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

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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.

Key words

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

Abbreviations

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

INTRODUCTION

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

The oral anaerobic rod, *Porphyromonas gingivalis*, is the organism most strongly associated with active periodontitis (1). This organism possesses a number of virulence determinants which potentially contribute to its pathogenicity including the ability to secrete a range of degradative proteinases (1); among these, the gingipains have been extensively studied (15). Furthermore, the pathogenic bacteria within the subgingival environment shed proinflammatory mediators such as lipopolysaccharide (LPS). LPS in turn triggers the secretion of chemokines/cytokines such as IL-1β, TNF-α, IL-6 and interleukin-8 (IL-8-72) from resident inflammatory cells, which contribute to the initial inflammatory response (20).

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

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

MATERIALS AND METHODS

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

HL60 cell cultures. The human pro-myelocytic cell line HL60 was purchased from the European collection of cell cultures (ECACC no 98070101) and maintained in RPMI-1640 medium supplemented with 10% heat inactivated foetal calf serum (FCS) and 1% penicillin/streptomycin at 37^0C in a 5% CO_2/ 95% air humidified atmosphere. The cells were induced to differentiate in the presence of 1.0% dimethyl sulfoxide (DMSO) for 5 days after seeding at cell density of 2×10^5 cells/ml.

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

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

Cultivation of Porphyromonas gingivalis Strain W83. Cultures were grown statically in 200ml of liquid medium containing 6.0g of trypticase soy broth (Difco, Detroit, Mich, U.S.A), 2.0 g of yeast extract, supplemented with 1mg of hemin, 200mg of L-cysteine, 20mg of di-thiothreitol and 0.5mg of menadione at 37^0C in an anaerobic atmosphere of 10% H_2, 10% CO_2, and 80% N_2 for 48h (miniMACS anaerobic workstation, Don Whitley Scientific).

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

Enzyme activity assays. The amidolytic activities of the purified Rgp and Kgp were measured with the substrate N_α-benzoyl-L-arginine p-nitroanilide hydrochloride (L-BAPNA). 100µl of Rgp and Kgp fractions were incubated with L-BAPNA (final concentration of 1mM) in 100µl of 0.2M Tris-HCl, 0.1M NaCl, 5mM $CaCl_2$, 10mM L-cysteine at pH 7.6 and 37^0C. After one-hour incubation, the reaction was stopped by addition of 10µl of glacial acetic acid. The optical density was measured at 405nm for each fraction and the values corrected by subtraction of negative control values (without proteinases).

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

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

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

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

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

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

RESULTS

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

Kgp and Rgp increase the chemotactic properties of biologically inactive IL-8-77. In order to determine the effect of Rgp and Kgp treatment on chemotactic properties of IL-8 isoforms, dHL60 cells/ primary neutrophils were allowed to undergo chemotaxis towards pre- or post-gingipain treated IL-8-72 and IL-8-77 isoforms. The effect of gingipain treatment in IL-8-dependent chemotaxis was corrected for chemotaxis towards either inactivated Rgp or inactivated Kgp, where migration was always lower towards gingipains than to LPS or IL-8 isoforms. IL-8-72 demonstrated higher chemotactic activity than the native IL-8-77 isoform. Both Rgp (Fig. 2A) and Kgp (Fig. 2B) treatments significantly decreased the chemotactic activity of IL-8-72 ($P<0.001$) towards HL60 cells. In contrast, Kgp treatment significantly increased IL-8-77 chemotactic properties ($P<0.05$). In order to compare the behaviour of dHL60 cells with peripheral blood neutrophils, chemotaxis of primary neutrophils towards IL-8 isoforms was measured. Confirming the observations in dHL60 cells, both Kgp- (Fig. 2C) and Rgp- (Fig. 2D) treatments significantly increased the chemotactic properties of IL-8-77 ($p<0.001$ and $p<0.01$ respectively) towards primary neutrophils.

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

Kgp and Rgp increase the priming effect of IL-8-77 for the respiratory burst in response to fMLP. The priming effect of IL-8 on the fMLP-induced respiratory burst was measured by lucigenin-dependant chemiluminescence using isolated peripheral blood neutrophils. IL-8-72 primed neutrophils for enhanced superoxide production after fMLP stimulation whereas neither IL-8-77 nor isolated gingipains had any priming effect (Fig. 4). However, both Rgp- (Fig. 4A) and Kgp- (Fig. 4B) treated IL-8-77 primed neutrophils for fMLP induced superoxide production, demonstrating significantly increased superoxide generation compared with native IL-8-77 ($p<0.05$). By contrast, gingipain treatment decreased the priming activity of IL-8-72 ($p<0.05$) for fMLP-stimulated superoxide production.

DISCUSSION

Infection of host tissue by pathogenic bacteria and/or stimulation by microbial components/virulence factors triggers the production of pro-inflammatory peptides that have the ability to activate and recruit neutrophilic polymorphonuclear leucocytes along a concentration gradient. Patients with periodontitis show increased numbers of neutrophils within periodontal tissues and pockets (21), and recent work has demonstrated baseline hyper-activity of peripheral blood neutrophils, with respect to extracellular ROS release (18) and proteolytic enzyme release (9), in periodontitis patients relative to matched healthy controls. Such mechanisms when co-active may explain a significant amount of the oxidative stress reported in periodontitis tissues (6). Therefore the influence of periodontal bacteria and their virulence factors on IL-8-mediated neutrophil chemotaxis and activation are important to elucidate. In contrast to biologically active IL-8-72, the IL-8-77 peptide produced by epithelial cells, fibroblasts and endothelial cells is resistant to a wide range of host proteinases; it has a low chemotactic activity (10) and less respiratory burst priming activity. In our experiments, we have used LPS as a positive control; LPS is a well known chemotactic bacterial component which requires serum components such as LPS-binding protein for receptor activation (28). However, as serum may also contain other chemotactic factors, it was excluded from our experiments, and the chemotactic activity of LPS in serum-free conditions here was greater than IL-8-77 but less than IL-8-72 towards primary neutrophils.

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

Our results demonstrate a significant increase in the chemotactic properties of IL-8-77 and a higher priming capability of IL-8-77 after incubation with L-cysteine-activated gingipains under the conditions described. Compared with primary neutrophils, dHL60 cells have low CXC2 receptor expression (26), and this may explain the lower rate of dHL60 cell migration towards IL-8. The corresponding increase in data variation may account for the lack of significant increase in migration of dHL60 towards Rgp-treated IL-8-77 when compared to the increased migration of primary neutrophils. Chemotactic properties and priming abilities of truncated, gingipain-treated forms of IL-8-77 were found to be 2-3-fold higher compared with non-treated IL-8-77. Using a neutralising antibody against the complete sequence of IL-8, we confirmed that the increased biological activity of IL-8-77 following gingipain treatment was due to the release of mature IL-8 rather than due to the release of small peptides identified by MS, as the neutralizing antibody (which does not recognize small peptide fragments) completely inhibited the increase in activity. Given that reported concentrations of reduced glutathione/cysteine in gingival fluids are 1000-fold higher than those of serum (7), this may represent a physiologically relevant mechanism whereby gingipains contribute to neutrophil recruitment and activation at *P. gingivalis* infected sites.

The extended amino terminus of IL-8-77 folds back to interact with the essential Glu^4-Leu^5-Arg^6 (ELR) sequence; this may protect the ELR from interaction with the receptor and may explain the low chemotactic activity of IL-8-77 compared to IL-8-72. The N-terminal amino acid sequence of IL-8-77 is AVLPRSAKELRCQCIKTYSK- [21]. Rgp has theoretical, specific amino-peptidase cleavage activity at Arg^5-Ser^6 and Arg^{11}-Cys^{12} [15] yielding peptides of 72 and 66 amino acid lengths. After Rgp treatment, we observed cleavage products of IL-8-77 by MS/MS of the 5 and 11 amino acids corresponding to the putative N-terminal cleavage sites. There was no evidence of low molecular weight peptides corresponding to cleavage at the putative C-terminal sites. Even though some reports suggest that ELR in IL-8 are necessary for high affinity binding to IL-8 receptor, recent studies have shown that IL-8-(66) has similar activity to IL-8-72 (8). Our observations do not fully support this later work; whilst an increase in activity of IL-8-77 is observed after Rgp treatment which results in products of 72 and 66 amino acid length, a decrease in IL-8-72 activity was observed after Rgp treatment, indicating that IL-8-66 is not biologically active and that the observed increase in activity after treatment of IL-8-77 with Rgp may be due to release of IL-8-72 alone. Active IL-8 requires a properly folded protein structure with a highly conserved ELR sequence near the N-terminus that is critical for its activity (16). Kgp, with its specificity for the Lys-X peptide bond, is predicted to cleave the IL-8-77 amino terminal sequence at Lys^{8}-Glu^{9}, and this product was observed in our studies by MS/MS. The resultant 69 amino acid long form of IL-8 shows enhanced biological activity compared with IL-8-77 in our studies of chemotactic activity and respiratory burst priming. In contrast, the more biologically active form of IL-8-72 showed reduced chemotactic activity after treatment with both Rgp and Kgp. Analysis of released peptides by MS/MS confirmed further cleavage of IL-8-72 releasing three peptides corresponding to the 15 N-terminal amino acids of IL-8-72. The presence of five amino acids at the N- terminus of IL-8-77 compared to IL-8-72 appears to modulate cleavage of the peptide by gingipains. The difference in IL-8-77 susceptibility to gingipain treatment compared with IL-8-72 may relate to either differences in three dimensional structures at the N-terminus or specific charge differences which contribute to change in altered accessibility and thus cleavage by gingipains. Previous studies have shown that prolonged incubation with Rgp or Kgp could result total IL-8-77 degradation (19); physiologically, a concentration gradient will exist, where released gingipain will be highest in closest proximity to the site of bacterial colonization and via diffusion, the concentration of gingipains will be lower further away from the site. Thus, whilst initial enzyme release may activate local IL-8-77 in the early stages of infection, the IL-8-77 may be completely degraded in the immediate locality over time. However, further from the site of infection, diffusing gingipain may cause activation of local IL-8-77. The importance of this observation should be addressed in vivo following specific inhibition of gingipain activity or expression.

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

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

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TABLE 1

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

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

FIGURE LEGENDS

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

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

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

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

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

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

increased hyperactivity of neutrophils in periodontitis. Prolonged exposure to gingipains may trigger further degradation of IL-8-77 which may reduce chemotaxis and neutrophil priming, thus prolonging the inflammatory lesion. Gingipain structure from www.caspases.org