick et al., 2011).

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

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

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signaling to uncouple bacterial clearance from inflammation and promote dysbiosis.

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Table 1. Peptides identified following Kgp digestion of CXCL14

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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.

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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 10^6, 1 x 10^7, or 1 x 10^8 *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.

**Figure 5.** Bactericidal activity of CXCL14. (A) *Escherichia coli*, (B) *P. gingivalis*, (C)
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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 10^4, 1 x 10^6, or 1 x 10^8 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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190x254mm (300 x 300 DPI)
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Author/s: Aw, J; Scholz, GM; Huq, NL; Huynh, J; O'Brien-Simpson, NM; Reynolds, EC

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