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Oral microbial biofilm stimulation of epithelial cell responses

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Abstract

Oral bacterial biofilms trigger chronic inflammatory responses in the host that can result in the tissue destructive events of periodontitis. However, the characteristics of the capacity of specific host cell types to respond to these biofilms remain ill-defined. This report describes the use of a novel model of bacterial biofilms to stimulate oral epithelial cells and profile select cytokines and chemokines that contribute to the local inflammatory environment in the periodontium. Monoinfection biofilms were developed with *Streptococcus sanguinis, Streptococcus oralis, Streptococcus gordonii, Actinomyces naeslundii, Fusobacterium nucleatum,* and *Porphyromonas gingivalis* on rigid gas-permeable contact lenses. Biofilms, as well as planktonic cultures of these same bacterial species, were incubated under anaerobic conditions with a human oral epithelial cell line, OKF4, for up to 24 h. Gro-1α, IL1α, IL-6, IL-8, TGFα, Fractalkine, MIP-1α, and IP-10 were shown to be produced in response to a range of the planktonic or biofilm forms of these species. *P. gingivalis* biofilms significantly inhibited the production of all of these cytokines and chemokines, except MIP-1α. Generally, the biofilms of all species inhibited Gro-1α, TGFα, and Fractalkine production, while *F. nucleatum* biofilms stimulated significant increases in IL-1α, IL-6, IL-8, and IP-10. *A. naeslundii* biofilms induced elevated levels of IL-6, IL-8 and IP-10. The oral streptococcal species in biofilms or planktonic forms were poor stimulants for any of these mediators from the epithelial cells. The results of these studies demonstrate that oral bacteria in biofilms elicit a substantially different profile of responses compared to planktonic bacteria of the same species. Moreover, certain oral species are highly stimulatory when in biofilms and interact with host cell receptors to trigger pathways of responses that appear quite divergent from individual bacteria.

Keywords

Oral bacteria; Biofilms; Epithelial cells; Cytokines; Chemokines

1. Introduction

Host gingival tissues respond to an array of microbial challenges in the oral cavity that are crucial for maintaining homeostasis within this constantly infected environment. These

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responses comprise a wide array of pro- and anti-inflammatory molecules produced by resident cells of the periodontium [1,2], as well as response molecules derived from infiltrating inflammatory and immune cells in the tissues [3,4]. While these innate and adaptive immune responses are generated to protect the host from the microbial burden juxtaposed to the tissues, magnitude of the burden and chronicity of the inflammatory response can result in both soft and hard tissue damage defined as periodontitis [5].

Many *in vitro* studies have reduced the complex *in situ* microbial infection into individual proposed pathogenic or commensal species as planktonic challenges of host cells [6–8]. These types of studies have frequently focused on a particular host target molecule [9,10], emphasized molecular aspects of Toll-like receptor engagement [11,12] and/or developed data to identify intracellular signaling pathways that account for the response profile of the cells [13]. Moreover, it appears clear that the net result of these host–bacterial interactions as maintaining health or manifesting disease is reflected in the relative distribution and abundance of a wide range of biomolecules with competing and complementary activities in the tissue microenvironment [14,15]. Many of these conclusions have resulted from studies that attempt to sample the oral environment and compare the patterns of various responses in health and disease [16], during progression of the periodontal lesion [17], or following clinically successful therapy of disease [18]. While providing a robust snapshot of the characteristics of the compendium of responses that can occur in the oral cavity, these types of studies are not able to delineate details of the microorganisms that have a predilection to elicit the particular responses or the relative contribution of individual host cell types to these response profiles.

It is clear that the bacteria inhabiting the oral cavity in contact with oral tissues reside in complex multispecies biofilms [19]. These biofilm structures arise via interaction with host substrates [20,21] and accrue and mature based upon the oral environment and specific interactions among individual species of bacteria [22,23]. However, there are very few reports that have evaluated the response profiles of specific host cells to oral bacteria in biofilms. Recently, Guggenheim and colleagues used a hydroxyapatite disc model to prepare oral multispecies biofilms and used these to challenge epithelial cell cultures [24,25]. While numerous species were used to create the biofilms, of the nine species used, *Porphyromonas gingivalis* and *Fusobacterium nucleatum* made up a rather small proportion of the overall microbial composite at a level approximating 1% of the total. Thus, while the architecture of these very complex biofilms was described using confocal scanning laser microscopy, it remains undetermined the density of the bacteria that interacted with the individual cells, nor the species that may have been primary participants in this process. Nevertheless, this report did document a range of host responses molecules produced in response to the biofilm challenge that occurred under aerobic conditions over 24 h. The primary findings were an apparent increase in apoptosis and degradation of IL-1β, IL-6 and IL-8 cytokines that were elicited from the epithelial cells that was predicted to be related to the presence of proteases, such as those produced by *P. gingivalis* in these biofilms.

The present report describes our use of a novel biofilm model, created on rigid gas permeable contact lens (RGPL) material [26,27] that were used to challenge oral epithelial cell cultures in an anaerobic environment that may better reflect the subgingival sulcus. We

evaluated patterns of cell responses to single species biofilms, compared with planktonic challenge with the same species, to elucidate unique features of the cellular responses to the biofilm challenge.

2. Materials and methods

2.1. Bacteria and culture conditions

P. gingivalis (FDC381), *F. nucleatum* ATCC 25586, *Actinomyces naeslundii* ATCC 49840, and *Streptococcus gordonii* ATCC 10558 were cultured in Brain Heart Infusion (Becton Dickinson and Company, Sparks, MD) medium supplemented with 5 µg hemin ml^{-1} and 1 µg menadione ml^{-1} under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37 °C. *Streptococcus sanguinis* ATCC 10556 and *Streptococcus oralis* ATCC 10557 were grown in Trypticase yeast extract salts (TYS) medium under anaerobic conditions. All bacterial strains used in this study have been described previously and were obtained from the ATCC, except *P. gingivalis* (FDC381) [26].

2.2. Biofilm growth conditions

Biofilms were grown on rigid gas permeable lenses (RGPLs) (Advanced Vision Technologies, Golden, CO) as previously described [26]. Briefly, prior to biofilm formation, RGPLs were coated with 1% fetal bovine serum (FBS; Invitrogen) to support the adherence of bacteria and incubated at room temperature until dry. Each RGPL was inoculated with a 5 ml of monospecies planktonic culture at 0.3 OD A_{600} in a single well of a 6-well polystyrene tissue culture plate (BD Falcon, Franklin Lakes, NJ) and incubated in an anaerobic chamber for 3 days for development of biofilms under static conditions. At each 24-h interval spent media was replenished by fresh medium. After incubation, RGPLs with adherent biofilms were washed in 1X PBS twice to remove loosely adherent cells and used in subsequent epithelial cell challenge. Biofilms grown on three additional RGPLs were used for bacterial enumeration by qPCR analysis as described previously [26].

2.3. OKF4 cell growth and bacterial challenge

An immortalized epithelial cell line OKF4 (Rheinwald 2002) was cultured in keratinocyte serum free medium (Invitrogen, Carlsbad, CA) and seeded into 48-well tissue culture plates (Costar, Cambridge, MA) at a density of 10^5 cells per well in a 1 ml volume and allowed to adhere for 24 h in a 5% CO_2 chamber at 37 °C to form a confluent monolayer. Planktonic, biofilm and control treatments were each carried out in six wells in 1 ml/well fresh media and continuously incubated for 6 h under anaerobic conditions (85% N_2, 5% CO_2, and 10% H_2). For planktonic challenge, overnight cultures were harvested by centrifugation and resuspended in keratinocyte medium. A 10^8, 10^7 and 10^6 cells/well challenge corresponding to a multiplicity of infection (MOI) at 1000:1, 100:1 and 10:1 was used to stimulate the OKF4 cells. The estimated MOI for the planktonic challenge represents the numbers of bacteria that could be predicted to be in association with the OKF4 cells interacting with the biofilms. Three day old biofilms grown on contact lens were overlaid with biofilm-side facing the epithelial cells. OKF4 cells with or without overlaid RGPL were used as controls for the biofilm or planktonic bacterial challenges, respectively. OKF4 cell supernatants from each of two wells were pooled and stored at −80 °C for cytokine determination. Previous

studies with this model system demonstrated that these biofilm challenges did not result in any obvious toxicity or cell death, as determined by both metabolic activity and housekeeping gene expression [27].

2.4. Inhibition of biofilm growth during the course of challenge

In order to constrain the biofilms from replicating in the keratinocyte media during the 24 h challenge with OKF4 cells, biofilms were treated with green fluorescent nucleic acid stain SYTO 24. SYTO 24 was chosen as it yielded lowest optical density with highest fluorescence intensity values for *S. gordonii*, *A. naeslundii* and *P. gingivalis* indicating that this stain inhibited replication while not affecting viability [26]. *S. oralis* and *S. sanguinis* biofilms were also treated with SYTO 24 to inhibit replication. 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 1X PBS twice to remove excess stain. *F. nucleatum* was not treated with any SYTO stains as it did not replicate in keratinocyte media.

2.5. Detection of cytokines/chemokines

The level of cells by 24 h was determined using a multiplexed beadlyte kit (R & D systems, Minneapolis, MN, USA) and a Luminex IS100 (Luminex, Inc., Austin, TX) instrument. The mean ± standard error of the mean of the planktonic and biofilm stimulation of OKF4 cells was compared with unchallenged and RGPL overlaid OKF4 cells, respectively. Statistical comparison of the data was accomplished using an ANOVA on ranks test with Dunn's test for multiple comparisons for multiple comparisons (SigmaStat 3.5; Systat Software, Inc., Chicago, IL).

3. Results

3.1. Characteristics of cytokine responses

An array of cytokines and cell communication factors produced by epithelial cells and having some potential role in responses to oral bacterial challenge was evaluated and included, IL-1α, IL-6, and TGFα. The results in Fig. 1A–C demonstrate the responses of the epithelial cells to challenge with the range of oral bacteria. Biofilms of *A. naeslundii* and all three streptococci provided minimal stimulus of IL-1α, while the planktonic challenge with these microorganisms appeared to inhibit production of this cytokine. The *F. nucleatum* biofilms significantly upregulated production of IL-1α, while *P. gingivalis* biofilms significantly inhibited this response. Of the planktonic bacteria only *P. gingivalis* appeared to have the capacity to induce IL-1α in this system.

The response of the epithelial in producing IL-6 was quite limited across these bacterial species. Only biofilms and planktonic *A. naeslundii* and *F. nucleatum* elicited this cytokine response, with the biofilms of each species inducing significantly greater quantities of IL-6 compared to the planktonic challenge.

As was noted with IL-1α, all of the bacterial biofilms inhibited the production of TGFα, with *P. gingivalis* totally eliminated. Planktonic bacterial challenge with *P. gingivalis* and *S. sanguinis* elicited elevated levels of this cell communication factor.

3.2. Characteristics of chemokine responses

This investigation also profiled a range of chemokines, Gro-1α, IL-8, Fractalkine, IP-10, and MIP-1α, RANTES and MCP-1 that are produced by epithelial cells and could be expected to provide early warning signals to the local tissue environment and the host immune system. The results in Fig. 2A–E provide a profile of these chemokine responses by the epithelial cells, with the exception of RANTES and MCP-1 which provided minimal detectable levels. Gro-1α production was inhibited by biofilms challenge with *P. gingivalis, F. nucleatum* and all three streptococci. Only planktonic challenge with *A. naeslundii* and *F. nucleatum* showed an induction of this chemokine. Interestingly, among the oral streptococci, *S. oralis* was completely nonreactive as a stimulus for this chemokine.

Significantly elevated production of IL-8 was observed with biofilms of *A. naeslundii* and *F. nucleatum*, as well as following challenge with planktonic versions of these species. In contrast, no effect was observed with the oral streptococci, and *P. gingivalis* inhibited the production of this critical chemokine.

The levels of Fractalkine were generally unaffected by challenge with all of the oral bacteria, except the lowest dose of planktonic *P. gingivalis*. A similar selective effect was observed with IP-10 and MIP-1α. Biofilms of *A. naeslundii* and *F. nucleatum* stimulated production of IP-10, as well as planktonic challenge with these species. Biofilms of *P. gingivalis* and the oral streptococci inhibited IP-10 production. Interestingly, with MIP-1α the biofilms of *P. gingivalis* increased the levels of this chemokine, as did selective doses of planktonic challenge with each of the other species of bacteria.

3.3. Profiles of cytokine/chemokine production by epithelial cells

Fig. 3A and B provides a summary of the patterns of responses of the epithelial cells to the individual biofilms and high and low dose challenge of the planktonic bacteria. The high dose planktonic challenge is similar to the overall numbers of the species in the biofilms, while the low dose planktonic challenge estimates the number of the bacteria in the layered biofilms that might be expected to be able to interact directly with the epithelial cells. Clearly, the profiles of stimulation are unique to each species, and the biofilms are unique compared to these doses of the planktonic stimulus across the species. The biofilms of *A. naeslundii* and *F. nucleatum* appear to be the most active with regards to breadth and quantity of mediators induced. In contrast, *P. gingivalis* biofilms were minimally reactive, and in most cases inhibited production of the cytokines and chemokines. Fig. 3B shows that generally the oral streptococci in any form were not particularly active in stimulating the epithelial cells. However, of interest was that while these are considered highly related species, there were distinct differences in the patterns of mediators induced by challenge with the planktonic forms of the bacteria. As noted, the streptococcal biofilms were not active in stimulating cytokines/chemokines and in most cases appeared to inhibit basal production by the epithelial cells.

4. Discussion

This report provides some of the first data documenting the patterns of cytokines and chemokines that are induced in oral epithelial cells following challenge with monospecies

oral bacterial biofilms. It is clear that 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 as biofilms [28,29]. Moreover, it is clear that with the two major oral diseases being infections, the characteristics of the microbial ecology changes when transitioning from health to disease [30]. Many investigations have reported the responses of various host cells, e.g. epithelial cells, fibroblasts, lymphocytes, following challenge with oral microorganisms in planktonic form from individual species [7,31–33]. Reducing the system even further, various studies have targeted selected biomolecules or structures of the oral bacteria, that in an isolated *in vitro* system can also stimulate an array of host responses [34,35]. Specifically, with respect to the characteristics of the interactions examined in this study, *P. gingivalis*, *F. nucleatum*, *Actinomyces* species, and oral streptococci have been shown to elicit IL-1α, IL-1β, IL-6, IL-8, IL-10, IL-8, LL37, HBD 1,2,3 from oral epithelial cells [31]. As noted in a number of these reports, the characteristics of the host cell responses to individual oral bacteria varies significantly across genera and species, as well as showing some differences when considering individual microorganisms as oral pathogens or commensals [7,8,31]. While these studies have some value in understanding the biology of host–bacterial interactions that occur *in vivo*, the challenge does not reflect the primary organization of the oral bacteria into complex multispecies biofilms that stimulate both non-immune and immune cells *in situ*. Recent reports have emphasized the important differences in oral microbial interactions with host tissues and cells when these bacteria exist in complex biofilms. Thus, it could be anticipated that these responses would vary due to interbacterial metabolic and virulence synergisms within biofilms. Additionally, differences in host responses would be expected related to the variations in bacterial gene expression profiles that contribute to the biofilm structure, enhanced resistance to antimicrobials, and physiologic microenvironments within the biofilms [36–40]. This study provides some foundational knowledge using a novel biofilm model system to demonstrate unique features of host response patterns to challenge across a range of oral bacteria and when comparing biofilms to planktonic forms of the bacteria.

A recent study has reported that complex multispecies biofilms stimulate a range of mediators from epithelial cells including IL-1β, IL-6, IL-8 [24] and RANKL/OPG [25]. These nine species biofilms provide support to the underlying premise that host cellular responses to biofilms versus planktonic bacteria are substantively different. However, these studies do not shed light on the characteristics of individual bacteria in these biofilms, whether or not they are even specifically interacting with the epithelial cell monolayer, and in general, these multispecies biofilms were vastly dominated by a very limited number of species [24]. Consequently, there remain unanswered questions regarding 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 a complex multispecies biofilm.

This report provides details on the use of a novel rigid gas-permeable lens material to build bacterial biofilms that were used to challenge oral epithelial cells. For this investigation we selected a group of cytokines and chemokines that have been reported to be induced by various stimuli interacting with a range of epithelial cell types [41,42]. IL-1α is a cytokine

produced by a range of cell types, with important functions in enhancement of inflammation and host defence [43]. IL-6 is a pleiotropic pro-inflammatory cytokine that regulates several biological functions including inflammatory responses and bone biology [44]. TGFα is produced by macrophages and keratinocytes, is closely related to epidermal growth factor (EGF), and can also bind to the EGF receptor leading to epithelial development [45]. The results demonstrated that each of these cytokines/growth factors was differentially induced by biofilms of the oral bacteria. Clearly, IL-1α was the most responsive to the biofilms, while IL-6 was selectively elicited by only *A. naeslundii* and *F. nucleatum* biofilms, and TGFα levels were inhibited below basal cell production by biofilms of all the oral bacteria. As was predicted the biofilm stimulation patterns were distinct from the planktonic bacteria, and in a number of instances, the level of stimulation was greater than planktonic challenge with approximately a two logs higher number of the bacteria.

Anticipating that a major role of the epithelial cells is to provide an "early warning" signal to the host inflammatory and innate and adaptive immune responses, we examined a range of chemokines that would be predicted to have a principle role for inducing the emigration of protective cells and biomolecules into the infected oral sites. IL-8 is a primary chemokine for attracting neutrophils into sites of inflammation, and is a hallmark of the chronic inflammatory response in periodontitis [46]. Gro-1α is a chemokine with multiple functions associated with atherosclerosis, angiogenesis and many inflammatory conditions [47]. Soluble Fractalkine is a potent chemoattractant for T cells and monocytes, although cell-bound Fractalkine promotes adhesion of leukocytes to activated endothelial cells [48]. IP-10 is secreted by monocytes, endothelial cells, epithelial cells and fibroblasts in response to interferon (IFN)γ. It is a chemoattractant for monocytes/macrophages, T cells, NK cells, and dendritic cells, and as with Fractalkine promotes T cell adhesion to endothelial cells [49]. MIP-1α is one of a family of chemokines produced by stimulated macrophages and epithelial cells in response to infection. This chemokine activates granulocytes contributing to acute inflammatory responses [50] and induces the synthesis of a range of pro-inflammatory molecules [51]. As was noted with the cytokines, the patterns of responses of the chemokines to the oral microbial biofilms were quite varied. Only IL-8 and IP-10 appeared to show elevated response levels to the biofilms, and this was limited to *A. naeslundii* and *F. nucleatum*. Additionally, with IP-10, planktonic forms of the *A. naeslundii* were significantly more stimulatory, while the *F. nucleatum* biofilms were most active for the chemokines compared to planktonic bacteria. While we also examined MCP-1 and RANTES in this study, neither of these analytes provided sufficient signal to identify any role they might have in the epithelial cell responses to these oral biofilms.

Our findings using planktonic forms of a range of species are consistent with those previous reports regarding epithelial cell responses. Of some interest was the variation in the dose response of a number of the mediators towards challenge with the planktonic bacteria. These results showed similar levels across the 2-log challenge, as well as instances where the highest or lowest challenge dose resulted in the greatest levels of individual cytokines/chemokines. Previous studies have identified that challenge of epithelial cell cultures with planktonic bacteria, irrespective of the MOI, results in a very disjointed host–bacterial interaction at the individual cell level. Thus, even though these are proposed to be identical

cells in culture, microscopic evaluation demonstrated some cells with tens to hundreds of bacteria coating their surface, while the juxtaposed neighboring cells have no bacteria attached to the host cell surface. Thus, population based evaluation of cellular responses can be affected by how the individual bacteria interact with the targeted host cells, as well as the relatively broad capacity of Toll-like receptors to interact with bacterial structures [52]. However, the novel inclusion of monospecies oral bacterial biofilms demonstrated response patterns, some of which at the bacterial species and/or analyte levels were parallel between the biofilms and planktonic bacteria. However, other bacterial-host interactions clearly showed that the biofilms stimulated unique patterns of responses, and in some cases as a biofilm the bacteria were significantly inhibitory to basal mediator production by the epithelial cells. An additional example of these variations was noted with different patterns of responses across the oral streptococcal species that were evaluated. While the oral viridans streptococci have been historically considered similar in their physiology and ecological niches within the commensal microbiota, recent genomic and functional studies have provided clear evidence the not only do these bacteria have some unique metabolic and structural characteristics, but also integrate into complex multispecies biofilms in unique ways [53–56]. Thus, variations in responses of the epithelial cells to these individual species should not be unexpected.

An interesting aspect of these studies was that the majority were conducted under standard aerobic tissue culture conditions. However, the literature supports that at the site of a periodontal disease lesion the ecology reflects an anaerobic microenvironment for the bacteria to interact with the host. A single recent study has been reported examining the variation in cellular response to oral bacterial challenge in aerobic versus anaerobic conditions [57]. This report showed that under reduced oxygen tension (i.e. 2% oxygen) selected oral bacteria, e.g. *Tannerella forsythia, P. gingivalis,* and *Prevotella intermedia* elicited elevated levels of IL-8 and TNFα with the highest levels in the low oxygen environment. Thus, with the variations in the subgingival microbial environment, including oxygen tension [58], presence of volatile sulfur compounds [59], and elevated pH [60], results are lacking regarding the impact of these environmental changes on the host–bacterial interactions related to periodontal disease. The model reported in this study will enable us to combine investigations of oral microbial biofilms, both monospecies and multispecies, to directly address the environmental control of host cell responses that could occur in the oral cavity.

While the mediators examined in this study do not cover the extent of response capacity of the oral epithelium, the variation even with two pro-inflammatory cytokines (IL-1α and IL-6) indicates that data is clearly needed to document the characteristics of responses of epithelial cells to the format in which oral bacteria exist *in situ* in the oral cavity. As importantly, these studies were limited in directly evaluating monospecies biofilms as a basis to understand the capabilities of individual bacteria, recognizing the ultimate need to delineate how these bacteria in complex multispecies biofilms would interact with and stimulate these host cells.

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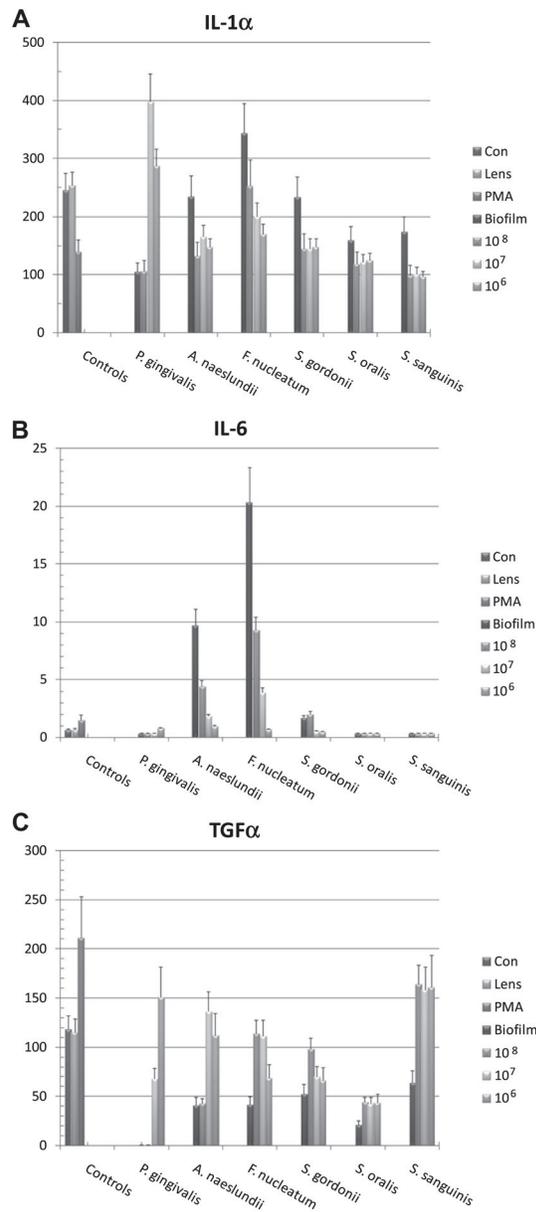
Fig. 1.
A–C: Levels of cytokines elicited by challenge of oral epithelial cells with biofilms or planktonic forms of various oral bacteria. Each bar denotes mean level of triplicate determinations for each condition and the vertical brackets signify 1 SD. The asterisk (*) denotes significantly different than controls (Con for planktonic; Lens for biofilms) at least at $p < 0.05$. The # signifies significantly greater that other stimuli for the particular microorganism at $p < 0.05$.
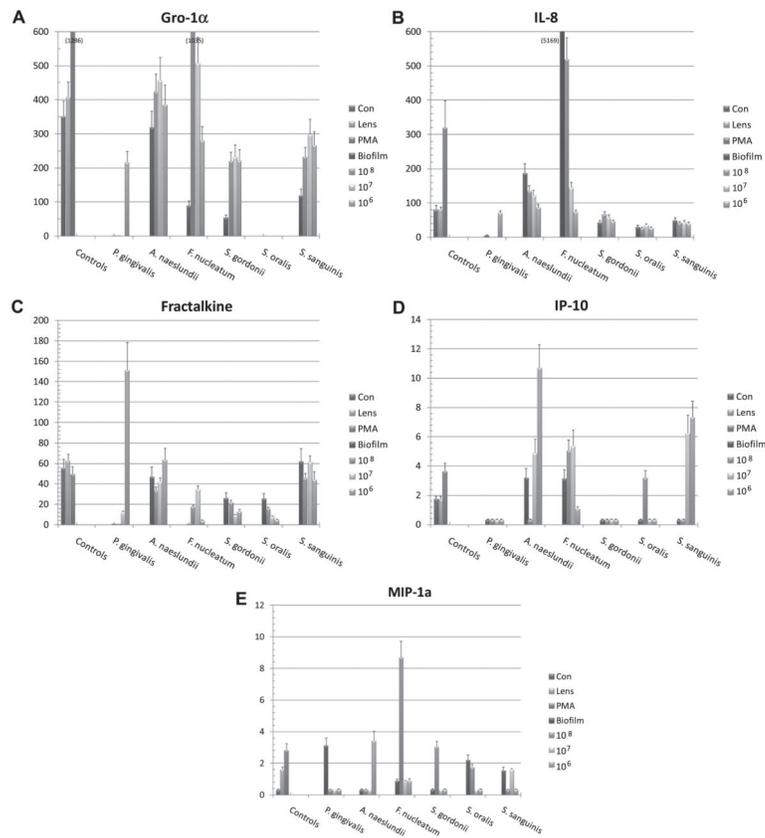
Fig. 2.
A–E: Levels of chemokines elicited by challenge of oral epithelial cells with biofilms or planktonic forms of various oral bacteria. Each bar denotes mean level of triplicate determinations for each condition and the vertical brackets signify 1 SD. The asterisk (*) denotes significantly different than controls (Con for planktonic; Lens for biofilms) at least at $p < 0.05$. The # signifies significantly greater that other stimuli for the particular microorganism at $p < 0.05$.
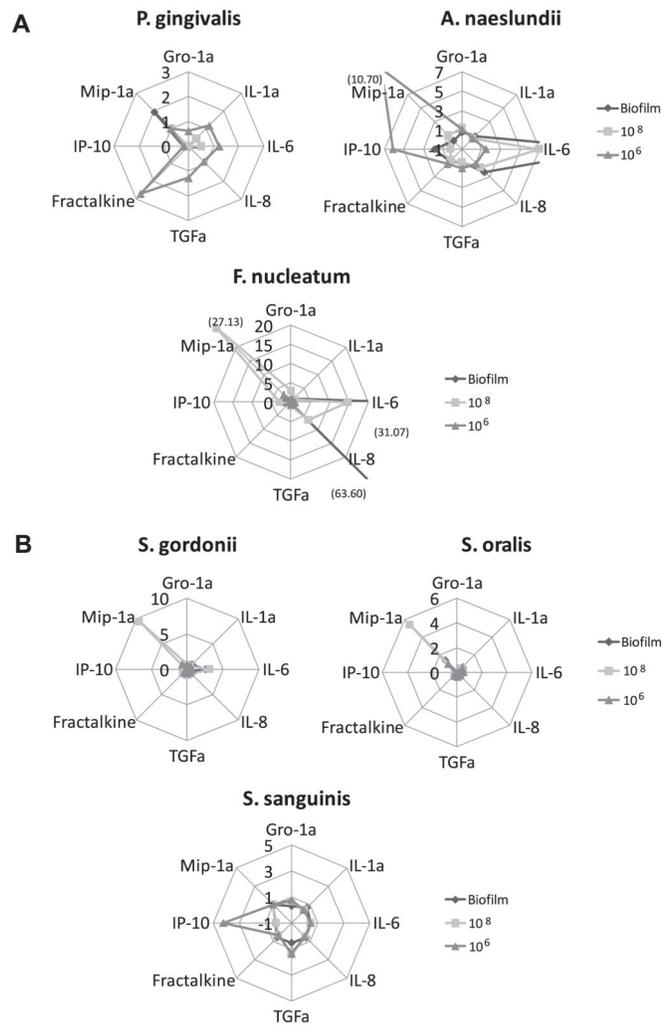
Fig. 3.
A and B: Summary of cytokine and chemokine response profiles for monospecies biofilms compared with response patterns for 10^8 or 10^6 planktonic forms for each bacterial species. The line peaks denote fold difference in response compared to control cell basal levels (planktonic comparison) or cells overlaid with a bacterial free RGPL (biofilms comparison). Numbers in parentheses denote fold changes beyond the scale of the graph.