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Streptococcus cristatus attenuates *Fusobacterium nucleatum*-induced cytokine expression by influencing pathways converging on nuclear factor-κB

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SUMMARY

We previously reported that *Streptococcus cristatus*, an oral commensal, was able to downregulate the interleukin-8 (IL-8) response to *Fusobacterium nucleatum*, a putative oral pathogen in oral epithelial cells. The aim of this study was to extend the understanding of how *S. cristatus* regulates cytokine expression in oral epithelial cells on a broad basis, and investigate whether the modulation of a Toll-like receptor (TLR) pathway was involved in this process. KB and TERT-2 cells were co-cultured with *F. nucleatum* and *S. cristatus*, either alone or in combination. Total RNA was extracted and pathway-specific focused microarrays were used to profile the transcriptional responses of various cytokine genes and those related to TLR-mediated signal transduction. Reverse transcription–polymerase chain reactions (RT-PCR) and protein assays were performed to confirm the microarray results for selected genes. We found that exposure to either *S. cristatus* or *F. nucleatum* alone led to distinct changes in cytokine expression patterns. *Fusobacterium nucleatum* induced a greater number of gene expression changes than *S. cristatus* (15% vs 4%, respectively). The presence of *S. cristatus* with *F. nucleatum* attenuated the expression of a number of inflammatory cytokines, and upregulated several anti-inflammatory mediators. The RT-PCR confirmed the messenger RNA attenuation of IL-1α, tumor necrosis factor-α and IL-6 by *S. cristatus*. Profiling of TLR-signaling-related genes revealed that *S. cristatus* most significantly impacted the downstream pathways, especially nuclear factor-κB, rather than altering TLRs and their adaptors and interacting proteins. Our data suggest that *S. cristatus* may attenuate the epithelial proinflammatory cytokine response to *F. nucleatum* by influencing pathways converging on nuclear factor-κB.

Keywords

cytokines; epithelial cells; *Fusobacterium nucleatum*; inflammatory response; nuclear factor-κB; *Streptococcus cristatus*

INTRODUCTION

Epithelial cells are the first line of host defense against physical, microbial and chemical insults that may cause local injury. Studies have shown that epithelial cells play an integral role in mucosal immune defense by sensing signals from the external environment, generating various molecules to affect growth, development and function of other cells, and maintaining the balance between health and disease (Kagnoff & Eckmann, 1997). A

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characteristic response of epithelial cells to bacterial stimuli is synthesis and release of cytokines, chemokines and other inflammatory mediators (Kagnoff & Eckmann, 1997). Putative oral pathogens, such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, have been shown to induce a wide array of proinflammatory cytokines such as interleukin-8 (IL-8), monocyte chemoattractant protein-1, IL-1β, IL-6 and tumor necrosis factor-α (TNF-α) from epithelial cells (Han *et al.*, 2000, 2003; Sandros *et al.*, 2000; Kusumoto *et al.*, 2004). There is emerging evidence that certain commensal oral streptococci behave in a manner different from that exhibited by the pathogenic species, in that they induce very low levels of proinflammatory cytokines (Cosseau *et al.*, 2008; Zhang *et al.*, 2008; Sliepen *et al.*, 2009). However, the cytokine expression patterns following contact with oral commensal micro-organisms are less studied, and conflicting results have been reported (Vernier *et al.*, 1996; Cosseau *et al.*, 2008; Zhang *et al.*, 2008).

The C-X-C family of cytokines, represented by IL-8, is generally produced at high levels by infected epithelial cells. They can initiate the mucosal influx of polymorphonuclear leukocytes to orchestrate innate mucosal inflammatory responses. The inflammatory process is critical to host defense, but deregulation of inflammatory cytokine production can cause epithelial damage. Excessive recruitment of polymorphonuclear leukocytes to the periodontium has been considered to contribute to the pathogenesis of periodontal disease (Van Dyke & Serhan, 2003). It therefore seems necessary to have regulatory mechanisms controlling innate immunity operating at the level of epithelial cells to prevent persistent inflammation. Recently, two strains of oral commensal streptococci, *Streptococcus cristatus* and *Streptococcus salivarius*, were found not to elicit proinflammatory responses by themselves, and were able to inhibit the IL-8 secretion induced by periodontal and respiratory pathogens, indicating a potential role of commensal bacteria in the maintenance of host–microbe homeostasis (Cosseau *et al.*, 2008; Zhang *et al.*, 2008).

Epithelial cells sense bacterial products through a variety of pattern recognition receptors, the best understood of which are Toll-like receptors (TLRs). Each TLR recognizes specific microbial components present on diverse microbes. For example, TLR2 recognizes peptidoglycan, in addition to the lipoproteins and lipopeptides of gram-positive bacteria, whereas TLR4 recognizes lipopolysaccharide from most gram-negative species (Takeuchi *et al.*, 1999). It is known that TLR2 and TLR4 are present on or in oral epithelial cells (Uehara *et al.*, 2001). In general, the cascade of events occurring following ligation of the different TLRs involves the activation of a common set of adapter proteins and protein kinases that results in the activation of nuclear factor-κB (NF-κB) to express cytokine genes relevant to inflammation (Kawai & Akira, 2005). Downregulation of TLR expression (Sweet *et al.*, 2001) or upregulation of inhibitory regulators of TLR signaling (Burns *et al.*, 2000) have been shown to be active in epithelial cells to mediate tolerance.

Given the amount of tissue or cells required for the variety of tests, traditional host–microbe interaction studies generally only focus on one or limited numbers of cytokines in a single assay. Transcriptional profiling using microarrays provides a way to monitor host responses on a broad scale (Mans *et al.*, 2006). Studies of the host transcriptional responses to oral bacteria have begun but the focus in these few studies is mostly on putative oral pathogens (Handfield *et al.*, 2005; Milward *et al.*, 2007). Using pathway-specific microarray technology in a dual infection model, here we demonstrated that the oral commensal species *S. cristatus* induces distinct cytokine expression patterns as opposed to the putative oral pathogen *F. nucleatum*, and dampens *F. nucleatum*-induced proinflammatory epithelial responses. Our data did not show altered expression of TLRs or their adaptors and interacting proteins as a mechanism for attenuation by *S. cristatus*. In contrast to the strong activation of elements downstream of the NF-κB pathway by *F. nucleatum*, the minimal NF-κB activation by *S. cristatus* raises the possibility that the mechanism of cytokine inhibition

in the presence of *S. cristatus* may be controlled at the level of the NF-κB transcription factor.

METHODS

Bacterial strains and culture conditions

Fusobacterium nucleatum (ATCC 10953) and *S. cristatus* CC5A (ATCC 49999) were routinely grown under anaerobic conditions (85% N_2, 10% H_2, 5% CO_2) at 37°C. The *F. nucleatum* was cultured in trypticase soy broth (BBL, Becton Dickinson, Sparks, MD) supplemented with 1 g yeast extract, 5 mg hemin and 1 mg menadione per liter; *S. cristatus* was grown in Todd–Hewitt broth (BBL).

Epithelial cell cultures

The epithelial carcinoma cell line KB was kindly provided by Dr Mark Herzberg (University of Minnesota). The immortalized normal oral epithelial cell line OKF6/TERT-2 was obtained under a materials transfer agreement from Dr James Rheinwald (Brigham and Women's Hospital, Boston, MA). All cell lines were maintained in 75-cm^2 flasks (Corning, Corning, NY) in a humidified atmosphere of 5% CO_2 at 37°C. KB cells were grown in minimal essential medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum, whereas OKF6/TERT-2 was cultured in keratinocyte serum-free medium (Invitrogen) supplemented with $CaCl_2$ (0.4 m_M), bovine pituitary extract (25 μg ml^{-1}) and epidermal growth factor (0.2 ng ml^{-1}). Twenty-four hours before infection, cells were trypsinized, centrifuged and seeded into triplicate wells in duplicate cell culture plates (Corning).

Bacterial infection procedure

Overnight broth cultures of bacteria were harvested by centrifugation at 5000 ***g*** for 20 min, washed twice with 1 × Dulbecco's phosphate-buffered saline, and then re-suspended in serum-free cell culture media. Bacterial concentrations were adjusted to 10^8 colony-forming units per ml by measuring optical density at 620 nm for each experiment, and confirmed by colony counting on agar plates. Then, *F. nucleatum* and/or *S. cristatus* was added to cell monolayers at a multiplicity of infection of 100 for each species, and incubated for 2 h at 37°C in 5% CO_2. Wells containing no bacteria served as negative controls. After stimulation, cell supernatants were collected for cytokine assays and cell monolayers were harvested for RNA extraction (see below). All assays were carried out in triplicate, and three independent experiments were performed. For all the experiments, the viability of infected cells was examined by trypan blue exclusion.

RNA extraction and array hybridization

Total RNA was extracted and purified from infected and control cells using the RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. The integrity, purity and quantification of RNA were evaluated on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA).

Pathway-specific, non-radioactive GEArray Q Series Human Inflammatory Cytokines & Receptors Gene Arrays (HS-015.2; SuperArray Inc., Bethesda, MD) containing 96 cytokine and receptor genes associated with the inflammatory response were used according to the manufacturer's instructions. Briefly, 3 μg total RNA from KB cells was used to synthesize biotin-16-dUTP-labeled (Roche, Indianapolis, IN) complementary DNA (cDNA) probes with an AmpoLabeling-LPR kit (L-03; SuperArray). Labeled cDNA probes were denatured and hybridized to GEArray membranes. After washing and blocking, the membranes were incubated with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate. Images

of the arrays were recorded by exposure to X-ray films (Kodak, Rochester, NY), and scanned by an imaging densitometer (Bio-Rad Laboratories, Hercules, CA). Scanned images were digitized using SCANALYZE software (free download from http://rana.stanford.edu/software), and data were analyzed on the GEARRAY analyzer program (SuperArray). The cDNA microarray experiments were carried out twice, using RNA isolated from two independent experiments.

Shortly after we finished the Inflammatory Cytokines & Receptors Array assays, SuperArray switched to the Oligo GEArrays® microarray system. To confirm the major cytokine responses obtained from KB cells and further analyse related signal transduction pathways, the Oligo GEArray® Human Toll-Like Receptor Signaling Pathway Microarray (EHS-018.2, SuperArray) was employed to assess the TLR-signaling-related gene expression in our dual infection model. OKF6/TERT2 cells were used because they had shown attenuation of the IL-8 response to *F. nucleatum* in a previous study (Zhang *et al.*, 2008). The Oligo GEArray® Human Toll-Like Receptor Signaling Microarray profiles the expression of 113 genes related to TLR-mediated signal transduction, including members of the TLR family, key mediators including adaptors and proteins that interact with TLR and effectors, and members of the NF-κB, c-JUN N-terminal kinase (JNK)/p38, nuclear factor-IL-6 (NF/IL6), and interferon regulatory factor (IRF) signaling pathways downstream of TLR signaling, including major proinflammatory cytokines that were also components of the Inflammatory Cytokines & Receptors Array. According to the manufacturer's instructions, 3 μg total RNA from infected and control TERT2 cells was used to synthesize biotin-16-UTP-labeled cRNA via an *in vitro* transcription labeling kit (TrueLabeling-AMP kit, GA-030, SuperArray). Labeled cRNA was then quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE) and hybridized to GEArray membranes. After washing and blocking, the membranes were incubated with alkaline phosphatase-conjugated streptavidin and CDP-Star substrate. Images of the arrays were recorded by exposure to X-ray films (Kodak), and scanned with an imaging densitometer (Bio-Rad). Scanned images were analysed using the specially designed web-based and completely integrated GEARRAY EXPRESSION ANALYSIS SUITE software (SuperArray). Oligo microarray assays were repeated three times, using RNA isolated from three independent experiments.

Analysis of microarray data

We first eliminated spots where the signals were so strong that they 'bled' into the background area, resulting in regions of high background values or non-specific background. According to the SuperArray software, a transcript's spot is considered 'absent' if the average density of the spot is less than 1.5 × the mean value of the local backgrounds of the lower 75th percentile of all non-bleeding spots. Consequently all transcripts that were 'absent' on any array under any experimental condition were removed from further analysis. The hybridization signal of each gene present was normalized to that of the internal positive control glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) observed on the same membrane for cytokine arrays, and to β_2-microglobulin for TLR arrays. The expression levels of different genes after bacterial exposure were compared with those from uninfected controls, and expressed as relative ratios. Results are presented as the average of the number of replicates noted above for each array. A more than two-fold increase or less than 0.5-fold decrease in signal intensity between experimental groups (bacteria-infected) and the uninfected control group was considered biologically significant. Statistical comparison between conditions was performed by comparing fold differences averaged over all genes expressed for each condition, by using repeated measures analysis of variance (ANOVA) followed by Duncan's multiple range test. For TLR array data, relative gene expression levels were converted to logs for statistical analysis.

Qualitative reverse transcriptase–polymerase chain reaction

A 3-μg sample of RNA from the same batch as that used for microarray assays was reverse transcribed using a random primer (Promega, Madison, WI) and Moloney monkey leukemia virus reverse transcriptase (Promega) using conditions described by the manufacturer. One microliter of each cDNA sample was used for routine polymerase chain reaction (PCR). The primers for IL-1α, IL-6, IL-8, TNF-α and GAPDH are listed in Table 1. Standard PCR was performed in a total volume of 25 μl with the following components: 1 × PCR buffer, 0.25 mM dNTPs, 2 mM $MgCl_2$, 0.5 μM of primers, and 1.5 U of platinum *Taq* polymerase (Invitrogen). The PCR amplicons were visualized in 1.5% agarose gels stained by ethidium bromide.

Luminex multiplex cytokine assays

Frozen cell supernatants were sent to the Cytokine Reference Laboratory at the University of Minnesota. Bead-based Fluorokine® MultiAnalyte Profiling Kits (R&D Systems, Minneapolis, MN) run on the Luminex® 100™ platform were used to measure the levels of selected cytokines including IL-1α, IL-8, interferon-γ (IFN-γ) and TNF-α simultaneously in a single sample. Cytokine concentrations were normalized to total protein levels as determined using the bicinchoninic acid protein assay (Pierce, Rockford, IL), and expressed as ng cytokine per mg total protein.

RESULTS

Differential cytokine expression profiles induced by *F. nucleatum* and *S. cristatus*

We first examined the effects of *F. nucleatum* and *S. cristatus* on 96 cytokine and receptor genes associated with an inflammatory response. After 2 h of exposure, *F. nucleatum* induced a potent proinflammatory cytokine response with 15% of all tested genes upregulated. No downregulated gene expression was found (Table 2). Among the 14 genes with high expression levels, *IL1α* was upregulated about 10-fold relative to the control. The relative messenger RNA (mRNA) expression of other upregulated genes varied from two- to approximately five-fold. Unfortunately, the manufacturer did not include the gene for IL-8 in this array. However, our previous work has shown *IL8* to be significantly upregulated by *F. nucleatum* (Zhang *et al.*, 2008).

A different gene expression pattern was found in cells treated with *S. cristatus* alone (Table 2). Only 4% (four genes) were modulated, with upregulation of three genes and downregulation of one. Among those, three genes exhibited more than a two-fold increase compared with the control, including *IL4*, *CCL1* and IL-10 receptor α (*IL-10RA*). One downregulated gene, *IL18*, was identified with a 2.3-fold decrease in mRNA expression. Duncan's multiple range test confirmed that the overall expression patterns between *F. nucleatum*-infected versus *S. cristatus*-treated cells were significantly distinct ($P = 0.0046$), with significantly higher expression levels in *F. nucleatum*-infected cells.

Streptococcus cristatus attenuated epithelial cytokine production induced by *F. nucleatum*

Next, the cytokine expression pattern induced by the co-infection of *F. nucleatum* and *S. cristatus* was analysed. We observed at least two-fold changes in transcription of 14 genes following 2 h of combined infection, with upregulation of 12 genes and downregulation of two genes (Table 2). According to their immunoregulatory effects in inflammation, those 14 genes were classified into five groups. Genes in the first group included those for IL-1α, CCL16, lymphotoxin-β, TNF-α and XCL2, which are recognized as proinflammatory cytokines. Their upregulation in response to *F. nucleatum* was attenuated at least 1.4-fold by the presence of *S. cristatus*. Exposure to the two organisms together also downregulated the expression of *IL18* and IL-6 signal transducer gp130 (*IL6*), which were not changed in the *F.*

nucleatum-infected cells. The third group comprised anti-inflammatory genes including *IL4* and *IL10RA*, whose expression remained upregulated in co-infection of KB cells with *F. nucleatum* and *S. cristatus* in an additive fashion, compared with the individual infection by either organism. The fourth group contained the chemokine genes *CCL1* and *CCL7* and chemokine receptor gene *CCR9*. In contrast to their response to *F. nucleatum* alone, their expression was upregulated with the addition of *S. cristatus*. The fifth group included chemokines *CCL2*, *CCL13*, *CCL15* and *CCL22*, which had similar levels of upregulation compared with *F. nucleatum* alone.

The ANOVA indicated that the overall cytokine expression patterns were significantly different between treatments ($P = 0.01$). Duncan's multiple range test further revealed that *F. nucleatum* and co-infection induced significantly higher gene expression levels than *S. cristatus* alone ($P = 0.0046$ and $P = 0.04$, respectively). However, the overall difference between *F. nucleatum* alone and co-infection was not significant ($P = 0.3$).

Modulation of TLR signaling pathway by *F. nucleatum* and *S. cristatus*

The previously reported IL-8 attenuation (Zhang *et al.*, 2008) and the cytokine array patterns pointed strongly toward *S. cristatus* having anti-inflammatory activities. Cytokine induction in response to micro-organisms is a recognized consequence of TLR activation in host cells (Akira & Takeda, 2004). To confirm the array results obtained from the KB cell model, and further explore the impacts of *F. nucleatum* and *S. cristatus* on TLR-signaling-related gene expression, we then profiled the expression of 113 genes related to TLR-mediated signal transduction in TERT2 cells. This array incorporated several cytokine genes, including those for IL-8 and IL-1β, which were not included in the cytokine array.

Among the 113 genes represented by the array, 7% (eight genes) were significantly modulated by *S. cristatus* alone. By contrast, exposure to *F. nucleatum* alone led to differential expression of 16% of genes (18 genes), and under combined infection this proportion was 12% (14 genes). These differentially expressed genes are listed in Table 3. As reported previously, no TLR expression was detected on the gene array, in contrast with their easy amplification by PCR (Durand *et al.*, 2006;Decanis *et al.*, 2009;Li & Dongari-Bagtzoglou, 2009). Such negative results have been attributed to the lower sensitivity resulting from the generation of the probes for the gene array (Luo *et al.*, 2002;Fuke *et al.*, 2004).

Compared with control cells, *S. cristatus*-stimulated cells never showed any notable changes in gene expression. Among the eight upregulated genes, modest increases were only seen in the expression of two genes: *NFKBIA* (NF-κB inhibitor α, 4.24-fold) and *NFKBIB* (4.10-fold), and the remaining six genes only had small increases in expression (from 2.02-fold to 2.49-fold). In contrast, *F. nucleatum*-exposed cells demonstrated a significant and dramatic increase in the expression of five genes: *CSF2* (colony stimulating factor 2, 17.96-fold), *IL1α* (11.16-fold), *IL8* (77.36-fold), *IRF1* (interferon regulatory factor 1, 19.87-fold) and *NFKBIA* (20.71-fold). Modest increases were seen in three genes: *CSF3* (4.13-fold), *IL1β* (5.12-fold) and *NFKB1* (3.99-fold), whereas the nine remaining genes only showed small increases (from 2.10-fold to 2.67-fold). Interestingly, we found that the addition of *S. cristatus* attenuated, to varying degrees (from 1.8-fold to 3-fold), the expression of almost every gene upregulated by *F. nucleatum* with the exception of *NFKBIB*, and ~ 60% of those genes were no longer upregulated.

Downregulation of TLR-related genes was not commonly seen with infection by either *F. nucleatum* or *S. cristatus* alone. Specifically, only one gene, *TOLLIP* (Toll interacting protein, 0.2-fold) was significantly downregulated in the presence of *F. nucleatum*. No gene was significantly downregulated by *S. cristatus*. In contrast, the combined infection of *F.*

nucleatum and *S. cristatus* led to significant downregulation of seven genes, the same number as were upregulated.

When overall gene expression levels were compared by repeat-measures ANOVA, there was a highly significant difference between groups ($P = 0.00002$). The *F. nucleatum* alone was significantly higher than *S. cristatus* alone ($P = 0.0004$), as well as than *F. nucleatum* plus *S. cristatus* ($P = 0.00007$). There was no significant difference found between *S. cristatus* alone and *F. nucleatum* plus *S. cristatus* ($P = 0.29$).

Immune function and pathway analysis of differentially expressed genes

The differentially expressed genes from TLR signaling arrays were further examined in the context of molecular function and pathway membership. In TERT2 cells, the majority of *F. nucleatum* upregulated genes were members of pathways downstream of TLR signaling (Table 3). Specifically, 10 genes were in the NF-κB pathway, two genes were in the IRF pathway and one gene was in the JNK/p38 pathway. The remaining five upregulated genes belong to the family of effectors, adaptors and TLR interacting proteins. *TOLLIP*, the only gene downregulated by *F. nucleatum*, is a negative regulator of TLR signaling.

Similar results were also found with *S. cristatus* alone, and the combined infection. Among the eight genes upregulated by *S. cristatus*, four were involved in the NF-κB pathway, two genes were in the JNK/p38 pathway, one was in the IRF pathway and one was in the family of adaptors and TLR-interacting proteins. On the other hand, the combined infection of *F. nucleatum* and *S. cristatus* modulated 10 genes involved in the NF-κB, IRF and JNK/p38 pathways, and four genes related to effectors, adaptors and TLR-interacting proteins.

Upon closer examination, we found that upregulation of key proinflammatory genes related to the NF-κB pathway (*CSF*, *IL1α*, *IL1β* and *IL8*) appeared as a major outcome following *F. nucleatum* stimulation of TERT2 cells, supporting the results obtained from inflammatory cytokine array analysis in KB cells. In the *S. cristatus* experiment, however, the expression levels of those genes did not change as much compared with control infected cells, except for a slight increase in *IL1α* level. The presence of *S. cristatus* clearly attenuated the degree of *F. nucleatum*-induced proinflammatory gene upregulation, which also confirmed the previous results.

Validation of array data of selected cytokine genes by RT-PCR and protein assays

To confirm the results of the array gene expression analysis, we performed qualitative RT-PCR to examine the mRNA expression changes of selected proinflammatory genes (*IL1α*, *IL6* and *TNFα*). As extensive assays had been performed in our previous study (Zhang *et al.*, 2008), IL-8 was not included in the validation. The RT-PCR was performed on the same set of RNA extracts used for arrays and the lowest possible number of PCR cycles was used to detect small differences. The RT-PCR results are shown in Fig. 1.

In KB cells, we found that *S. cristatus* alone barely stimulated *IL6* and *TNFα* expression, but was able to attenuate the robust expression of both cytokine genes induced by *F. nucleatum*. Constitutive expression of *IL1α* was detected in unstimulated KB cells and *S. cristatus* only induced a subtle increase of *IL1α* expression as opposed to the significant upregulation caused by *F. nucleatum*. In agreement with the array results, *F. nucleatum*-induced *IL1α* upregulation was inhibited in the presence of *S. cristatus*. The same expression patterns and *S. cristatus*-elicited attenuation effects were also observed in TERT2 cells for all three cytokines, although control TERT2 cells demonstrated a baseline constitutive expression of these genes.

To validate and extend the results of mRNA analysis, we further determined the secreted protein levels of four selected genes from KB cells using the Luminex multiplex cytokine assay (Fig. 2). Those included modulated genes such as those for IL-1α and TNF-α, a non-modulated gene for IFN-γ, and IL-8 as a positive control. No TNF-α protein was detected in any sample, levels were below the limit of detection. Interleukin-1-α was only detected in *F. nucleatum*-infected cell supernatants, and fell below the limit of detection in all the other three conditions. Interleukin-8 was significantly elevated following stimulation with *F. nucleatum*, and this elevation was attenuated in the presence of *S. cristatus*. Secretion of IFN-γ was detected in all samples, and as with the array, no difference was found among the conditions. Results with the proteins selected for analysis were consistent with the data obtained by microarrays.

DISCUSSION

In an effort to characterize the repertoire of cytokine responses of oral epithelia, in this study we have applied pathway-specific microarrays to the mRNA expression analysis of 96 cytokine genes in epithelial cells exposed to two common organisms, *S. cristatus*, a commensal oral viridans streptococcoccus, and *F. nucleatum*, a putative oral pathogen. We demonstrated that *F. nucleatum* and *S. cristatus* induce differential cytokine expression patterns in epithelial cells, and *S. cristatus* is able to inhibit inflammatory responses provoked by *F. nucleatum*. By further profiling the expression of 113 genes related to TLR-mediated signal transduction, we confirmed the cytokine-modulating effect of *S. cristatus*, and our results suggest that *S. cristatus* exerts its anti-inflammatory activities through influencing pathways converging on NF-κB.

It is well recognized that *F. nucleatum* can stimulate a wide array of cytokines from oral epithelial cells (Ji *et al.*, 2007; Milward *et al.*, 2007; Peyret-Lacombe *et al.*, 2009). Our microarray data confirmed the upregulation of the genes for IL-1α, IL-1β, IL-8 and TNF-α, and identified a number of upregulated genes whose mRNA expression after *F. nucleatum* infection had not previously been investigated, including various CC, CXC and C chemokines such as CCL16, CCL2, CXCL11 and XCL2. These chemokines are chemoattractants of monocytes/macrophages, eosinophils and subpopulations of T cells. It is speculated that the expression of various chemokines by activated epithelial cells plays a role in orchestrating the initiation of mucosal inflammatory and immune responses in which a number of different cell types participate (Kagnoff & Eckmann, 1997). Other than the proinflammatory cytokines and chemokines, it is noteworthy that *IL4* and *IL10RA* were also found to be up-regulated to various degrees in *F. nucleatum*-infected KB cells. Interleukin-4 is known to be a potent anti-inflammatory cytokine that acts by inhibiting the synthesis of proinflammatory cytokines such as IL-1, TNF-α, IL-6 and IL-8 by hematopoietic cells (D'Andrea *et al.*, 1995) and keratinocytes (Raingeaud & Pierre, 2005). Although mainly produced by T helper type 2 lymphocytes and mast cells, expression of IL-4 was also observed in bile duct epithelial cells (Chedid *et al.*, 1999), indicating that IL-4 might be able to exert its activity directly at epithelial surfaces. Interleukin-10 is another potent immunosuppressive cytokine that initiates a wide variety of anti-inflammatory activities in cells of different lineages when it binds to its cellular receptor complex. The IL-10 receptor complex is a heterodimer consisting of the specific IL-10R1 (alias of IL10RA) and the common IL-10R2, but only the IL-10R1 is essential for initial ligand binding and receptor signaling (Moore *et al.*, 2001). Primarily expressed by hematopoietic cells such as B cells, T cells, natural killer cells, monocytes and macrophages, *IL10R1* expression had also been described in keratinocytes (Michel *et al.*, 1997) and colonic epithelium (Denning *et al.*, 2000). The presence of IL-10RA in KB cells is an addition to this short list of non-hematopoietic cells. Upregulation of *IL4* and *IL10RA* induced by *F. nucleatum* indicated that pathogenic bacteria not only activate a proinflammatory state of acute-phase response but

also trigger a potentially compensatory antagonistic mechanism involving anti-inflammatory mediators such as IL-4 or IL-10 receptor.

The cytokine response to oral streptococci is less studied, and the results have been conflicting (Vernier *et al.*, 1996; Scannapieco *et al.*, 2001; Ji *et al.*, 2007; Peyret-Lacombe *et al.*, 2009). In contrast to *F. nucleatum*, here we demonstrated that *S. cristatus* did not provoke a strong host response in either KB or TERT2 cells, which was in agreement with most previous studies (Scannapieco *et al.*, 2001; Hasegawa *et al.*, 2007; Ji *et al.*, 2007; Cosseau *et al.*, 2008; Peyret-Lacombe *et al.*, 2009). Only four genes were altered following *S. cristatus* infection. Among those was a proinflammatory cytokine, IL-18, which was down-regulated. It has been reported that oral epithelial cells can produce IL-18 (Rouabhia *et al.*, 2002). Accumulated IL-18 is positively correlated to unresolved periodontal inflammation (Johnson & Serio, 2005). Therefore, downregulation of IL-18 may indicate an anti-inflammatory mechanism exerted by *S. cristatus*. Two of the three genes upregulated after exposure to *S. cristatus* were again those for IL-4 and IL-10R, which further suggested that *S. cristatus* induces an anti-inflammatory response in epithelial cells. The distinct transcriptional responses to *F. nucleatum* and *S. cristatus* are relevant to their biological behaviors, and provide experimental support for their distinct roles in health and disease. It appears that *S. cristatus* is a 'true' commensal that has developed a balanced relationship with the epithelial cells, whereas *F. nucleatum* is a potential threat to the host, and has to be monitored closely.

Communities in the oral cavity are polymicrobial, and host immune responses to complex microbial communities may differ greatly from those to single species. Oral bacteria implicated in periodontal diseases appear to have the capacity to downregulate the cytokine response of the host to other pathogenic species through various components, which has been related to periodontal pathogenesis (Huang *et al.*, 2004a; Asai *et al.*, 2005). Little is known about the interactions between oral commensal bacteria and pathogens in the host cytokine response. In this study we found that the presence of *S. cristatus* decreased the levels of epithelial proinflammatory cytokine production induced by *F. nucleatum*, and upregulated anti-inflammatory mediators such as IL-4 and IL-10RA. Such community-based responses are of clinical significance. In the oral cavity, oral mucosa coexists in intimate contact with a diverse bacterial flora including putative periodontal pathogens but dominated by commensal streptococci (Rudney *et al.*, 2005). We previously demonstrated that *S. cristatus* could be transported into KB and TERT-2 epithelial cells by invasive *F. nucleatum* through a combination of coaggregation and invasion mechanisms (Edwards *et al.*, 2006); however, the inhibitory effect of *S. cristatus* on *F. nucleatum*-induced IL-8 was independent of interspecies' coaggregation and appeared to require bacterial contact with host cells (Zhang *et al*., 2008). The inhibition of an acute inflammatory response demonstrated by *S. cristatus* might represent an effective anti-inflammatory mechanism for polymicrobial flora to live in harmony with the host on epithelial surfaces. On the other hand, the mucosal hyporesponsiveness to invasive bacteria may also provide a protected environment for these microbes to later colonize the gingival crevice, and even remote locations.

Cytokine synthesis in response to microorganisms is largely a consequence of TLR activation (Kawai & Akira, 2009). Activation of TLR is essential for provoking the innate response and enhancing adaptive immunity against pathogens, but if it is left unchecked, the host would be overwhelmed by immune activation. Mechanisms exist to tightly regulate TLR signaling and functions at multiple levels (Liew *et al.*, 2005). As no TLR genes were detected on our arrays, no conclusions could be drawn regarding the differential effects of bacteria on TLR expression in our model system. Further analysis of the TLR array data revealed that most of the *F. nucleatum* up-regulated genes identified in our study are NF-κB-dependent proinflammatory genes, which was generally in agreement with previous reports

(Huang *et al.*, 2004b; Milward *et al.*, 2007). Typically pathogenic bacteria can prevent or limit the inflammatory response by targeting NF-κB (Schesser *et al.*, 1998). Here we observed that almost all of *F. nucleatum* upregulated NF-κB-dependent genes were attenuated by the presence of *S. cristatus* (some of those genes were also upregulated by *S. cristatus* alone, but at much lower levels). In support of this observation, it was recently reported that nonpathogenic/commensal gut flora are also able to act on the NF-κB activation pathway, either inducing its precocious nuclear clearance (Kelly *et al.*, 2004) or preventing the nuclear translocation of its active dimer (Neish *et al.*, 2000), to attenuate proinflammatory cytokine expression induced by various inflammatory stimuli. Given the limited number of NF-κB pathway-related genes activated by *S. cristatus* alone, and its attenuation of most NF-κB-targeted genes induced by *F. nucleatum*, we speculate that *S. cristatus* may also exert its attenuating activity through interfering with the signal transduction pathways of the host cells.

Other pathways stimulated by either species alone and attenuated in combination were mitogen-activated protein kinases (MAPK) and interferon-regulatory factor (IRF) signal transduction. We have not been able to locate any previous reports of IRF pathway induction by *F. nucleatum* or *S. cristatus*, so the role of the IRF pathway remains elusive. Considerably more information is available for the MAPK pathway. For example, MAPK–JNK/p38 pathways, rather than NF-κB, were involved in the induction of an antimicrobial peptide human β-defensin-2 in epithelial cells by *F. nucleatum* (Krisanaprakornkit *et al.*, 2000; Chung & Dale, 2008). Largely mediated by NF-κB activation, IL-8 upregulation by *F. nucleatum* also involved MAPK p38 and MAPK kinase/extracellular regulated kinase pathways (Huang *et al.*, 2004b). As cross-talk between various pathways in mammalian cells can regulate the amplitude of cytokine responses in inflammation, differences in the cytokine expression profiles between *F. nucleatum* alone and *F. nucleatum* with the addition of *S. cristatus* may be related to differential regulation of individual components of various pathways. The upregulation of c-Fos mRNA by *S. cristatus*, but not by *F. nucleatum*, was of interest. c-Fos is a transcription factor that, together with c-Jun, composes activator protein-1 (AP-1). It can be induced by a variety of stimuli in diverse cell types including keratinocytes. It was reported that c-Fos may function as an anti-inflammatory transcription factor to suppress systemic inflammatory response to endotoxin through inhibiting NF-κB activity (Ray *et al.*, 2006). In *Drosophila*, the lipopolysaccharide response of NF-κB target genes can be downregulated by AP-1 (Kim *et al.*, 2005). Interestingly, MAPK signaling pathways regulate AP-1 activity by increasing transcription and by phosphorylation of AP-1. Based on these findings, it is tempting to speculate that the addition of *S. cristatus* induces a negative cross-talk between the c-Fos/AP-1 and NF-κB signaling modules, and consequently, the amplitude of cytokine responses in inflammation is dampened. Further study is warranted for confirmation.

In conclusion, the data presented here extend our previous study on the effects of *S. cristatus* on epithelial responses to *F. nucleatum*, and highlight many previously unreported findings. It is clear from our results that oral pathogens and commensals can induce distinct cytokine expression patterns in oral epithelial cells, and *S. cristatus* is able to attenuate the acute inflammatory responses to *F. nucleatum*, probably by influencing pathways converging on NF-κB.

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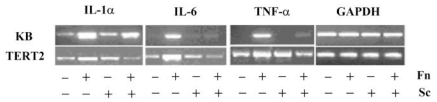

Figure 1.

Qualitative reverse transcription–polymerase chain reaction (RT-PCR) analysis of selected cytokine gene expression in infected KB and TERT2 cells. KB and TERT2 cells were co-cultured for 2 h with medium; *Fusobacterium nucleatum* (Fn); *Streptococcus cristatus* (Sc); or *S. cristatus* and *F. nucleatum*. The same set of total RNA as that used for array analysis was analysed by RT-PCR. Representative gel images of triplicate analysis are shown.

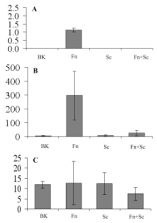

Figure 2.

Selected cytokine protein levels in KB cells following bacterial challenge. KB cells were co-cultured for 2 h with medium (Blank); *Fusobacterium nucleatum* (Fn); *Streptococcus cristatus* (Sc); or *F. nucleatum* plus *S. cristatus* (Fn + Sc). Cell supernatants were then collected, and levels of the indicated cytokines were analysed by multiplexed Luminex assay. Cytokine concentrations were normalized to total protein levels, and expressed as ng cytokine per mg total protein. (A) Interleukin-1α (IL-1α); (B) IL-8; (C) interferon-γ (IFN-γ). Statistical analysis confirmed that the significant upregulation of IL-8 by *F. nucleatum* ($P < 0.01$) was significantly attenuated to background level by the presence of *S. cristatus* ($P < 0.01$).

Table 1

The sequences of primers used for reverse transcription–polymerase chain reaction

Gene	Forward primer sequence	Reverse primer sequence
IL1α	CAC TCC ATG AAG GCT GCA TGG	ACC CAG TAG TCT TGC TTT GTG G
IL6	GTG TGA AAG CAG CAA AGA GG	TGG ACT GCA GGA ACT CCT T
IL8	GAG ACA GCA GAG CAC ACA AGC	TTC TCA GCC CTC TTC AAA AAC T
TNF-α	CACCAGCTGGTTATCTCTCAGCTC	GGGACGTGGAGCTGGCCGAGGAG
GAPDH	GAC CCC TTC ATT GAC CTC AAC TAC	AGC CTT CTC CAT GGT GGT GAA GAC

Table 2

Gene expression profile in KB cells induced by *Fusobacterium nucleatum* and/or *Streptococcus cristatus*, as determined by cytokine arrays[1]

Gene[2]	Description	Expression ratio[3]			Fn/Fn+Sc ratio[4]
		Fn	Sc	Fn+Sc	
IL1α	Interleukin 1, alpha	**9.92**	1.41	**2.53**	3.92
IL6	Interleukin 6	1.16	0.65	**0.38**	3.08
IL18	Interleukin 18	0.77	**0.44**	**0.40**	1.90
CCL16	Chemokine ligand 16	**3.84**	1.31	**2.65**	1.45
LTB	Lymphotoxin beta	**3.56**	1.61	**2.51**	1.41
TNFα	Tumor necrosis factor,	**2.32**	0.85	1.64	1.41
XCL2	Chemokine ligand 2	**2.56**	1.06	1.84	1.40
CCL19	Chemokine ligand 19	**2.44**	1.28	1.84	1.32
IL11	Interleukin 11	**2.17**	1.21	1.65	1.31
CXCL11	Chemokine ligand 11	**2.31**	0.92	1.77	1.30
CCL2	Chemokine ligand 2	**2.50**	1.31	**2.11**	1.19
CCL13	Chemokine ligand 13	**2.68**	1.49	**2.32**	1.15
CCL22	Chemokine ligand 22	**2.93**	1.89	**2.84**	1.03
CCL15	Chemokine ligand 15	**2.12**	1.48	**2.08**	1.02
CCL7	Chemokine ligand 7	1.93	1.35	**2.06**	0.94
IL10Rα	Interleukin 10 receptor, alpha	**4.82**	**2.06**	**6.64**	0.73
CCR9	Chemokine receptor 9	1.47	1.59	**2.04**	0.72
CCL1	Chemokine ligand 1	1.50	**2.79**	**2.52**	0.60
IL4	Interleukin 4	**2.75**	**4.89**	**5.86**	0.47
Overall mean[5]		2.83 ± 0.86	1.56 ± 0.97	2.40 ± 1.46	

[1] After background subtraction, the signal intensity of each gene present was normalized to that of *GAPDH*. The expression levels of different genes after bacterial exposure were compared with those from uninfected controls and expressed as relative ratios. Only the genes that had a fold change > 2-fold or < 0.5-fold in any condition (shown in bold) were included for analysis.

[2] Genes whose expression levels were significantly modulated by any experimental treatment. They were sorted in a descending order according to the Fn/Fn+Sc ratio.

[3] Values represent mean relative ratios from two independent experiments.

[4] Fold changes in expression levels when comparing *F. nucleatum* and *F. nucleatum* plus *S. cristatus*. Fn, *F. nucleatum*; Sc, *S. cristatus*; Fn+Sc, *F. nucleatum* plus *S. cristatus*.

[5] Values represent the mean ± SD for each ratio column. Statistical comparison showed that the overall expression level induced by *S. cristatus* was significantly lower than *F. nucleatum* ($P = 0.0046$), as well as lower than *F. nucleatum* plus *S. cristatus* ($P = 0.04$). However, the difference between *F. nucleatum* and *F. nucleatum* plus *S. cristatus* was not significant.

Table 3

Gene expression profile in TERT2 cells induced by *Fusobacterium nucleatum* and/or *Streptococcus cristatus*, as determined by Toll-like receptor (TLR) arrays

Gene[1]	Pathway	Expression ratio[2]			Fn/Fn+Sc ratio[3]
		Fn	Sc	Fn+Sc	
GPC1	Adaptors & TLR Interacting	**2.41**	0.67	0.87	3.06
HMGB1	Adaptors & TLR Interacting	**2.25**	1.40	1.18	3.04
HSPA1A	Adaptors & TLR Interacting	**2.45**	**2.49**	1.24	3.03
HSPA4	Adaptors & TLR Interacting	0.72	0.84	**0.36**	3.00
HSPD1	Adaptors & TLR Interacting	**2.10**	0.60	0.82	2.97
IL1A	NF-κB Pathway	**11.16**	**2.20**	**3.83**	2.95
IL1B	NF-κB Pathway	**5.12**	1.01	1.73	2.92
IL8	NF-κB Pathway	**77.36**	1.61	**38.46**	2.91
CSF2	NF-κB Pathway	**17.96**	1.77	**8.54**	2.89
CSF3	NF-κB Pathway	**4.13**	0.79	1.36	2.81
IRAK1	Effectors	**2.20**	0.61	0.73	2.76
IRF1	IRF Pathway	**19.87**	**2.26**	**6.62**	2.56
IRF3	IRF Pathway	**2.59**	1.08	1.08	2.44
FADD	Effectors	0.70	1.35	**0.46**	2.41
JUN	JNK/p38 Pathway	0.68	0.93	**0.46**	2.39
MAPK9	JNK/p38 Pathway	**2.50**	**2.02**	0.86	2.25
MYD88	Adaptors & TLR Interacting	1.14	1.35	0.50	2.10
NFKB1	NF-κB Pathway	**3.99**	**2.23**	**2.27**	2.04
NFKBIA	NF-κB Pathway	**20.71**	**4.24**	**6.97**	2.04
NFKBIB	NF-κB Pathway	**2.67**	**4.10**	**2.85**	2.01
RELA	NF-κB Pathway	0.58	1.07	**0.31**	1.90
RELB	NF-κB Pathway	**2.34**	1.10	0.76	1.86
FOS	JNK/p38 Pathway	0.89	**2.39**	0.72	1.76
TOLLIP	Adaptors & TLR Interacting	**0.20**	0.56	**0.10**	1.54
UBE2V1	Effectors	0.81	0.77	**0.33**	1.48
TNFRSF	NF-κB Pathway	1.32	0.78	**0.47**	1.23

Gene[1]	Pathway	Expression ratio[2]			Fn/Fn+Sc ratio[3]
		Fn	Sc	Fn+Sc	
Overall mean[4]		7.26 ± 15.49	1.55 ± 0.98	3.23 ± 7.53	

After background subtraction, the expression level of each gene present was normalized to β_2-microglobulin. The expression levels of different genes after bacterial exposure were compared with those from uninfected controls and expressed as relative ratios. Only the genes which had a fold change > 2-fold or < 0.5-fold in any condition (shown in bold) were included for analysis.

[1]Genes whose expression levels were significantly modulated by any experimental treatment. They were sorted in a descending order according to the Fn/Fn+Sc ratio.

[2]Values represented mean relative ratios from three independent experiments. Statistical comparison was performed for log-transformed data, and showed that the overall expression level induced by *F. nucleatum* was significantly higher than *S. cristatus* (P = 0.00042), as well as *F. nucleatum* plus *S. cristatus* (P = 0.00007). However, the difference between *S. cristatus* and *F. nucleatum* plus *S. cristatus* was not significant.

[3]Fold changes in expression levels when comparing *F. nucleatum* and *F. nucleatum* plus *S. cristatus.* Fn, *F. nucleatum*; Sc, *S. cristatus*; Fn+Sc, *F. nucleatum* plus *S. cristatus*.

[4]Values represented mean ± SD for each ratio column. Statistical comparison was performed for log-transformed data, and showed that the overall expression level induced by *F. nucleatum* was significantly higher than *S. cristatus* (P = 0.00042), as well as *F. nucleatum* plus *S. cristatus* (P = 0.00007). However, the difference between *S. cristatus* and *F. nucleatum* plus *S. cristatus* was not significant.