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Biological

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Oral Epithelial Cell Responses to Multispecies Microbial Biofilms

ABSTRACT

This report describes the use of a novel model of multispecies biofilms to stimulate profiles of cytokines/chemokines from oral epithelial cells that contribute to local inflammation in the periodontium. *Streptococcus gordonii* (Sg)/*S. oralis* (So)/*S. sanguinis* (Ss) and Sg/*Fusobacterium nucleatum* (Fn)/*Porphyromonas gingivalis* (Pg) biofilms elicited significantly elevated levels of IL-1α and showed synergistic stimulatory activity compared with an additive effect of the 3 individual bacteria. Only the Sg/*Actinomyces naeslundii* (An)/Fn multispecies biofilms elicited IL-6 levels above those of control. IL-8 was a primary response to the Sg/An/Fn biofilms, albeit the level was not enhanced compared with a predicted composite level from the monospecies challenges. These results represent some of the first data documenting alterations in profiles of oral epithelial cell responses to multispecies biofilms.

KEY WORDS: oral health, cytokines, chemokines, host-pathogen interactions, gingivitis, periodontitis.

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INTRODUCTION

Microbiological studies have expanded knowledge of the genera and species that form the human oral microbiome (Zaura *et al*., 2009; Jakubovics, 2010; Kolenbrander *et al.*, 2010). A range of gingival cells responds to this microbial challenge in the oral cavity (Kebschull and Papapanou, 2011). To maintain homeostasis, these responses have evolved with pro- and anti-inflammatory molecules and cell communication molecules arising from the resident cells of the periodontium (Bodet *et al*., 2006; Stathopoulou *et al.*, 2010). Additional molecules derive from infiltrating inflammatory and immune cells in the affected tissues. Current evidence supports that these responses can result in chronic inflammation that generates both soft- and hard-tissue damage, defined as periodontitis (Preshaw and Taylor, 2011).

Historically, most *in vitro* studies have evaluated how individual planktonic bacteria or their component products would trigger host responses (Ji *et al*., 2007; Stathopoulou *et al*., 2010). The investigations were designed to identify specific response molecules (Ji *et al.*, 2007; Mans *et al*., 2009), as well as the receptors and ligand interactions that account for the responses (Hayashi *et al*., 2010). These studies have provided some understanding of the capacity of individual micro-organisms or bacterial consortia to elicit patterns of responses from specific host cell types, the net result of which are identified by *in situ* response profiles. However, there are few data elucidating specific stimulatory capabilities of oral multispecies biofilms or comparing these responses with the individual bacteria within the microbial complexes (Guggenheim *et al.*, 2009; Belibasakis *et al.*, 2011). This report describes seminal data on the use of a novel multispecies biofilm model, created on rigid gas-permeable contact lens (RGPL) material, used to challenge oral epithelial cell cultures and contrasting responses with those occurring after challenge with monospecies biofilms of the component bacteria (Peyyala *et al.*, 2011a,b).

MATERIALS & METHODS

Bacteria and Biofilm Growth

Bacterial strains have been described previously: *Fusobacterium nucleatum* ATCC 25586, *Actinomyces naeslundii* ATCC 49840, *Streptococcus gordonii* ATCC 10558, *S. sanguinis* ATCC 10556, *S. oralis* ATCC 1055, and *Porphyromonas gingivalis* FDC38 (Peyyala *et al*., 2011b). Bacteria were grown in Brain Heart Infusion (Becton Dickinson, Sparks, MD, USA) medium supplemented with 5 µg hemin mL^{-1} and 1 µg menadione mL^{-1} (*F. nucleatum, A. naeslundii, S. gordonii*) or Trypticase Yeast Extract salts medium (*S. sanguinis, S. oralis*) under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37ºC (Peyyala *et al*., 2011b).

Biofilms were grown on Rigid Gas Permeable Lenses (RGPL) (Advanced Vision Technologies, Golden, CO, USA), 10.5 mm in diameter, in a single well of a 48-well plate, which allows the RGPLs to cover the entire surface

of the well. Prior to biofilm formation, RGPLs were coated with 1% fetal bovine serum (Invitrogen, Carlsbad, CA, USA) diluted in PBS to support the adherence of bacteria and incubated at room temperature for 2 to 4 hrs until dry, then stored at room temperature until further use. A 5-mL quantity of monospecies planktonic culture at 0.3 OD A_{600} or multispecies planktonic culture where an equal volume of each representative monospecies planktonic culture at 0.3 OD A_{600} was mixed to obtain a mixed culture was used to inoculate each RGPL in a single well of a 6-well polystyrene tissue culture plate (BD Falcon, Franklin Lakes, NJ, USA) and incubated in an anaerobic chamber for 3 days for static development of biofilms. At each 24-hour interval, a 3-mL quantity of culture fluid was aspirated from each well and replaced with 4 mL of media to replenish nutrients. After incubation, RGPLs with adherent biofilms were washed in PBS twice to remove loosely adherent cells. Biofilms grown on 3 additional RGPLs were used for bacterial enumeration by qPCR analysis (Peyyala *et al.*, 2011b). The monospecies biofilms contained from 6-16 x 10^8 bacteria, dependent upon the species. The *Sg/So/Ss* multispecies biofilms were approximately 3 x 10^8 total bacteria (ratio: 2%/2%/96%); the *Sg/An/Fn* biofilms approximated 1-2 x 10^9 total bacteria (ratio: 29%/63%/8%); and the *Sg/Fn/Pg* biofilms were 2-3 x 10^9 total bacteria (ratio: 92%/2%/6%).

To constrain the multispecies biofilms from replicating in the keratinocyte media during the 24-hour challenge, biofilms were treated with green fluorescent nucleic acid stain SYTO 24. SYTO 24 was chosen to treat all 3 multispecies biofilms since it yielded the lowest optical density with high fluorescence intensity for the bacteria, indicating that this stain inhibited replication, while not affecting viability (Peyyala *et al.*, 2012). Prior to challenging OKF4 cells, biofilms were immersed in 10 µg/mL SYTO 24 stain in keratinocyte media for 5 min, after which they were immersed in PBS twice to remove excess stain.

OKF4 Challenge

An immortalized epithelial cell line, OKF4 (Rheinwald *et al.*, 2002), was cultured to form a confluent monolayer (Peyyala *et al.*, 2011a). Biofilm and control treatments were each carried out in 6 wells in 1-mL/well fresh media and continuously incubated for 6 hrs under anaerobic conditions (85% N_2, 5% CO_2, and 10% H_2). Three-day-old biofilms grown on contact lenses were overlaid with the biofilm surface juxtaposed to the epithelial cells. OKF4 cells with or without overlaid RGPL were used as controls and maintained high viability and function for the 24-hour experimental interval (Peyyala *et al.*, 2011a).

Detection of Cytokines/Chemokines

Interleukin (IL)1α, IL-6, IL-8, transforming growth factor (TGF)α, Gro-1α (CXCL1), regulated upon activation, normal T-cell expressed and secreted (RANTES; CCL5), Fractalkine (CX3CL1), interferon gamma-induced protein (IP)-10 (CXCL10), monocyte chemotactic protein (MCP)-1 (CCL2), and macrophage inhibitory protein (Mip)-1α (CCL3) levels that accumulated in the supernatants were determined (R&D Systems, Minneapolis, MN, USA) with a Luminex IS100 instrument. The amount of cytokine/chemokine produced was also evaluated relative to the total bacterial challenge that was administered. The wet weight of each bacterial species contained in the monospecies and multispecies biofilms was estimated from cultivated bacteria and the cytokine/chemokine level expressed *per* mg wet weight of the species. The means ± standard error of the biofilm stimulation of OKF4 were compared by an analysis of variance (ANOVA) on ranks test, with Dunn's test for multiple comparisons to evaluate the data from stimulated cells compared with unchallenged and RGPL-overlaid OKF4 cells (SigmaStat 3.5; Systat Software, Inc., Chicago, IL, USA).

RESULTS

Selected cytokine/cell communication factor responses of the epithelial cells to challenge with the 3 multispecies biofilms, compared with a composite summation of responses to the individual bacterial biofilms, are demonstrated in Figs. 1A-1C. The Sg/So/Ss and Sg/Fn/Pg biofilms elicited significantly elevated levels of IL-1α and showed synergistic stimulatory activity compared with that expected from an additive effect of the individual bacteria. As importantly, both of the multispecies biofilms stimulated significantly greater IL-1α production than the mass of the bacteria that was present (*i.e.*, biofilm pg/mL *vs*. biofilm pg/mg wet weight). The Sg/An/Fn multispecies biofilms were generally inactive in enhancing production of this cytokine. However, we observed that a summation of IL-1α levels with the monospecies biofilms related to the mass of the challenge was significantly elevated and directly related to the capacity of *F. nucleatum* to stimulate IL-1α (Peyyala *et al.*, 2012).

The Sg/So/Ss and Sg/An/Fn biofilms induced elevated IL-6 levels compared with the monospecies biofilms. The Sg/An/Fn biofilms were very active in inducing IL-6, as both monospecies and multispecies biofilms, with *F. nucleatum* demonstrating the greatest stimulatory capacity. Finally, the Sg/Fn/Pg multispecies biofilms were inactive compared with levels expected from a summation of the individual species. The multispecies and monospecies biofilms showed an overall inhibition of production of TGFα below basal levels of the cells.

IL-8 was a primary response to the Sg/An/Fn biofilms, albeit the level to the multispecies biofilm was not enhanced compared with a predicted composite level from the individual monospecies challenges (Fig. 2A). The *F. nucleatum* monospecies biofilms induced exceptional levels of IL-8 (Peyyala *et al.*, 2011a), which is reflected in the composite values for the monobiofilms. The Sg/So/Ss biofilms appeared to inhibit the production of IL-8. Finally, minimal responses were observed with the Sg/Fn/Pg biofilms, and the combination of these micro-organisms lowered the levels that were expected from challenge with the individual bacterial biofilms, primarily elicited by Fn.

No Gro-1α was observed with either the Sg/So/Ss or Sg/Fn/Pg multispecies biofilms (Fig. 2B). There was a more dramatic decrease from basal production than that which occurred with the monospecies biofilms of the individual bacteria. This chemokine was detected in supernatants of the Sg/An/Fn biofilms, with a level significantly lower than basal epithelial cell production. While the levels were consistently below the basal levels

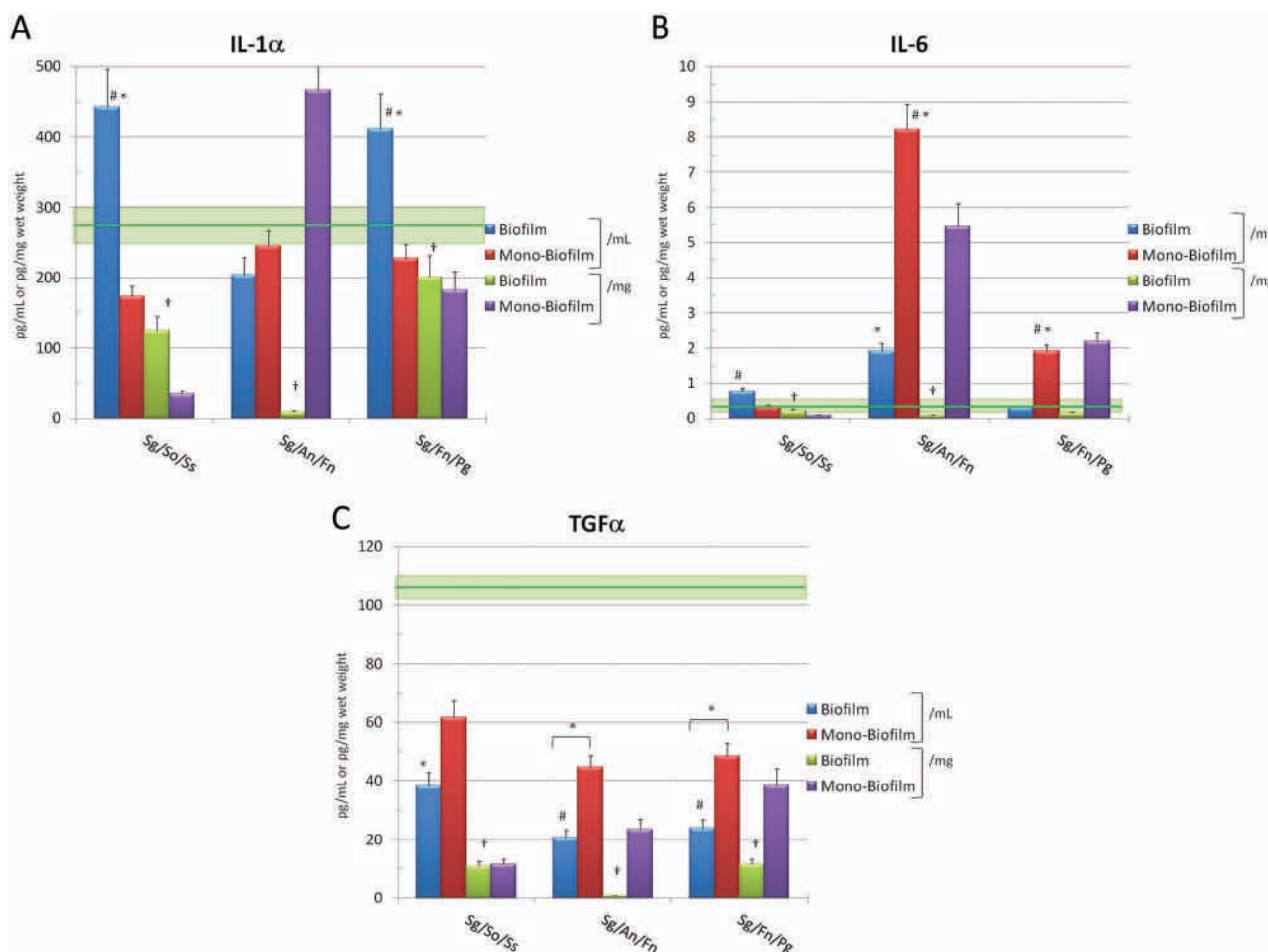

Figure 1. Levels of cytokines elicited by challenge of oral epithelial cells with multispecies biofilms (Biofilm). Mono-Biofilm denotes a composite of responses for the 3 monospecies biofilms represented in the multispecies biofilms. Each bar denotes mean level of triplicate determinations for each condition, and the vertical brackets signify 1 SD. The asterisk (*) denotes a significant difference from basal controls (RGPL-overlaid epithelial cells) at least at $p < 0.05$. The green horizontal line denotes mean of cells incubated in media and overlaid with RGPL absent bacteria for comparison of responses, and the light green box denotes 1 SD. The pound sign (#) denotes significant difference between multispecies biofilms and composite sum of monospecies biofilms for the 3 bacteria at least at $p < 0.05$. Basal epithelial cell levels of IL-1α = 245 ± 27; IL-6 = 0.64 ± 0.12; TGFα = 118 ± 16.

produced by the cells, it appeared that higher levels were detected with the multispecies biofilms considered as a unit (*i.e.*, pg/mL) *vs.* expression of the levels related to the mass of the bacteria (pg/mg wet weight).

The Sg/An/Fn biofilms demonstrated an elevated level of Fractalkine compared with what would have been predicted from the individual monospecies biofilms (Fig. 2C). A substantial inhibition was observed with the Sg/Fn/Pg biofilms that showed less than 5% of the basal levels of this chemokine. Of interest was that with both the pioneer and bridging microorganism biofilms, the levels related to the biofilms as a unit (*i.e.*, pg/mL) were consistently increased compared with the levels of this chemokine expressed *per* mass of the bacteria (pg/mg wet weight). Generally, IP-10 levels were triggered by all the multispecies biofilms to a significantly greater level than noted with individual bacterial biofilms (Fig. 2D). As noted with the other chemokines, levels observed related to the multispecies biofilms as a unit were routinely increased when compared with levels related to simply the mass of the bacteria in the biofilms. MIP-1α, RANTES, and MCP-1 were present in minimal levels in the basal cell cultures, as well as following stimulation of the cells with various combinations of bacteria (data not shown).

DISCUSSION

This report extends our findings using novel RGPL material to build oral bacterial biofilms that were used to challenge epithelial cells. The oral bacterial ecology is acquired early in life and evolves over time with the host to form a complex of numerous genera and species occupying the various ecological niches in the oral cavity (Paster *et al.*, 2006; Kishi *et al.*, 2009). We provide some unique data documenting the patterns of cytokines/chemokines that are induced in oral epithelial cells following challenge with targeted multispecies bacterial biofilms, representing bacterial consortia associated with periodontal health,

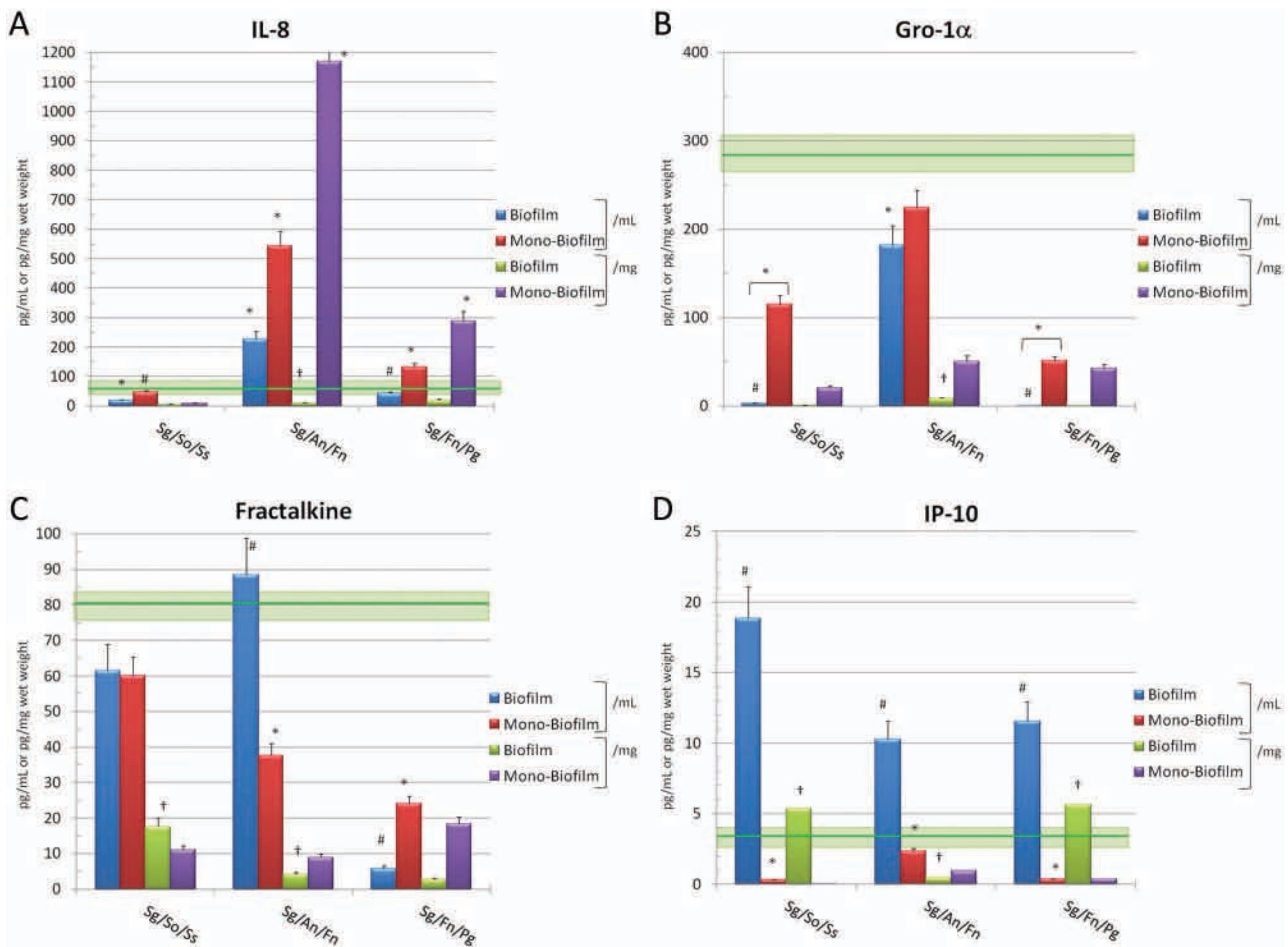

Figure 2. Levels of chemokines elicited by challenge of oral epithelial cells with multispecies biofilms (Biofilm). Mono-Biofilm denotes a composite of responses for the 3 monospecies biofilms represented in the multispecies biofilms. Each bar denotes mean level of triplicate determinations for each condition, and the vertical brackets signify 1 SD. The asterisk (*) denotes a significant difference from basal controls (RGPL-overlaid epithelial cells) at least at $p < 0.05$. The green horizontal line denotes mean of cells incubated in media and overlaid with RGPL absent bacteria for comparison of responses, and the light green box denotes 1 SD. The pound sign (#) denotes significant difference between multispecies biofilms and composite sum of monospecies biofilms for the 3 bacteria at least at $p < 0.05$. Basal epithelial cell levels of IL-8 = 81 ± 11; Gro-1α (CXCL1) = 321 ± 43; Fractalkine (CX3CL1) = 56 ± 17; IP-10 (CXCL10) = 1.8 ± 0.5.

gingivitis, and periodontitis (Socransky and Haffajee, 2005). Cytokines/chemokines that have been reported to be induced by various stimuli interacting with a range of epithelial cell types were targeted, including IL-1α, IL-6, TGFα, IL-8, Gro-1α, Fractalkine, IP-10, MIP-1α, RANTES, and MCP-1 (Gemmell *et al.*, 2001; Ji *et al.*, 2007; Pathirana *et al.*, 2010).

Extensive results have documented the responses of epithelial cells after challenge with planktonic oral bacteria (Huang *et al.*, 2004; Hasegawa *et al.*, 2007; Ji *et al.*, 2007; Stathopoulou *et al.*, 2010). However, current models of the host-microbe interactions in the oral cavity emphasize the critical nature of complex microbial biofilms that form unique microbial ecologies (Colombo *et al.*, 2009; Darveau, 2009) and that change during transition from health to disease (Marsh, 2010). Importantly, we currently know very little concerning how this multicellular organized microbial structure, *i.e.*, biofilm, alters the characteristics of the host responses to individual bacterial species. The results demonstrated unique characteristics of the multispecies biofilms dependent upon the composition of the ecology, and whether the biofilms reflected bacteria associated with periodontal health, gingivitis, or periodontitis. Moreover, we identified specific mediators that were significantly elevated with the multispecies biofilms compared with what would have been expected based upon a simple additive effect of individual monospecies biofilms challenge. Finally, a number of the biofilms appeared to inhibit either the normal basal level of message, basal level of translation and secretion, and/or degradation of various mediators in this biofilm model system. This was often in contrast to mediator levels expected from the monospecies biofilms, as well as the levels in response to planktonic bacterial challenge (Peyyala *et al.*, 2012).

A few recent reports of hydroxyapatite discs used to create multispecies biofilms determined levels of IL-1ß, IL-6, IL-8 (Guggenheim *et al*., 2009), and RANKL/OPG (Belibasakis *et al*., 2011) produced by epithelial cells, periodontal ligament cells, or dental pulp cells in response to biofilms with multiple

species. Analysis of the data indicated an apparent increase in apoptosis and degradation of Il-1ß, IL-6, and IL-8 in this system. These complex multispecies biofilms were vastly dominated by a very limited number of species, and the model did facilitate identification of the types of responses that might occur to various members of the ecology that are observed with disease. Consequently, the stimulatory capacity of individual bacteria in biofilms, how they compare with planktonic stimuli, and how these responses would be altered in the presence of complex multispecies biofilms remain to be elucidated.

We have evaluated the interactions of the host cells with monospecies biofilms created from all 6 of the oral species used in these multispecies biofilms and noted that these bacteria form multiple formats/architecture that would be detected by the cells (Peyyala *et al*., 2012). The results of these studies demonstrated clear differences in the profiles of epithelial cell responses to bacteria in monospecies biofilms compared with planktonic forms of the bacteria. Moreover, selected biofilms significantly up-regulated a range of mediators, while certain of the bacterial biofilms, *e.g., P. gingivalis*, appeared to adversely affect even basal production of the cytokines/chemokines, as has been suggested from previous studies (Huang *et al*., 2004; Guggenheim *et al.*, 2009). Thus, it was of particular interest to determine how these bacteria would interact with the host cells in complex biofilms, and whether there would be synergistic, additive, or subtractive outcomes for specific cytokines/chemokines. As noted in the results, examples of each of these outcomes could be identified compared with the monospecies biofilms created with each of the species. Specifically, we included biofilms composed of a mixture of oral streptococci that have been identified as prominent members of the subgingival ecology in biofilms from healthy sulci (Zijnge *et al.*, 2010). The Sg/An/Fn biofilm was created to reflect changes that have been suggested to occur early in the ecological shift related to gingival inflammation (Teles *et al.*, 2007). Finally, the Sg/Fn/Pg biofilm incorporates representative bacteria considered pioneer species that directly colonize the tooth surface (*i.e., S. gordonii*), a species (*i.e., F. nucleatum*) that increases with inflammation and demonstrates a significant capacity to co-aggregate with both pioneer streptococcal species, as well as providing bridging cognate interaction with pathogens (*i.e., P. gingivalis*) in the climax community of diseased periodontal pockets (Jakubovics and Kolenbrander, 2010).

These studies are also novel in that the host-biofilm interactions were conducted under anaerobic conditions that reflect the ecological microenvironment at the site of a periodontal disease lesion. While the vast majority of *in vitro* studies have been conducted under aerobic conditions (Hasegawa *et al*., 2007; Ji *et al.*, 2007; Guggenheim *et al*., 2009), a recent study reported that under reduced oxygen tension (*i.e.*, 2% oxygen), selected oral bacteria, including *P. gingivalis*, elicited elevated levels of cytokines/chemokines in a low oxygen environment (Grant *et al.*, 2010). This model system will enable us to explore variations in the oxygen level, volatile sulfur compounds, and elevated pH on the host-bacterial biofilm interactions that would be expected to occur in periodontal disease.

This novel biofilm model should also facilitate evaluation of variations in response profiles with differences in epithelial cell features (Seifi *et al.*, 2011). This investigation also provided some fundamental biologic knowledge of host response outcomes to multispecies biofilms, facilitating future examination of the attributes of intracellular signaling pathways that respond to these complexes.

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