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Fusobacterium nucleatum enhances invasion of human gingival epithelial and aortic endothelial cells by Porphyromonas gingivalis

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Bacterial invasion; Porphyromonas gingivalis; Fusobacterium nucleatum; endothelial; gingival epithelial; polymicrobial infection

Short running title:
Polymicrobial infection of endothelial cells

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Abstract
Invasion by Porphyromonas gingivalis has been proposed as a possible mechanism of pathogenesis in periodontal and cardiovascular diseases. *P. gingivalis* have direct access to the systemic circulation and endothelium in periodontitis patients by transient bacteremia. Periodontitis can be described as one of the predominant polymicrobial infections of humans. In the present study, *P. gingivalis* strains were tested for their ability to invade a human gingival epithelial cell line (Ca9-22) and human aortic endothelial cells (HAEC) in co-infection with Fusobacterium nucleatum using antibiotic protection assays. Co-infection with *F. nucleatum* resulted in 2- to 20- fold increase in invasion of host cells by *P. gingivalis* strains. The invasive abilities of *P. gingivalis* strains were significantly greater when incubated with a *F. nucleatum* clinical isolate (which possesses strong biofilm-forming ability), than when incubated with *F. nucleatum* type strain. In inhibition assays with metabolic inhibitors, difference in inhibition profiles was noted between mono- and polymicrobial infections. Collectively, our results suggest that *F. nucleatum* facilitates invasion of host cells by *P. gingivalis*. Investigations of polymicrobial infection of host cells should improve our understanding of the role of *P. gingivalis* in periodontal infection and proatherogenic mechanisms.
Introduction

Epidemiological studies have demonstrated a positive association between periodontitis and cardiovascular diseases (Beck et al., 1996). It has been suggested that one of the linkage between these diseases is directly infectious, i.e., bacteria within the atheroma may be involved in the development of the atherosclerotic plaque (Chiu, 1999; Lalla et al., 2003; Gibson III et al., 2004). Periodontal pathogens including Porphyromonas gingivalis have been detected in atherosclerotic plaques in humans using PCR techniques (Haraszthy et al., 2000; Ishihara et al., 2004, Kozarov et al., 2006). P. gingivalis elicits a proatherogenic response in endothelial cells in the form of increased leukocyte adhesion with concomitant up-regulation of adhesion molecules, heightened production of proinflammatory cytokines and chemokines, as well as an induction of prothrombotic properties (Kang & Kuramitsu, 2002; Roth et al., 2007). Interestingly, these effects on endothelial cells cannot be attributed to a sole effect of stimulation by bacterial cell-surface components, but may require the invasion of host cells by viable bacteria (Darveau et al., 2002; Roth et al., 2007).

Invasion by P. gingivalis has been proposed as a possible mechanism of pathogenesis in periodontal and cardiovascular diseases (Lamont et al., 1995; Deshpande et al., 1998). P. gingivalis has direct access to the systemic circulation and the endothelium in periodontitis patients, as transient bacteremias are common (Kinane et al., 2005), and the ability of P. gingivalis, detected at the sites of atherosclerotic disease, to invade host cells has been demonstrated (Kozarov et al., 2005).

Periodontitis can be described as one of the predominant polymicrobial infections of humans (Brogden et al., 2005). Since periodontal diseases result from complex interactions of multiple microorganisms, it is essential to investigate interactions between different periodontal bacteria and host cells. Bacterial species in subgingival
plaque have been shown to fall into five distinctive complexes of closely related species (Socransky et al., 1998). Bacteria in one of the complexes referred to as “red complex” are commonly associated with periodontal lesions. However, bacteria in this complex such as *P. gingivalis* are usually detected in the presence of bacteria from a closely related complex referred to as “orange complex” comprising e.g. *Fusobacterium nucleatum* (Socransky et al., 1998, Kesavalu et al., 2007). Antagonistic and synergistic physiologic mechanisms, as well as environmental selection are thought to be involved in such relationships (Kesavalu et al., 2007). *F. nucleatum* initially adheres to early colonizers, including gram-positive cocci, and enhances the adherence of other periodontopathic bacteria including *P. gingivalis* (Kolenbrander, 2000).

In polymicrobial infections by bacterial enteropathogens, it has been shown that the ability of *Campylobacter jejuni* to invade cultured epithelial cells is significantly enhanced by the presence of other enteropathogens as coinfectants (Bukholm & Kapperud, 1987). Whether similar interaction occurs in periodontopathogens is unknown. Data on the potential of *P. gingivalis* invasion into host cells in polymicrobial infection are scarce; the present study therefore sought to investigate the capacity of *P. gingivalis* to invade human gingival epithelial and aortic endothelial cells in co-infection with *F. nucleatum*. 
Materials and Methods

Bacterial strains and growth conditions

*P. gingivalis* ATCC 33277, *P. gingivalis* W83 (ATCC BAA-308), *F. nucleatum* TDC100 [a clinical isolate and working strain in our laboratory (Saito et al., 2008)] and *F. nucleatum* ATCC 25586 were routinely maintained on tryptic soy agar (Difco Laboratories, Detroit, MI) supplemented with 10% defibrinated horse blood, hemin (5 µg mL\(^{-1}\)) and menadione (0.5 µg mL\(^{-1}\)) at 37 °C under anaerobic conditions.

*Escherichia coli* SCS110 and DH5α strains were used as a control in antibiotic protection assays.

Cells and culture conditions

Human gingival epithelial cell line, Ca9-22, was purchased from Health Science Research Resources Bank (Osaka, Japan). Ca9-22 is an established transformed human gingival cell line, which has been used in previous studies as a culture model of oral epithelial cells (Hirose et al., 1999; Ohshima et al., 2001; Takeuchi et al., 2008). The Ca9-22 cells were maintained in Eagle’s minimal essential medium (MEM) supplemented with glutamine (0.6 g L\(^{-1}\)), heat-inactivated 10 % fetal calf serum, and gentamicin (50µg mL\(^{-1}\)) / amphotericin B (50ng mL\(^{-1}\)) (Cascade Biologics, Portland, OR) at 37 °C in 5 % CO\(_2\).

Human aorta endothelial cells (HAEC) were supplied by Kurabo Inc. (Osaka, Japan) and maintained in HuMedia-EG2 (Kurabo) under an atmosphere of 5% CO\(_2\) and 95 % air at 37 °C. Cells from passages 4 through 9 were tested for viability and morphology prior to seeding in appropriate tissue culture plates and allowed to reach near-confluency before assay.

Invasion procedures

Invasion of bacteria was quantitated by the standard antibiotic protection assay (Lamont
et al, 1995, Deshpande et al., 1998). Briefly, epithelial cells were seeded in 12-well flat-bottom culture plate (Iwaki, Chiba, Japan) at a cell density of 2.0 x 10^5 cells per well. Prior to infection, the cells were washed twice with phosphate-buffered saline (PBS, pH 7.4) and incubated further 2 h in MEM without antibiotics. The multiplicity of infection (MOI) was calculated based on the number of cells per well at confluence. *P. gingivalis* and *F. nucleatum* strains were inoculated into brain heart infusion broth (Becton Dickinson, Sparks, MD) supplemented with 0.5 % of yeast extract, hemin (5 μg mL⁻¹) and menadione (0.5μg mL⁻¹), and grown for 2 days until the optical density at 660 nm reached 1.0. After washing with PBS, bacterial cells were resuspended in MEM. Bacterial suspensions (2.0 x 10^7 cells per well) were added to confluent Ca9-22 monolayers (MOI=100) and incubated at 37°C in 5 % CO₂ for 2h. After incubation, unattached bacteria were removed following washing of the monolayers 3 times with PBS. External adherent cells were then killed by incubating the infected monolayers with MEM containing 200 μg mL⁻¹ of metronizazole and 300 μg mL⁻¹ of gentamicin for 1 h. This concentration of antibiotic was sufficient to completely kill 10^8 bacteria per mL in 1h. Controls for antibiotic killing of bacteria were included in all experiments. After exposure to antibiotic, monolayers were washed twice with PBS, and lysed in 1 mL of sterile distilled water per well. Cells were incubated aerobically for 30 min, during which they were disrupted by repeated pipetting. Lysates were diluted and plated on blood agar plates, and incubated anaerobically at 37°C for 10 days. CFU of invasive organisms were then enumerated. Invasion efficiency was expressed as the percentage of the initial inoculum recovered after antibiotic treatment and Ca9-22 lysis.

The invasion assay with HAEC was performed using the same procedure as above with EG2 medium.
**Polymicrobial infection**

Following demonstration of monomicrobial infections with *P. gingivalis* strains, we performed experiments to develop a model of polymicrobial periodontal infection using *P. gingivalis* and *F. nucleatum* as members of a prototype consortium, and examined the invasion characteristics and interactions of these organisms. For polymicrobial infection, *P. gingivalis* (1 x 10^7 cells mL^-1) was gently mixed with an equal volume of *F. nucleatum* (1 x 10^7 cells mL^-1) and the organisms were allowed to interact for 5 min. For the monomicrobial control infection, *P. gingivalis* was mixed with an equal volume of MEM or EG2 medium. As a control for polimicrobrial infection, *E. coli* SCS110 or DH5α was pre-incubated with *P. gingivalis*. The poly- and monomicrobial inocula were added to Ca9-22 or HAEC monolayers.

The bacterial culture growth phase, viability, counts, interaction times, suspension medium, infection dose, and infection procedures were all standardized; i.e., the same preparation and infection protocols were used for all invasion assays throughout the study.

**Inhibition of bacterial invasion**

For inhibition assays with antiserum, *P. gingivalis* or *F. nucleatum* cells were preincubated with the indicated dilution of rabbit polyclonal anti-*P. gingivalis* serum for 30 min at RT prior to use in assays.

*P. gingivalis* or *F. nucleatum* cells were also preincubated with indicated concentrations of D-galactose (inhibitor of *F. nucleatum* adhesion/invasion) for 15 min at RT prior to use in assays.

To dissect the biochemical pathways involved, the effect of a group of metabolic inhibitors on invasion was investigated. The following inhibitors, in the solvent and at the final concentration indicated, were used. Cytochalasin D, 1 µg mL^-1 in dimethyl
sulfoxide (DMSO); nococazole, 10 µg mL⁻¹ in DMSO; staurosporine, 0.5 µM in DMSO; cycloheximide, 100 µg mL⁻¹ in ethanol. The inhibitors were preincubated with the host cells for 60 min prior to addition of the bacteria and remained present throughout the invasion assay. All potential inhibitors were tested at the concentration used for possible adverse effects on the host cells, through comparison to cells without inhibitors, by examining the morphology of the cells and the confluency of the monolayer.

**Data and statistical analysis**

All experiments were performed in duplicate or triplicate for each condition and repeated at least three times. Statistical comparisons were performed using a software package (InStat 3.0, GraphPad Software Inc., La Jolla, CA). Mann-Whitney U test or analysis of variance (ANOVA) with Bonferroni post test (for multiple comparisons) was used.
Results

Monomicrobial infection

There was strain to strain variability in the ability of \textit{P. gingivalis} to invade HAEC and Ca9-22 (Fig. 1). For both HAEC and Ca9-22, \textit{P. gingivalis} strain 33277 had greater invasive abilities than strain W83. For HAEC, mean invasion efficiencies for \textit{P. gingivalis} 33277 and W83 were 1.58 % and 0.52 %, respectively (Fig. 1a). The ability of \textit{P. gingivalis} 33277 to invade Ca9-22 was essentially equal to its ability to invade HAEC (Fig. 1b); however, \textit{P. gingivalis} W83 invaded HAEC approximately 3-fold over Ca9-22.

Polymicrobial infection with \textit{P. gingivalis} and \textit{F. nucleatum}

In polymicrobial infection experiments with \textit{P. gingivalis} and \textit{F. nucleatum}, viable counts of \textit{P. gingivalis} strains recovered from HAEC or Ca9-22 cells were used to determine bacterial invasion. Co-incubation of \textit{P. gingivalis} 33277 with \textit{F. nucleatum} significantly boosted \textit{P. gingivalis} invasion of host cells, resulting in 2- to 20-fold increase in invasion efficiencies (Fig. 1). Co-incubation with \textit{E. coli} SCS110 or DH5α exerted no effect on \textit{P. gingivalis} invasion of HAEC or Ca9-22.

Invasion of host cells by \textit{P. gingivalis} 33277 was statistically significantly higher in the presence of \textit{F. nucleatum} TDC100 (invasion efficiency: 12 %) than \textit{F. nucleatum} 25586 (6 %) \((P < 0.01)\) (Fig. 1a). In the presence of \textit{F. nucleatum}, \textit{P. gingivalis} W83 which showed low invasive ability in monomicrobial infection, demonstrated dramatic increase in invasion. Invasion of HAEC with \textit{P. gingivalis} W83 was significantly more enhanced in the presence of \textit{F. nucleatum} TDC100 (invasion efficiency: 8 %) than \textit{F. nucleatum} 25586 (2 %) \((P < 0.01)\). Similarly, \textit{F. nucleatum} significantly enhanced invasion of \textit{P. gingivalis} strains to Ca9-22 cells (Fig. 1b).
Inhibition of bacterial invasion

*P. gingivalis* invasion of HAEC was inhibited by anti-*P. gingivalis* serum (diluted 1:100) by approximately 70% (Table 1). The inhibition was significant, but relatively low when co-incubated with *F. nucleatum*. A similar trend was observed with Ca9-22 cells (data not shown).

To determine whether the enhanced invasion of *P. gingivalis* in polymicrobial infection involves a lectin-like adhesin(s), sugar inhibition assay was performed. Incubation with D-galactose resulted in decreased invasion by *P. gingivalis* in polymicrobial infection experiments (Fig.2).

Various metabolic inhibitors previously reported to reduce *P. gingivalis* or *F. nucleatum* invasion were assessed for the ability to inhibit *Fusobacterium*-enhanced *P. gingivalis* invasion. In mono- and polymicrobial infection experiments, invasion of the host cells by *P. gingivalis* required multiple components of the host including actin, microtubule, and protein kinases (Table 2). One notable difference in inhibition profiles was observed between mono- and polymicrobial infections. Cycloheximide (which target host cell protein synthesis) significantly reduced invasion by *P. gingivalis* in polymicrobial infection experiments. This inhibitor has previously been shown to inhibit *F. nucleatum* invasion but not *P. gingivalis* invasion.
Discussion
In the present study, we first explored the abilities of different *P. gingivalis* strains to invade gingival epithelial and endothelial cells. *P. gingivalis* W83 has been shown to be highly virulent in experimental animal models (Neiders *et al.*, 1989, Genco *et al.*, 1991). In our experimental set-up, invasive ability of *P. gingivalis* W83 into host cells was relatively low when compared to *P. gingivalis* 33277, which has been shown to be highly fimbriated but less virulent. Furthermore, *P. gingivalis* W83 displayed an invasive ability that differed in the tested cell types. Fimbriae are considered important in adherence and invasion by *P. gingivalis*. However, it has been shown that the presence and expression of *fimA* is not sufficient for *P. gingivalis* invasion of endothelial and epithelial cells (Dorn *et al.*, 2000; Umeda *et al.*, 2006). The differential invasion efficiency observed for different cell types is likely due to different interactions between *P. gingivalis* and the types of cell surface receptors present on the different cell types that are involved in the invasion process.

There is increasing evidence in the literature for the importance of polymicrobial infections in which selected microorganisms interact in a synergistic or antagonistic fashion, impacting on pathogenesis of periodontal disease (Chen *et al.*, 1996; Feuille *et al.*, 1996; Kesavalu *et al.*, 2007). Synergistic interactions in virulence between *F. nucleatum* and *P. gingivalis* have been observed *in vitro* and in animal models (Feuille *et al.*, 1996; Ebersole *et al.*, 1997). In this study, we demonstrated that *F. nucleatum* enhances the ability of *P. gingivalis* to invade host cells. To the best of our knowledge, this study is the first to demonstrate such an interaction between *F. nucleatum* and *P. gingivalis*.

In polymicrobial infection experiments, *F. nucleatum* TDC 100 enhanced *P. gingivalis* invasion of host cells significantly more than *F. nucleatum* type strain. A
previous study from our group has demonstrated that \textit{F. nucleatum} TDC 100 has a synergistic relationship with \textit{P. gingivalis} and a strong biofilm forming ability (Saito \textit{et al.}, 2008). Han \textit{et al.} (2000) reported that a spontaneous \textit{lam} mutant \textit{F. nucleatum}, defective in aggregation with human lymphocytes and coaggregation with \textit{P. gingivalis}, was defective in attachment to and invasion of human gingival epithelial cells, suggesting that same bacterial determinants are involved in aggregation properties and ability to invade host cells. \textit{F. nucleatum} and \textit{P. gingivalis} are strong coaggregating pairs, and the coaggregation may have the capacity to alter the expression of virulence factors in individual microorganisms (Feuille \textit{et al.}, 1996). Coaggregation between \textit{P. gingivalis} and \textit{F. nucleatum} is mediated by a galactoside moiety on the \textit{P. gingivalis} surface and a lectin on the \textit{F. nucleatum}, and inhibited by lactose, galactose and related monosaccharides (Kolenbrander \& Anderson, 1989; Kinder \& Holt, 1993). We have observed a strong coaggregation reaction between \textit{P. gingivalis} 33277 and \textit{F. nucleatum} TDC 100, that is inhibitable by galactose (data not shown). The synergistic interactions between \textit{F. nucleatum} and \textit{P. gingivalis} observed in the present study could be partly explained by coaggregating effect between these organisms, as galactose also inhibited \textit{F. nucleatum} enhanced \textit{P. gingivalis} invasion.

In the present study, co-infection with \textit{F. nucleatum} strains markedly enhanced invasion of host cells by \textit{P. gingivalis} W83, a strain we have shown to be minimally invasive in monomicrobial infection of host cells. Rudney \textit{et al.} (2005) showed that intracellular infections of buccal epithelial cells with periodontal pathogens were uniformly polymicrobial, and proposed several scenarios regarding invasion of host cells by a consortium of oral bacteria. Invasiveness might be limited to a subset of oral species that use it as a virulence factor. Alternatively, a wide range of oral bacteria which principally live in biofilm might be capable of invasion as a means of persisting.
Since species interaction appears to be widespread in oral biofilm (Cook et al., 1998; Palmer et al., 2001; McNab et al., 2003), another alternative could be that non-invasive species gain entrance to cells by forming consortia with invasive species. It has been reported that *F. nucleatum* transports noninvasive *Streptococcus cristatus* into human epithelial cells (Edwards et al., 2006). In the present study, polymicrobial infection of the host cells by *P. gingivalis* and *F. nucleatum* not only facilitated *P. gingivalis* invasion, but also resulted in the invasion by *F. nucleatum*, although the extent of *F. nucleatum* invasion was relatively low, when compared to that of *P. gingivalis* 33277 (data not shown). Although anti-*P. gingivalis* serum abrogated *P. gingivalis* invasion of host cells in a monomicrobial infection setting, the extent of inhibition was less in polymicrobial infection. These results suggested that mechanisms other than adherence signal induced by *P. gingivalis* are likely to be involved, and that interaction(s) between *F. nucleatum* and host cells may play a significant role in fusobacterium-enhanced *P. gingivalis* invasion.

Also in the inhibition experiment, cycloheximide significantly reduced invasion by *P. gingivalis* in polymicrobial infection. As this inhibitor has previously been shown to inhibit *F. nucleatum* invasion (Han et al., 2000) but not *P. gingivalis* invasion (Deshpande et al., 1998), it is conceivable that infection by *F. nucleatum* may pave the way for increased invasion of *P. gingivalis*.

We cannot yet clarify whether the effect exerted by the coinfectant is directed at the host cell or the *P. gingivalis*. Invasive bacteria generally gain entry by co-opting and re-directing host cell mechanisms such as endocytosis (Lamont et al., 1995; Sandros et al., 1996; Progulske-Fox et al., 1999; Meyer et al., 1999; Han et al., 2000). Immunomodulating roles of *F. nucleatum* have been suggested by previous studies (Feuille et al., 1996; Choi et al., 2001). Polymicrobial infections may actually modulate
the adaptive host responses, leading to more effective evasion of protective immune responses.

In summary, this report demonstrates that *F. nucleatum* facilitates *P. gingivalis* invasion of human gingival epithelial and endothelial cells. The significance of this increased ability to invade cells in progression of periodontal as well as cardiovascular diseases needs to be elucidated in future studies. We are currently investigating molecular mechanisms involved in this relationship, and whether other periodontopathogens in a consortium are able to induce the synergistic effects.

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Fig. 1

Invasion of (a) HAEC (b) Ca9-22 by *P. gingivalis* 33277 or *P. gingivalis* W83 in mono- or polymicrobial infection with *F. nucleatum* strains. (MOI=1:100, infected for 2h). Invasion efficiency (%) was expressed as percentage of the inoculum (*P. gingivalis*) protected from the antibiotic killing for 1h. Values are the means ± standard deviations of triplicate independent determinations from a typical experiment. *Statistically significantly different (*P* < 0.01, ANOVA with Bonferroni multiple comparisons test).
Fig. 2
Inhibition of fusobacterial enhanced *P. gingivalis* invasion by D-galactose. The effects of increasing concentrations of D-galactose on *P. gingivalis* invasion of HAEC were assessed for monomicrobial (*P. gingivalis* only; open circles), or polymicrobial (*P. gingivalis* and *F. nucleatum* together; filled squares) infections. Values are the means ± standard deviations of triplicate independent determinations from a typical experiment. *Statistically significantly different from control (P < 0.01, Mann-Whitney U test).
**Table 1.** Effect of anti-*P.gingivalis* serum on *P.gingivalis* invasion of HAEC by mono- or polymicrobial infection

<table>
<thead>
<tr>
<th>Pre-incubation</th>
<th>Invasion of HAEC (% of control)(^a)</th>
<th>Mono-infection (Pg)(^b)</th>
<th>Poly-infection (Pg + Fn)(^c)</th>
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<tbody>
<tr>
<td>Control (Pre-immune serum)</td>
<td>100 ± 12</td>
<td></td>
<td>100 ± 21</td>
</tr>
<tr>
<td>Anti-Pg serum (dilution 1:1000)</td>
<td>79 ± 12*</td>
<td></td>
<td>89 ± 18</td>
</tr>
<tr>
<td>Anti-Pg serum (dilution 1:100)</td>
<td>29 ± 20*</td>
<td></td>
<td>58 ± 11*</td>
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\(^a\) Invasion of *P.gingivalis* 33277 relative to the level obtained in the absence of antiserum (pre-immune serum control). Values given as means ± standard deviations of triplicate independent determinations from a typical experiment.

\(^b\) Cells were infected by *P. gingivalis* 33277

\(^c\) Cells were infected by *P. gingivalis* 33277 and *F. nucleatum* TDC100

*Statistically significantly different from control (*P < 0.01*) by ANOVA with Bonferroni multiple comparisons test
**Table 2.** Effects of metabolic inhibitors on *P. gingivalis* invasion of HAEC by mono- or polymicrobial infection

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Target</th>
<th>Invasion of HAEC (% of untreated control) a</th>
<th>Mono-infection (Pg) b</th>
<th>Poly-infection (Pg + Fn) c</th>
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<tbody>
<tr>
<td>Cytochalasin D</td>
<td>Actin</td>
<td>11.5 ± 3.5*</td>
<td>23.0 ± 11.1*</td>
<td></td>
</tr>
<tr>
<td>Nocodazole</td>
<td>Microtubule</td>
<td>14.8 ± 5.0*</td>
<td>18.2 ± 16.4*</td>
<td></td>
</tr>
<tr>
<td>Saturosporine</td>
<td>Protein kinase</td>
<td>54.4 ± 5.5*</td>
<td>22.7 ± 17.9*, †</td>
<td></td>
</tr>
<tr>
<td>Cycloheximide</td>
<td>Protein synthesis</td>
<td>91.7 ± 5.5</td>
<td>25.7 ± 8.3*, †</td>
<td></td>
</tr>
</tbody>
</table>

a Invasion of *P. gingivalis* 33277 relative to the level obtained in the absence of inhibitor (medium control). Values given as means ± standard deviations of triplicate independent determinations from a typical experiment.

b Cells were infected by *P. gingivalis* 33277

c Cells were infected by *P. gingivalis* 33277 and *F. nucleatum* TDC100

*Statistically significantly different from control (P < 0.01) by ANOVA with Bonferroni multiple comparisons test

†Statistically significantly different from monomicrobial infection (P < 0.01) by Mann-Whitney U test