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HOST RESPONSE AND INFLAMMATION

Treponema denticola Induces Interleukin-36γ Expression in Human Oral Gingival Keratinocytes via the Parallel Activation of NF-κB and Mitogen-Activated Protein Kinase Pathways

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ABSTRACT The oral epithelial barrier acts as both a physical barrier to the abundant oral microbiome and a sentry for the immune system that, in health, constrains the accumulation of the polymicrobial plaque biofilm. The immune homeostasis during gingivitis that is largely protective becomes dysregulated, unproductive, and destructive to gingival tissue as periodontal disease progresses to periodontitis. The progression to periodontitis is associated with the dysbiosis of the oral microbiome, with increasing prevalences and abundances of periodontal pathogens such as *Treponema denticola*. Despite the association of *T. denticola* with a chronic inflammatory disease, relatively little is known about gingival epithelial cell responses to *T. denticola* infection. Here, we characterized the transcriptome of gingival keratinocytes following *T. denticola* challenge and identified interleukin-36γ (IL-36γ) as the most differentially expressed cytokine. IL-36γ expression is regulated by p65 NF-κB and the activation of both the Jun N-terminal protein kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) pathways downstream of Toll-like receptor 2 (TLR2). Finally, we demonstrate for the first time that mitogen- and stress-activated kinase 1 (MSK1) contributes to IL-36γ expression and may link the activation of MAPK and NF-κB signaling. These findings suggest that the interactions of *T. denticola* with the gingival epithelium lead to elevated IL-36γ expression, which may be a critical inducer and amplifier of gingival inflammation and subsequent alveolar bone loss.

KEYWORDS pathogenesis, gingival, inflammation, cytokine, periodontitis, *Treponema denticola*

Chronic periodontitis is an inflammatory disease that weakens tooth-supporting tissues and is characterized by inflammation of the gingival epithelium and alveolar bone resorption that may result in tooth loss (1). In gingivitis, the subgingival microbiome is constrained by, largely protective, homeostatic inflammation. However, in susceptible individuals, periodontitis progresses from a dysbiotic microbial community and dysregulated, unproductive inflammation (2). Chronic inflammation associated with periodontitis also contributes to systemic inflammation and increases the risk of systemic conditions such as diabetes, atherosclerosis, Alzheimer's disease, and some cancers (3–5). A better understanding of the immunopathology of the disease may allow therapeutic interventions to control inflammation that have both local and systemic health benefits.

Treponema denticola is a strictly anaerobic spirochete and a pathobiont associated with periodontitis (6). The prevalence and abundance of *T. denticola* are highly elevated at diseased sites and are associated with clinical markers of periodontitis such as increased pocket depth and bleeding upon probing (7). *T. denticola* has several well-characterized virulence factors, which include the major sheath protein (Msp) and dentilisin (chymotrypsin-like protease

Editor Denise Monack, Stanford University

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The authors declare no conflict of interest.

Received 22 June 2022
Returned for modification 31 July 2022
Accepted 16 August 2022
Published 30 August 2022

[CTLP] complex), which degrades the extracellular matrix (ECM) and serum proteins (8–11). *T. denticola* can attach to and invade epithelial cells (12), fibroblasts (13, 14), and periodontal ligament cells (15; reviewed in reference 16), and both dentilisin and Msp are cytotoxic (8, 17). Interactions between *T. denticola* and host cells have been shown to induce the expression and secretion of the proinflammatory cytokines interleukin-6 (IL-6) and IL-8 (18–20). Furthermore, the stimulation of Toll-like receptor 2 (TLR2) during *T. denticola* infection promotes matrix metalloprotease (MMP) activity, which contributes to extracellular matrix breakdown and inflammation (21, 22). Considering the close association of *T. denticola* with the progression of periodontitis, a chronic inflammatory disease, the influence of *T. denticola* on the immunopathology of periodontitis remains poorly characterized. Here, we sought to utilize RNA sequencing (RNAseq) to identify the transcriptomic responses of gingival epithelial cells (GECs) to *T. denticola* infection that may promote gingival inflammation.

IL-36 cytokines are recently described members of the IL-1 superfamily that are overexpressed in gingival tissues and show elevated protein levels in the gingival crevicular fluid (GCF) of patients with periodontitis (23, 24). Additionally, IL-36 cytokines are elevated during pulmonary infections and bacterial vaginosis (25–27), and IL-36γ is highly associated with noninfectious chronic inflammatory diseases such as psoriasis, rheumatoid arthritis (RA), and inflammatory bowel disease (IBD) (reviewed in reference 28). IL-36 signaling is composed of three agonists (IL-36α, -β, and -γ) that all bind the same heterodimeric receptor composed of the IL-36 receptor (IL-36R or IL-1RL2) and the IL-1 receptor accessory protein (IL-1RAP or IL-1RAcP) (29). Signaling through the IL-36R is antagonized by the IL-36 receptor antagonist (IL-36RN) and possibly IL-38, which binds to the IL-36R that blocks the recruitment of the coreceptor and prevents signaling. The activation of the IL-36R by the agonists activates the NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways and induces proinflammatory responses such as IL-6 and IL-8 responses (30). Epithelial barriers, including the gingival epithelium, are the primary sources of IL-36 cytokine expression (30). IL-36γ is thought to be an alarmin that senses pathogen-dependent proteolytic activity and pathogen-induced cell damage to initiate immunopathology in epithelial tissues that can distinguish between harmless and harmful microbes (31). Recent studies suggest that IL-36 cytokines are important autocrine and paracrine regulators of periodontal inflammation that induce inflammatory markers (e.g., IL-6 and IL-8), display reciprocal positive-feedback and feed-forward loops with IL-1β (30) and IL-17A (32), and influence the receptor activator of NF-κB ligand (RANKL)/osteoprotegerin (OPG) ratio to support osteoclastogenesis and alveolar bone loss (23).

In this study, we characterized the gingival epithelial cell responses to *T. denticola* infection using transcriptomics and identified IL-36γ as the most differentially expressed cytokine. Our study elucidated that *T. denticola* induction of IL-36γ is mediated through the stimulation of TLR2/6 and involves the activation of the phosphatidylinositol 3-kinase (PI3K), MAPK, and NF-κB signaling pathways. Finally, we also demonstrate, for the first time, that mitogen- and stress-activated kinase 1 (MSK1) regulates IL-36γ expression.

RESULTS

Epithelial cell responses to *T. denticola* challenge. RNA sequencing (RNAseq) was utilized to demonstrate the influence of *T. denticola* on the host transcriptomic responses of gingival epithelial cells. We challenged human immortalized gingival keratinocytes (HIGKs) with *T. denticola* at a multiplicity of infection (MOI) of 100 for 6 h, and the cells were incubated for an additional 18 h prior to the harvest of RNA for sequencing, while uninfected cells were used as the negative controls. Principal-component analysis (PCA) plots showed tight clustering within experimental conditions and a strong separation between uninfected HIGKs and HIGKs challenged with *T. denticola* (Fig. 1A). Genes with a minimum of 10 average reads per sample were determined to be differentially expressed in the *T. denticola*-infected cells, with a $\log_2$ fold change of ± 1 and a significant difference (adjusted *P* value of ≤ 0.05). Differential expression analysis revealed expression differences in 1,240 genes (851 upregulated and 389 downregulated) between *T. denticola*-infected and uninfected HIGKs (Fig. 1B; see also Table S1 in the supplemental material). Gene ontology (GO) analysis and KEGG pathway analysis revealed the robust downregulation of genes involved in cell cycle regulation

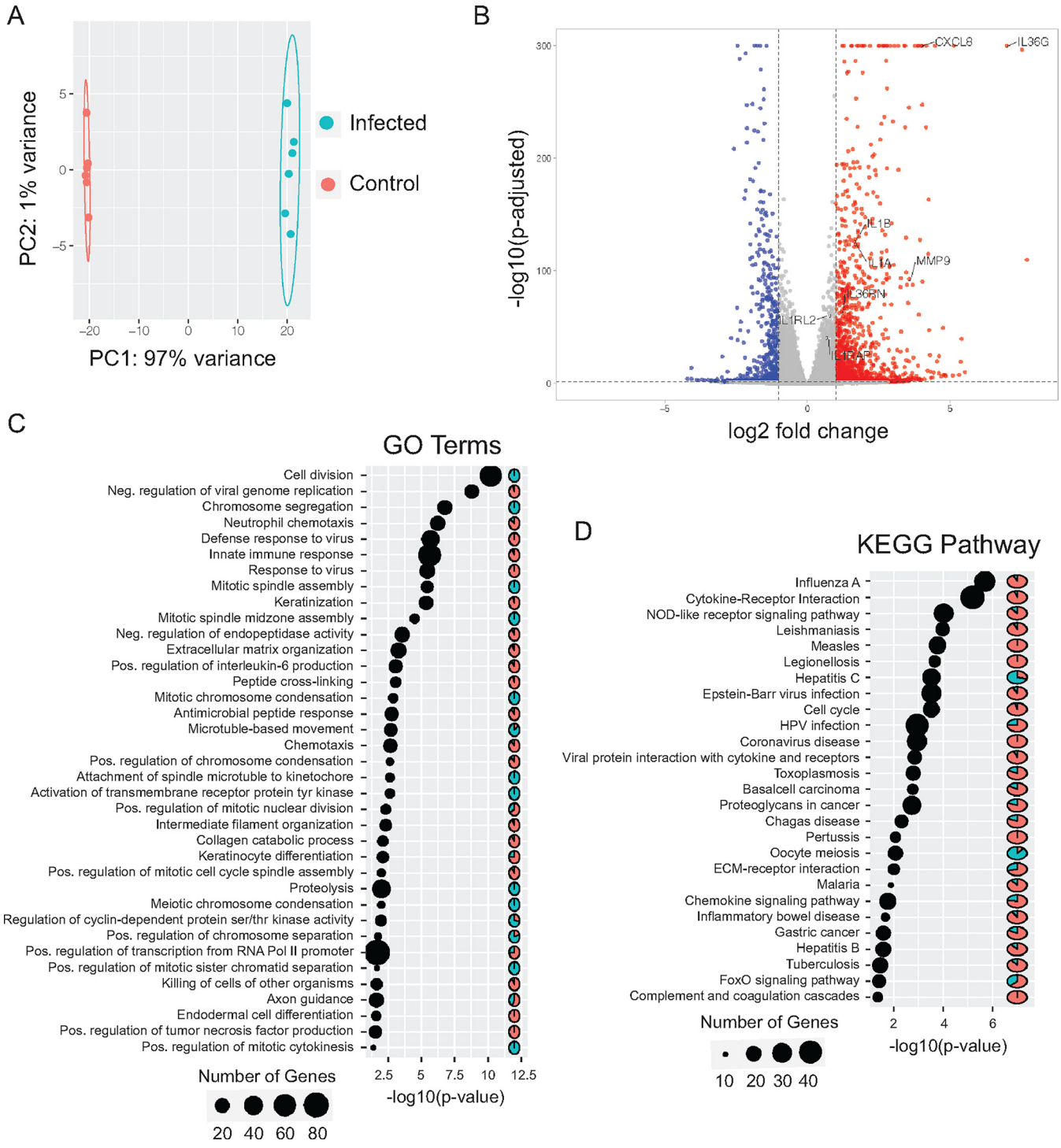

FIG 1 Transcriptomic responses of HIGKs to *T. denticola* include innate immune responses, cytokine signaling, and extracellular matrix rearrangement. (A) Principal-component plot generated with FPKM (fragments per kilobase per million) values showing strong clustering of the control and *T. denticola*-infected groups. (B) Scatterplot of differentially expressed genes (± 1 $\log_2$ fold change with a q value of <0.05), with downregulated genes in blue and upregulated genes in red. IL-36γ is the most differentially expressed cytokine. (C and D) Pathway analysis was performed using all differentially expressed genes to identify gene ontology terms (C) and KEGG pathways (D) enriched during *T. denticola* infection. The size of the dot for each pathway indicates the number of differentially expressed genes, and the pie charts represent the percentages of genes that were upregulated (red) or downregulated (green). Pol II, polymerase II; HPV, human papillomavirus.

and cell division (Fig. 1C and D). *T. denticola* infection significantly enriched for genes involved in extracellular matrix (ECM) remodeling, including the expression of several matrix metalloproteases (MMP-1, -2, -3, -9, -10, -12, -13, and -28) by HIGKs. Interestingly, challenge with *T. denticola* stimulated multiple innate inflammatory pathways (e.g., type I interferon,

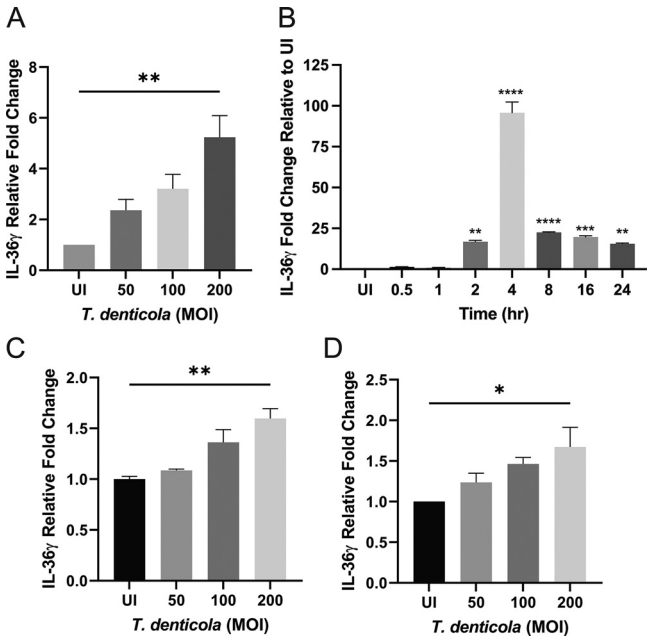

FIG 2 *T. denticola* rapidly induces the expression of IL-36γ in human keratinocytes. (A) HIGK cells were infected with *T. denticola* at increasing MOIs for 6 h and incubated in fresh medium for 18 h prior to the quantification of IL-36γ expression by qRT-PCR. (B) HIGK cells were infected with *T. denticola* (MOI of 200) for various durations, and IL-36γ expression was determined by qRT-PCR. (C and D) TIGK (C) and nHOK (D) cells were infected with *T. denticola* at increasing MOIs for 6 h and incubated in fresh medium for 18 h prior to the quantification of IL-36γ expression by qRT-PCR. In all cases, IL-36γ expression was normalized to GAPDH expression and was determined relative to the values for the uninfected controls (UI). The results are the averages from 3 biological replicates with standard errors of the means (SEM) and were analyzed by one-way ANOVA with Dunnett's *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

cytokine-cytokine receptor signaling, and Toll-like receptor [TLR] signaling). Of particular interest, IL-36γ was the most upregulated cytokine in response to *T. denticola* infection. IL-36γ is a recently characterized member of the IL-1 family of cytokines, and IL-36γ is abundant in GCF and overexpressed in gingival tissues from patients with periodontitis (23, 24). Additionally, IL-36γ has been shown to be induced by other oral microbes such as *Porphyromonas gingivalis* and *Candida albicans* (33, 34).

Next, we sought to further characterize the impact of *T. denticola* infection on the expression of IL-36γ. Quantitative reverse transcription-PCR (qRT-PCR) confirmed the upregulation of IL-36γ in both a time- and dose-dependent manner, and the secretion of IL-36γ may also increase with increasing *T. denticola* MOIs by an enzyme-linked immunosorbent assay (ELISA) (Fig. 2 and Fig. S1A). IL-36 cytokine signaling involves three agonists (IL-36α, -β, and -γ) that all bind the same heterodimeric receptor (IL-36R) (IL-1RL2 and IL-1AcR), while IL-36 signaling is inhibited by the IL-36RN antagonist. The expression of IL-36α and -β was not detected by RNAseq, and we found no change in expression following *T. denticola* infection by qRT-PCR (Fig. S1A). Furthermore, both genes encoding the IL-36R were induced by *T. denticola* infection but not to the level of statistical significance by RNAseq. The expression of IL-36RN was also scantly elevated following infection (Fig. S1A). We examined IL-36γ induction by *T. denticola* in multiple gingival epithelial cell lines and primary cells (Fig. 2). Here, we demonstrated that *T. denticola* infection of telomerase-immortalized gingival keratinocytes (TIGKs) and normal human gingival keratinocytes (nHOKs) led to a modest but significant expression of IL-36γ by qRT-PCR. We then sought to determine the expression of IL-36γ in response to multiple *T. denticola* strains and isogenic mutants (Fig. 3). All strains tested, including the naturally low-dentilisin-activity strains SP50 and SP55, significantly induced IL-36γ expression in HIGK cells. All isogenic mutants tested were derived from the 35405 background, and all mutants tested significantly induced IL-36γ expression. Strains with either a deletion of *prtP* (part of the dentilisin complex) or a disruption of the major sheath protein (Msp) resulted in the additional expression of IL-36γ relative to the

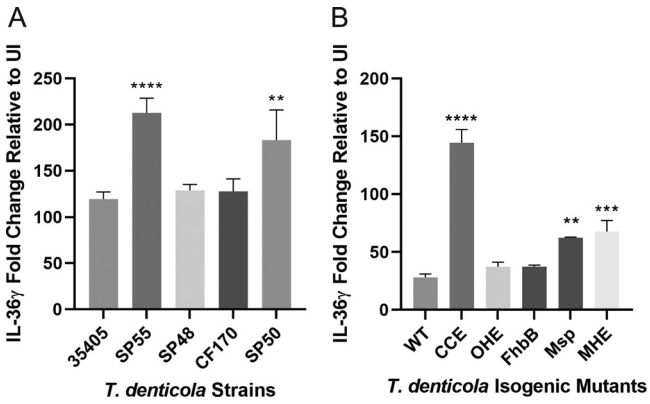

FIG 3 *T. denticola*-mediated IL-36γ induction is attenuated by dentilisin and Msp. HIGKs were infected with multiple strains (A) and isogenic mutants (B) of *T. denticola* for 4 h at an MOI of 200 prior to the quantification of IL-36γ expression by qRT-PCR. Expression was normalized to GAPDH expression and determined relative to the values for the uninfected controls (UI). The results are the averages from 3 biological replicates with SEM and were analyzed by one-way ANOVA with Dunnett's *post hoc* test compared to 35405 (A) and the wild type (WT) (B) (**, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.001$).

wild-type 35405 strain. As both Msp and dentilisin are known cytotoxins, we hypothesized that differences in IL-36γ expression may be due to reduced cytotoxicity in the absence of cytotoxins. However, we did not observe significant differences in the cell cytotoxicity of any mutant compared to the parental 35405 strain (Fig. S2). Collectively, these results demonstrate that *T. denticola* infection of gingival keratinocytes leads to the induction of IL-36γ gene expression.

TLR2/6 but not TLR4 is critical for *T. denticola* induction of IL-36γ expression. Next, we sought to determine the *T. denticola*-induced GEC signaling pathways involved in IL-36γ expression. Previous studies of bacterially induced IL-36γ, including the periodontal pathogen *P. gingivalis*, suggest that TLR2 and TLR4 are activators of IL-36γ induction. To that end, we interrogated the role of TLR signaling in IL-36γ expression by RNA interference (RNAi). Prior to infection with *T. denticola*, HIGKs were transfected with either TLR2- or TLR4-specific small interfering RNA (siRNA). As a control, a nontargeting siRNA was used. Confirmation of the siRNA knockdown of all genes is shown in Fig. S3. The knockdown of TLR2 resulted in a 3-fold decrease in IL-36γ expression compared to cells treated with the control siRNA (siControl) following *T. denticola* infection (Fig. 4). The knockdown of TLR4 showed a slight but statistically insignificant impact on IL-36γ expression. These experiments demonstrate that *T. denticola* infection stimulates IL-36γ expression in HIGKs largely via the activation of TLR2, consistent with the results of previous studies of *P. gingivalis*. We further demonstrated that RNAi of TLR6 but not TLR1 reduced IL-36γ expression, suggesting that signaling operates largely through the TLR2/6 and not the TLR1/2 heterodimer. During *P. gingivalis* infection of HIGKs, the expression of IL-36γ is dependent on the transcriptional regulator interferon regulatory factor 6 (IRF6) downstream of TLR2 activation. Thus, we asked if IRF6 plays a role in IL-36γ expression in the context of *T. denticola* infection by knocking down IRF6 gene expression by siRNA prior to infection (Fig. S4). Unlike for *P. gingivalis*, we found that IRF6 does not significantly impact IL-36γ expression in *T. denticola* infection.

IL-36γ expression is regulated by NF-κB, PI3K, and MAPK signaling pathways. Next, we sought to determine the signaling pathways activated by *T. denticola* downstream of TLR2 that may impact IL-36γ expression. Multiple epithelial signaling pathways have been shown to influence IL-36γ expression, so we started by pharmacologically inhibiting the NF-κB, MAPK, and PI3K/Akt pathways. HIGK cells were treated with either SB203580 (p38), PD98059 (MEK1/2), SP600125 (Jun N-terminal protein kinase [JNK]), or dimethyl sulfoxide (DMSO) as a vehicle control to inhibit each respective MAPK branch prior to or throughout infection with *T. denticola* (Fig. 5). Here, we found that the inhibition of the p38 and JNK MAPK pathways attenuated IL-36γ expression, while the inhibition of extracellular signal-regulated kinase 1/2 (ERK1/2) resulted in a 4-fold increase in IL-36γ expression compared to the infected vehicle control. We then investigated the involvement of the C-JUN (member of AP-1) and

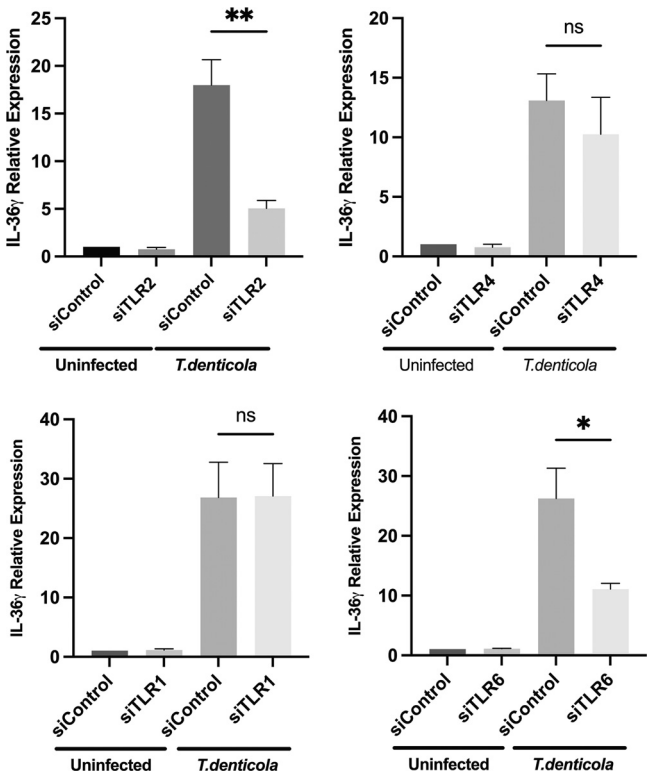

FIG 4 TLR2/6 but not TLR4 is critical for the induction of IL-36γ in the context of *T. denticola* infection. TLR1, TLR2, TLR4, and TLR6 gene expression was knocked down by RNA interference prior to infection with *T. denticola* at an MOI of 200 for 4 h. As controls, HIGKs were treated with nontargeting siRNAs (siControl). The expression of IL-36γ was determined by qRT-PCR, normalized to GAPDH expression, and calculated relative to the values for uninfected cells treated with the siControl. The results are the averages from 3 biological replicates with SEM and were analyzed by one-way ANOVA with Tukey's *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ns, not significant).

SP-1 transcriptional regulators downstream of p38 MAPK by RNA interference. We found that knocking down the expression of C-JUN or SP-1 had no impact on IL-36γ during *T. denticola* infection (Fig. S4). We sought to elucidate the role of NF-κB signaling in IL-36γ induction following *T. denticola* infection. First, we pharmacologically inhibited IκB kinase β (IKKβ) using BAY11-7082 prior to *T. denticola* infection and demonstrated a 40% decrease in IL-36γ expression at 10 μM relative to the DMSO vehicle control (Fig. 5). BAY11-7082 is an IKK inhibitor but also has broad-spectrum anti-inflammatory effects; as such, we confirmed the role of NF-κB signaling by RNAi prior to infection with *T. denticola*. Here, only cells treated with RelA (p65) siRNA showed significantly reduced IL-36γ expression following *T. denticola* infection, while the knockdown of RelB, p50, and p52 had no significant impact on IL-36γ expression (Fig. 6), confirming the role of p65 NF-κB in *T. denticola*-mediated IL-36 induction. Finally, we demonstrated the role of PI3K/Akt signaling in the induction of IL-36γ by treating HIGK cells with LY294002. These results demonstrate a 59% reduction in IL-36γ expression following PI3K/Akt inhibition at 50 μM (Fig. 5). Collectively, these data suggest that multiple signaling pathways that lead to elevated IL-36γ levels are induced by *T. denticola*.

MSK1 influences IL-36γ expression. The pharmacological inhibition of both MAPK and PI3K/Akt signaling attenuated IL-36γ expression, and both signaling pathways can ultimately lead to the activation of p65. Mitogen- and stress-activated kinase 1 (MSK1) is a nuclear kinase that is activated by ERK1/2 upon stimulation with growth factors and p38 in response to stress. MSK1 can phosphorylate transcription factors such as CREB, ATF1, STAT3, and β-catenin along with Akt, histone H3 on serine 10 (H3S10), and H3S28 (35, 36). A recent report demonstrated that MSK1 can phosphorylate p65 in the nucleus on S276, acting as a secondary stimulus to promote p65-mediated gene expression (37). However, the role of MSK1 in IL-36γ expression has never been investigated. To explore whether MSK1

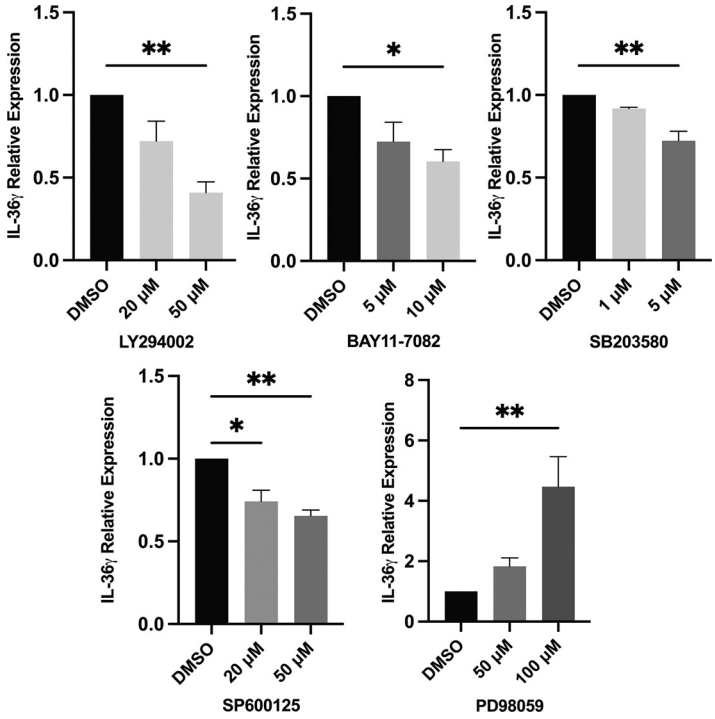

FIG 5 IL-36γ expression is regulated by MAPK, PI3K, and NF-κB signaling. Pharmacological inhibition of PI3K (LY294002), NF-κB (BAY11-7082), p38 (SB203580), and JNK1/2 (SP600125) prior to *T. denticola* infection for 4 h at an MOI of 200 reduced IL-36γ expression, while inhibition of MEK1/2 (PD98059) enhanced the *T. denticola*-mediated induction of IL-36γ expression by qRT-PCR. Expression data were normalized to GAPDH expression and were determined relative to the values for the vehicle (DMSO)-treated cells. The results are the averages from 3 biological replicates with SEM and were analyzed by one-way ANOVA with Dunnett's *post hoc* test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

influences *T. denticola*-mediated IL-36γ expression, we knocked down MSK1 gene expression using RNAi and performed qRT-PCR. Cells were treated with MSK1-specific siRNA to knock down MSK1 prior to infection with *T. denticola*. We found that knocking down MSK1 resulted in a 51% decrease in IL-36γ expression relative to the siControl (Fig. 7). This is the first time that MSK1 has been shown to play a role in IL-36γ expression; however, the mechanism of MSK1 regulation remains unclear and will be explored in future studies.

DISCUSSION

Here, we report the transcriptomic responses of gingival keratinocytes to infection by the pathobiont *T. denticola* that is associated with the progression of periodontitis. We clearly demonstrate that *T. denticola* induces a proinflammatory immune response from HIGKs. Previously characterized periodontitis-associated markers such as IL-8 and MMPs are differentially expressed during infection; however, our analysis also revealed that IL-36γ is the most upregulated cytokine in HIGKs in response to *T. denticola*. IL-36 signaling plays a central pathogenic role in psoriasis and is thought to contribute to other chronic inflammatory diseases (e.g., rheumatoid arthritis and IBD) (28). More recently, IL-36 has been implicated in infection-driven inflammation associated with pulmonary infections, bacterial vaginosis, and periodontitis. IL-36γ is a global alarmin in epithelial tissues that discriminates between commensal colonization and pathogenic invasion (31). Despite mounting evidence that IL-36 cytokines are elevated during infections and are drivers of bacterially induced inflammation, few studies have detailed the signaling and regulatory pathways that control IL-36 cytokines in response to bacterial challenge. We found the HIGK cells express IL-36γ only under normal conditions, as IL-36α and IL-36β were nearly undetectable. The expression levels of the different IL-36 isoforms are variable between cell types, under different physiological conditions, and in response to challenges with different bacterial pathogens despite their biological functions being nearly identical. It is worth considering

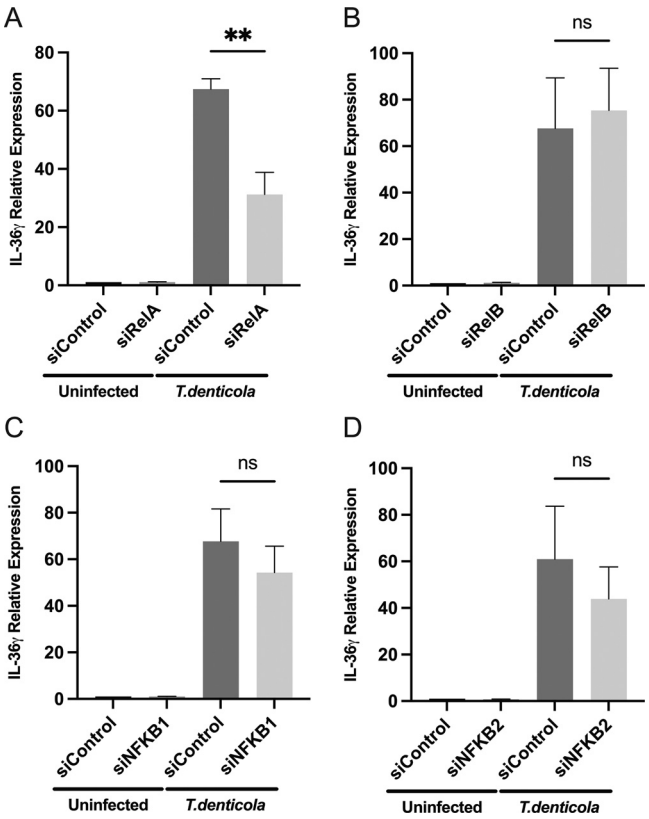

FIG 6 RelA (p65) is critical for IL-36γ expression during *T. denticola* infection. HIGKs were treated with nontargeting siRNA (siControl) or gene-specific siRNA to knock down the expression of RelA (p65) (A), RelB (B), NFKB1 (p105/p50) (C), or NFKB2 (p100/p52) (D) prior to infection with *T. denticola* (MOI 200) for 4 h. The expression of IL-36γ was determined by qRT-PCR, normalized to GAPDH expression, and calculated relative to the values for uninfected cells treated with the siControl. The results are the averages from 3 biological replicates with SEM and were analyzed by one-way ANOVA with Tukey's *post hoc* test (**, $P < 0.01$).

that we examined the responses of only gingival keratinocytes in the current study, and *T. denticola* or other periodontal bacteria may influence IL-36 expression in other oral cell types such as T cells, neutrophils, or dendritic cells (reviewed in reference 29). The levels of both genes in the IL-36R complex were elevated but not to the level of significance, while the expression of the IL-36R antagonist was significantly induced during infection. Yet the expression ratio of IL-36γ to IL-36RN following *T. denticola* infection was 10:1, suggesting

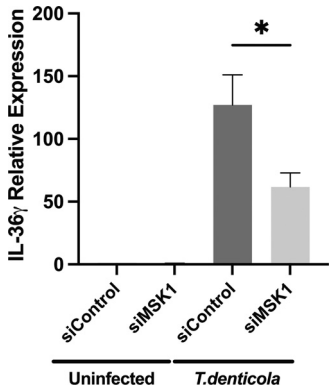

FIG 7 MSK1 influences the expression of IL-36γ. MSK1 gene expression in HIGKs was knocked down by RNAi for 48 h prior to infection with *T. denticola* (MOI of 200) for 4 h. Following infection, the expression of IL-36γ was determined by qRT-PCR. Expression was normalized to GAPDH expression and was determined relative to the values for uninfected cells treated with the siControl. The results are the averages from 3 biological replicates with SEM and were analyzed by one-way ANOVA with Tukey's *post hoc* test (*, $P < 0.05$).

a profound potential to impact IL-36-mediated signaling. We demonstrated IL-36γ induction in two gingival keratinocyte cell lines and primary cells along with every strain of *T. denticola* tested. Analysis of the effects of isogenic mutants of several treponemal virulence factors on IL-36γ expression showed that Msp- and dentilisin-deficient strains induce more IL-36γ gene expression than the parental strain. As both Msp and dentilisin are known cytotoxins, we anticipated that this effect was likely due to reduced cytotoxicity; however, we found no significant impact of any mutant on cytotoxicity after 4 h compared to the parental strain. Dentilisin and Msp physically interact on the outer sheath of *T. denticola*, and both are dominant virulence factors. Thorough characterizations of the dentilisin complex demonstrate that deletions of *prcA* and *prtP* abolish Msp expression, while the deletion of *pcrB* does not significantly reduce the surface localization of Msp. Coimmunoprecipitation and mutational studies suggest that native levels of Msp oligomerization and surface presentation require direct interactions with the dentilisin component PrcA (38–40). The two *T. denticola* strains tested, SP50 and SP55, that lack dentilisin activity also displayed more pronounced IL-36γ expression (10). The expression of *prcA* and *prtP* in the SP50 and SP55 strains is uncharacterized. However, the reduced dentilisin activity in these strains suggests that the common deficiency in all strains and mutants that displayed variable IL-36γ expression may be the reduced Msp on the surface of the treponemes. We hypothesize that the deletion of the most abundant protein produced by *T. denticola* (Msp) or its interacting partner (dentilisin) exposes a less abundant lipoprotein that is a more potent inducer of TLR2 signaling. However, it is also possible that Msp functions to manipulate cell signaling that blunts the signaling pathways involved in regulating IL-36γ expression. Despite the abundant data on the roles of Msp and dentilisin in *T. denticola* pathogenesis, more studies are required to understand the role of *T. denticola* virulence factors in regulating GEC immune signaling.

In the gingival epithelium, IL-36 cytokines contribute to periodontal disease development at the oral-mucosal barrier (23, 33, 34, 41–43). A recent study found that most patients (70%) with chronic periodontal disease had an IL-36 agonist/IL-36RN ratio of $>$1.5 (23). In psoriasis, the same agonist-to-antagonist ratio is elevated in 93% of patients, while it is elevated in fewer than 30% of patients with RA and Crohn's disease. The same study found high interindividual variability in IL-36γ expression, which is supported by a recent single-cell RNAseq (scRNAseq) study that identified several types of keratinocytes within the gingiva, and one subtype displayed robust expression of IL-36γ, potentially limiting the value of IL-36γ as a diagnostic marker (44).

Alarmins are endogenous immunomodulatory molecules released by damaged/dying or pathogen-activated cells that recruit and activate antigen-presenting cells (45). The IL-1 family members IL-1α, IL-33, and IL-36γ have been described as alarmins that have significant impacts on both innate and adaptive immune responses. Stimulation of keratinocytes with IL-36γ induces proinflammatory signaling in a MYD88-dependent manner, which leads to the activation of MAPK and NF-κB signaling (30, 46). In dermal keratinocytes, interferon receptors and type I and type II interferon-responsive genes were differentially expressed in response to IL-36γ (30). Our RNAseq data highlight the significant induction of interferon signaling and cellular responses to virus following *T. denticola* infection (Fig. 1). Future studies will seek to determine if IL-36γ is critical for *T. denticola*-mediated interferon responses. Furthermore, IL-36γ has also been shown to amplify cytokine production by keratinocytes, macrophages, neutrophils, dendritic cells, and Th17 cells, leading to elevated expression levels of periodontitis-associated markers such as IL-1α, IL-1β, IL-6, IL-8, CCL20, tumor necrosis factor alpha (TNF-α), and RANKL, the dominant osteoclastic cytokine (23, 30, 33, 42, 43, 47). Our ongoing and future studies will seek to define the role of IL-36γ-stimulated GECs in activating neutrophils and the differentiation and activation of osteoclasts, as osteoclasts lack IL-36R expression. IL-36γ increases the expression of TLR2 and itself in epidermal and gingival keratinocytes (23), suggesting that a positive-feedback loop may lead to an uncontrolled inflammatory cascade. This is consistent with the hypothesis that IL-36γ is an initial inducer and amplifier of periodontal inflammation. There is also reciprocal stimulatory cross talk between IL-36γ and IL-17A, which is important, as IL-17A is a significant contributor to the

immunopathology of periodontitis (23, 32, 48). The mechanisms of IL-36γ in periodontal immunity and inflammation may be multiplicative. Our future studies will examine experimental periodontitis using mice deficient in IL-36γ and IL-36R. Establishing models of IL-36γ-driven periodontitis will allow targeted therapeutic testing for preventing dysregulated inflammation and alveolar bone loss.

The production and secretion of IL-36γ by keratinocytes remain poorly understood. Like IL-1β, IL-36 cytokines are thought to be secreted in a Golgi-independent manner that involves packaging into microparticles, and release may be stimulated by secondary signals such as the interaction of ATP with the P2X7 ligand-gated ion channel in monocytes and macrophages (49–51). The expression of the IL-36γ gene requires the activation of caspase-1, while secretion requires the activation of caspase-3/7, independent of ATP (52). The protein secretion of IL-36γ is also signal dependent, as keratinocytes stimulated with both poly(I·C) and flagellin overexpressed the IL-36γ gene, but only poly(I·C) stimulated protein secretion (52). Collectively, these studies demonstrated that IL-36γ secretion is complex and poorly understood. In our studies, we routinely failed to detect secreted IL-36γ above the level of detection by the ELISA in either infected or uninfected control cells. Limited attempts yielded significant secretion of IL-36γ from *T. denticola*-infected cells compared to the uninfected controls, but due to the inconsistent nature of this detection, we opted to include these data in Fig. S1B in the supplemental material. Additionally, previous studies did not demonstrate IL-36γ secreted by GECs in response to *P. gingivalis* and *C. albicans* (33, 34). Further investigation is required to better understand the nature of IL-36γ secretion by GECs and whether the costimulation of GECs with multiple oral pathogens may increase IL-36γ secretion.

Like *P. gingivalis*, *T. denticola* stimulates cells to express IL-36γ through the induction of TLR2 and likely not through TLR4 (33). Here, we further describe the involvement of TLR6 but not TLR1, suggesting that the stimulation of the TLR2/6 heterodimer and not the TLR2/1 heterodimer is involved in the regulation of IL-36γ mRNA levels. As reported previously by Huynh et al., *P. gingivalis* induction of IL-36γ is potentially mediated via IRF6; however, IRF6 does not play a significant role in the regulation of IL-36γ in the context of *T. denticola* infection (33). This suggests that multiple periodontal pathogens likely stimulate IL-36γ expression in the gingiva, and induction may be synergistic and mediated by multitiered regulatory signaling pathways. Using pharmacological inhibitors, we found that the induction of IL-36γ is regulated by both JNK and p38 MAPK signaling and NF-κB signaling pathways. Interestingly, the inhibition of MEK1/2 led to increased IL-36γ expression. This finding is consistent with the results of a recent study demonstrating that epidermal growth factor receptor (EGFR)/MEK/ERK inhibition leads to increased Krüppel-like factor 4 (KLF4) activity and subsequent IL-36γ induction (53). The phosphorylation of the S132 residue of KLF4 by ERK1/2 results in nuclear export; thus, we hypothesize that the inhibition of ERK leads to the nuclear accumulation of the KLF4-enhanced induction of IL-36γ gene expression (54). The regulation and activity of KLF4 are unstudied in the context of *T. denticola* infection but are worthy of future investigation, as the induction of KLF4 may impact IL-36γ expression but has also been shown to prevent the G_1-to-S transition during cell cycle progression (55, 56), and our data (Fig. 1) demonstrate that *T. denticola* infection dramatically alters cell cycle progression.

The NF-κB transcription factor p65 (RelA) regulates many proinflammatory genes, including IL-36γ, via the canonical activation of NF-κB signaling (29, 53). Here, we demonstrate that p65 is critical for the induction of IL-36γ in response to *T. denticola*. RelA can bind DNA to regulate transcription as either a homodimer or a heterodimer with p50. The knockdown of p105/p50 (NFKB1) did not impact IL-36γ expression, suggesting that *T. denticola* stimulation may induce p65 homodimer formation; however more, work is needed to be certain of the nature of NF-κB-driven regulation. We also demonstrated, for the first time, the involvement of mitogen- and stress-activated protein kinase 1 (MSK1) in the regulation of IL-36γ. MSK1 is activated by either ERK1/2 or p38 and functions as a nuclear kinase that interacts with CREB, NF-κB, ATF1, and p53 as well as in the direct phosphorylation of histone H3 at serine 10 (H3S10) and H3S28 (36, 57). MSK1 has been shown to enhance the transcriptional

activity of p65 by the phosphorylation of S276 in the nucleus, acting as a secondary stimulus (37, 58). We hypothesize that *T. denticola* infection stimulates the activation of MSK1 through p38, which ultimately enhances p65 regulation of IL-36γ. Alternatively, MSK1 may promote epigenetic modifications within the IL-36γ promoter to enhance gene expression. The exact nature of the influence of MSK1 on the regulation of IL-36γ will be determined in future studies.

Overall, these findings report the global transcriptomic responses of gingival keratinocytes to the periodontal pathogen *T. denticola*. We show that *T. denticola* induces robust proinflammatory responses while dramatically manipulating cell cycle regulation. IL-36γ is the most differentially expressed cytokine in response to *T. denticola* infection and displays a dose response that peaks shortly after infection. Our study is the first to demonstrate the detailed regulation of IL-36γ in response to oral bacterial challenge and identify MSK1 as a novel regulator of IL-36γ gene expression. Further characterizing IL-36γ and uncovering its role in periodontitis immunopathology may lead to novel therapeutic approaches to reduce gingival inflammation and treat periodontal disease.

MATERIALS AND METHODS

Bacterial strains and eukaryotic cells. The *T. denticola* strains and eukaryotic cells used in this study are listed in Table S2 in the supplemental material. Bacterial strains were grown in new oral spirochete (NOS) medium at 37°C for 3 to 4 days under anaerobic conditions (90% N_2, 5% H_2, 5% CO_2). Gingival epithelial cells (GECs) were maintained at 37°C with 5% CO_2 in DermaLife serum-free basal medium (Lifeline Cell Technology) with DermaLife K LifeFactors (5 μg/mL recombinant human insulin, 6 mM L-glutamine, 1 μM epinephrine, 5 μg/mL apo-transferrin, 0.5 ng/mL recombinant human transforming growth factor alpha [TGF-α], 0.4% extract P, 100 ng/mL hydrocortisone). Epithelial cells at between 70 and 90% confluence were counted using the Countess FL2 system (Thermo) and treated as described below for the individual experiments.

RNA sequencing. GECs were infected with *T. denticola* ATCC 35405 at an MOI of 100 for 6 h or incubated with medium alone, after which nonadherent bacteria were removed and washed away with 3 washes in phosphate-buffered saline (PBS); fresh medium was then added; and the cells were incubated for 18 h. After incubation, RNA was extracted using the Qiagen RNeasy Plus kit. The TruSeq stranded total mRNA with poly(A) enrichment kit (Illumina) was used to generate a sequencing library from 500 ng of total RNA. Paired-end sequencing was performed by the University of Louisville Center for Genetics and Molecular Medicine using the Illumina NextSeq 500 and high-output kit (75 cycles). After quality control, trimming, and merging of paired sequence reads using FastQC (59), cutadapt (60), and FLASH (61), respectively, there were on average 33,197,127 reads per control sample and 28,569,667 reads per *T. denticola*-infected sample. Gene assignment was performed using Bowtie 2 (62) against the transcriptome of *Homo sapiens* (assembly GRCh38.p14) downloaded from the NCBI GenBank database. Gene expression profiles were normalized by a variance-stabilizing transformation using the varianceStabilizingTransformation function, and the dissimilarity of the profiles was visualized with the plotPCA function in the DESeq2 package (63) in R software. Differentially expressed genes were tested using the DESeq function in the DESeq2 package and were input into DAVID bioinformatics resources (64) for pathway enrichment analysis. Significantly enriched KEGG pathways and GO terms with false-discovery-rate-adjusted *P* values of ≤0.05 were visualized by pie plots.

Pharmacological treatments, RNA interference, and transfections. HIGKs were grown to 60 to 70% confluence and incubated with pharmacological inhibitors (Table S2). To inhibit IKK and p38, cells were treated with BAY11-7821 and SB203580, respectively, for 2 h at various concentrations and removed immediately prior to a 4-h incubation with *T. denticola*. To inhibit JNK, MEK1/2, and PI3K/Akt, cells were treated with SP600125, PD980059, and LY294002, respectively, for 1 h at various concentrations prior to infection and throughout the 4-h incubation with *T. denticola*. Cells were grown to 50 to 60% confluence for siRNA transfection. Transfections were done using Lipofectamine RNAiMAX (Invitrogen) for siRNA for 48 h, and medium was replaced following bacterial challenge. Confirmation of siRNA knockdowns is shown in Fig. S3.

Quantitative reverse transcription-PCR. RNA was isolated using the RNeasy Plus minikit (Qiagen), RNA concentrations were quantified using the NanoDrop One system (Thermo Scientific), and cDNA was synthesized from total RNA (1 μg RNA per reaction) using a high-capacity cDNA reverse transcription (RT) kit (Applied Biosystems). Quantitative RT-PCR (qRT-PCR) was performed with 10 ng cDNA and TaqMan Fast advanced PCR master mix with TaqMan gene expression assays (Thermo Fisher) using an Applied Biosystems QuantStudio 3 system. The cycle threshold (C_T) values were determined, and mRNA expression levels were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression and expressed relative to the values for noninfected controls according to the $2^{-\Delta\Delta CT}$ method.

Statistical analyses. Assays were performed with a minimum of three biological replicates. GraphPad Prism software was used for statistical analyses, and data were evaluated by a *t* test or one-way analysis of variance (ANOVA) with either Tukey's or Dunnett's multiple-comparison test as described in the figure legends for each experiment.

Data availability. Raw data and processed data files have been deposited in the Gene Expression Omnibus under accession number GSE207003.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.5 MB.

SUPPLEMENTAL FILE 2, XLSX file, 12.6 MB.

ACKNOWLEDGMENTS

We thank the NIH/NIDCR for support through DE028166 (Z.R.F.), DE011111 (R.J.L.), and DE028346 (D.P.M.) and VCU School of Medicine VETAR funding for supporting this work.

We declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

A.N.H., C.G.H., C.S.B., Z.R.F., and D.P.M. participated in the experiments and analyzed the data. B.Z. and G.B. performed the bioinformatics analysis of the RNAseq results. A.N.H., R.J.L., and D.P.M. developed the idea for this study and wrote and revised the manuscript. All authors contributed to the article and approved the submitted version.

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