

1 **Gingipains from *Porphyromonas gingivalis* increase chemotactic and**
2 **respiratory burst-priming properties of IL-8-77**

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12 **Short Title – Gingipains increase the biological activity of IL-8-77**

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ABSTRACT

Porphyromonas gingivalis, a gram negative anaerobe, which is implicated in the etiology of active periodontitis, secretes degradative enzymes (gingipains) and sheds proinflammatory mediators (e.g. lipopolysaccharides; LPS). LPS triggers the secretion of interleukin-8 from immune (IL-8-72) and non-immune (IL-8-77) cells. IL-8-77 has low chemotactic and respiratory burst-inducing activity but is susceptible to cleavage by gingipains. This study shows that both R- and K-gingipain treatments of IL-8-77 significantly enhance burst-activation by fMLP and chemotactic activity ($p < 0.05$), but decrease burst-activation and the chemotactic activity of IL-8-72 towards neutrophil-like HL-60 cells and primary neutrophils ($p < 0.05$). Using MS/MS we have demonstrated that R-gingipain cleaves 5 and 11 amino acid peptides from the N-terminal of IL-8-77, and resultant peptides are biologically active, whilst K-gingipain removes an 8 amino acid N-terminal peptide yielding a 69 amino acid isoform of IL-8 which shows enhanced biological activity. During periodontitis, secreted gingipains may differentially affect neutrophil chemotaxis and activation in response to IL-8 according to the cellular source of the chemokine.

1

2 **Key words**

3 Chemotaxis, Interleukin-8, Gingipain, inflammation, neutrophils, respiratory burst

4

5 **Abbreviations**

6 Lipopolysaccharide (LPS), Interleukin-8 (IL-8), Arginine-specific gingipain (Rgp), Lysine-
7 specific gingipain (Kgp), *Porphyromonas gingivalis* (*Pg*), formyl-methionine-leucine-
8 phenylalanine (fMLP).

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1 **INTRODUCTION**

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3 Inflammatory periodontal diseases have an infectious aetiology and are
4 characterised by excess inflammation within the periodontal tissues, which can
5 progress to alveolar bone loss and ultimately tooth loss (4). The primary
6 aetiological agent for periodontitis is the subgingival plaque biofilm and disease
7 progression is associated with an ecological shift in biofilm composition to a
8 predominantly anaerobic flora (5, 27). Evidence indicates that this in turn triggers
9 the host response, which in susceptible patients is abnormal, involving excess
10 generation of proteolytic enzymes (9) and reactive oxygen species (ROS; (18))
11 both of which are important determinants of disease progression and severity (5,
12 11). Neutrophilic inflammation is the major source of the tissue destructive
13 species (6) and recent studies have demonstrated that peripheral blood
14 neutrophils from periodontitis patients are both hyper-reactive to Fc γ -receptor
15 stimulation and also demonstrate baseline hyper-activity, with respect to
16 extracellular ROS release (17, 18). The extracellular ROS production from
17 neutrophil infiltrates into the periodontium is significant (17) but modest, however,
18 any process which results in enhanced polymorphonuclear leukocyte recruitment
19 to, or retention (e.g. delayed apoptosis) within the periodontal tissues may
20 contribute to ROS-mediated tissue damage.

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22 The oral anaerobic rod, *Porphyromonas gingivalis*, is the organism most strongly
23 associated with active periodontitis (1). This organism possesses a number of

1 virulence determinants which potentially contribute to its pathogenicity including
2 the ability to secrete a range of degradative proteinases (1); among these, the
3 gingipains have been extensively studied (15). Furthermore, the pathogenic
4 bacteria within the subgingival environment shed proinflammatory mediators
5 such as lipopolysaccharide (LPS). LPS in turn triggers the secretion of
6 chemokines/cytokines such as IL-1 β , TNF- α , IL-6 and interleukin-8 (IL-8-72) from
7 resident inflammatory cells, which contribute to the initial inflammatory response
8 (20).

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10 IL-8 is a major chemokine with potent stimulatory effects on neutrophils including
11 chemotaxis, degranulation, and cytoplasmic Ca²⁺ elevation. IL-8 is a small
12 polypeptide with a molecular weight of 8-10kD (22) that was originally isolated
13 from monocytes (2). Subsequent studies have shown that IL-8 is also produced
14 from a wide range of cell types including fibroblasts, epithelial cells/keratinocytes,
15 lymphocytes, endothelial cells and neutrophilic polymorphonuclear leukocytes
16 (neutrophils). In response to stimulus, IL-8 is produced as a 99 amino acid long
17 precursor polypeptide (2), which is subsequently processed into a biologically
18 active peptide. IL-8 varies in length from 79 amino acids through to 77, 72, 71, 70
19 and 69 amino acid variants (23). Although IL-8 is subject to variable processing
20 at the N-terminus, the 72 amino acid long (IL-8-72) and 77 amino acid long (IL-8-
21 77) peptides have been identified as the predominant variants. The major form
22 of IL-8-72 has been extensively studied for its potent ability to prime neutrophils
23 to stimulate the respiratory burst to a secondary stimulus, such as fMLP (12, 14).

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1 IL-8-77 is recognised as a less potent variant for neutrophil activation. It exhibits
2 reduced chemotactic properties and attenuates neutrophil adhesion to
3 endothelial cell walls (10). Recent reports have suggested that this longer amino
4 acid form is susceptible to cleavage by proteolysis into a biologically active form
5 (13, 24, 25). It has also been shown that IL-8-77 is susceptible to cleavage by
6 gingipains, the principal secreted cysteine proteases of *P.gingivalis* (19). The
7 potential for gingipains to enhance the activity of IL-8-77, presents an additional
8 mechanism of the organism's pathogenicity, whereby *P.gingivalis* promotes
9 enhanced neutrophil recruitment, activation and further local tissue degradation.
10 To date the effect of gingipain processing of IL-8 on subsequent chemotactic
11 activity or respiratory burst priming has not been determined. Therefore, this
12 report investigates whether gingipains from *P. gingivalis* can modify IL-8-77
13 chemotactic and priming activities using primary neutrophils and differentiated
14 neutrophil-like HL-60 cell line as responder cells.

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1 **MATERIALS AND METHODS**

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3 **Materials.** *P.gingivalis* W83 was kindly provided by Dr. A. Roberts (Periodontal
4 Research Group, School of Dentistry, University of Birmingham, U.K.). All
5 reagents were obtained from Sigma Chemical Company (Poole, U.K.) and
6 solvents from Fisher (Loughborough, U.K.) unless otherwise stated. RPMI 1640,
7 foetal bovine serum and penicillin (1000 U ml⁻¹)/ streptomycin (10,000 µg ml⁻¹)
8 were obtained from GibcoBRL (Paisley, U.K.). Recombinant IL-8-72 endothelial
9 derived recombinant IL-8-77 and monoclonal anti-human IL-8 antibody, clone
10 6218, were purchased from R&D systems (Abingdon, U.K.).

11

12 **HL60 cell cultures.** The human pro-myelocytic cell line HL60 was purchased
13 from the European collection of cell cultures (ECACC no 98070101) and
14 maintained in RPMI-1640 medium supplemented with 10% heat inactivated foetal
15 calf serum (FCS) and 1% penicillin/streptomycin at 37⁰C in a 5% CO₂/ 95% air
16 humidified atmosphere. The cells were induced to differentiate in the presence of
17 1.0% dimethyl sulfoxide (DMSO) for 5 days after seeding at cell density of 2 ×10⁵
18 cells/ml.

19

20 **Collection and isolation of peripheral blood Neutrophils.** Venous blood was
21 collected from systemically and periodontally healthy donors into 4% sodium
22 citrate (w/v) in PBS, with a ratio of citrate: blood of 1:9 and neutrophils were
23 isolated as described in Matthews et al 2007 (7). Isolated cells were washed and
24 resuspended in physiological salt solution (PSS: 115mM NaCl, 5mM KCl, 1mM

1 KH_2PO_4 , 10mM glucose, 1mM MgSO_4 , 1.25mM CaCl_2 , 25mM HEPES-Na
2 supplemented with 0.1% bovine serum albumin (BSA) at pH 7.4).

3

4 **Cultivation of Porphyromonas gingivalis Strain W83.** Cultures were grown
5 statically in 200ml of liquid medium containing 6.0g of trypticase soy broth (Difco,
6 Detroit, Mich, U.S.A), 2.0 g of yeast extract, supplemented with 1mg of hemin,
7 200mg of L-cysteine, 20mg of di-thiothreitol and 0.5mg of menadione at 37°C in
8 an anaerobic atmosphere of 10% H_2 , 10% CO_2 , and 80% N_2 for 48h (miniMACS
9 anaerobic workstation, Don Whitley Scientific).

10

11 **Isolation of Arg(R)-gingipain and Lys (K)-gingipain.** Gingipains were isolated
12 according to the method described in Yun et al 1999 (29) using a 5ml packed
13 volume arginine-sepharose column for the final affinity purification stage. Column
14 fractions (1ml) containing Kgp (eluted with 0.75M L-lysine) and Rgp (eluted with
15 1M L-arginine) were dialysed against Tris buffer overnight at 5°C.

16

17 **Enzyme activity assays.** The amidolytic activities of the purified Rgp and Kgp
18 were measured with the substrate N_α -benzoyl-L-arginine p-nitroanilide
19 hydrochloride (L-BAPNA). 100 μl of Rgp and Kgp fractions were incubated with L-
20 BAPNA (final concentration of 1mM) in 100 μl of 0.2M Tris-HCl, 0.1M NaCl, 5mM
21 CaCl_2 , 10mM L-cysteine at pH 7.6 and 37°C. After one-hour incubation, the
22 reaction was stopped by addition of 10 μl of glacial acetic acid. The optical density

1 was measured at 405nm for each fraction and the values corrected by
2 subtraction of negative control values (without proteinases).

3

4 **Proteolytic degradation of IL-8 by purified gingipains.** Pooled fractions for
5 each gingipain were activated as described in Mikolajczyk-Pawlinska et al (18).
6 Rgp and Kgp were adjusted to equimolar concentrations in Tris buffer, pH7.6.
7 Activated gingipains (3mM) were mixed with 1.65µM IL-8-77 or IL-8-72 and
8 incubated at 37°C for 30min. Enzyme activity was terminated post-incubation by
9 addition of 1 µl of a protease inhibitor cocktail containing leupeptin hemisulphate.

10

11 **MS/MS analysis of Kgp and Rgp treated IL-8-77 and IL-8-72.** Kgp or Rgp
12 treated IL-8-77 and IL-8-72 (0.14pg/µl) was diluted in 50% methanol in water with
13 acetic acid (1%) to enhance ionisation and subjected to mass analysis after
14 injection at 1µl/ min using a Thermo LTQ MS/MS in electrospray mode. The
15 machine was externally mass-calibrated using peptides, caffeine and Ultramark
16 1621TM (ABCR GmbH & Co.). The acceleration voltage was set at 20 kV and
17 data was collected as the average total scan of 100 scans with the scan range
18 set from 100-2000 m/z to search for fragmented IL-8-77 and IL-8-72. Multiply
19 charged molecular ions were subjected to collision-induced dissociation (CID)
20 with argon gas and the resulting MS/MS data were recorded for comparison
21 against SWISSPROT predicted cleavage sites in IL-8

22

1 **Neutralisation of IL-8-77 peptide chemotactic activity.** In order to investigate
2 whether the enhanced biological activity of Kgp or Rgp treated IL-8 could be
3 ascribed to the resultant formation of IL-8-72 rather than to the released peptides,
4 neutralising anti-human IL-8 antibody (5µg/ml) which recognises the whole
5 molecule, was added into Kgp or Rgp treated IL-8-77 (8.25nM) for 30 min at
6 room temperature prior to addition of neutralised IL-8 to the lower chambers for
7 chemotaxis experiments.

8

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10 **Chemotaxis assay.** dHL60 cell/neutrophil chemotaxis was measured by
11 Boyden's technique using (2µm or 5µm pore size respectively) PVP-free
12 polycarbonate filters in 96 multiwell chamber (Neuroprobe Inc.). dHL60
13 cells/neutrophils (1×10^5) were washed and re-suspended in PSS. Pre- and post-
14 gingipain-treated IL-8 (8.25nM) samples, gingipain treated and antibody
15 neutralised IL-8-77 and untreated gingipains were added to the lower wells of the
16 chamber, the filter was fixed in place and upper wells were loaded with 1×10^5
17 dHL-60 cells/neutrophils in 100µl of PSS at 37°C for 90 minutes. After
18 chemotaxis, cell-containing buffer from the upper chamber was removed and the
19 top of the filter was washed with PSS. The microplate/filter assembly was
20 centrifuged at 400g for 10min. The filter was carefully removed and cell counts in
21 the lower chamber were taken by flow cytometry (Coulter Epics XL). Results
22 were expressed as specific cell migration after subtraction of background
23 migration. *Escherichia Coli* LPS (serotype 0111:B4, 1µg/ml) was used as a

1 positive control in all assays and Rgp and Kgp alone in the presence of protease
2 inhibitor cocktail acted as a negative control.

3

4 **Chemiluminescent assay for respiratory burst activity.** Chemiluminescence
5 assays were performed using lucigenin to detect total superoxide production by
6 neutrophils or dHL60 cells. Assays were performed (37°C) using a Berthold
7 microplate luminometer (LB96v). Neutrophils (5×10^5 cells) were added to each
8 well containing 100 μ M lucigenin in PSS and incubated for 30 min at 37°C. Light
9 emission in relative light units (RLUs) was recorded during the 30-min pre-
10 stimulation period to established steady baseline. Cells were then incubated with
11 gingipain-treated or untreated IL-8 isoforms for 10 min prior to stimulation with
12 1 μ M fMLP. The RLU peak values were analysed for each treatment, and time to
13 peak for each stimulus was recorded.

14

15 **Data analysis.** Statistical analysis was performed by one-way ANOVA followed
16 by Tukey's comparison test analysis. A $P < 0.05$ was considered as significant.

17

1 **RESULTS**

2

3 **Kgp and Rgp specifically cleave the N-terminus of IL-8-77.** Rgp has two
4 theoretical, amino peptidase cleavage sites for IL-8-77 and Kgp has three amino
5 peptidase cleavage sites in IL-8-77 (Fig 1). At the N-terminus of IL-8-72, Rgp has
6 one theoretical cleavage site whilst Kgp has three sites. To investigate the
7 effects of gingipain activity on IL-8-77, released N-terminal peptides
8 corresponding in mass to Rgp and Kgp cleaved IL-8-77 isoforms were
9 investigated by MS/MS with CID. Rgp treatments preferentially cleaved the N-
10 terminus of IL-8-77 releasing peptides of m/z 555.7 and 703.82 corresponding to
11 residues 1-5 and 6-11 of the N-terminal region with the sequences reported in
12 Table 1, to produce 72 and 66 amino acid long IL-8 peptides. Rgp treatment also
13 released a peptide of m/z 703.41 and sequence SAKELR from IL-8-72 to yield an
14 IL-8-66 polypeptide. However, Kgp treatment of IL-8-77 released an 8 amino acid
15 long polypeptide of 842.03 m/z (AVLPRSAK) from the N-terminus resulting in a
16 69 amino acid long IL-8 polypeptide. Kgp treatment of IL-8-72 released peptides
17 of 305.18, 1135.4 and 498.5 m/z resulting in 69, 61 and 57 amino acid long
18 peptides respectively.

19

20 **Kgp and Rgp increase the chemotactic properties of biologically inactive**
21 **IL-8-77.** In order to determine the effect of Rgp and Kgp treatment on
22 chemotactic properties of IL-8 isoforms, dHL60 cells/ primary neutrophils were
23 allowed to undergo chemotaxis towards pre- or post-gingipain treated IL-8-72
24 and IL-8-77 isoforms. The effect of gingipain treatment in IL-8-dependent

1 chemotaxis was corrected for chemotaxis towards either inactivated Rgp or
2 inactivated Kgp, where migration was always lower towards gingipains than to
3 LPS or IL-8 isoforms. IL-8-72 demonstrated higher chemotactic activity than the
4 native IL-8-77 isoform. Both Rgp (Fig. 2A) and Kgp (Fig. 2B) treatments
5 significantly decreased the chemotactic activity of IL-8-72 ($P < 0.001$) towards
6 HL60 cells. In contrast, Kgp treatment significantly increased IL-8-77 chemotactic
7 properties ($P < 0.05$). In order to compare the behaviour of dHL60 cells with
8 peripheral blood neutrophils, chemotaxis of primary neutrophils towards IL-8
9 isoforms was measured. Confirming the observations in dHL60 cells, both Kgp-
10 (Fig. 2C) and Rgp- (Fig. 2D) treatments significantly increased the chemotactic
11 properties of IL-8-77 ($p < 0.001$ and $p < 0.01$ respectively) towards primary
12 neutrophils.

13

14 **N-terminal-shortened peptide fragments of IL-8-77 account for the**
15 **increased chemotactic activity.** To investigate whether N-terminal-shortened
16 IL-8-77 peptides accounted for the observed chemotactic activity, gingipain-
17 treated IL-8-77 was neutralised with anti-human IL-8 antibody prior to chemotaxis
18 assay. Both antibody-neutralised Rgp-treated IL-8-77 (Fig 3A) and Kgp-treated
19 IL-8-77 (Fig 3B) showed significantly decreased chemotactic activity towards
20 dHL60 cells ($p < 0.05$ and $p < 0.01$ respectively) when compared with gingipain-
21 treated IL-8-77 in the absence of neutralising antibody. Similarly, when the
22 experiment was repeated using neutralised Kgp- or Rgp-treated IL-8-77 as a
23 chemotaxin for neutrophils, again, the enhanced biological activity of gingipain-
24 cleaved IL-8-77 was not evident (Fig 3C and 3D).

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2 **Kgp and Rgp increase the priming effect of IL-8-77 for the respiratory burst**

3 **in response to fMLP.** The priming effect of IL-8 on the fMLP-induced respiratory

4 burst was measured by lucigenin-dependant chemiluminescence using isolated

5 peripheral blood neutrophils. IL-8-72 primed neutrophils for enhanced superoxide

6 production after fMLP stimulation whereas neither IL-8-77 nor isolated gingipains

7 had any priming effect (Fig. 4). However, both Rgp- (Fig. 4A) and Kgp- (Fig. 4B)

8 treated IL-8-77 primed neutrophils for fMLP induced superoxide production,

9 demonstrating significantly increased superoxide generation compared with

10 native IL-8-77 ($p < 0.05$). By contrast, gingipain treatment decreased the priming

11 activity of IL-8-72 ($p < 0.05$) for fMLP-stimulated superoxide production.

12

13 **DISCUSSION**

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15 Infection of host tissue by pathogenic bacteria and/or stimulation by microbial

16 components/virulence factors triggers the production of pro-inflammatory

17 peptides that have the ability to activate and recruit neutrophilic

18 polymorphonuclear leucocytes along a concentration gradient. Patients with

19 periodontitis show increased numbers of neutrophils within periodontal tissues

20 and pockets (21), and recent work has demonstrated baseline hyper-activity of

21 peripheral blood neutrophils, with respect to extracellular ROS release (18) and

22 proteolytic enzyme release (9), in periodontitis patients relative to matched

23 healthy controls. Such mechanisms when co-active may explain a significant

24 amount of the oxidative stress reported in periodontitis tissues (6). Therefore the

1 influence of periodontal bacteria and their virulence factors on IL-8-mediated
2 neutrophil chemotaxis and activation are important to elucidate. In contrast to
3 biologically active IL-8-72, the IL-8-77 peptide produced by epithelial cells,
4 fibroblasts and endothelial cells is resistant to a wide range of host proteinases; it
5 has a low chemotactic activity (10) and less respiratory burst priming activity. In
6 our experiments, we have used LPS as a positive control; LPS is a well known
7 chemotactic bacterial component which requires serum components such as
8 LPS-binding protein for receptor activation (28). However, as serum may also
9 contain other chemotactic factors, it was excluded from our experiments, and the
10 chemotactic activity of LPS in serum-free conditions here was greater than IL-8-
11 77 but less than IL-8-72 towards primary neutrophils.

12

13 In this study we have investigated a possible mechanism by which *P.gingivalis*
14 could manipulate IL-8 cytokine-mediated neutrophil chemotaxis using a dHL60
15 cell model and also primary human neutrophils. Gingipains increased the priming
16 activity induced by IL-8-77 on the fMLP-induced oxidative burst in primary
17 neutrophils, data that confirms previous studies measuring elastase release from
18 neutrophils, where IL-8-77-induced release was shown to be increased after
19 gingipain treatment (19).

20

21 Our results demonstrate a significant increase in the chemotactic properties of IL-
22 8-77 and a higher priming capability of IL-8-77 after incubation with L-cysteine-
23 activated gingipains under the conditions described. Compared with primary
24 neutrophils, dHL60 cells have low CXCR2 receptor expression (26), and this may

1 explain the lower rate of dHL60 cell migration towards IL-8. The corresponding
2 increase in data variation may account for the lack of significant increase in
3 migration of dHL60 towards Rgp-treated IL-8-77 when compared to the increased
4 migration of primary neutrophils. Chemotactic properties and priming abilities of
5 truncated, gingipain-treated forms of IL-8-77 were found to be 2-3-fold higher
6 compared with non-treated IL-8-77. Using a neutralising antibody against the
7 complete sequence of IL-8, we confirmed that the increased biological activity of
8 IL-8-77 following gingipain treatment was due to the release of mature IL-8 rather
9 than due to the release of small peptides identified by MS, as the neutralizing
10 antibody (which does not recognize small peptide fragments) completely inhibited
11 the increase in activity. Given that reported concentrations of reduced
12 glutathione/cysteine in gingival fluids are 1000-fold higher than those of serum
13 (7), this may represent a physiologically relevant mechanism whereby gingipains
14 contribute to neutrophil recruitment and activation at *P. gingivalis* infected sites.

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16 The extended amino terminus of IL-8-77 folds back to interact with the essential
17 Glu⁴-Leu⁵-Arg⁶ (ELR) sequence; this may protect the ELR from interaction with
18 the receptor and may explain the low chemotactic activity of IL-8-77 compared to
19 IL-8-72. The N-terminal amino acid sequence of IL-8-77 is
20 AVLPRSAKELRCQCIKTYSK- [21]. Rgp has theoretical, specific amino-peptidase
21 cleavage activity at Arg⁵-Ser⁶ and Arg¹¹-Cys¹² [15] yielding peptides of 72 and 66
22 amino acid lengths. After Rgp treatment, we observed cleavage products of IL-8-
23 77 by MS/MS of the 5 and 11 amino acids corresponding to the putative N-
24 terminal cleavage sites. There was no evidence of low molecular weight peptides

1 corresponding to cleavage at the putative C-terminal sites. Even though some
2 reports suggest that ELR in IL-8 are necessary for high affinity binding to IL-8
3 receptor, recent studies have shown that IL-8-(66) has similar activity to IL-8-72
4 (8). Our observations do not fully support this later work; whilst an increase in
5 activity of IL-8-77 is observed after Rgp treatment which results in products of 72
6 and 66 amino acid length, a decrease in IL-8-72 activity was observed after Rgp
7 treatment, indicating that IL-8-66 is not biologically active and that the observed
8 increase in activity after treatment of IL-8-77 with Rgp may be due to release of
9 IL-8-72 alone. Active IL-8 requires a properly folded protein structure with a
10 highly conserved ELR sequence near the N-terminus that is critical for its activity
11 (16). Kgp, with its specificity for the Lys-X peptide bond, is predicted to cleave the
12 IL-8-77 amino terminal sequence at Lys⁸-Glu⁹, and this product was observed in
13 our studies by MS/MS. The resultant 69 amino acid long form of IL-8 shows
14 enhanced biological activity compared with IL-8-77 in our studies of chemotactic
15 activity and respiratory burst priming. In contrast, the more biologically active
16 form of IL-8-72 showed reduced chemotactic activity after treatment with both
17 Rgp and Kgp. Analysis of released peptides by MS/MS confirmed further
18 cleavage of IL-8-72 releasing three peptides corresponding to the 15 N-terminal
19 amino acids of IL-8-72. The presence of five amino acids at the N- terminus of IL-
20 8-77 compared to IL-8-72 appears to modulate cleavage of the peptide by
21 gingipains. The difference in IL-8-77 susceptibility to gingipain treatment
22 compared with IL-8-72 may relate to either differences in three dimensional
23 structures at the N-terminus or specific charge differences which contribute to
24 change in altered accessibility and thus cleavage by gingipains. Previous studies

1 have shown that prolonged incubation with Rgp or Kgp could result total IL-8-77
2 degradation (19); physiologically, a concentration gradient will exist, where
3 released gingipain will be highest in closest proximity to the site of bacterial
4 colonization and via diffusion, the concentration of gingipains will be lower further
5 away from the site. Thus, whilst initial enzyme release may activate local IL-8-77
6 in the early stages of infection, the IL-8-77 may be completely degraded in the
7 immediate locality over time. However, further from the site of infection, diffusing
8 gingipain may cause activation of local IL-8-77. The importance of this
9 observation should be addressed in vivo following specific inhibition of gingipain
10 activity or expression.

11

12 Previous studies have shown that the capacity of gingipains to manipulate the
13 host cytokine network is partly due to degradation of other cytokines such as IL-
14 1β , IL-6, and TNF- α (3) .Therefore, it has been suggested that the ability to
15 inactivate cytokines by *P.gingivalis* in the early stages of pathogenesis is
16 advantageous for the organism. Rgp has recently been shown to digest secretory
17 leukocyte protease inhibitor (SLPI) released from neutrophils thus reducing the
18 protective effect against bacterial pro-inflammatory molecules by which disease
19 in periodontal tissues may be accelerated [21]. In contrast, degradation of pro-
20 inflammatory cytokines could diminish neutrophil chemotaxis towards infected
21 periodontal sites by lowering inflammatory cytokine secretion. However, in
22 periodontal patients, neutrophil recruitment to the gingival crevice is maintained
23 despite the presence of gingipains.

24

1 It is probable that an alternative mechanism exists to promote neutrophil
2 chemotaxis and activity at periodontitis sites which may involve the secretion of
3 the longer form of IL-8-77 by non-immune cells. This *in vitro* study provides a
4 possible mechanism for *P.gingivalis* manipulated neutrophil chemotaxis into
5 periodontal pockets via activation of IL-8-77 as illustrated by figure 5. In
6 conclusion, products from *P. gingivalis* may regulate host neutrophil
7 accumulation at infected periodontal sites by initially stimulating the production of
8 IL-8-77 by non-immune cells (e.g. epithelium) and promoting gingipain-
9 dependent modification of IL-8-77 into a more biologically active chemokine
10 which promotes neutrophil chemotaxis and priming. Thereafter, after prolonged
11 degradation by gingipains, the modified IL-8-77 may reduce chemotaxis and
12 neutrophil priming, thus prolonging the inflammatory lesion. Such a model (figure
13 5) is worthy of further investigation.

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3

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5

1 **TABLE 1**

2 Gingipain treatment of IL-8-77 and IL-8-72 released several amino terminal
 3 peptides as determined by MS/MS. The table documents peptide mass to charge
 4 (m/z) ratios, amino acid positions and sequences of peptides released post-Kgp
 5 treatment of IL-8-77.

6
 7
 8

IL-8 isoform	Gingipain treatment	m/z	Start	End	Sequence
IL-8-77	Rgp	555.7020	1	5	AVLPR
		703.8212	6	11	SAKELR
	Kgp	842.0352	1	8	AVLPRSAK
IL-8-72	Rgp	703.4097	1	6	SAKELR
	Kgp	305.1819	1	3	SAK
		1135.4	4	11	ELRCQCIK
		498.5	12	15	TYSK

1 **FIGURE LEGENDS**

2 **FIG. 1. N-terminal amino acid sequence of IL-8 and possible Rgp and Kgp**
3 **cleavage sites.** IL-8-77 has a five amino acid extended peptide sequence to IL-
4 8-72. Rgp has specific amino peptidase activity to R-X peptide bonds and Kgp
5 has specific amino peptidase activity to K-X peptide bonds.

6

7 **FIG. 2. Gingipain treatment increases chemotactic activity of IL-8-77**
8 **towards dHL60 cells and neutrophils.** IL-8 isoforms of 72 and 77 amino acid
9 long peptides were treated with 10mM cysteine activated Kgp or Rgp for 30 min.
10 Chemotaxis (corrected for background) of 1×10^5 dHL60 cells through a $2 \mu\text{m}$ filter
11 towards Kgp (A) and Rgp (B) treated or untreated IL-8 isoforms was observed for
12 90 min in a multiwell chemotaxis chamber. Specific chemotaxis of 1×10^5 primary
13 neutrophils towards Kgp (C) and Rgp (D) treated IL-8 isoforms through a $5 \mu\text{m}$
14 filter was observed for 90 min in multiwell chemotaxis chamber. Cell counts in the
15 lower wells were taken by flow cytometry and are expressed as mean cell
16 number migrated \pm S.E.M, where $n=3$ independent experiments performed in
17 triplicate. * $p < 0.01$, ** $p < 0.001$ and NS = not significant using Tukey's multiple
18 comparison test.

19

20 **FIG. 3. Major IL-8 peptides released from gingipain treatment of IL-8-77 are**
21 **responsible for enhanced chemotactic activity of dHL60 cells and primary**
22 **neutrophils.** IL-8 isoforms of 72 and 77 amino acid long peptides were treated
23 with 10mM cysteine activated Kgp or Rgp for 30 min. After neutralisation of
24 gingipains, resultant peptides were subsequently incubated with neutralising anti-

1 IL-8. Chemotaxis (corrected for background) of 1×10^5 dHL60 cells through a $2 \mu\text{m}$
2 filter towards Kgp-treated-IL-8-77 and Kgp-treated-IL-8-77 + IL-8 neutralised
3 isoforms (A) or Rgp-treated-IL-8-77 and Rgp-treated-IL-8-77 + IL-8 neutralised
4 isoforms (B) was observed for 90 min in a multiwell chemotaxis chamber.
5 Specific chemotaxis of 1×10^5 primary neutrophils towards Kgp-treated-IL-8-77
6 and Kgp-treated-IL-8-77 + IL-8 neutralised isoforms (C) and Rgp-treated-IL-8-77
7 and Rgp-treated-IL-8-77 + IL-8 neutralised isoforms (D) through a $5 \mu\text{m}$ filter was
8 observed for 90 min in a multiwell chemotaxis chamber. Cell counts in the lower
9 wells were taken by flow cytometry and are expressed as mean cell number
10 migrated \pm S.E.M, where $n=3$ independent experiments performed in triplicate. **
11 $p < 0.001$ using Tukey's multiple comparison test.

12

13 **FIG. 4. Gingipain treatment enhances the priming effect of IL-8-77 on**
14 **neutrophils.** Neutrophils (5×10^5) were primed with (a) Rgp-treated or untreated
15 IL-8 isoforms or (B) Kgp- treated or untreated IL-8 isoforms for 10min prior to
16 stimulation with $1 \mu\text{M}$ fMLP. Mean peak (RLU \pm S.E.M., $n=9$) chemiluminescence
17 generated by neutrophils was recorded. Significant differences were calculated (*
18 $p < 0.05$) using Tukey's multiple comparison test. NS= not significant .

19

20 **Fig. 5: Schematic representation of gingipain modulated IL-8 response.** *Pg*
21 stimulates production of host pro-inflammatory mediators including IL-8. IL-8-77
22 secreted by host epithelial cells can be cleaved into more active, truncated forms.
23 Collectively with IL-8-72, these truncated forms may recruit more neutrophils to
24 the site of infections and also prime their activation which may contribute to the

1 increased hyperactivity of neutrophils in periodontitis. Prolonged exposure to
2 gingipains may trigger further degradation of IL-8-77 which may reduce
3 chemotaxis and neutrophil priming, thus prolonging the inflammatory lesion.

4 Gingipain structure from www.caspases.org

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6