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Epithelial cell pro-inflammatory cytokine response differs across dental plaque bacterial species

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Abstract

Aim: The dental plaque is comprised of numerous bacterial species, which may or may not be pathogenic. Human gingival epithelial cells (HGECs) respond to perturbation by various bacteria of the dental plaque by production of different levels of inflammatory cytokines, which is a putative reflection of their virulence. The aim of the current study was to determine responses in terms of interleukin (IL)-1β, IL-6, IL-8 and IL-10 secretion induced by *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, *Fusobacterium nucleatum* and *Streptococcus gordonii* in order to gauge their virulence potential.

Materials and Methods: HGECs were challenged with the four bacterial species, live or heat killed, at various multiplicity of infections and the elicited IL-1β, IL-6, IL-8 and IL-10 responses were assayed by enzyme-linked immunosorbent assay.

Results: Primary HGECs challenged with live *P. gingivalis* produced high levels of IL-1β, while challenge with live *A. actinomycetemcomitans* gave high levels of IL-8. The opportunistic pathogen *F. nucleatum* induces the highest levels of pro-inflammatory cytokines, while the commensal *S. gordonii* is the least stimulatory.

Conclusion: We conclude that various dental plaque biofilm bacteria induce different cytokine response profiles in primary HGECs that may reflect their individual virulence or commensal status.

Key words: *A. actinomycetemcomitans*; cytokines; epithelial cells; *F. nucleatum; P. gingivalis; S.gordonii*

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Dental plaque is a polymicrobial biofilm that is considered the primary aetiologic factor in chronic inflammatory periodontal disease (Loe et al. 1965, Kinane & Attstrom 2005). It is estimated that over 700 different species are capable of colonizing the oral cavity (Aas et al. 2005), and at least 400 species can be found in subgingival plaque (Paster et al. 2001). These can be classified as commensals, which can co-exist with the host without causing disease, opportunistic pathogens, which can be found in health and can cause disease under certain conditions, or periodontal pathogens, which are only found in disease states and initiate and exacerbate chronic periodontitis.

The gingival epithelial cells of the superficial layer of the gingival epithelium are the first host cells that come in contact with bacteria of the dental plaque biofilm and their byproducts. Once exposed to a bacterial stimulus, the gingival epithelial cells can elicit a wide array of responses including cytokines and chemokines that recruit inflammatory and immune cells to indirectly eliminate the infection (Kinane et al. 2008). Clinical studies have shown that pro-inflammatory cytokines and chemokines are present in the gingival crevicular fluid, both in health and disease, and are elevated in sites exhibiting clinical signs of inflammation (Figueredo et al. 1999, Sakai et al. 2006, Zhong et al. 2007).

In order to study the host-bacterial interactions that initiate periodontal disease, four different oral bacteria were used in the present study: *Streptococcus gordonii*, *Fusobacterium nucleatum*, *Aggregatibacter actinomycetemcomitans* and *Porphyromonas gingivalis*. *S. gordonii* is an early colonizer of oral biofilms that is considered a commensal in the oral cavity, because it has been

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frequently isolated from dental plaque samples of healthy subjects (Paster et al. 2001, Aas et al. 2005) and can co-exist with the host without causing oral disease (Handfield et al. 2008). *F. nucleatum* is present in both health (Lo Bue et al. 1999, Paster et al. 2001, Aas et al. 2005) and disease (Dzink et al. 1985, Moore et al. 1985) and its persistence is associated with treatment failure (Van Dyke et al. 1988, Paster et al. 2001, Van der Velden et al. 2003), suggesting a role as an opportunistic pathogen (Handfield et al. 2008). In oral epithelial cells, *F. nucleatum* and its cell wall components can act as potent stimulators of pro-inflammatory cytokines [interleukin (IL)-1β, IL-6)], chemokines (IL-8) and antimicrobial peptide (hBD-2) expression (Krisanaprakornkit et al. 2000, Huang et al. 2004, Hasegawa et al. 2007). *A. actinomycetemcomitans* is a putative periodontal pathogen strongly associated with certain types of localized aggressive periodontitis (AAP 1996) and is a potent stimulator of the chemokine IL-8 in gingival epithelial cells, with minimal effects on the pro-inflammatory cytokine IL-1β (Uchida et al. 2001). *P. gingivalis* is another putative periodontal pathogen with strong evidence to support its role as an aetiologic agent in chronic inflammatory periodontal diseases (AAP 1996), as it is uncommon in health (Aas et al. 2005) but frequently found in sites with periodontitis (Paster et al. 2001). *P. gingivalis* can induce a strong cytokine and chemokine response in gingival epithelial and other host cells, which has been shown to positively correlate with the adhesive/invasive potential of the infecting strain (Sandros et al. 2000).

Our hypothesis is that the various bacteria of the dental plaque biofilm may induce different inflammatory responses that may reflect their individual virulence. Thus, the aim of the current study was to characterize the IL-1β, IL-6, IL-8 and IL-10 secretion by *P. gingivalis*, *A. actinomycetemcomitans*, *F. nucleatum* and *S. gordonii*.

Materials and Methods

Cell isolation and culture

Gingival tissue biopsies were obtained with informed consent from three periodontally healthy patients undergoing crown-lengthening procedures at the University of Louisville School of Dentistry Graduate Periodontics Clinic, according to an IRB approval. The gingiva was treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C and human gingival epithelial cells (HGECs) were isolated as described previously (Shiba et al. 2005). The three patients provided one biopsy each from which we derived three HGEC primary cultures for the current experiments and these had a median response pattern to microbial perturbation as discussed in more detail in our previous reports (Stathopoulou et al. 2009a, Stathopoulou et al. 2009b). The authenticity of the gingival epithelial cells was confirmed by immunohistochemistry with monoclonal antibody against human pankeratin (Dako, Carpinteria, CA, USA) and histologically by cell morphology. HGECs were seeded in 60 mm plastic tissue culture plates coated with type-I collagen (BD Biocoat, Franklin Lakes, NJ, USA) and incubated in 5% CO_2 at 37°C using K-SFM medium (Invitrogen, Carlsbad, CA, USA) containing 10 μg/ml of insulin, 5 μg/ml of transferrin, 10 μM of 2-mercaptoethanol, 10 μM of 2-aminoethanol, 10 mM of sodium selenite, 50 μg/ml of bovine pituitary extract, 100 units/ml of penicillin/streptomycin and 50 ng/ml of fungizone (complete medium). When the cells reached sub-confluence, they were harvested and sub-cultured as described previously (Feng et al. 1993).

Bacterial strains and conditions

P. gingivalis ATCC 33277 was purchased from the ATCC (Manassas, VA, USA) and was grown in GAM media (Nissui Pharmaceutical, Tokyo, Japan) at low passage under anaerobic conditions (85% N_2, 10% CO_2 and 10% H_2; Coy Laboratory, MI, USA) for 2 days. *A. actinomycetemcomitans* Y4 (rough strain), *S. gordonii* DL-1 and *F. nucleatum* 364 were kindly provided by Dr. D. Demuth (University of Louisville School of Dentistry). *A. actinomycetemcomitans* were grown in brain heart infusion (Difco Laboratories, Detroit, MI, USA) supplemented with 40 mg of $NaHCO_3$ per liter at 37°C in an atmosphere of 5% CO2. *F. nucleatum* were grown in brain heart infusion broth (Difco Laboratories) supplemented with 0.2% yeast extract (Difco Laboratories), 0.5 mg of L-cysteine hydrochloride, and freshly prepared 0.5% sodium bicarbonate under anaerobic conditions for 3–4 days. *S. gordonii* were cultured in brain heart infusion broth supplemented with 1% yeast extract aerobically without shaking for 16 h at 37°C. After cultivation, the bacteria were harvested by centrifugation, washed in phosphate-buffered saline (PBS) (pH 7.4) and used immediately for the live cell challenge or heat inactivated for 1 h at 60°C. Heat-killed bacteria were used in order to exclude the effects of the proteases and other proteins produced by the bacteria. Furthermore, a substantial number of studies in the literature have used heat-killed bacteria (Sandros et al. 2000, Eskan et al. 2008), hence the present study can provide direct comparability.

Bacterial challenge

HGEC cultures at the fourth passage were harvested and seeded at a density of 0.5×10^5 cells/well in a six-well culture plate coated with type-I collagen, and maintained in 2 ml of complete medium. When they reached confluence (approximately 10^6 cells/well), the cells were washed twice with fresh media and were challenged with live or heat-inactivated bacteria in antibiotic-free medium at an multiplicity of infection (MOI) of 1:10 (10^7 bacteria/well) or MOI 1:100 (10^8 bacteria/well) at 37°C in 5% CO_2 for 4 or 24 h. For each experiment the final concentration of the suspension was determined by measurement of A_{600} and appropriate dilutions were made to achieve the desired MOI. The bacterial number was confirmed by viable counting of colony forming units on species-specific agar plates incubated anaerobically at 37°C. The ratio of live: dead bacteria was determined using a Petroff-Hausser counting chamber. Each assessment confirmed that live bacteria comprised 95% of total bacterial cells counted. IL-1β, IL-6, IL-8 and IL-10 were measured in the final supernatant by enzyme-linked immunosorbent assay (ELISA) using a commercially available kit (BD OptEIA, BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The positive controls were recombinant purified human IL-1 β, IL-6, IL-8 and IL-10. The minimum detectable level was 3.9, 4.7, 3.1 and 7.8 pg/ml, respectively. Briefly, 96-well plates were coated with anti-human monoclonal capture antibody against IL-1β, IL-6, IL-8 and IL-10, respectively, and were incubated overnight at 4°C. After washing with PBS/0.05% Tween three times, the plates were blocked with PBS/10%

FBS for 1 h at room temperature. After washing three times, the standards and samples were added and incubated for 2 h at room temperature. After washing five times, the biotinylated anti-human monoclonal detection antibody was added and incubated for 1 h at room temperature. After washing five times, the streptavidin-horseradish peroxidase conjugate was added and incubated for 30 min at room temperature. After washing seven times with 1-min soaking, the tetramethylbenzidine/hydrogen peroxide substrate solution was added for 30 min at room temperature. The reaction was stopped with 2 N H_2SO_4 solution and the absorbance was read at 450 nm.

Statistical analysis

All data are expressed as the mean ± SD. Statistical analyses were performed by one-way analysis of variance using the InStat program (GraphPad, San Diego, CA, USA) with Bonferroni correction. Statistical differences were considered significant at the $p < 0.05$ level.

Results

Live *P. gingivalis* induce a characteristic cytokine response in HGECs that is reversed by heat killing. HGECs from three different donors were challenged with live or heat-killed *P. gingivalis* 33277 at an MOI 1:10 or MOI 1:100 for 4 and 24 h. Unchallenged cells were used as a negative control. Secreted IL-1β, IL-6, IL-8 and IL-10 were measured in the supernatant by ELISA. For MOI 1:10, there was no statistically significant cytokine response at any time point and under any conditions (Figs 1a, 2a, 3a). At MOI 1:100, live *P. gingivalis* elicited a high IL-1β response at 24 h (Fig. 1a, $p < 0.001$), while the IL-6 and IL-8 response was completely abrogated (Figs 2a and 3a, $p < 0.001$). Heat killing reversed both the primary and secondary cytokine response, i.e. negatively affected IL-1β levels (Fig. 1a) and positively affected IL-6 and IL-8 levels (Figs 2a and 3a). The anti-inflammatory cytokine IL-10 was below the detection level under all conditions (data not shown).

Live *A. actinomycetemcomitans* induce a high chemokine response in HGECs that is reversed by heat killing. HGECs from three different donors were challenged with live or heat-killed *A. actinomycetemcomitans* Y4 at an MOI 1:10 or MOI 1:100 for 4 and 24 h. Unchallenged cells were used as a negative control. Secreted IL-1β, IL-6, IL-8 and IL-10 were measured in the supernatant by ELISA. For MOI 1:100 at 24 h, live *A. actinomycetemcomitans* elicited a low IL-1β response (Fig. 1b) that was not statistically significant, and a high IL-6 and IL-8 response (Fig. 2b and c, $p < 0.001$). Heat killing negatively affected the elevated response while under all other conditions there was no statistically significant cytokine response. The anti-inflammatory cytokine IL-10 was below the detection level under all conditions tested.

F. nucleatum induce the highest pro-inflammatory cytokine and chemokine response in HGECs. HGECs from three different donors were challenged with live or heat-killed *F. nucleatum* 364 at an MOI 1:10 or MOI 1:100 for 4 and 24 h. Unchallenged cells were used as a negative control. Secreted IL-1β, IL-6, IL-8 and IL-10 were measured in the supernatant

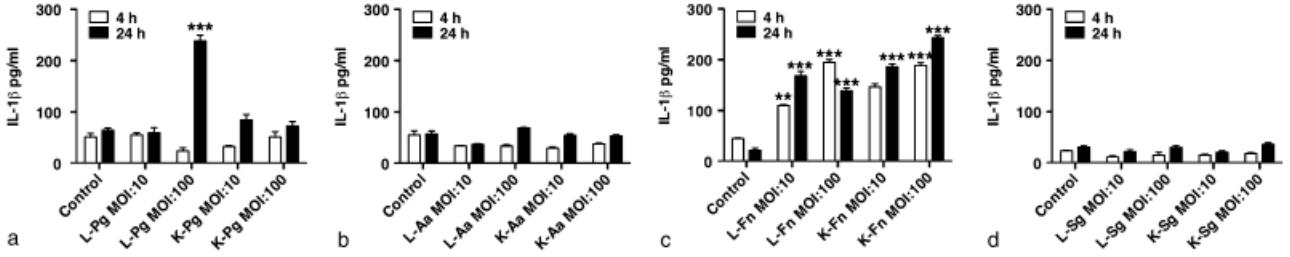

Fig 1. IL-1β enzyme-linked immunosorbent assay of supernatant from human gingival epithelial cells (HGECs) challenged with live (L) or heat-killed (K) *Porphyromonas gingivalis* 33277 (a), *Aggregatibacter actinomycetemcomitans* Y4 (b), *Fusobacterium nucleatum* 364 (c) and *S. gordonii* DL-1 (d) multiplicity of infection (MOI) 1:10 or MOI 1:100 for 4 and 24 h. The negative control was unchallenged HGECs in media. Values represent the means ± SD of three experiments from three different donors. Statistical comparisons are to the unchallenged negative control cells ($^{**}p < 0.01$, $^{***}p < 0.001$).

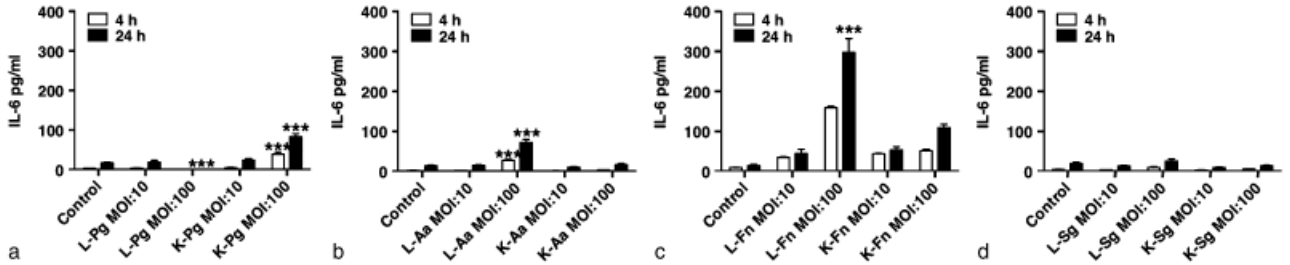

Fig. 2. IL-6 enzyme-linked immunosorbent assay of supernatant from human gingival epithelial cells (HGECs) challenged with live (L) or heat-killed (K) *Porphyromonas gingivalis* 33277 (a), *Aggregatibacter actinomycetemcomitans* Y4 (b), *Fusobacterium nucleatum* 364 (c) and *S. gordonii* DL-1 (d) multiplicity of infection (MOI) 1:10 or MOI 1:100 for 4 and 24 h. The negative control was unchallenged HGECs in media. Values represent the means ± SD of three experiments from three different donors. Statistical comparisons are to the unchallenged negative control cells ($^{***}p < 0.001$).

by ELISA. Under all conditions *F. nucleatum* elicited a high IL-1β, IL-6 and IL-8 response (Figs 1c, 2c, 3c) that was dose- and time-dependent for live and heat-killed bacteria. The only exception that did not follow the dose- and time-response was the IL-1β response to live *F. nucleatum* at MOI 1:100 and at 24 h (Fig. 1c). Heat killing negatively affected the elevated IL-6 and IL-8 response (Figs 2c and 3c). The anti-inflammatory cytokine IL-10 was below detection level under all conditions tested. Live *F. nucleatum*, an opportunistic pathogen of the dental plaque, was the most stimulatory bacterium for the secondary cytokine response, compared with *P. gingivalis*, *A. actinomycetemcomitans* and *S. gordonii*.

S. gordonii induce the lowest pro-inflammatory cytokine response in HGECs. HGECs from three different donors were challenged with live or heat-killed *S. gordonii* DL-1 at an MOI 1:10 or MOI 1:100 for 4 and 24 h. Unchallenged cells were used as a negative control. Secreted IL-1β, IL-6, IL-8 and IL-10 were measured in the supernatant by ELISA. Under all conditions *S. gordonii* had no significant effect on the cytokine response (Figs 1d, 2d, 3d). The only exception was the IL-8 response to live *S. gordonii* at MOI 1:100 and at 24 h (Fig. 3d), which was significantly elevated compared with the unchallenged control ($p<0.05$), and negatively affected by heat killing. The anti-inflammatory cytokine IL-10 was below detection level under all conditions tested. *S. gordonii*, a commensal of the dental plaque, induced a minimal chemokine response in HGECs, much lower than *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum*.

Discussion

We demonstrate that various dental plaque bacteria induce different cytokine response profiles in primary HGECs that may reflect their virulence. Bacteria considered putative periodontal pathogens elicit a strong inflammatory response, while those considered commensals, trigger a minimal inflammatory response.

In the present study, primary HGECs challenged with live *P. gingivalis* MOI 1:100 elicited a high IL-1β response at 24 h, while the IL-6 and IL-8 response was completely abrogated (Figs 1a, 2a, 3a). Heat killing reversed both the primary and secondary cytokine response profile, i.e. negatively affected IL-1β levels and positively affected IL-6 and IL-8 levels. For MOI 1:10, there was no statistically significant cytokine response at any time point and under any conditions, suggesting that even pathogenic bacteria at low concentrations may co-exist with the host for prolonged periods without eliciting an inflammatory response. Although the model used in the present study is an in vitro one, the data presented suggest that once the bacteria to host cells ratio reaches a critical threshold the epithelial cells are triggered to secrete pro-inflammatory cytokines (IL-1β) and to recruit professional immune and inflammatory cells to the site. The presence of this primary cytokine in the culture media indicates that gingival epithelial cells possess the ability to mount an inflammatory response, while the lack of secondary cytokines suggests that the bacteria have developed mechanisms to reduce this response. It has been previously shown that gingipains, proteases secreted by *P. gingivalis*, are such a mechanism that can degrade secondary cytokines at a higher rate than the primary cytokine, IL-1β (Stathopoulou et al. 2009a) and that the cytokine downregulation does not happen at the transcriptional level (Stathopoulou et al. 2009a). The anti-inflammatory cytokine IL-10 was below the detection level under all conditions and for all bacteria tested (data not shown). This suggests that epithelial cells either do not secrete IL-10 or the dose and time of bacterial exposure were not adequate for an anti-inflammatory response. The reasons for lack of IL-10 expression are complex and may involve transcriptional or translational repression.

The cytokine response of HGECs to another putative periodontal pathogen, *A. actinomycetemcomitans* (AAP 1996), although elevated, exhibited a distinct profile that may indicate that different pathogens exhibit different virulence factors, which is consistent with previous reports (Handfield et al. 2005). HGECs challenged with live *A. actinomycetemcomitans* MOI 1:100 for 24 h elicited a low IL-1β response (Fig. 1b) and a high IL-6 and IL-8 response (Figs 2b and 3b). Heat killing negatively affected the elevated response, while at a lower bacterial concentration (MOI 1:10), similarly to *P. gingivalis*, there was no statistically significant cytokine response. The highly elevated levels of IL-8, a strong chemoattractant of leucocytes and activator of neutrophils, may be needed by the host to off-set the fact that *A. actinomycetemcomitans* has the ability to kill leucocytes with its primary virulence factors, leukotoxin and Cdt.

Surprisingly, the bacterium most effective in inducing an inflammatory response was *F. nucleatum*, because it is considered an opportunistic pathogen

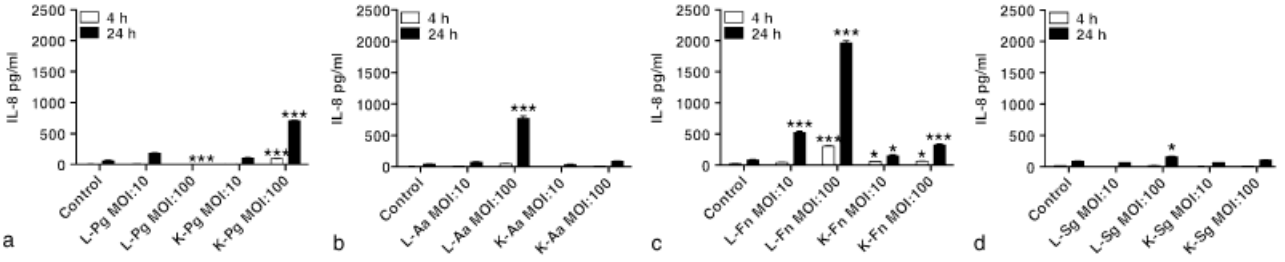

Fig. 3. IL-8 enzyme-linked immunosorbent assay of supernatant from human gingival epithelial cells (HGECs) challenged with live (L) or heat-killed (K) *Porphyromonas gingivalis* 33277 (a), *Aggregatibacter actinomycetemcomitans* Y4 (b), *Fusobacterium nucleatum* 364 (c) and *S. gordonii* DL-1 (d) multiplicity of infection (MOI) 1:10 or MOI 1:100 for 4 and 24 h. The negative control was unchallenged HGECs in media. Values represent the means ± SD of three experiments from three different donors. Statistical comparisons are to the unchallenged negative control cells ($^{*}p<0.05$, $^{***}p<0.001$).

(Handfield et al. 2008). HGECs challenged with *F. nucleatum* elicited a high IL-1β, IL-6 and IL-8 response (Figs 1c, 2c, 3c) that was generally dose- and time-dependent for live and heat-killed bacteria, and this is in agreement with previous studies (Huang et al. 2001, Hasegawa et al. 2007). Although *F. nucleatum* is detected not only in active periodontitis sites (Dzink et al. 1985, Moore et al. 1985) but also in healthy subjects and inactive sites (Lo Bue et al. 1999, Paster et al. 2001, Aas et al. 2005), its highly stimulatory effect on epithelial cells suggests that even minimal changes in the host-bacterial interface may be sufficient for this opportunistic pathogen to initiate the disease process and/or these findings may be explained by subtle but important differences between bacterial strains of the same species.

On the other hand, *S. gordonii*, considered a dental plaque commensal (Aas et al. 2005), induced a minimal chemokine response in HGECs at MOI 1:100 and at 24 h, while under all other conditions *S. gordonii* had no significant effect on the cytokine response (Figs 1d, 2d, 3d). The HGECs' cytokine response to *S. gordonii* was the lowest compared with the putative pathogens *P. gingivalis*, *A. actinomycetemcomitans* and opportunistic pathogen *F. nucleatum*, and this is in agreement with previous studies focusing on the transcriptional profiles of epithelial cells exposed to the same bacteria (Handfield et al. 2005, Hasegawa et al. 2007).

The differential cytokine response to various oral bacteria can be attributed to the specificity conferred to the innate immune system by a wide range of pattern-recognition receptors that recognize highly conserved microbe-associated molecular patterns (Medzhitov & Janeway 2000) such as the Toll-like receptors (TLRs), the nucleotide-binding oligomerization domain proteins (Nods) and the G-protein-coupled receptors. Triggering of these receptors activates the mitogen-activated protein kinases (MAPK) signaling pathway that leads to nuclear factor κB nuclear translocation and subsequent regulation of the cytokine expression (Dong et al. 2002). The lipopolysaccharide of the Gram-negative *A. actinomycetemcomitans* and *F. nucleatum* exert their biological functions by signaling via TLR-4, while *P. gingivalis* lipopolysaccharide and also peptidoglycan and lipoteichoic acid from the cell wall of the Gram-positive *S. gordonii* signal via TLR-2. *P. gingivalis*, however, can also activate TLR-4 via their fimbriae (Hajishengallis et al. 2004), as well as protease-activated receptors via their gingipains (Lourbakos et al. 2001, Chung & Dale 2004). Furthermore, bacteria with an invasive potential such as *P. gingivalis* (Lamont et al. 1995, Madianos et al. 1996). *A. actinomycetemcomitans* (Meyer et al. 1991) and *F. nucleatum* (Han et al. 2000) can activate the cytosolic Nods. The combined effect of the aforementioned activations and interactions can be responsible, at least in part, for the specificity of the epithelial cell response to various oral bacteria.

In conclusion, the present study provides evidence that the bacteria of the dental plaque induce a different cytokine response in primary HGECs that may reflect their virulence. Cytokine profiles indicate that putative periodontopathogens have the potential to elicit a high inflammatory response that is further tailored by their specific virulence mechanisms, while commensals elicit a minimal inflammatory response. These findings underline the complexity of modeling multi-species challenges to epithelial cells pertinent to our understanding of multi-species biofilm interactions with epithelial cells.

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Clinical Relevance

Scientific rationale for the study: Subgingival plaque has multiple bacterial species, which may be pathogenic or commensal. To diagnose and treat periodontal disease we should know the main disease causing bacteria such that we can effectively target them.

Principal findings: Of the four species studied, and based on their ability to elicit pro-inflammatory cytokine responses in gingival epithelial cells, *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* have pathogenic properties whereas *S. gordonii* appears to be a commensal.

Practical implications: Conformation that *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* have pathogenic attributes supports an antibacterial strategy in periodontal therapy.