HHS Public Access

Author manuscript

Mol Oral Microbiol. Author manuscript; available in PMC 2020 February 01.

Published in final edited form as:

Mol Oral Microbiol. 2019 February ; 34(1): . doi:10.1111/omi.12251.

Biofilm Induced Profiles of Immune Response Gene Expression by Oral Epithelial Cells

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SUMMARY

Objective: This study examined the oral epithelial immunotranscriptome response patterns modulated by oral bacterial planktonic or biofilm challenge.

Methods: We assessed gene expression patterns when epithelial cells were challenged with a multispecies biofilm composed of *S. gordonii, F. nucleatum*, and *P. gingivalis* representing a type of periodontopathic biofilm compared to challenge with the same species of planktonic bacteria.

Results: Of the 579 human immunology genes, a substantial signal of the epithelial cells was observed to 181 genes. Biofilm challenged stimulated significant elevations compared to planktonic bacteria for IL32, IL8, CD44, B2M, TGFBI, NFKBIA, IL1B, CD59, IL1A, CCL20 representing the top 10 signals comprising 55% of the overall signal for the epithelial cell responses. Levels of PLAU, CD9, IFITM1, PLAUR, CD24, TNFSF10, and IL1RN were all elevated by each of the planktonic bacterial challenge versus the biofilm responses. While the biofilms upregulated 123/579 genes (>2-fold), fewer genes were increased by the planktonic species [36 (*S. gordonii*), 30 (*F. nucleatum*), 44 (*P. gingivalis*)].

Conclusions: A wide array of immune genes were regulated by oral bacterial challenge of epithelial cells that would be linked to the local activity of innate and adaptive immune response components in the gingival tissues. Incorporating bacterial species into a structured biofilm dramatically altered the number and level of genes expressed. Additionally a specific set of genes were significantly decreased with the multispecies biofilms suggesting that some epithelial cell biologic pathways are down-regulated when in contact with this type of pathogenic biofilm.

INTRODUCTION

Recent evidence has demonstrated the critical nature of the human gut microbiome as a component of the maintenance of health at this mucosal surface. This feature of the host-bacterial interactions has been shown to interact with and regulate epithelial cell functions in the gut. Additionally, these interactions have been documented to be a significant component of the characteristics and maturation of the host immune system both locally and

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systemically [1–3]. However, while clear data delineate the relationship of selected gut bacteria in driving this communication with the immune system, similar data from other mucosal tissues, including the oral cavity is much more limited.

Historically the epithelium and epithelial cells were viewed more as a mechanical barrier in innate immunity, including the routine sloughing of the cells removing the bound bacteria from the mucosal surface. However, more recent information has provided insights into the numerous biological functions of epithelial cells. These include the production of a constitutive and induced profile of antimicrobial peptides [4–6], various cell growth and communication factors to help maintain the integrity of the epithelium [7–9], and an array of pro- and anti-inflammatory chemokines and cytokines to inform the cells of the immune system concerning the epithelial cell interactions with the juxtaposed microbiome [7,8,10].

Previous results have demonstrated a limited response profile of oral epithelial cells following challenge with various planktonic oral bacteria [11–13]. We and others have also provided novel data regarding the influence of various mono- and multispecies biofilms on these response profiles with results demonstrating significant differences in planktonic versus biofilm challenge within a bacteria species, and a different profile of responses to the multispecies biofilms that varied from simply a summation of the individual bacterial components [14–16]. This report describes our studies focusing on the capabilities of the epithelial cells to activate an array of immune response pathway signals that would prime the gingival tissues for responses to the oral microbiome. We assessed these gene expression patterns related to challenge of the epithelial cells with a multispecies biofilm composed of *Streptococcus gordonii, Fusobacterium nucleatum*, and *Porphyromonas gingivalis* representing a type of periodontopathic biofilm [16,17]. These response profiles were compared to challenge with the same species of planktonic bacteria reflecting the ongoing process of detachment and dispersal that would occur with oral bacteria throughout the oral cavity to establish biofilms at other sites [18–20].

MATERIALS and METHODS

Growth of bacteria and multispecies biofilms

F. nucleatum ATCC 25586, *S. gordonii* ATCC 10558, and *P. gingivalis* FDC381) were grown in Brain Heart Infusion (Becton Dickinson, 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 as we have described previously [15,17] . Biofilms were grown on Rigid Gas Permeable Lenses (RGPL) (Advanced Vision Technologies, Golden, CO), 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) and monospecies planktonic cultures of the 3 bacteria were mixed and used to create the biofilms, with bacterial input from $1\text{-}11\times10^8$ [21]. Our previous studies of these biofilms have shown an approximate composition of the final biofilms at 3.4×10^9 with 92% *S. gordonii*, 2% *F. nucleatum*, and 6% *P.* gingivalis.

Oral epithelial cell culture model

An immortalized epithelial cell line OKF6 [22] was cultured in standard KFM media to form a confluent monolayer [17]. Planktonic bacteria and biofilm challenge, and control treatments were each carried out in 6 wells in 1ml/well fresh media seeded with 5×10^4 OKF6 cells, and continuously incubated for 12 h under anaerobic conditions (85% N_2, 5% CO_2, and 10% H_2). The results of gene expression levels in the epithelial cells that were challenged with the planktonic bacteria at an MOI of 1:50-1:100 were combined since no significant differences were generally noted in response profiles with these 2 doses [23]. Three day old biofilms grown on contact lenses were overlaid with the biofilm surface juxtaposed to the epithelial cells. OKF6 cells with or without overlaid RGPL were used as controls and maintain high viability (XTT conversion and level of housekeeping gene expression[23]) and function for the 24 hr. experimental interval [15,24]. Based upon estimated calculations of the area of the biofilms on the RGPL [23] and the surface area of an epithelial cell, we estimated that the direct interaction of the biofilm surface with the epithelial cells would approximate an MOI of 10:1 to 50:1 bacterial cells on the surface of the biofilms were in contact with an individual epithelial cell.

NanoString analysis

Gene expression profiles of the oral epithelial cells exposed to the biofilms and bacteria were assessed using the n Counter Human Immunology Kit panel (NanoString, Seattle, WA; https://www.nanostring.com/products/gene-expression-panels/ncounter-immunology-panels) containing a set of 579 genes representing pathways that cover an array of inflammatory, and innate and adaptive immune responses. After exposure of cell cultures to the bacteria, media only or RGPL, total mRNA was isolated using the Pure Link RNA Mini (Life Technologies, NY, USA) kit following the manufacturer's instructions. RNA (100ng) with integrity numbers of 9-10 from each sample was hybridized with the reporter code set beads [25] in a final volume of 30 μl at 65°C for 12 hours and processed using the NanoString Cell Prep Station. Data normalized to total RNA levels was collected using the NanoString Digital Analyzer (NanoString Technologies, Seattle, WA, USA) through the Microarray Core facility at the University of Kentucky.

Statistical analysis:

The mean ± standard deviation of the bacteria/biofilm stimulation of OKF6 were compared using an ANOVA on ranks test with Dunn's test for multiple comparisons to evaluate the data from stimulated cells compared with unchallenged cells or RGPL overlaid OKF6 cells (Sigma Stat 3.5; Systat Software, Inc., Chicago, IL).

RESULTS

Comparison of gene expression profiles of oral epithelial cells to planktonic bacteria and biofilms

Figure 1 provides an overview of the primary response profiles of the oral epithelial cells to challenge with each of the three planktonic bacteria and the biofilms organized based upon the magnitude of gene expression with the biofilm. Of the 579 genes in the NanoString

human immunology portfolio, substantial signal (>20 copies under one or more conditions) of the epithelial cells was observed to 181 genes. Highlighted on the figure are genes that were most greatly affected (fold differences >2) by the bacterial challenge. Summation of the mRNA signal across the 181 genes showed that this set of response genes accounted for 98.2% of total biofilm mRNA signal, and 98.9%, 98.8%, and 98.3% of the mRNA signals from the epithelial cells stimulated with *S. gordonii, F. nucleatum*, and *P. gingivalis*, respectively. Many of these host response genes were at an increased or decreased signal level following challenge with *S. gordonii*, *F. nucleatum*, or *P. gingivalis* as planktonic bacteria. Of note was the high gene expression of IL32 following biofilm challenge, and the substantially lower level of IL8, IL1B, CCL20, PML, LTB4R2, LIF, SOCS3, and MBP induced by all the planktonic bacteria versus the biofilm. In contrast, levels of PLAU, CD9, IFITM1, PLAUR, CD24, TNFSF10, and IL1RN were all elevated by each of the planktonic bacterial challenge versus the biofilm responses.

Figure 2 displays the fold changes in response patterns of the oral epithelial cells following interaction with serum coated or biofilms on rigid gas permeable lens (RGPL). Generally the RGPL interaction resulted in minimal gene expression differences compared to the media control cell cultures. Of note was the large array of genes (those elevated by ≥16-fold highlighted) that were elevated following the biofilm challenge representing various pathways of host response in which the epithelial cells may be participating to help maintain homeostasis and communicate with immune system cells.

Figure 3 summarizes the differences in gene expression profiles of the multispecies biofilm with each of the individual planktonic microorganisms used to challenge the epithelial cells. Fig. 3A highlights major gene differences between the biofilm and challenge with *S. gordonii*. Beyond these targeted genes, the profile demonstrates an upregulation of >2-fold with 123 of the 181 highly expressed genes (>20 copies) with the biofilm and only 36 with *S. gordonii*. Of these elevated responses, only IL8, CCL20, CXCL2, PSMB5, CXCl1, ICAM1, MBP, IL23A, CXCL11, CXCL10, and CCL5 were increased >8-fold. Nineteen genes showed expression levels decreased by >2-fold following challenge with *S. gordonii*. Fig. 3B provides a similar profile comparing the biofilm responses to those elicited by challenge with *F. nucleatum*. We observed 30 genes with expression levels >2-fold increased to *F. nucleatum* with only MBP, IL23A, IL1R2, and TRAF1 being substantially increased and NOTCH1, and MAF being decreased by >2-fold compared to control cells. Interestingly, Fig. 3C presents patterns comparing the biofilm challenge to responses following infection with planktonic *P. gingivalis*. Few genes were elevated beyond 16-fold with *P. gingivalis* and included IL23A, CXCL11, CXCL10, and TRAF1. As observed previously [26] the planktonic bacteria increased PLA2G2A by over 3000-fold that appeared to be attenuated when *P. gingivalis* was in a biofilm milieu. The expression of 11 genes was decreased by >2-fold compared to control cells. Moreover, of the genes that were decreased by the planktonic bacteria, only MX1, STAT1, and POU2F2 were down-regulated by both *S. gordonii* and *P. gingivalis*.

Figure 4 summarizes the relationship of the up-regulated genes between the biofilm and the individual planktonic species. Fig. 4A compares the unique and overlapping genes between across the bacterial challenge conditions. The data shows that the majority of the genes that

were up-regulated by the individual planktonic bacteria were also increased by the biofilm, albeit, nearly 60% of the gene up-regulated by the biofilms were not represented across the planktonic species. Fig. 4B depicts the features of altered gene expression across the planktonic species. Only 1 gene (ICAM1) was up-regulated with all species, which was lost when the bacteria were in a biofilm. Additionally, *P. gingivalis* demonstrated a feature of a larger set of unique genes that were increased versus the other species (eg. IL32, CTSC, NOTCH1, C3, CD14, NOD2).

Target genes affected by planktonic bacteria and biofilms

Figure 5A-C focuses on specific gene profiles that were elevated by at least 2 times with the biofilms compared to any of the planktonic bacteria driven gene expression. As such, 72/579 of the human immunology genes were specifically elevated by the multispecies biofilm challenge compared to all planktonic challenges. The Protein ANalysis THrough Evolutionary Relationships [PANTHER; [27]] classification system was used to assess the gene ontology of these profiles. The pathways that were significantly over-represented are shown in Table 1. These included an array of pathways directly involved in communicating the cellular response to infection to immune system components. Additionally, this periodontopathic biofilm surrogate elicited various pathways controlling cell survival and apoptotic processes.

Figure 6 provides a depiction of the group of genes (n=17) in which the response to one or more of the individual planktonic bacteria was greater than the biofilm challenge, or in many cases multiple planktonic bacteria elicited elevated responses that appeared to be decreased when the bacteria were in the biofilm structure. Table 1 provides a pathway analysis of the over-represented responses, which were limited to inflammation mediated chemokine/cytokine signaling pathways. This could be interpreted that these bacteria in biofilms, while upregulating a number of chemokines/cytokines appeared to “block” the increase in the expression of these genes, for example CCL5, CXCL10, CXCL11, ICAM1, IL23A, PLA2G2A, and PSMB5.

DISCUSSION

Epithelial cells that provide a mechanical barrier at mucosal surfaces are gaining a stronger recognition regarding their ability to respond to both the commensal microbiome and deleterious microbial insults, and are involved in regulating mucosal inflammatory and immune responses. In this study, we evaluated gene expression profiles of oral epithelial cells, specifically targeting genes that could be more directly involved in communicating regulatory signals to the inflammatory, innate, and adaptive immune system in the periodontium. The specific hypothesis to be tested was that distinct differences would be observed in response profiles of oral epithelial cells reacting to challenge with planktonic bacteria, representing the characteristics of detached and dispersed microorganisms throughout the oral cavity, and the response patterns to these species when in organized biofilms.

Numerous reports have documented responses of oral epithelial cells to bacterial challenge generally with a biased sampling of a very limited number of host response biomolecules

[10,28–30]. This study examined a broad array of genes and gene pathways that describe the capacity of epithelial cells to defend and communicate against a noxious microbial challenge. We identified that >98% of the basal mRNA signal within this array of 579 genes was accounted for by 181 genes. Interestingly, the message signal for basal epithelial cell responses and control reactions to the biofilm carrier (RPGL) within this immunology array showed that the top 20 gene signals (TGFB1, B2M, IL32, ITGB1, APP, CD59, ITGA6, CTNNB1, ITGA5, CD44, GPI, PSMB7, CD99, CD81, ETS1, FN1, BCAP31, CD9, CTSC, IL6ST) comprised >60% of the overall message for both conditions. In contrast, these 20 genes only accounted for 31%, 27%, 47%, and 55% of the total response message to the biofilm, *S. gordonii, F. nucleatum*, and *P. gingivalis*, respectively. This supports that the bacterial challenge was not only substantially increasing the level of selected genes, but triggering a much broader array of responses from the epithelial cells that could be engaged in host responses to the infection. Also of interest was the identification of some of these major responses, for example IL32, amyloid beta precursor protein (APP), PSMB7 (Proteasome Subunit Beta 7; component of the 20S core proteasome complex), ETS1 (ETS Proto-Oncogene 1, Transcription Factor), and IL6ST (interleukin 6 signal transducer) that have not been well described in the oral epithelium.

Biofilm challenge of the oral epithelial cells induced up regulation of a wide array of the immune response-related genes with >16-fold up regulation of cytokines/chemokines (IL32, IL8, IL1B, CCL20, CXCL2, CXCL1, CXCL11, CXCL10, CCL5, LIF). Interestingly, IL32 and LIF (leukemia inhibitory factor) have not been previously described in oral epithelial cell biology. IL32 has been implicated in the pathogenesis and progression of various chronic inflammatory disorders [31–33] and is induced by microbial ligands via TLR pathways [34]. The IL32 gene leads to 9 splice variants and isoforms that have been shown to have varied activities under different cellular conditions [32,35,36]. This molecule has been described as a pro-inflammatory cytokine that induces differentiation of monocytes to macrophages [37], as well as up-regulation of other pro-inflammatory cytokines in these cells [38]. Recently, it was shown that IL-32 levels were elevated in saliva and GCF in periodontitis [39] and is consistent with elevated levels of IL-32 in gingival tissues in periodontitis [40]. LIF (Leukemia inhibitory factor) regulates cellular differentiation, growth and inflammation [41]. It is a member of the IL-6 family of cytokines with pro-inflammatory capabilities [42]. This molecule has been shown to be produced by the epithelial cells in the lung and protect against bacterial pneumonia infections [42,43]. However, the same ligand has been shown to induce the production of an array of pro-inflammatory molecules (eg. IL-1, IL-6, IL-8, inflammatory lipids) by gut [44], uterine [45], corneal [46], nasal airway and bronchial epithelial cells [47], and keratinocytes [41,48]. This activity appears to occur through the LIF receptor and JAK1-STAT3 signaling pathway [45,46]. Literature on its role in the oral mucosa is more limited with decreased levels in periodontitis GCF [49], and production by gingival fibroblasts in response to host pro-inflammatory signals [50]. Thus, this report of the substantial transcription of the LIF gene by oral epithelial cells in response to biofilm challenge provides new information on this potentially important molecule in gingival tissue responses.

IL-23 activates STAT4 inducing IFNγ, preferentially targeting memory $CD4^+$ T cells and promoting the production of various proinflammatory cytokines [51]. This cytokine is directly

linked to Th17 T cell functions and production of IL-17 [52], and the IL-23/IL-17 axis has been reported as a major host response abnormality in leukocyte adhesion deficiency type 1 [53] with a clear impact on expression of early onset severe periodontal disease [54]. Information on this cytokine in periodontitis has been accumulating and is clearly interconnected with the importance of IL-17 in the inflammatory changes in periodontitis [55–58]. Additional studies have shown that antigen-presenting cells (eg. macrophages, dendritic cells) challenged with *P. gingivalis* or *Prevotella* spp. demonstrated increases in IL-23 within the broader repertoire of inflammatory mediator responses [59–61], and *P. endodontalis* LPS elicited IL-23 linked to enhanced osteoclastogenesis [62], the cytokine cascade of granuloma tissues [63] and in responses of periodontal ligament cells to LPS [62]. However, IL-23 in responses of oral epithelial cells is sparse. A single report describes IL-23 elevations in immortalized gingival keratinocytes following infection with *P. gingivalis* [61]. The interesting findings with this important regulatory cytokine were its elevated expression with each of the planktonic species that was decreased substantially with the combined biofilms.

An interesting profile of the cytokine family of genes was also noted to be significantly elevated in the infected epithelial cells. IL8 was a major gene induced by the biofilms, albeit its constitutive production was rather low [24,64]. CCL20 (MIP3a) is a chemokine for dendritic cells, and can recruit both Th17 and Treg cells to sites of inflammation. Previous studies have shown increased CCL20 expression by epithelial cells stimulated with oral bacteria [13,59,65] , and is elevated in periodontitis tissues [66,67] . CXCL1 (Groα) together with IL-8 are major chemotaxins for neutrophils. This chemokine is upregulated in gingival epithelial cells [68], and Ramage et al. [14] have shown that both CXCL1 and CXCL2 are expressed in the junctional epithelium potentially contributing to attempts to maintain homeostasis [69]. Gingival fibroblasts were reported to produce CXCL11 (SCYB11; I-TAC) in response to challenge with muramydipeptide [70], while macrophages stimulated with *P. gingivalis* also upregulated this chemokine. Thus, with each of these cytokines/chemokines/signaling factors that were elevated in the oral epithelial cells there is a biological consideration regarding roles in the gingival communication of innate immune system processes.

CSF2 (colony stimulating factor 2; GMCSF) is a cytokine associated with functions of granulocytes and macrophages [71]. It has been shown to be elevated in gingival tissues of periodontitis [72] and was suggested to drive MMP-12 production in diseased tissues [73]. Interestingly, CSF2 was overexpressed by gingival epithelial cells following challenge with *A. actinomycetemcomitans* [74] and was one of the target cytokines that were increased in oral epithelial cell cultures treated with mono- and multi-species biofilms [14], particularly associated with a mixed species biofilm containing *F. nucleatum* and *P. gingivalis*. In this study, the biofilms upregulated this gene by 30-fold; however, of interest was that only *S. gordonii* (7.6-fold) and *F. nucleatum* (11.3-fold) in planktonic form stimulated expression of this important cell communication factor compared with *P. gingivalis* (−1.07-fold). Thus, the biofilms appear to be reflecting a synergistic stimulation of CSF2 when these species are organized into this type of structure.

Additionally a number of altered genes following biofilm challenge were directly related to control and regulation of the NF-κB pathway and as an activator of genes involved in both innate and acquired immune responses by binding to an interferon-stimulated response

element (ISRE) in their promoters., including: TNFAIP3 [A20; [75]], NFKBIZ (Iκbζ) [76–78], SOCS3 (suppressor of cytokine signaling 3) [79] and IRF1 [Interferon Regulatory Factor 1; [80]]. While there exists some information on certain of these regulatory factors in periodontitis, their combined expression profiles by epithelial cells in response to biofilm challenge has not be described. TNFAIP3 has not been reported related to oral epithelial cell biology, some recent studies suggest a role in dampening osteoclastogenesis [81], and elevations in gingival tissue related to decreased periodontitis coupled with TLR9 activity [82]. NFKBIZ regulates antimicrobial peptide production by epithelial cells [77,78] and has recently been confirmed as a major factor in epithelial cell functions, controlling communication signals with inflammatory/immune cells [83]. In recent studies IRF1 showed a decreased expression in chronic periodontitis [84,85], although at the cellular level, how epithelial cells were affected has not be described. Finally, there is a robust literature on SOCS3 in periodontal disease, including the identification of this regulatory molecule in human periodontal tissues [86] and rodent models of periodontal disease [87–89], as well as responses of periodontal ligament cells [90] and osteoblasts [91]. However, minimal data is available regarding the role of the inflammation regulatory molecule in the oral epithelium.

Comparison of the biofilm challenge for gene expression profiles with the individual planktonic bacteria provided a somewhat differential pattern with each bacterial species. Substantial overlap in up-regulated genes was noted between the biofilm and *S. gordonii* challenge. However, PSMB5, ICAM1, MBP, IL23A, CXCL11, CXCL10 and CCL5 were significantly increased with the *S. gordonii* challenge versus the biofilm containing this species. In addition, CXCL10 and CXCL11 were also increased by *P. gingivalis* infection. IL23A was the only gene that was substantially increased by all the planktonic bacteria compared to the biofilms. This cassette of genes that appears to be affected most by challenge with the planktonic bacteria comprises some related activities in control of local immune response. MBP (myelin basic peptide) is involved in signaling pathways in T-cells and can induce T-cell proliferation. This is coupled with CXCL11 that is chemotactic for interleukin-activated T-cells, CCL5 that is a chemo attractant for monocytes and memory T-helper cells, CXCL10 that binds to CXCR3 and stimulates monocytes, natural killer and T-cell migration, and modulation of adhesion molecule expression, such as ICAM1. ICAM1 is expressed on endothelial and immune system cells engaging integrins of the CD11/CD18 type that are implicated in interactions with monocytes, macrophages and granulocytes, as well as binding to the iC3b fragment of the third complement component. Finally, PSMB5 is a component of the core proteasome complex involved in the proteolytic degradation of most intracellular proteins. This type of proteolysis is necessary for generation of a subset of MHC class I-presented antigenic peptides in adaptive immune responses. Thus, it appears that this group of genes depicts the potential for this commensal bacterium to communicate to both innate and adaptive immune mechanisms to potentially regulate the symbiotic relationship with the host.

Also of interest was the distribution of genes whose levels were substantially decreased by treatment of the epithelial cells with the planktonic or biofilm bacteria. *S. gordonii* challenged decreased 24 genes by 2-fold or greater versus the basal cell levels, with *P. gingivalis* down-regulating only 11 genes and *F. nucleatum* only 2 genes. Interestingly, there was minimal overlap in this gene expression inhibition across the planktonic species. In

contrast, of these 579 immune response associated genes, only TGFBR1 was down-regulated by the biofilms at approximately 2-fold.

This study provided a robust assessment of major gene expression patterns of host response system biomolecules associated with the biology of the epithelium. Importantly, the breadth of gene expression and up-regulation following challenge with the individual planktonic species and the multispecies biofilms emphasized a critical role for epithelial cell responses in the periodontium enabling both direct interactions with the microbial insult, as well as a sophisticated communication and regulatory system for the inflammatory and immune infiltrate to reestablish homeostasis. More generally, the pathway analysis did provide some insight into the features of the pathogenic biofilm stimulation of a breadth of epithelial cell responses that signal the immune system. These pathways included chemokines/cytokines that would communicate effective host responses to T cells, B cells, and endothelial cells, as well as controlling cell behavior including survival and replication. Nevertheless, limitations of these types of *in vitro* studies are the capacity to truly model the complex microbial biofilms that occur *in situ*, as well as fully understanding the dynamics of interactions between the bacterial biofilms and individual epithelial cells that are critical for maintaining homeostasis. The character of these cellular responses in health, and the changes that occur with disease initiation still remain to be fully elucidated. The data also identified some unique gene profiles for the oral epithelial cells including IL32 and LIF as cytokines that have not been linked to major responses of these cells to microbial challenge. Thus, the potential for a unique role of these host response factors to pathogenic biofilms may provide additional insights into the underlying biologic mechanisms of the chronic inflammation in periodontitis.

ACKNOWLEDGEMENTS

We want to acknowledge support from U.S.P.H.S. grant P30 GM103538 from the National Institute of General Medical Sciences and the Center for Oral Health Research in the University of Kentucky College of Dentistry. We also thank Dr. Kuey Chen in the Department of Pharmacology, College of Medicine, University of Kentucky for support with the NanoString analyses. The authors explicitly state no conflicts of interest in connection with this report.

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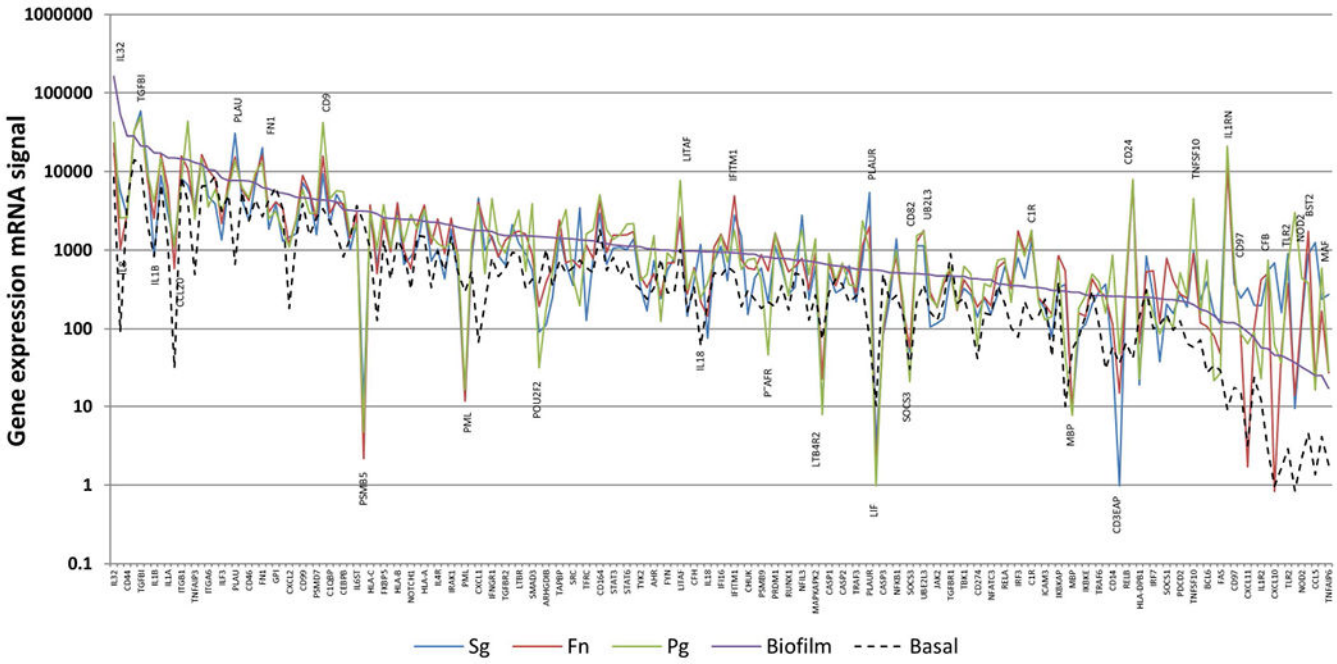


Figure 1:

Gene expression mRNA signal of genes expressed by oral epithelial cells with signal >100 to biofilm and/or planktonic bacterial challenge. The genes are ordered based upon the magnitude of signal with the biofilm challenge. The highlighted genes are those expressed by planktonic challenge with all the species that resulted in substantially (>4-fold) increased or decreased levels compared to the biofilm signal. Values denote the mean of values from 5 independent cell culture wells for the biofilms and basal levels (cells only), and duplicate cell culture wells for the planktonic bacteria.

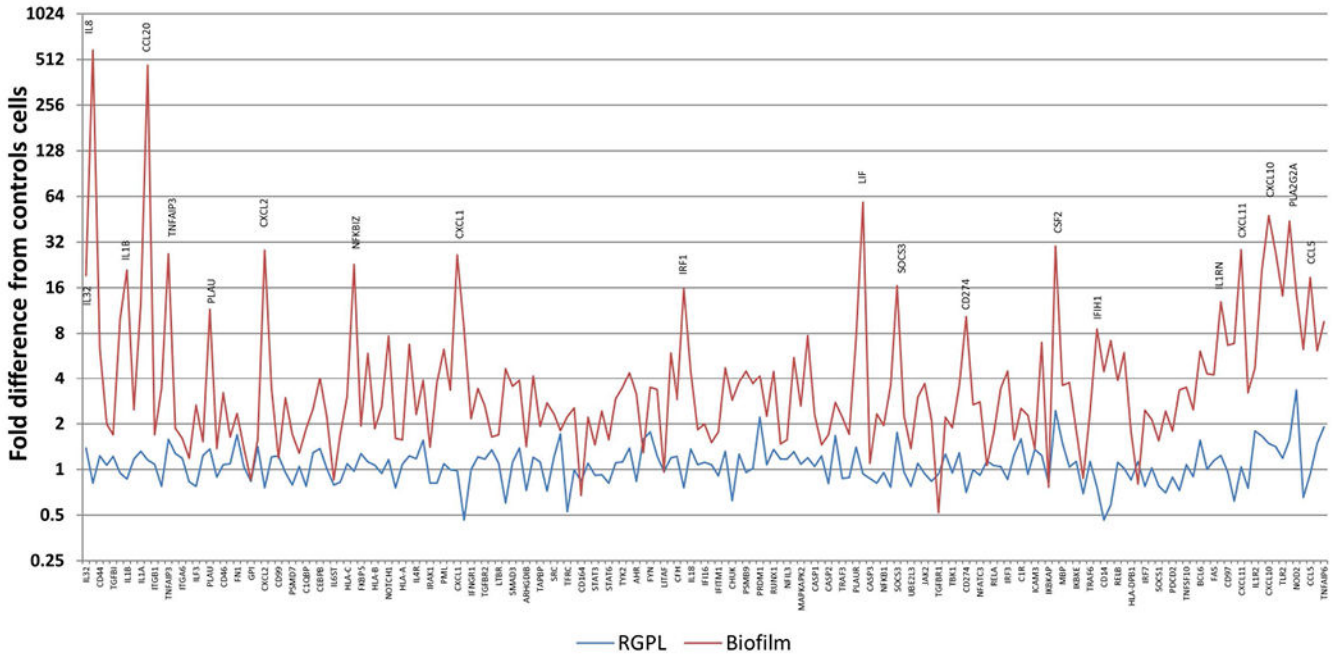
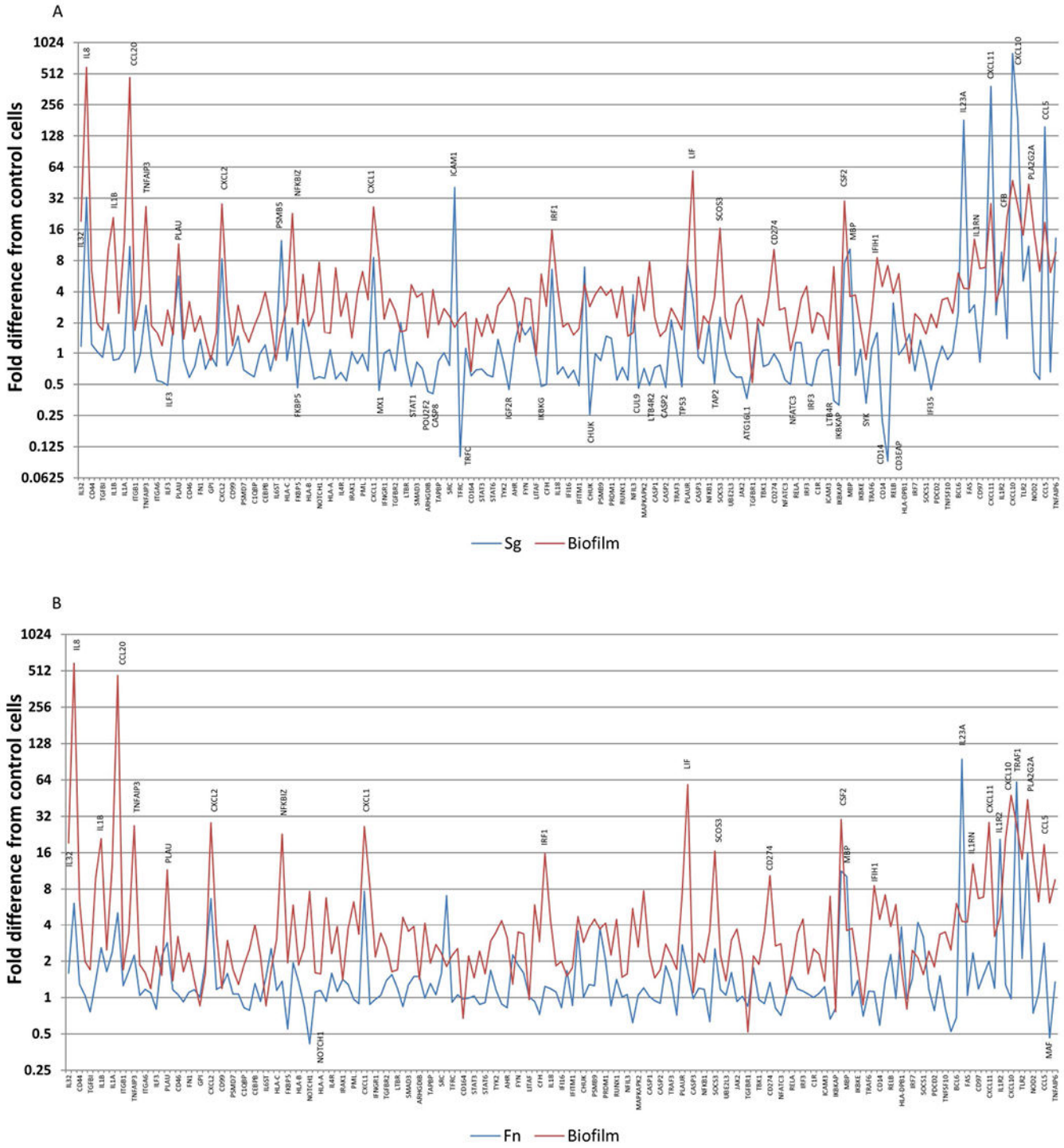


Figure 2:

Comparison of fold difference in gene expression with biofilm or RGPL interaction with the oral epithelial cells. Genes are ordered based upon the magnitude of signal expression following biofilm challenge. Control cells represented basal production in media. Values denote the mean of 5 values for the biofilms and 3 for the RGPL.



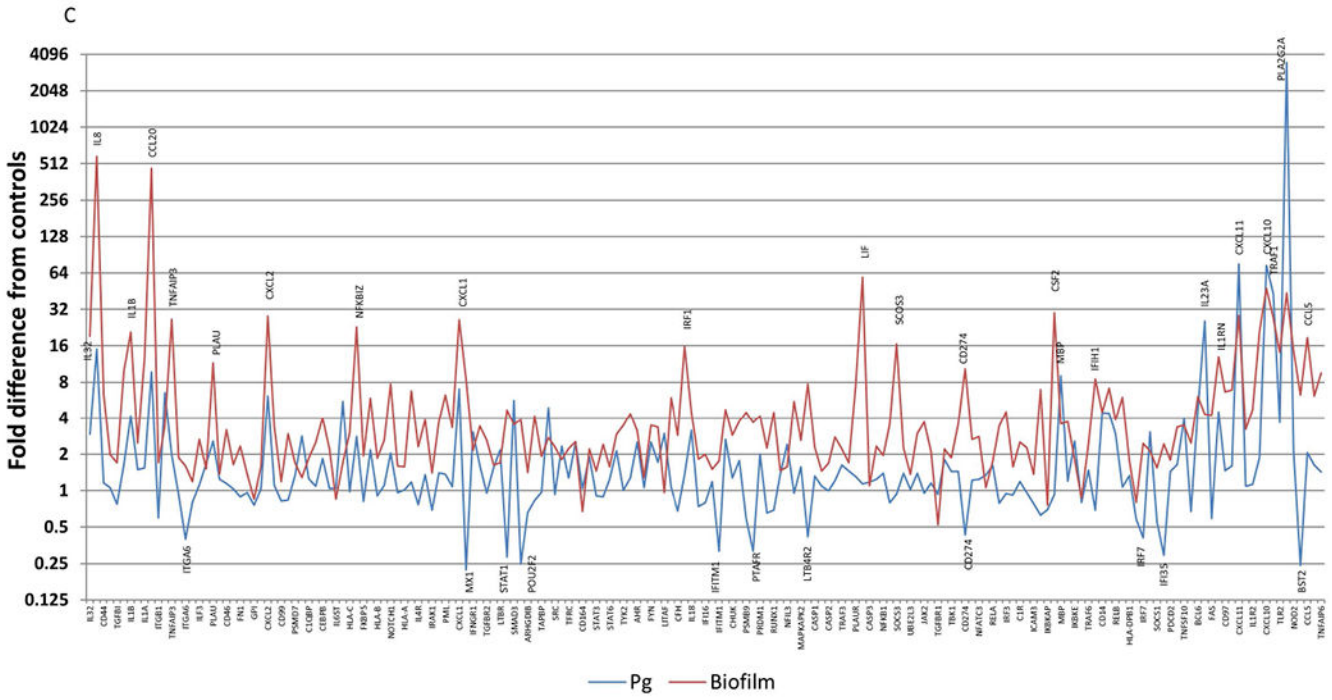


Figure 3A-C:

Comparison of fold differences in gene expression with biofilms compared to individual planktonic bacteria, *S. gordonii* (**A**), *F. nucleatum* (**B**), and *P. gingivalis* (**C**). Highlighted genes are those increased by >8-fold or decreased by >2-fold. Values denote the mean of 5 values for the biofilms and duplicates for the planktonic bacteria.

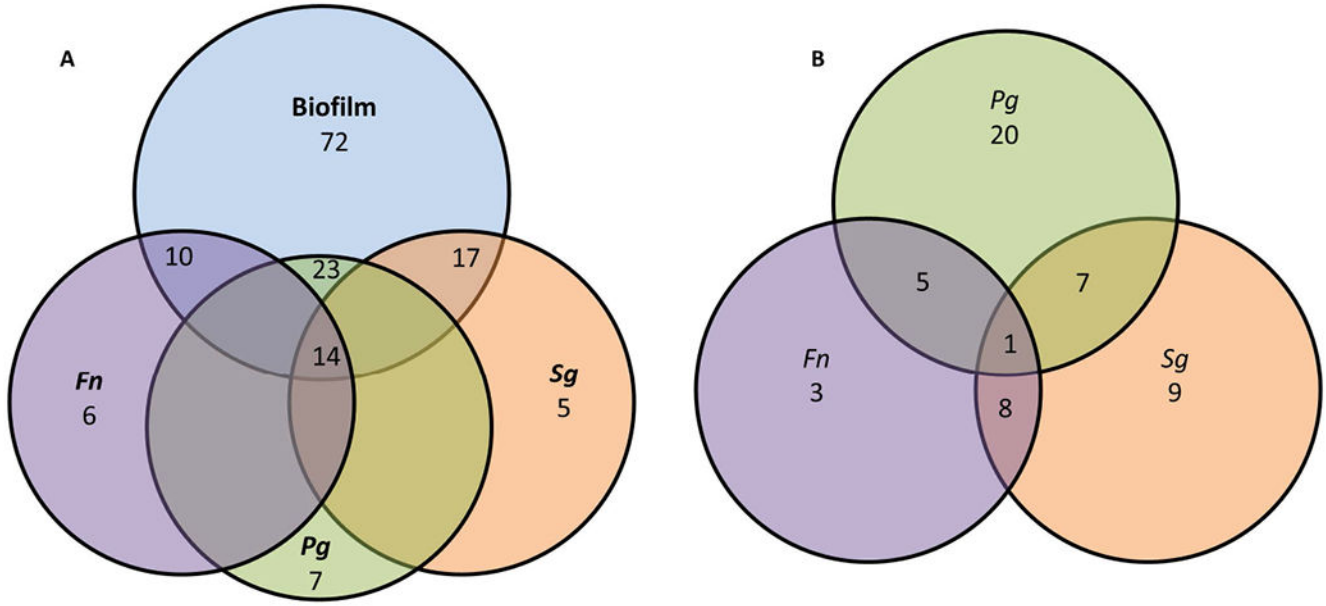
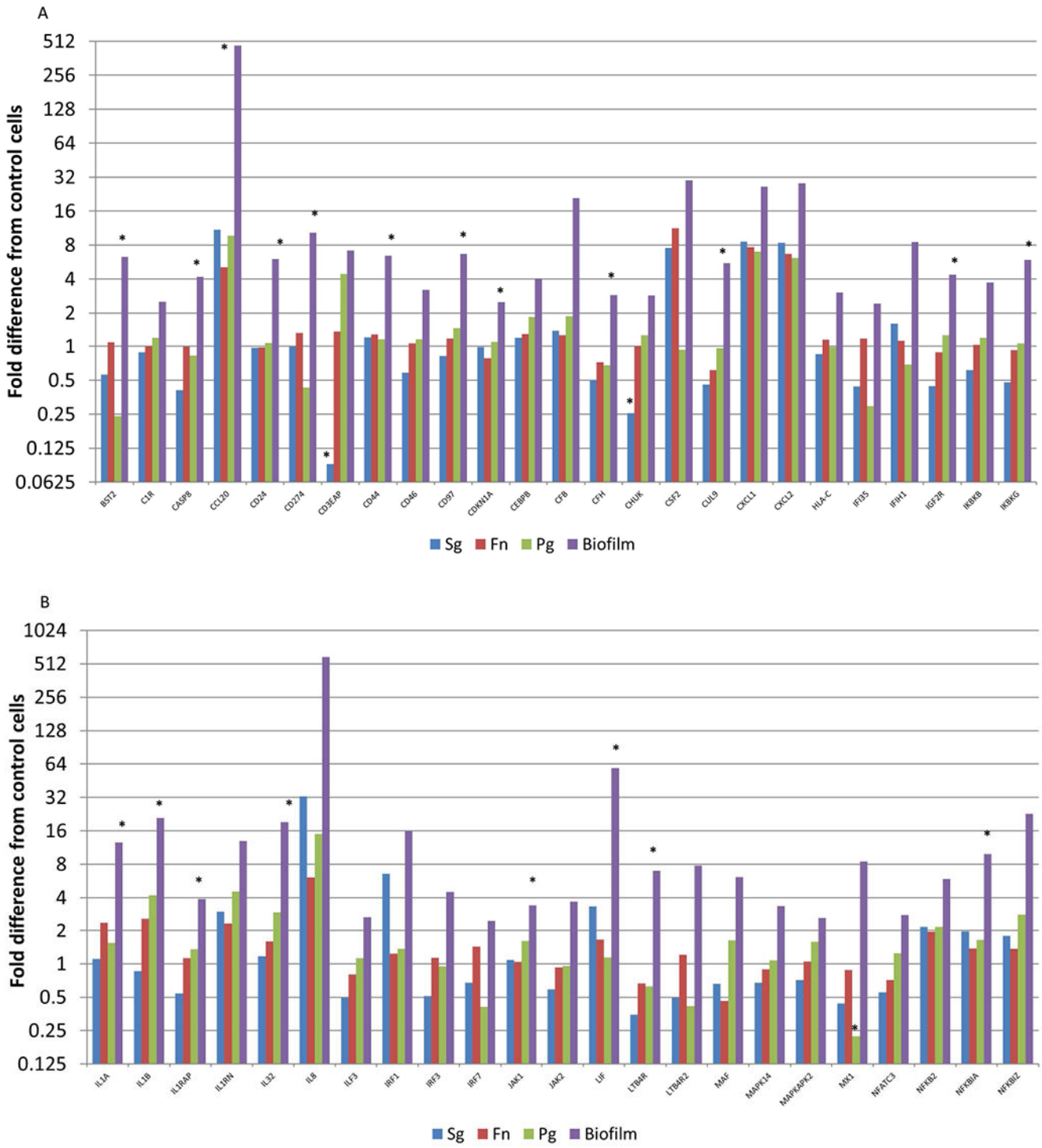


Figure 4:

Venn diagram depicting the number of genes upregulated by (**A**) biofilm or planktonic bacteria and identifying overlap or unique alteration of the expression levels. (**B**) Provides similar comparison of gene IDs upregulated among the planktonic bacteria only.



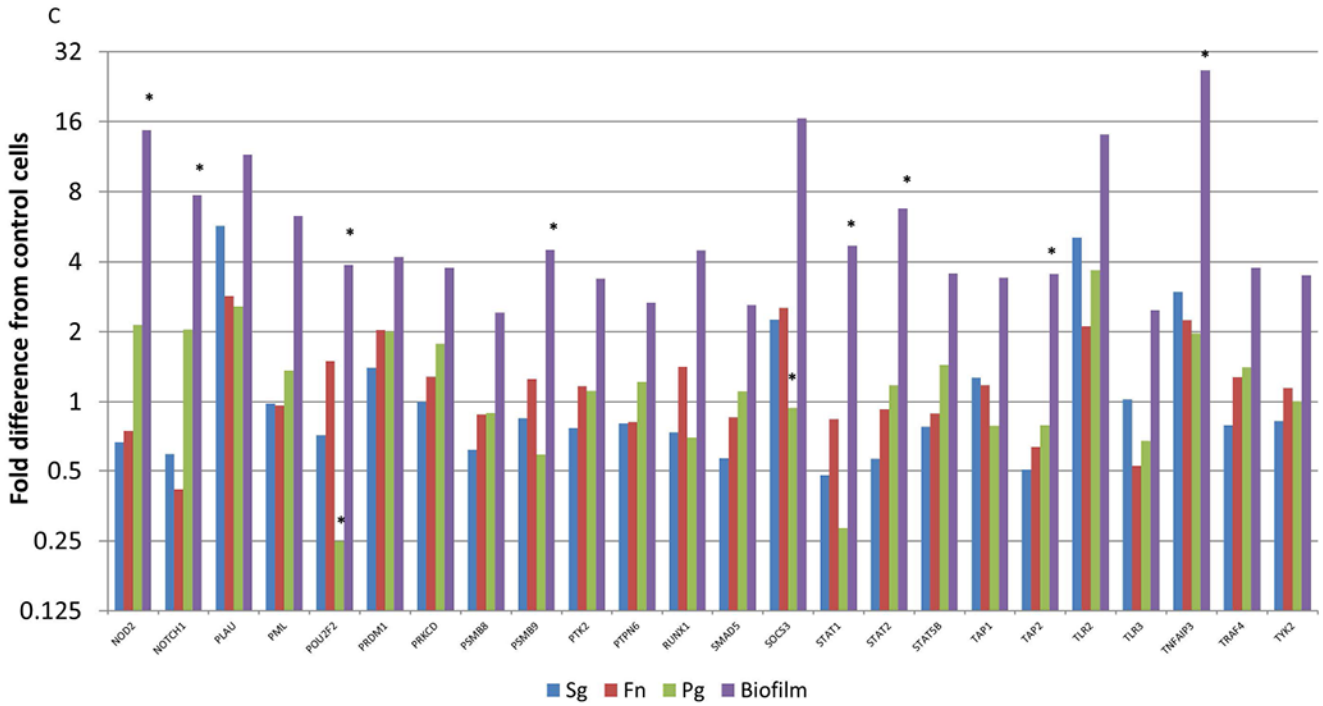


Figure 5A-C:

Identification of genes with at least 2-fold increased expression induced by biofilms compared to all of the planktonic species. Values denote the mean of 5 values for the biofilms and duplicates for the planktonic bacteria. Asterisk (*) denotes at least $p<0.01$ from the other conditions.

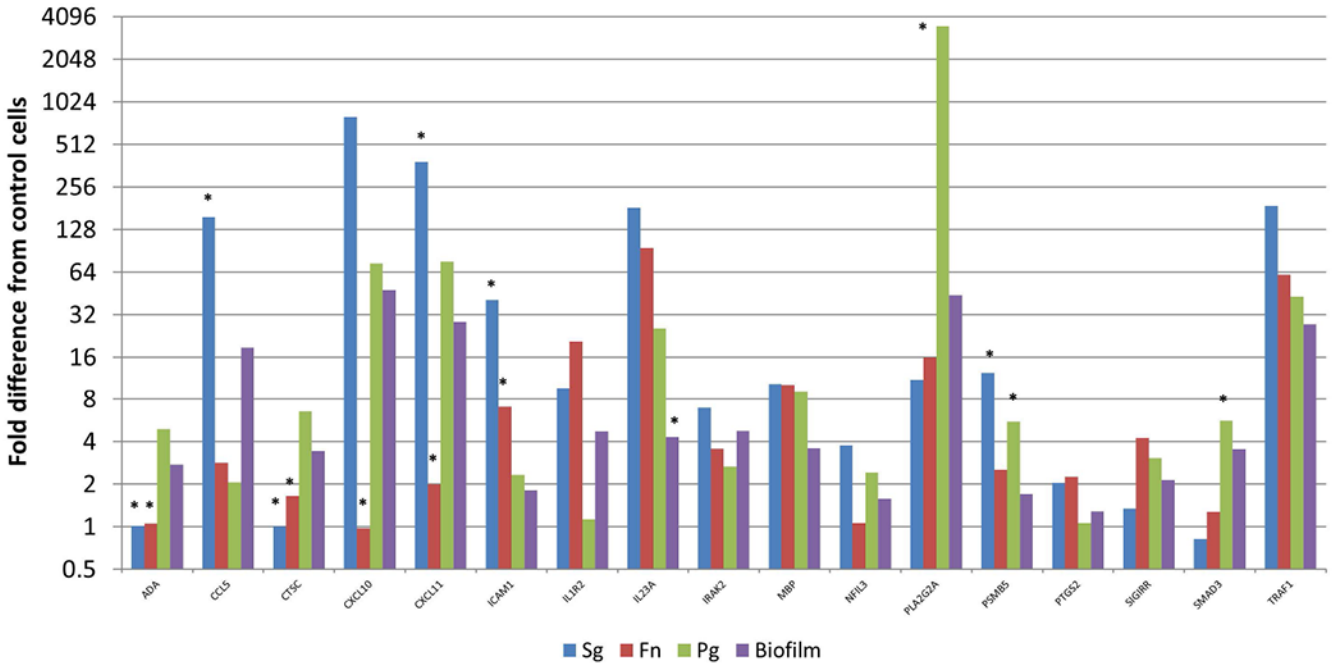


Figure 6:

Identification of genes with at least 2-fold increased expression induced by one or more planktonic bacteria compared to the multispecies biofilm. Values denote the mean of 5 values for the biofilms and duplicates for the planktonic bacteria. Asterisk (*) denotes at least $p<0.01$ from the other conditions.

Table 1:

Major pathways of immunology gene upregulation by bacterial challenge of oral epithelial cells.

PANTHER Pathways	*H. sapiens* Genome #	Biofilms #	Expected	Fold Enrichment	Raw *P*-value	FDR
Biofilm						
JAK/STAT signaling pathway	17	6	.05	>100	6.22E-11	2.03E-09
Interferon-gamma signaling pathway	34	6	.10	57.59	2.27E-09	5.29E-08
Toll receptor signaling pathway	64	11	.20	56.09	3.74E-16	3.04E-14
B cell activation	69	8	.21	37.84	8.66E-11	2.35E-09
Interleukin signaling pathway	94	10	.29	34.72	7.16E-13	2.92E-11
p53 pathway feedback loops 2	49	3	.15	19.98	5.40E-04	5.87E-03
VEGF signaling pathway	68	4	.21	19.20	6.98E-05	8.75E-04
T cell activation	88	5	.27	18.54	9.61E-06	1.31E-04
PDGF signaling pathway	142	8	.44	18.39	1.82E-08	3.70E-07
Apoptosis signaling pathway	129	7	.40	17.71	1.91E-07	2.83E-06
Inflammation mediated by chemokine/ cytokine signaling pathway	286	15	.88	17.12	1.41E-14	7.66E-13
Angiogenesis	169	8	.52	15.45	6.60E-08	1.19E-06
CCKR signaling map	180	8	.55	14.50	1.05E-07	1.71E-06
RAS pathway	76	3	.23	12.88	1.82E-03	1.75E-02
EGF receptor signaling pathway	153	5	.47	10.66	1.22E-04	1.42E-03
Planktonic						
Inflammation mediated by chemokine/ cytokine signaling pathway	286	5	.22	23.18	1.81E-06	2.94E-04