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HOST RESPONSE AND INFLAMMATION

Streptococcus gordonii-Induced miRNAs Regulate CCL20 Responses in Human Oral Epithelial Cells

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ABSTRACT The mechanisms through which oral commensal bacteria mitigates uncontrolled inflammatory responses of the oral mucosa remain unknown. Here, we show that representative oral bacterial species normally associated with oral health [*S. gordonii* (*Sg*), *V. parvula* (*Vp*), *A. naeslundii* (*An*), *C. sputigena* (*Cs*), and *N. mucosa* (*Nm*)] enhanced differential chemokine responses in oral epithelial cells (OECs), with some bacteria (*An, Vp, and Nm*) inducing higher chemokine levels (CXCL1, CXCL8) than others (*Sg, Cs*). Although all bacterial species (except *Cs*) increased CCL20 mRNA levels consistent with protein elevations in cell lysates, only *An, Vp, and Nm* induced higher CCL20 secretion, similar to the effect of the oral pathogen *F. nucleatum (Fn)*. In contrast, most CCL20 remained associated with OECs exposed to *Sg* and negligible amounts released into the cell supernatants. Consistently, *Sg* attenuated *An*-induced CCL20. MiR-4516 and miR-663a were identified as *Sg*-specifically induced miRNAs modulating validated targets of chemokine-associated pathways. Cell transfection with miR-4516 and miR-663a decreased *An*- and *Fn*-induced CCL20. MiRNA upregulation and attenuation of *An*-induced CCL20 by *Sg* were reversed by catalase. Up-regulation of both miRNAs was specifically enhanced by oral streptococci H_2O_2-producers. These findings suggest that CCL20 levels produced by OECs in response to bacterial challenge are regulated by *Sg*-induced miR-4516 and miR-663a in a mechanism that involves hydrogen peroxide. This type of molecular mechanism could partly explain the central role of specific oral streptococcal species in balancing inflammatory and antimicrobial responses given the critical role of CCL20 in innate (antimicrobial) and adaptive immunity (modulates Th17 responses).

KEYWORDS oral epithelial cells, oral commensal bacteria, miRNA, CCL20, hydrogen peroxide, chemokine regulation

A balanced cross talk between oral epithelial cells (OECs) and bacteria is crucial to avoid destructive inflammation of the gingival tissues and alveolar bone loss. An extensive number of studies have demonstrated that recognition of oral bacteria by OECs, programs them to produce soluble factors (e.g., antimicrobial peptides and cytokines/chemokines) with the ability to initiate and modulate various immune responses. Nevertheless, these OEC responses have been predominantly evaluated with oral pathogenic species such as *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum* (1–3). In contrast, despite there being significantly higher numbers of commensal bacteria in the microbial ecology, biofilms at the sites of periodontal lesions, significantly fewer studies have elucidated the important role of commensal bacterial species in maintaining oral health or in contributing to a disease process (4, 5).

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Evidence suggests that OECs may discriminate between commensal and pathogenic bacteria, inducing varied response patterns associated with antimicrobial peptides (AMPs), cytokines, and chemokines production (6, 7). Specifically, human β-defensins and CC chemokine ligand 20 (CCL20), also known as macrophage inflammatory protein-3 alpha (MIP-3α), are critical components of innate immune responses against microbial invasion (8, 9) and are widely expressed in oral mucosa (10, 11). CCL20 binds exclusively to CC chemokine receptor 6 (CCR6), the predominant chemokine receptor expressed by T helper 17 (Th17) cells. Therefore, CCL20 appears as a critical chemokine for driving Th17 cell migration to inflamed sites. CCL20 also recruits T regulatory cells (Tregs), which impact both innate and adaptive immune responses (12). Accordingly, dysregulated CCL20/CCR6 binding has been linked to a variety of inflammatory and autoimmune conditions, e.g., rheumatoid arthritis (13). Similarly, elevated CCL20 levels have been shown in OECs with periodontal disease and correlated with clinical periodontal inflammation (11, 14, 15).

MicroRNAs (miRNAs) are small, noncoding RNAs that regulate gene expression through degradation of target mRNA or inhibition of translation (16). Growing evidence suggests that miRNAs dynamically control mucosal immunity (17), and variations in their expression suggest that miRNAs could play important roles in the pathogenesis of inflammatory diseases (18) including periodontitis (19–22). In particular, miRNAs have been shown to regulate cytokine/chemokine expression by gingival epithelial cells in response to oral pathogenic bacteria (23–25); however, less is known about the effect of oral commensal bacteria on the expression of miRNAs to regulate epithelial chemokine responses required for homeostasis. Herein, we sought to evaluate the effect of representative bacterial species of the four most abundant phyla in the oral cavity that are early colonizers and normally associated with oral health (i.e., Firmicutes: *Streptococcus gordonii* and *Veillonella parvula*; Actinobacteria: *Actinomyces naeslundii*; Bacteroidetes: *Capnocytophaga sputigena*; *and* Proteobacteria: *Neisseria mucosa*) (26–29) on CCL20 responses by OECs and the potential regulatory role for miRNAs in these responses.

RESULTS

Effect of bacteria in chemokine expression and secretion in OECs. In general, CCL20 mRNA and protein levels produced by OECs (i.e., OKF6 cells) in response to all tested oral bacterial species except *C. sputigena* were increased at 24 h. CCL20 supernatant levels were significantly higher in OECs exposed to *A. naeslundii*, *V. parvula*, *F. nucleatum*, and *N. mucosa*, compared to *S. gordonii*, which induced a negligible increase (Fig. 1). Interestingly, CCL20 constitutive levels and responses to bacterial challenge remained mostly associated with the OECs in contrast to other chemokines (CXCL1, CXCL8, and CXCL10), which were efficiently secreted after bacterial exposure (Fig. S1 in the supplemental material). Further, evaluation of the effect of different bacterial amounts confirmed a disconnect between the transcriptional activation of CCL20 induced by *S. gordonii* and the protein levels that contrasted with the pattern of *F. nucleatum* showing lower mRNA chemokine levels (2- to 4-fold less) accompanied by an enhanced CCL20 secretion (Fig. 2). Both *S. gordonii* and *F. nucleatum* increased IL-8 transcription and protein production/secretion by OECs, which was used as a control for bacteria-induced chemokine responses. Consistent with the ability of *Sg* to attenuate CCL20 responses, OECs exposed to *A. naeslundii* in the presence of *S. gordonii* produced and released lower amounts of CCL20, even at low OEC:*Sg* ratios (1:2 and 1:5) (Fig. 3).

Role of miRNAs in oral bacterial-induced CCL20. To determine a possible involvement of miRNAs in regulating CCL20 levels produced by OECs in response to *S. gordonii*, miRNA global expression analysis was first performed. *S. gordonii* upregulated 115 out of 2,578 miRNAs ($\geq$2-fold) in a dose-dependent manner (Table S1). Bioinformatics analysis of upregulated miRNAs suggested that miR-4516 (2.5-fold) and miR-663a (14.3-fold) had predicted and validated target genes involved in Toll-like receptor and cytokine-induced chemokine transcription and translation (i.e., JunB and Stat3) (Fig. 4A). Further, qRT-PCR analysis confirmed that the expression of miR-4516 and miR-663a was significantly

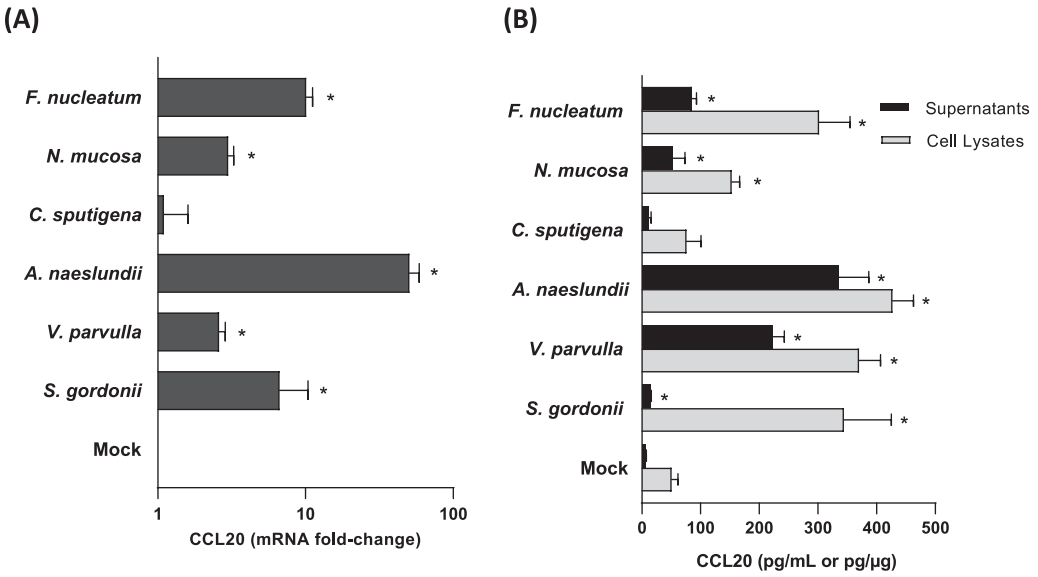

FIG 1 CCL20 expression and secretion are differentially induced by oral bacteria in oral epithelial cells (OECs). (A) mRNA and (B) Protein CCL20 levels from supernatants and cell lysates produced by unstimulated (Mock) OECs or in response to representative oral commensal bacterial species [MOI = 1:50] after 24 h of bacterial challenge were determined as described in methods. Mock: unstimulated OECs. Normalized amounts of CCL20 in cell lysates per 100 μg of total protein are shown. The means $\pm$ standard deviation of three or four replicates from each treatment condition from at least two independent experiments are shown. *, $P \leq 0.05$ when bacteria-treated groups compared with unstimulated (Mock) cells.

higher in OECs challenged with *S. gordonii* compared to either *A. naeslundii* or *F. nucleatum* (Fig. 4B). Interestingly, OECs transfected with the miR663a and miR4516 secreted significantly lower amounts of CCL20 induced by *F. nucleatum* and *A. naeslundii* (Fig. 4C). In contrast, OECs transfected with miR-4516 and miR-663a inhibitors did not rescue CCL20 secretion in cells exposed to *S. gordonii* (Fig. S2A).

The experimental conditions used with miRNAs yielded high transfection efficiency (99.9%) confirmed by immunofluorescence microscopy and flow cytometry using a fluorescently labeled control siRNA **(**Fig. S2B**)**. In addition, a significant decrease in bacterial-induced miR-663a and IL-8 expression in OECs confirmed the functionality of transfected small miRNAs (i.e., siRNA for IL-8 or miR-663a inhibitor) (Fig. S2C and S2D).

Role of *S. gordonii*-produced hydrogen peroxide (H_2O_2) in miRNA and CCL20 responses. *S. gordonii* is an H_2O_2 producer, and miRNA expression can be triggered by H_2O_2. Therefore, we tested the potential role of H_2O_2 produced by *S. gordonii* to induce miR-663a and miR4516 expression in OECs using catalase. Upregulation of both miR-663a and miR-4516 induced by *S. gordonii* was significantly decreased in cells incubated with catalase (Fig. 5A). Upregulation of miRNAs was similarly observed in OECs exposed to *S. sanguinis* (another oral *streptococcus* H_2O_2-producer), but not by oral streptococci *S. mutans* or *S. salivarius* (H_2O_2 nonproducers) (Fig. 5B). Since transfection of OECs with miR-663a and miR-4516 decreased bacterial-induced CCL20 and H_2O_2 produced by oral streptococci was involved in upregulation of these miRNAs, the presence of catalase should rescue bacteria-induced CCL20. As expected, CCL20 induced by *A. naeslundii* were decreased by *S. gordonii* and *S. sanguinis*, and were rescued in the presence of catalase in both supernatants and cell lysates (Fig. 5C).

In general, *S. gordonii*, *S. mutans*, and *S. salivarius* did not significantly affect viability of oral epithelial cells after 24 h, except when cells were exposed to higher MOI [1:50] with *S. gordonii* and *S. mutans*, where viability was reduced by about 15–20% (Fig. S3). Among all tested oral streptococcal species, *S. sanguinis* exhibited a higher cytotoxicity in OECs, reducing cell viability between 10% and 50% in a dose-dependent manner (Fig. S3).

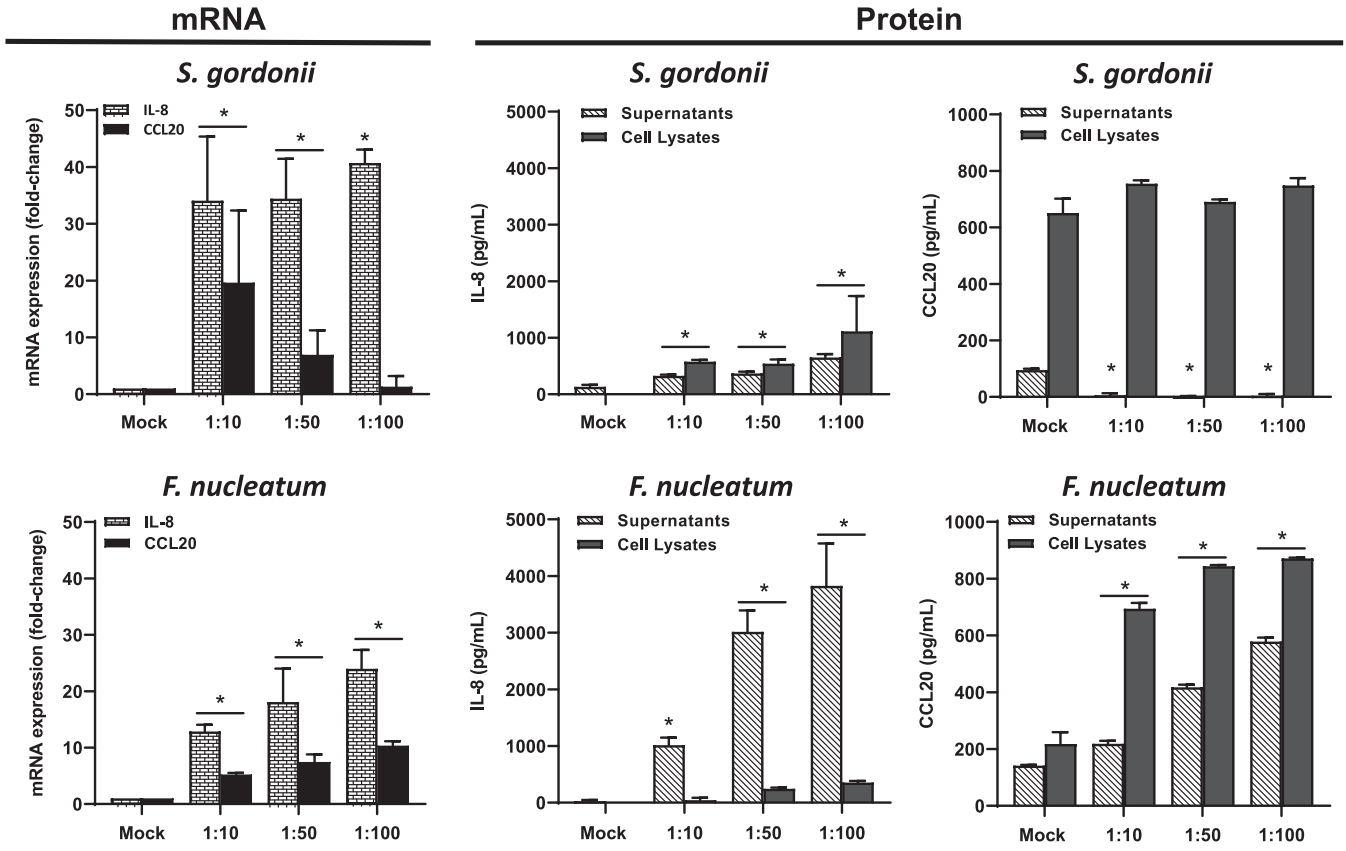

FIG 2 CCL20 secretion by oral epithelial cells (OECs) in response to *S. gordonii* is impaired. CCL20 and IL-8 mRNA and protein levels in supernatants or cell lysates produced by unstimulated (Mock) OECs or cells exposed to several MOIs of *S. gordonii for* 24 h are shown. OECs exposed to *F. nucleatum* using similar MOIs were used as a positive control for chemokine expression/secretion. Normalized amounts of CCL20 and IL-8 in cell lysates per 100 μg of total protein are shown. The means ± standard deviation of three replicates from each treatment condition from at least two independent experiments are shown. *, $P \leq 0.05$ when bacteria-treated groups were compared with unstimulated (Mock) cells.

Effect of miR-663a and miR-4516 in oral bacterial-induced transcription factor activation. Since JunB and Stat3 are validated targets for miR-663a and miR-4516 respectively (30, 31), we evaluated the effect of these miRNAs to inhibit bacterial-induced expression of these transcription factors. OECs transfected with miR-663a exhibited a reduced expression of constitutive JunB compared to cells transfected with the siRNA scramble negative control. Likewise, JunB expression induced by *A. naeslundii* was inhibited in cells transfected with miR-663a (Fig. 6A and B). In contrast, Stat3 constitutive levels were not significantly affected by miR-4516 transfection alone or after challenge with *A. naeslundii* in OECs (Fig. 6C and 6D).

DISCUSSION

The production of chemokines by OECs in response to commensal bacteria continually colonizing the oral mucosa, needs to be tightly regulated in order to avoid undesirable gingival inflammatory responses contributing to an immunopathology, while protecting the host from opportunistic pathobionts. Nevertheless, the cellular and molecular mechanisms regulating OEC chemokine responses to commensal bacteria remain not fully understood.

Initial evaluation of the effect of representative oral commensal bacterial species on OEC chemokine responses, suggested that in general all tested bacteria increased production and secretion of CXCL1 (GROα) and CXCL8 (IL-8) although at different levels. In some cases, commensal-induced CXCL1 and CXCL8 levels were comparable or greater than the levels induced by the oral pathogen *F. nucleatum*. These chemokines attract mainly neutrophils (32), thus commensal oral bacterial species such as *A. naeslundii* and *V. parvula* could also be contributing to enhanced gingival inflammatory responses with

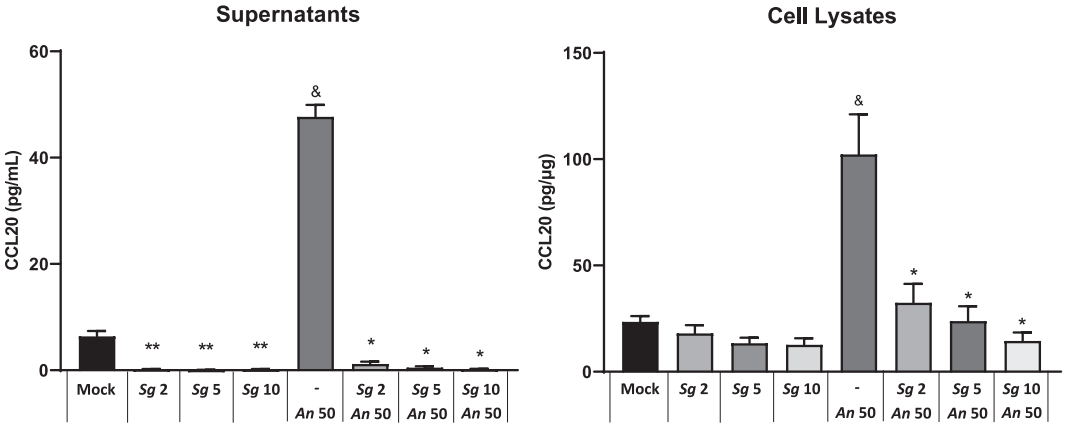

FIG 3 *S. gordonii* inhibits bacterial-induced CCL20 expression and secretion in oral epithelial cells (OECs). CCL20 levels in supernatants and cell lysates from unstimulated (Mock) OECs or cells exposed to *A. naeslundii* (*An*) (1:50) in presence or absence of different MOIs of *S. gordonii* (*Sg*) for 24 h is shown. Normalized amounts of CCL20 in cell lysates per 100 μg of total protein are shown. The means ± standard deviations of three replicates from each treatment condition from at least two independent experiments are shown. **, $P \leq 0.05$ *Sg* versus unstimulated (Mock); &, $P \leq 0.05$ *An* versus Mock; *, $P \leq 0.05$ *An* versus *Sg*+*An* groups.

pathogens such as *F. nucleatum* during dysbiotic events. CXCL10 (IP-10) responses were similarly induced by all bacterial species. This observation suggests that bacterial-induced CXCL10 could be an innate OEC response critical to maintain oral homeostasis, particularly mediated by Th1 and NK cells, which are specifically recruited by this chemokine. Although variability in OEC chemokine responses induced by the tested oral

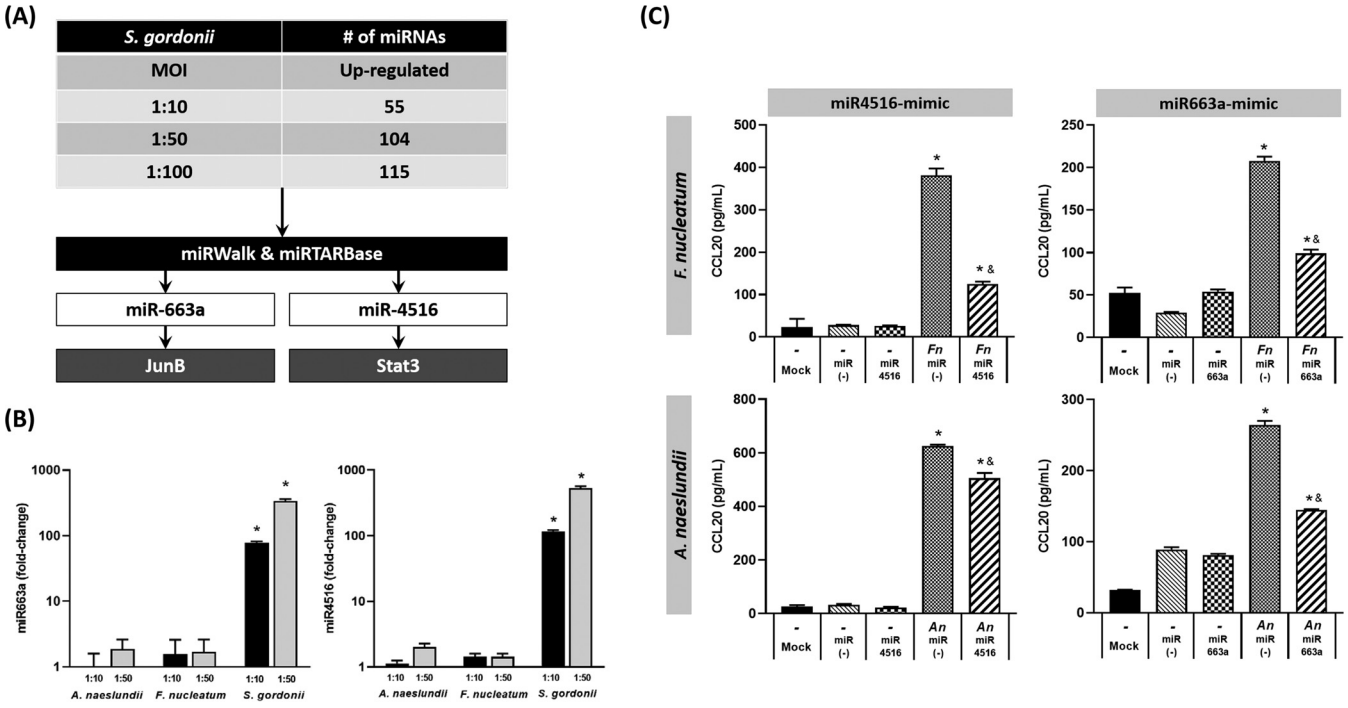

FIG 4 Oral bacteria-induced CCL20 secretion is regulated by *S. gordonii*-induced miRNAs in oral epithelial cells. (A) Number of upregulated (≥ 2-fold) miRNAs in response to *S. gordonii* with respect to unstimulated cells were determined by microarray as described in methods, with each condition by duplicate. Bioinformatics analysis using the database software tools MiRWalk and miRTARBase was used to identify miR-663a and miR-4516 with validated targets for proteins (JunB and Stat3) involved in chemokine expression. (B) Validation of microarray findings for *S. gordonii*-induced miR-663a and miR-4516 was done by RT-qPCR in cells exposed to oral bacteria for 24 h. *, $P \leq 0.05$ *Sg* versus *An* or *Fn*. (C) Bacteria-induced CCL20-secreted levels into supernatants from oral epithelial cells transfected with miR-4516, miR-663a, or scrambled miRNA negative control (miR (−)) followed by challenge with *F. nucleatum (Fn)* or *A. naeslundii (An)* were determined by ELISA. Cells were exposed to bacteria (MOI = 1:50) for 24 h after transfection. Cells without bacterial challenge or miRNA transfection (Mock) were used as control. The means ± standard deviations of a representative experiment of two to three replicates from each treatment condition are shown. *, $P \leq 0.05$ bacteria-treated groups versus corresponding non-bacteria-treated groups. &, $P \leq 0.05$ Bacteria + miR663a/miR4516 versus Bacteria + miR (−).

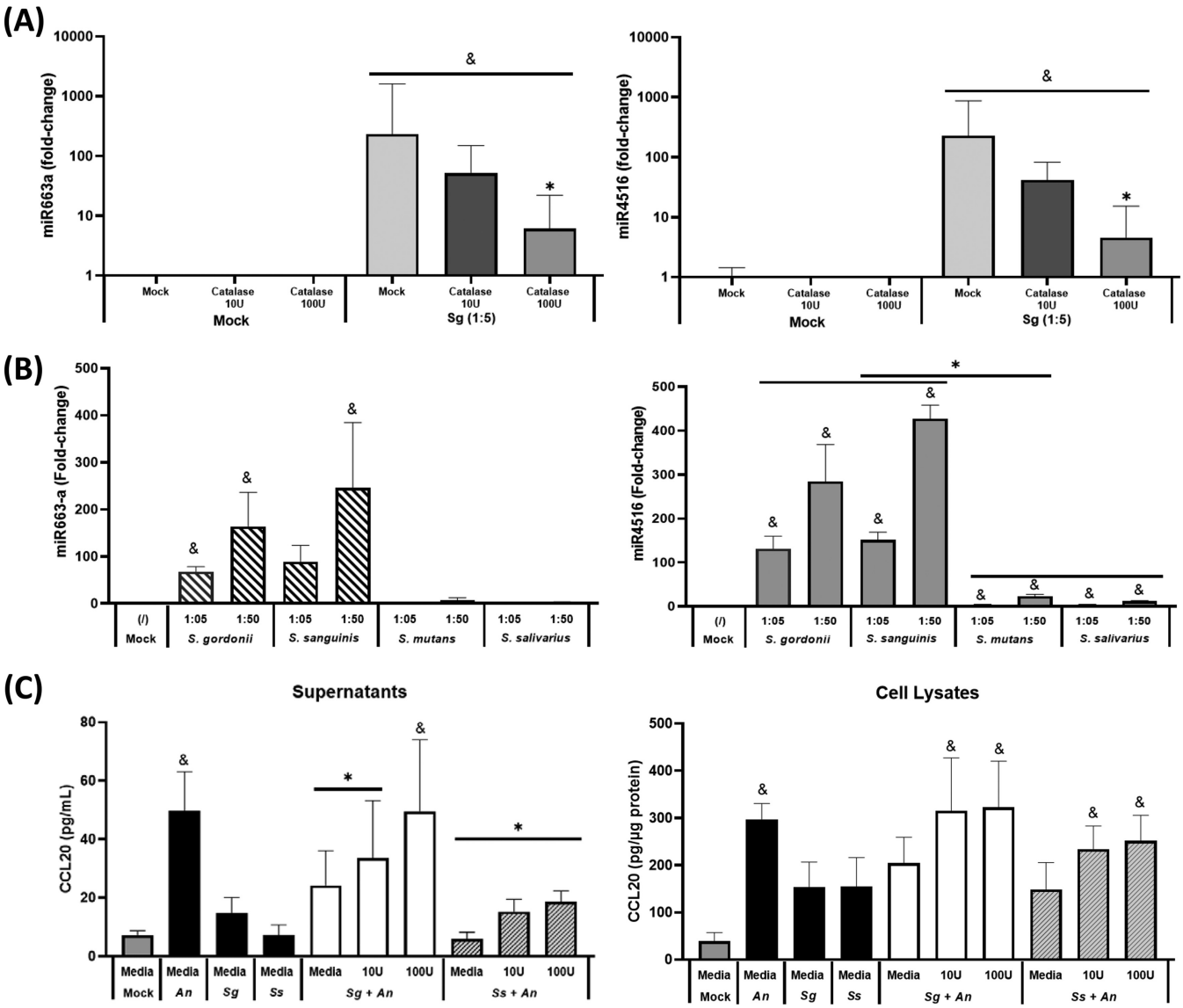

FIG 5 Hydrogen Peroxide (H_2O_2) produced by oral streptococci is involved in miRNAs and CCL20 expression regulation in oral epithelial cells (OECs). (A) Effect of catalase in *S. gordonii* (*Sg*)-induced miRNAs expression after 24 h by OKF6 cells. *, $P \leq 0.05$ *Sg* in media versus *Sg* + catalase. (B) Effect of oral streptococci H_2O_2 producers (*S. gordonii* & *S. sanguinis*) and nonproducers (*S. mutans* & *S. salivarius*) in miRNAs expression after 24 h. *, $P \leq 0.05$ H_2O_2 producers versus nonproducers. (C) Effect of catalase (10 or 100 Units) in rescuing *A. naeslundii* (*An*)-induced CCL20 expression inhibited by *S. gordonii* (*Sg*) and *S. sanguinis* (*Ss*). MOI 1:50 was used for *An* and MOI 1:5 for *S. gordonii* and *S. sanguinis*. CCL20 levels evaluated at 24 h. Unstimulated OECs (Mock) were used as negative control. *, $P \leq 0.05$ *Sg/Ss*+*An* versus *An-Media*. The means ± standard deviations of three replicates from each treatment condition from two independent experiments is shown. &, $P \leq 0.05$ bacteria-treated versus Media/Mock corresponding groups in all panels.

commensal bacterial species likely involve differential recognition of specific bacterial antigens, differences in metabolic activity during OEC stimulation of some bacterial strains (e.g., *C. sputigena*) that can grow in both capnophilic (presence of oxygen and carbon dioxide) and anaerobic environments, could also be involved in the observed chemokine responses.

While all bacterial species stimulated CCL20 expression, the bulk of chemokine was found in cell lysates, although some bacteria effectively enhanced its secretion (*A. naeslundii*, *V. parvula*, *N. mucosa*, and *F. nucleatum*). The expression pattern of CCL20, with the majority of this chemokine associated with the cells, is interesting given the antimicrobial and paracrine immunomodulatory functions that have been described for this chemokine (8, 12). Thus, increased intracellular CCL20 levels could protect epithelial surfaces from invasive pathogenic bacteria, whereas secreted CCL20 would be predicted to impact features of the oral microbiome through killing susceptible bacterial

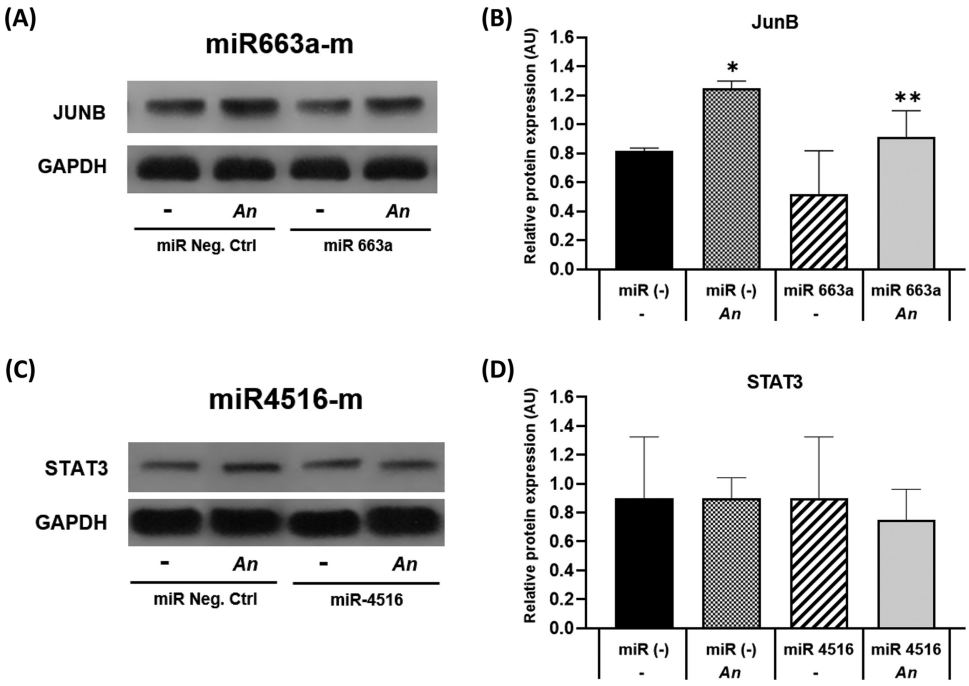

FIG 6 Effect of miR-663a and miR-4516 in *A. naeslundii*-induced expression of JunB and STAT3 in oral epithelial cells (OECs). Protein expression of (A) JunB and (C) Stat3 in OECs previously transfected with miR-663a (miR-663a) or miR-4516 (miR-4516) respectively for 24 h and exposed or not to *A. naeslundii* (1:50) for an additional 24 h was determined by Western blotting as described in methods. (B and D) Transcription factor level intensities from panels A and B normalized to GAPDH levels were determined using image J software. The means ± standard deviations of two independent experiments are shown. *, $P \leq 0.05$ *An*-treated groups versus Media (−). **, $P \leq 0.05$ miR (−) *An* versus miR 663a-m *An*.

species, as well as regulating the recruitment of specific immune cells (e.g., Th17 cells, $\gamma\delta$ T-cells, and regulatory T cells). Therefore, local variations in extracellular CCL20 derived from OECs could be related to oral dysbiosis and inflammatory disease.

Interestingly, although *S. gordonii*, also increased CCL20 expression; this chemokine was completely relegated to cell lysates with only negligible levels detected in supernatants. A similar disconnection between mRNA expression and CCL20 secretion by OECs in response to *S. gordonii* has been previously reported; however, the mechanisms involved in this response have not been described (33). Our finding suggested a potential regulatory effect of *S. gordonii* in OEC CCL20 responses, which was consistent with almost a complete abrogation of *A. naeslundii*-induced CCL20 levels by *S. gordonii*. Thus, *S. gordonii* could be activating specific regulatory mechanisms in OECs to mitigate CCL20 responses induced by other bacterial species.

A growing body of evidence indicates that the expression of cytokines/chemokines is highly transcriptionally and post-transcriptionally regulated through different mechanisms including miRNAs and RNA-binding proteins (34). Herein we hypothesized that expression of specific miRNAs induced by *S. gordonii* in OECs could be involved in regulating CCL20 responses. MiRNA expression analysis in OECs indicated that *S. gordonii* enhanced a significant expression of several miRNAs including miR-4516 and miR-663a, which have validated targets involved in cytokine/chemokine expression and secretion (30, 31). In contrast, *F. nucleatum* and *A. naeslundii* –both strong inducers of CCL20 secretion-, had a significantly lower effect on the expression levels of these miRNAs. Further transfection of OECs with miR-663a and miR-4516 significantly decreased CCL20 secretion induced by *F. nucleatum* and *A. naeslundii*, supporting the potential role for these *S. gordonii*-induced miRNAs to regulate OEC CCL20 responses. These findings reinforce the concept that normal oral bacterial colonizers could be contributing to maintaining health and regulating inflammatory mucosal responses

induced by other opportunistic or pathogenic bacteria, in this case through specific miRNAs in OECs.

Transfection of OECs with miR-4516 and miR-663a inhibitors did not rescue CCL20 secretion in cells exposed to *S. gordonii*. Possibly other miRNAs or regulatory mechanisms could be involved in the failure of OECs to secrete CCL20 in response to *S. gordonii*. Proteins involved in cytokine/chemokine vesicular secretory transport would be plausible targets for future studies (35).

Several miRNAs have also been reported to target different transcription factors as a mechanism for regulating inflammatory responses (36). The inhibitory effect of miR-663a on *A. naeslundii*-induced CCL20 secretion by OECs appears to involve the regulation of JunB expression. This transcription factor is involved in CCL20 expression and can be activated by toll-like receptors and pro-inflammatory cytokine receptors.(37) Although, miR-4516 has been shown to target STAT3 in a keratinocyte cell line (38), we did not see a significant effect on STAT3 expression in OECs transected with this miRNA. This discrepancy could be explained by differences in the cell types evaluated. OKF6 cells express high basal constitutive levels of STAT3 and perhaps regulation of this transcription factor in oral epithelial cells could involve different molecular mechanisms.

Various oral streptococcal species including *S. gordonii* are strong producers of hydrogen peroxide (H_2O_2) (39), which plays a crucial role in bacteria-bacteria as well as bacteria-host interactions. Specifically, killing or inhibiting growth of pathobionts and downregulating host inflammatory responses that allow some commensal bacteria to survive and colonize epithelial surfaces can occur (40, 41). Likewise, increased H_2O_2 levels stimulate the expression of miRNAs (42). Consistent with these observations, we found that expression of miR-663a and miR4516 induced by *S. gordonii* was abrogated by catalase treatment. Moreover, catalase treatment rescued *A. naeslundii*-induced CCL20 secretion that was inhibited by *S. gordonii* as well as *S. sanguinis* (another oral *streptococcus* H_2O_2 producer). These findings support a new potential mechanism through which oral streptococcal species could be regulating host pro-inflammatory responses in the oral epithelium through H_2O_2 production, which can contribute to maintain an oral symbiotic environment in a similar manner as recently described in the gut (40).

CCL20 expression and secretion is also stimulated by inflammatory cytokines (e.g., IL-1β and TNF-α) and human beta defensins 2 and 3 in OECs via a mechanism that appears to involve activation of the MAPKs (i.e., ERK1/2 and p38), as well as regulation of the vesicle transport secretory pathway (10). Therefore, bacterial-induced CCL20 secretion observed in these studies could also involve the production of pro-inflammatory cytokines by OECs in response to specific bacteria such as *F. nucleatum* and *A. naeslundii*. Since JunB is also activated by pro-inflammatory cytokines (37), *S. gordonii*-induced miR-663a could be attenuating CCL20 secretion during inflammation.

It is also well known that miRNAs can regulate more than one gene/protein target, and target mRNA sequences for degradation as a post-transcriptional regulatory mechanism, which could explain decreases in CCL20 mRNA levels induced by *S. gordonii* (16). In particular, miR-4516 has also been shown to regulate apoptosis-related proteins, and miR-663a downregulates miR-155 expression, which is a central regulator of immunoinflammatory responses (30, 38). Future studies evaluating the regulation of these pathways by planktonic and biofilm models of oral streptococci H_2O_2-producers through these miRNAs in OECs are warranted. Although at lesser extent, IL-8 mRNA and secreted protein levels also showed a partial disconnection. Whether similar miRNAs-associated regulatory mechanisms are associated with other chemokines needs to be further evaluated.

Finally, how regulation of CCL20 secreted levels by *S. gordonii* associate with oral health or inflammatory disease, or regulate or enhance pathobionts (43), requires future *in vivo* mechanistic studies. Evidence suggests that increases of CCL20 are associated with periodontal disease (11, 14, 15). Thus, elevations in OEC-secreted CCL20

levels induced by specific oral bacteria could contribute not only to oral dysbiosis, but also fostering Th17/Treg dysregulated gingival responses, which have been broadly described in periodontal disease (44). The ability of miR-663a and miR-4516 to regulate bacterial-induced CCL20 secretion makes these miRNAs interesting candidates for future research exploring their capacity to positively modulate oral epithelial anti-microbial and inflammatory responses.

MATERIALS AND METHODS

Oral epithelial cells and bacterial strains. The immortalized oral keratinocyte cell line OKF6/hTERT was used and grown in serum-free keratinocyte medium (Ker-SFM, Gibco, Carlsbad, CA) supplemented with 25 μg/mL bovine pituitary extract and 0.2 ng/mL human recombinant epidermal growth factor, as previously reported (45, 46).

The following bacterial strains were used: Firmicutes: *Streptococcus gordonii* (ATCC10558), *Streptococcus sanguinis* (ATCC10556), *Streptococcus mutans* (ATCC25175), *Streptococcus salivarius* (ATCC BAA-2593), *Veillonella parvula* (ATCC10790); Actinobacteria: *Actinomyces naeslundii* (ATCC49340); Bacteroidetes: *Capnocytophaga sputigena* (ATCC33612), and Proteobacteria: *Neisseria mucosa* (ATCC25998). *Fusobacterium nucleatum* (ATCC25586) was used as a positive control for CCL20 expression (10). All bacterial strains were initially grown on blood agar plates (BBL, Becton, Dickinson, Sparks, MD) from a frozen stock and incubated in appropriate aerobic or anaerobic conditions as previously described (46). Each bacterial strain was further grown in 2–3 mL of appropriate broth media to reach logarithmic growth, centrifuged, and resuspended in OEC media (Ker-SFM). *V. parvula* was grown in Clostridial medium with 60% sodium lactate, *C. sputigena* in Brain Heart Infusion (BHI) broth with supplements and 1% Glucose. All other strains were grown in BHI with supplements. OECs at a concentration of 1×10^5 were incubated overnight and challenged with bacteria growing exponentially at different multiplicity of infections (MOIs) and times. MOIs were determined by counting bacteria under the microscope. Media supernatants or OEC lysates obtained with 0.1% Triton in water in the presence of protease inhibitors (PI) or with TRIzol (Qiagen, Germantown, MD, USA), were further used to measure chemokine protein and mRNA levels respectively. OECs exposed to bacteria in the presence or absence of catalase (10-100 Units) (Millipore Sigma, Burlington, MA, USA) were used to test the role of hydrogen peroxide (H_2O_2) produced by oral streptococci in miRNA and CCL20 responses. Cell viability after different treatments was determined through trypan blue exclusion using the Lifestyle Cell Counting (Countess II FL Life Technologies, Thermo Scientific Technologies, USA).

Chemokine mRNA and protein levels. Transcriptional activation of CCL20 and IL-8 was evaluated by RT-qPCR in OKF6 cells with different bacterial challenges. RNA was extracted using RNeasy minikit (Qiagen, Germantown, MD) and then reverse-transcribed using qScript cDNA Synthesis Kit (QuantaBio, Beverly, MA). RTqPCR assays were run using PerfeCTa SYBR Green SuperMix (Quanta, San Diego, CA) in a LightCycler480 (Roche, Basel, Switzerland) using the following cycling conditions: Initial denaturation: 95°C, 3 min followed by PCR cycling: 45 cycles (95°C, 15 s, 60°C, 30 s). Relative quantification method (based on $2^{-\Delta\Delta CT}$) was used to calculate fold differences using GAPDH as an endogenous control. Primers used were: CCL20: forward 5′-CTGGCTGCTTTGATGTCAGT-3′ and reverse 5′-CGTGTGAAGCCCACAATAAA-3′, IL-8: forward 5′-CTGCGCC AACACAGAAATTAT-3′ and reverse 5′-AAACTTCTCCACAACCCTCTG-3′, and GAPDH: forward 5′-CACCC ACTCTTCCACCTTC-3′ and reverse 5′-CCTGTTGCTGTAGCCAAATTC-3′. Protein levels of chemokines either in cell lysates or supernatants were detected by ELISA (BioLegend, San Diego, CA) or Luminex (Millipore Sigma, Burlington, MA) following manufacturer instructions.

Evaluation of miRNA expression. Global expression analysis of miRNAs (2578 miRNAs) in OKF6 cells exposed or not to *S. gordonii* was performed using the GeneChip miRNA 4.0 Array (ThermoFisher Scientific, Waltham, MA). RNA was isolated using miRNeasy kit (Qiagen, Valencia, CA) and quantified using Nanodrop 1000 (ThermoFisher Scientific). Briefly, 500 ng of total RNA per sample was processed using the Flash-tag Biotin HSR kit (ThermoFisher), miRNA arrays were hybridized at 48°C for 16 h, washed, stained using Affymetrix Fluidics Station 450, and scanned using the Affymetrix 7G Scanner. Data was normalized using the Expression Console Software (Thermofisher). Bioinformatics analysis was performed using the database software tools miRWalk (47) and miRTarbase (48) to identify upregulated miRNAs ($\geq$2-fold) with predicted/validated gene targets involved in cytokine/chemokine expression.

The effect of oral bacteria on miR-663a and miR-4516 expression was validated using the miRCURY Lock Nucleic Acid (LNA) Universal Reverse Transcriptase (RT) microRNA PCR System (Exiqon/Qiagen, Germantown, MD) according to the manufacturer's instructions. Briefly, 5 ng/mL of total RNA from cells, stimulated or not with oral bacteria, were used for cDNA synthesis. Further, 3 μL of diluted cDNA (1:60) was amplified by PCR using miR-663a or miR-4516 specific forward and universal reverse primers provided by the manufacturer. Addition of UniSp6 RNA spike-in (small RNA) to each reaction tube was used as internal control for miRNA stability during RT and PCR amplification. RT-qPCR was performed in the LightCycler 480 for 45 cycles (95°C/2 min, 95°C/10 s, 56°C/60 s). Concentration ratios for the target miRNAs were calculated by normalizing to the endogenous control miR103a-3p using the $2^{-\Delta\Delta CT}$ method.

Transfection with miRNAs. OKF6 cells (1×10^5) were seeded in 24-well plates in 1 mL Ker-SFM overnight. Then, cells were transfected with Syn-has-mirR4516 or Syn-has-mirR663a; miScript anti-has-mirR4516 or anti-has-mirR663a inhibitors; or a non-silencing siRNA negative control at final concentration of 100 nM (Qiagen). Briefly, 2 μL per well Hi-Perfect transfection reagent (Qiagen) and 2.5 μL of each miRNA (ThermoFisher Scientific) were separately diluted in 100 μL Opti-MEM medium, mixed by vortexing and incubated for 10 min at room temperature. The complexes were then added dropwise to

the cells and plates incubated for 24 h at 37°C. Transfected cells were finally exposed to bacteria, and media supernatants were used for CCL20 and IL-8 determination levels by ELISA and cell lysates for transcription factor expression analysis by Western blotting. Transfection efficiency was assessed by fluorescence microscopy and flow cytometry in cells transfected with the siRNA-AF488 (50 nM) (Qiagen) as well as determination of CXCL8 mRNA and protein expression levels by qPCR and ELISA in OECs transfected with a specific siRNA for IL-8 (Ambion/ThermoFisher Scientific). The efficiency of miRNA inhibitors to reduce bacterial-induced miRNA levels was evaluated by RT-qPCR as a control using cells transfected with the miR-663a-inhibitor.

Western blot. After harvesting media supernatants, cells were washed with 1X PBS and lysed with 300 μL RIPA buffer for 30 min on ice followed by centrifugation at 14,000 rpm at 4°C. Total protein was determined using the DC protein assay kit (Bio-Rad, Hercules, CA). For Western blot, 10 μg of total protein was separated by electrophoresis on a 10% polyacrylamide gel (Bio-Rad) and transferred onto a Polyvinylidene Fluoride (PVDF) membrane. Nonspecific binding sites were blocked using nonfat dry milk for 1 h at room temperature. The membrane was then incubated with specific rabbit IgG primary antibodies for each transcription factor, anti-JunB (1:2000) (Catalog #3753S) or anti-Stat3 (1:1000) (Catalog # 30835S) (Cell Signaling, Danvers, MA) overnight at 4°C followed by washes and addition of the corresponding secondary antirabbit IgG (1:2000) (Catalog # 7074S). Anti-GAPDH (diluted 1:5000) (Catalog # 2118S) was used as a loading control (Cell Signaling). Protein bands were revealed by chemiluminescence using the ECL revelation kit (Bio-Rad) and quantified by densitometry using Image-J software (National Institutes of Health, Bethesda, MD).

Statistical analysis. To account for heterogeneity of variance across treatment groups and potential correlation among samples taken from the same experiment, linear mixed models were fit for each outcome variable to test for differences across treatment groups. Random effects were included for wells and adjustments were made to account for differences across experiments, as necessary. In addition, a log transformation was used to help stabilize the variance across treatment groups. For models containing a significant overall fixed effect for treatment, pairwise comparisons and/or subset mixed models were run, as appropriate. Kenward-Roger adjustments were used to correct for negative bias in the standard errors and degrees of freedom calculations induced by small sample sizes. These analyses were completed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA). Differential miRNA expression ($\geq$2-fold) of cells exposed to bacteria versus unstimulated cells was evaluated using one-way between-subject ANOVA (unpaired) through the Transcriptome Analysis Console (TAC) (ThermoFisher Scientific). Across all analyses, a *P*-value of less than 0.05 was considered significant.

Data availability. The microarray data were deposited in GEO under accession number GSE192388.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

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V.T.E.A. contributed to data acquisition, analysis, and interpretation, and drafted the manuscript; A. A.-A., Y.A., M.M., S.K., and A.M. contributed to data acquisition and interpretation. G.S.H. reviewed/performed statistical analysis, S.S. and J.L.E. contributed to data analysis and interpretation and critically revised the manuscript. O.A.G. contributed to conception, design, data analysis and interpretation, and writing of the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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