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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 (HGEs) 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 IL-1 β , 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—HGEs were challenged with the four bacterial species, live or heat-killed, at various MOIs (multiplicity of infection) and the elicited IL-1 β , IL-6, IL-8 and IL-10 responses were assayed by ELISA.

Results—Primary HGEs challenged with live *P. gingivalis* produced high levels of IL-1 β , 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 human gingival epithelial cells that may reflect their individual virulence or commensal status.

Keywords

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

Introduction

Dental plaque is a polymicrobial biofilm that is considered the primary etiologic factor in chronic inflammatory periodontal disease (Kinane and Attstrom 2005, Loe et al. 1965). 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.

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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 (GCF), 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, since it has been frequently isolated from dental plaque samples of healthy subjects (Aas et al. 2005, Paster et al. 2001) and can co-exist with the host without causing oral disease (Handfield et al. 2008). *F. nucleatum* is present in both health (Aas et al. 2005, Lo Bue et al. 1999, Paster et al. 2001) and disease (Dzink et al. 1985, Moore et al. 1985) and its persistence is associated with treatment failure (Paster et al. 2001, Van der Velden et al. 2003, Van Dyke et al. 1988), 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 proinflammatory cytokines (IL-1 β , IL-6), chemokines (IL-8) and antimicrobial peptide (hBD-2) expression (Hasegawa et al. 2007, Huang et al. 2004, Krisanaprakornkit et al. 2000). *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 proinflammatory cytokine IL-1 β (Uchida et al. 2001). *P. gingivalis* is another putative periodontal pathogen with strong evidence to support its role as an etiologic agent in chronic inflammatory periodontal diseases (AAP 1996), since 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-1 β , 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 previously described (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 (21, 23). The authenticity of the gingival epithelial cells was confirmed by immunohistochemistry with monoclonal antibody against human pankeratin (Dako, Carpinteria, CA) 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% CO₂ 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 previously described (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 % N₂, 10 % CO₂ and 10 % H₂; Coy Laboratory) for 2 days. *Aggregatibacter actinomycetemcomitans* Y4 (rough strain), *Streptococcus gordonii* DL-1 and *Fusobacterium nucleatum* 364 were kindly provided by Dr. D. Demuth (University of Louisville School of Dentistry). *A. actinomycetemcomitans* were grown in brain heart infusion (Difco, Detroit, MI) supplemented with 40 mg of NaHCO₃ per liter at 37°C in an atmosphere of 5% CO₂. *F. nucleatum* were grown in brain heart infusion broth (Difco Laboratories, Detroit, MI) supplemented with 0.2% yeast extract (Difco), 0.5 mg of L-cysteine hydrochloride, and freshly prepared 0.5% sodium bicarbonate under anaerobic conditions for 3 to 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 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, Eskin 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 6-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 MOI (multiplicity of infection) of 1:10 (10^7 bacteria/well) or MOI 1:100 (10^8 bacteria/well) at 37°C in 5% CO₂ for 4 or 24 hours. For each experiment the final concentration of the suspension was determined by measurement of A₆₀₀ and appropriate dilutions were made to achieve the desired MOI. The bacterial number was confirmed by viable counting of colony forming units (cfu) 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 ELISA (Enzyme-Linked Immunosorbent Assay) 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 hour at room temperature. After washing three times, the standards and samples were added and incubated for 2 hours at room temperature. After washing five times, the biotinylated anti-human monoclonal detection antibody was added and incubated for 1 hour 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-minute soaking, the tetramethylbenzidine/hydrogen peroxide

substrate solution was added for 30 min at room temperature. The reaction was stopped with 2N H₂SO₄ solution and the absorbance was read at 450 nm.

Statistical analysis

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

Results

Live *P. gingivalis* induce a characteristic cytokine response in HGEs that is reversed by heat-killing

HGEs 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 hours. Unchallenged cells were used as a negative control. Secreted IL-1 β , 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 (Fig. 1A, 2A, 3A). At MOI 1:100, live *P. gingivalis* elicited a high IL-1 β response at 24 hours (Fig. 1A, $P < 0.001$), while the IL-6 and IL-8 response was completely abrogated (Fig. 2A, 3A, $P < 0.001$). Heat killing reversed both the primary and secondary cytokine response, i.e. negatively affected IL-1 β levels (Fig. 1A) and positively affected IL-6 and IL-8 levels (Fig. 2A, 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 HGEs that is reversed by heat killing

HGEs 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 hours. Unchallenged cells were used as a negative control. Secreted IL-1 β , IL-6, IL-8 and IL-10 were measured in the supernatant by ELISA. For MOI 1:100 at 24 hours, live *A. actinomycetemcomitans* elicited a low IL-1 β response (Fig. 1B) that was not statistically significant, and a high IL-6 and IL-8 response (Fig. 2B, 2C, $P < 0.001$). 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 HGEs

HGEs 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 hours. Unchallenged cells were used as a negative control. Secreted IL-1 β , IL-6, IL-8 and IL-10 were measured in the supernatant by ELISA. Under all conditions *F. nucleatum* elicited a high IL-1 β , IL-6 and IL-8 response (Fig. 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-1 β response to live *F. nucleatum* at MOI 1:100 and at 24 hours (Fig. 1C). Heat killing negatively affected the elevated IL-6 and IL-8 response (Fig. 2C, 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 to *P. gingivalis*, *A. actinomycetemcomitans* and *S. gordonii*.

***S. gordonii* induce the lowest pro-inflammatory cytokine response in HGEs**

HGEs 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 hours. Unchallenged cells were used as a negative control. Secreted IL-1 β , 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 (Fig. 1D, 2D, 3D). The only exception was the IL-8 response to live *S. gordonii* at MOI 1:100 and at 24 hours (Fig. 3D), which was significantly elevated compared to 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 HGEs, 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 human gingival epithelial cells 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 HGEs challenged with live *P. gingivalis* MOI 1:100 elicited a high IL-1 β response at 24 hours, while the IL-6 and IL-8 response was completely abrogated (Fig. 1A, 2A, 3A). Heat killing reversed both the primary and secondary cytokine response profile, i.e. negatively affected IL-1 β 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-1 β) 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-1 β (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 HGEs 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). HGEs challenged with live *A. actinomycetemcomitans* MOI 1:100 for 24 hours elicited a low IL-1 β response (Fig. 1B) and a high IL-6 and IL-8 response (Fig. 2B, 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 leukocytes and activator of neutrophils, may be needed by the host to off-set the fact that *A. actinomycetemcomitans* has the ability to kill leukocytes with its primary virulence factors, leukotoxin and Cdt.

Surprisingly, the bacterium most effective in inducing an inflammatory response was *F. nucleatum*, since it is considered an opportunistic pathogen (Handfield et al. 2008). HGEs challenged with *F. nucleatum* elicited a high IL-1 β , IL-6 and IL-8 response (Fig. 1C, 2C, 3C) that was generally dose- and time-dependent for live and heat-killed bacteria, and this is in agreement with previous studies (Hasegawa et al. 2007, Huang et al. 2001). 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 (Aas et al. 2005, Lo Bue et al. 1999, Paster et al. 2001), 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 HGEs at MOI 1:100 and at 24 hours, while under all other conditions *S. gordonii* had no significant effect on the cytokine response (Fig. 1D, 2D, 3D). The HGEs' cytokine response to *S. gordonii* was the lowest compared to 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 (PRRs) that recognize highly conserved microbe-associated molecular patterns (MAMPs) (Medzhitov and Janeway 2000) such as the Toll-like receptors (TLRs), the Nucleotide-binding oligomerization domain proteins (Nods) and the G-protein-coupled receptors (GPCRs). Triggering of these receptors activates the mitogen-activated protein kinases (MAPK) signaling pathway that leads to NF- κ B 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* LPS 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 (PARs) via their gingipains (Chung and Dale 2004, Loubakos et al. 2001). 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 human gingival epithelial cells 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.

Clinical Relevance

Scientific Rationale: 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 proinflammatory 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: Confirmation that *P. gingivalis*, *A. actinomycetemcomitans* and *F. nucleatum* have pathogenic attributes supports an antibacterial strategy in periodontal therapy.

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Conflict of Interest and Sources of Funding Statement

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References

- AAP. Consensus report. Periodontal diseases: pathogenesis and microbial factors. *Ann Periodontol*. 1996; 1:926–932. [PubMed: 9118284]
- Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol*. 2005; 43:5721–5732. [PubMed: 16272510]
- Chung WO, Dale BA. Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species. *Infect Immun*. 2004; 72:352–358. [PubMed: 14688115]
- Dong C, Davis RJ, Flavell RA. MAP kinases in the immune response. *Annu Rev Immunol*. 2002; 20:55–72. doi:10.1146/annurev.immunol.20.091301.131133 091301.131133 [pii]. [PubMed: 11861597]
- Dzink JL, Tanner AC, Haffajee AD, Socransky SS. Gram negative species associated with active destructive periodontal lesions. *J Clin Periodontol*. 1985; 12:648–659. [PubMed: 3863838]
- Eskan MA, Benakanakere MR, Rose BG, Zhang P, Zhao J, Stathopoulou P, Fujioka D, Kinane DF. Interleukin-1beta modulates proinflammatory cytokine production in human epithelial cells. *Infect Immun*. 2008; 76:2080–2089. doi:IAI.01428-07 [pii] 10.1128/IAI.01428-07. [PubMed: 18332211]
- Feng L, Sun W, Xia Y, Tang WW, Chanmugam P, Soyoola E, Wilson CB, Hwang D. Cloning two isoforms of rat cyclooxygenase: differential regulation of their expression. *Arch Biochem Biophys*. 1993; 307:361–368. [PubMed: 8274023]
- Figueredo CM, Ribeiro MS, Fischer RG, Gustafsson A. Increased interleukin-1beta concentration in gingival crevicular fluid as a characteristic of periodontitis. *J Periodontol*. 1999; 70:1457–1463. [PubMed: 10632521]
- Galicía JC, Benakanakere MR, Stathopoulou PG, Kinane DF. Neutrophils rescue gingival epithelial cells from bacterial-induced apoptosis. *J Leukoc Biol*. 2009; 86:181–186. doi:jl.b.0109003 [pii] 10.1189/jlb.0109003. [PubMed: 19389800]
- Hajishengallis G, Sojar H, Genco RJ, DeNardin E. Intracellular signaling and cytokine induction upon interactions of *Porphyromonas gingivalis* fimbriae with pattern-recognition receptors. *Immunol Invest*. 2004; 33:157–172. [PubMed: 15195695]
- Han YW, Shi W, Huang GT, Kinder Haake S, Park NH, Kuramitsu H, Genco RJ. Interactions between periodontal bacteria and human oral epithelial cells: *Fusobacterium nucleatum* adheres to and invades epithelial cells. *Infect Immun*. 2000; 68:3140–3146. [PubMed: 10816455]
- Handfield M, Baker HV, Lamont RJ. Beyond good and evil in the oral cavity: insights into host-microbe relationships derived from transcriptional profiling of gingival cells. *J Dent Res*. 2008; 87:203–223. [PubMed: 18296603]
- Handfield M, Mans JJ, Zheng G, Lopez MC, Mao S, Progulske-Fox A, Narasimhan G, Baker HV, Lamont RJ. Distinct transcriptional profiles characterize oral epithelium-microbiota interactions. *Cell Microbiol*. 2005; 7:811–823. [PubMed: 15888084]

- Hasegawa Y, Mans JJ, Mao S, Lopez MC, Baker HV, Handfield M, Lamont RJ. Gingival epithelial cell transcriptional responses to commensal and opportunistic oral microbial species. *Infect Immun*. 2007; 75:2540–2547. [PubMed: 17307939]
- Huang GT, Kim D, Lee JK, Kuramitsu HK, Haake SK. Interleukin-8 and intercellular adhesion molecule 1 regulation in oral epithelial cells by selected periodontal bacteria: multiple effects of *Porphyromonas gingivalis* via antagonistic mechanisms. *Infect Immun*. 2001; 69:1364–1372. [PubMed: 11179300]
- Huang GT, Zhang HB, Dang HN, Haake SK. Differential regulation of cytokine genes in gingival epithelial cells challenged by *Fusobacterium nucleatum* and *Porphyromonas gingivalis*. *Microb Pathog*. 2004; 37:303–312. [PubMed: 15619426]
- Kato S, Nakashima K, Inoue M, Tomioka J, Nonaka K, Nishihara T, Kowashi Y. Human epithelial cell death caused by *Actinobacillus actinomycetemcomitans* infection. *J Med Microbiol*. 2000; 49:739–745. [PubMed: 10933260]
- Kinane DF, Attstrom R. Advances in the pathogenesis of periodontitis. Group B consensus report of the fifth European Workshop in Periodontology. *J Clin Periodontol*. 2005; 32(Suppl 6):130–131. [PubMed: 16128834]
- Kinane DF, Galicia J, Gorr SU, Stathopoulou P, Benakanakere MM. *P. gingivalis* interactions with epithelial cells. *Front Biosci*. 2008; 13:966–984. [PubMed: 17981604]
- Krisanaprakornkit S, Kimball JR, Weinberg A, Darveau RP, Bainbridge BW, Dale BA. Inducible expression of human beta-defensin 2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. *Infect Immun*. 2000; 68:2907–2915. [PubMed: 10768988]
- Lamont RJ, Chan A, Belton CM, Izutsu KT, Vasel D, Weinberg A. *Porphyromonas gingivalis* invasion of gingival epithelial cells. *Infect Immun*. 1995; 63:3878–3885. [PubMed: 7558295]
- Lo Bue AM, Nicoletti G, Toscano MA, Rossetti B, Cali G, Condorelli F. *Porphyromonas gingivalis* prevalence related to other micro-organisms in adult refractory periodontitis. *New Microbiol*. 1999; 22:209–218. [PubMed: 10423739]
- Loe H, Theilade E, Jensen SB. Experimental Gingivitis in Man. *J Periodontol*. 1965; 36:177–187. [PubMed: 14296927]
- Lourbakos A, Potempa J, Travis J, D'Andrea MR, Andrade-Gordon P, Santulli R, Mackie EJ, Pike RN. Arginine-specific protease from *Porphyromonas gingivalis* activates protease-activated receptors on human oral epithelial cells and induces interleukin-6 secretion. *Infect Immun*. 2001; 69:5121–5130. doi:10.1128/IAI.69.8.5121-5130.2001. [PubMed: 11447194]
- Madianos PN, Papapanou PN, Nannmark U, Dahlen G, Sandros J. *Porphyromonas gingivalis* FDC381 multiplies and persists within human oral epithelial cells in vitro. *Infect Immun*. 1996; 64:660–664. [PubMed: 8550223]
- Medzhitov R, Janeway C Jr. Innate immunity. *N Engl J Med*. 2000; 343:338–344. [PubMed: 10922424]
- Meyer DH, Sreenivasan PK, Fives-Taylor PM. Evidence for invasion of a human oral cell line by *Actinobacillus actinomycetemcomitans*. *Infect Immun*. 1991; 59:2719–2726. [PubMed: 1855989]
- Middleton J, Patterson AM, Gardner L, Schmutz C, Ashton BA. Leukocyte extravasation: chemokine transport and presentation by the endothelium. *Blood*. 2002; 100:3853–3860. doi:10.1182/blood.V100.12.3853 100/12/3853 [pii]. [PubMed: 12433694]
- Moore WE, Holdeman LV, Cato EP, Smibert RM, Burmeister JA, Palcanis KG, Ranney RR. Comparative bacteriology of juvenile periodontitis. *Infect Immun*. 1985; 48:507–519. [PubMed: 3988344]
- Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. Bacterial diversity in human subgingival plaque. *J Bacteriol*. 2001; 183:3770–3783. [PubMed: 11371542]
- Sakai A, Ohshima M, Sugano N, Otsuka K, Ito K. Profiling the cytokines in gingival crevicular fluid using a cytokine antibody array. *J Periodontol*. 2006; 77:856–864. [PubMed: 16671879]
- Sandros J, Karlsson C, Lappin DF, Madianos PN, Kinane DF, Papapanou PN. Cytokine responses of oral epithelial cells to *Porphyromonas gingivalis* infection. *J Dent Res*. 2000; 79:1808–1814. [PubMed: 11077999]

- Shiba H, Venkatesh SG, Gorr SU, Barbieri G, Kurihara H, Kinane DF. Parotid secretory protein is expressed and inducible in human gingival keratinocytes. *J Periodontol Res.* 2005; 40:153–157. [PubMed: 15733150]
- Stathopoulou PG, Benakanakere MR, Galicia JC, Kinane DF. The host cytokine response to *Porphyromonas gingivalis* is modified by gingipains. *Oral Microbiol Immunol.* 2009a; 24:11–17. doi:OMI467 [pii] 10.1111/j.1399-302X.2008.00467.x. [PubMed: 19121064]
- Stathopoulou PG, Galicia JC, Benakanakere MR, Garcia CA, Potempa J, Kinane DF. *Porphyromonas gingivalis* induce apoptosis in human gingival epithelial cells through a gingipain-dependent mechanism. *BMC Microbiol.* 2009b; 9:107. doi:1471-2180-9-107 [pii] 10.1186/1471-2180-9-107. [PubMed: 19473524]
- Uchida Y, Shiba H, Komatsuzawa H, Takemoto T, Sakata M, Fujita T, Kawaguchi H, Sugai M, Kurihara H. Expression of IL-1 beta and IL-8 by human gingival epithelial cells in response to *Actinobacillus actinomycetemcomitans*. *Cytokine.* 2001; 14:152–161. [PubMed: 11396993]
- Van der Velden U, Varoufaki A, Hutter JW, Xu L, Timmerman MF, Van Winkelhoff AJ, Loos BG. Effect of smoking and periodontal treatment on the subgingival microflora. *J Clin Periodontol.* 2003; 30:603–610. [PubMed: 12834497]
- Van Dyke TE, Offenbacher S, Place D, Dowell VR, Jones J. Refractory periodontitis: mixed infection with *Bacteroides gingivalis* and other unusual *Bacteroides* species. A case report. *J Periodontol.* 1988; 59:184–189. [PubMed: 3162983]
- Zhong Y, Slade GD, Beck JD, Offenbacher S. Gingival crevicular fluid interleukin-1beta, prostaglandin E2 and periodontal status in a community population. *J Clin Periodontol.* 2007; 34:285–293. [PubMed: 17378884]

Fig 1.

IL-1 β ELISA of supernatant from HGECS challenged with live (L) or heat-killed (K) *P. gingivalis* 33277 (A), *A. actinomycetemcomitans* Y4 (B), *F. nucleatum* 364 (C) and *S. gordonii* DL-1 (D) MOI 1:10 or MOI 1:100 for 4 and 24 hours. The negative control was unchallenged HGECS in media. Values represent the means $\pm$ 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 ELISA of supernatant from HGECS challenged with live (L) or heat-killed (K) *P. gingivalis* 33277 (A), *A. actinomycetemcomitans* Y4 (B), *F. nucleatum* 364 (C) and *S. gordonii* DL-1 (D) MOI 1:10 or MOI 1:100 for 4 and 24 hours. The negative control was unchallenged HGECS in media. Values represent the means $\pm$ SD of three experiments from three different donors. Statistical comparisons are to the unchallenged negative control cells (***) $P < 0.001$).

Fig. 3.

IL-8 ELISA of supernatant from HGECS challenged with live (L) or heat-killed (K) *P. gingivalis* 33277 (A), *A. actinomycetemcomitans* Y4 (B), *F. nucleatum* 364 (C) and *S. gordonii* DL-1 (D) MOI 1:10 or MOI 1:100 for 4 and 24 hours. The negative control was unchallenged HGECS in media. Values represent the means $\pm$ SD of three experiments from three different donors. Statistical comparisons are to the unchallenged negative control cells (* $P < 0.05$, *** $P < 0.001$)