

## *Treponema denticola* Suppresses Expression of Human $\beta$ -Defensin-3 in Gingival Epithelial Cells through Inhibition of the Toll-Like Receptor 2 Axis<sup>∇</sup>

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We reported previously that *Treponema denticola*, one of the periodontal pathogens, suppresses the expression of human  $\beta$ -defensins (HBDs) in human gingival epithelial cells. To identify the mechanisms involved in this suppression, immortalized and normal human gingival epithelial cells were infected with live or heat-killed *T. denticola* for 24 h, and then the expression of HBDs was examined by real-time RT-PCR. Live *T. denticola* suppressed the expression of HBD-3 substantially and also suppressed the expression of HBD-1 and HBD-2. However, heat-killed bacteria did not produce a suppressive effect but instead slightly upregulated the levels of HBD-2 and HBD-3. In contrast to live *T. denticola*, which reduced the activation of mitogen-activated protein kinase (MAPK) and NF- $\kappa$ B within an hour of infection, heat-killed bacteria did not show any inhibitory effect on the MAPK and NF- $\kappa$ B signaling pathways. Knockdown of Toll-like receptor 2 (TLR2) via RNA interference abolished the suppressive effect of *T. denticola* on the expression of HBD-3. Heat-killed *T. denticola* but not live bacteria could activate TLR2 in CHO/CD14/TLR2 reporter cells, suggesting that *T. denticola* contains a heat-labile inhibitor(s) of TLR2 in addition to ligands recognized by TLR2. Indeed, live *T. denticola* was able to inhibit TLR2 activation by Pam<sub>3</sub>CSK. In conclusion, *T. denticola* suppressed the expression of HBD-3 by inhibiting the TLR2 axis in gingival epithelial cells. These results may provide new insight into the pathogenesis of periodontitis caused by *T. denticola*.

Periodontitis is the inflammation of periodontal tissue that results from the interaction of plaque-associated bacteria with the host immune system. During the transition from health to periodontitis, the amount of total bacteria and the proportion of periodontopathic bacteria in the plaque microflora increase (22). Among the periodontitis-associated bacteria, the members of the so-called red complex triad (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) are the most well-studied periodontal pathogens, and these bacteria have been characterized as the primary causal agents of periodontal disease (10, 22). The gingival epithelium provides the first line of defense against these plaque bacteria by forming a physical and chemical barrier. The barrier function of epithelia is attributed to the unique architectural integrity of the cells and to the production of antimicrobial peptides, such as human  $\beta$ -defensins (HBDs) and the cathelicidin LL-37 (9). Patients with Kostmann syndrome lack LL-37 in their saliva and develop severe periodontitis in young adulthood (21), suggesting the importance of antimicrobial peptides in the maintenance of periodontal health.

The regulation of HBD expression has been studied extensively. HBD-1 is expressed constitutively, whereas the expression of HBD-2 and HBD-3 is under the control of the NF- $\kappa$ B and/or mitogen-activated protein kinase (MAPK) signaling pathway (11). At the receptor level, induction of HBD-2 ex-

pression is mediated via pattern recognition receptors (PRRs) and proinflammatory cytokine receptors that respond to microbe-associated molecular patterns and cytokines such as tumor necrosis factor alpha, interleukin-1 (IL-1), and IL-17 (11). PRRs that mediate HBD upregulation in various epithelial tissues include Toll-like receptors (TLRs) and NOD2 (nucleotide-binding oligomerization domain 2) (11).

In gingival epithelial tissues, components of several species of oral bacteria, including *Streptococcus sanguinis*, *S. gordonii*, *Veillonella atypica*, *Fusobacterium nucleatum*, *Prevotella intermedia*, and *P. gingivalis*, have been shown previously to induce HBD-2 and/or HBD-3 expression (6, 13, 17, 24, 29). Protease-activated receptor 2 (PAR2) has been shown to be partially involved in the upregulation of HBD-2 by *P. gingivalis* (7). We have recently demonstrated that NACHT-LRR- and pyrin domain-containing protein 2 (NALP2) and TLR2 mediate the induction of HBD-2 and HBD-3 by *F. nucleatum* in gingival epithelial cells (14).

Pathogens often subvert host immune responses, and downregulation of HBD expression by bacteria has also been demonstrated. Virulent *Shigella flexneri* has been shown to suppress transcription of HBD-3 in human intestinal cells (23). In our previous study, we demonstrated that *T. denticola* suppressed the expression of HBD-1 and HBD-3 in gingival epithelial cells (13). Compared with the mechanisms involved in bacterium-induced upregulation of antimicrobial peptides, the mechanisms for the suppression of antimicrobial peptides by pathogens are poorly understood. The aim of this study was to delineate the mechanism(s) involved in the suppression of HBD expression by *T. denticola* in gingival epithelial cells.

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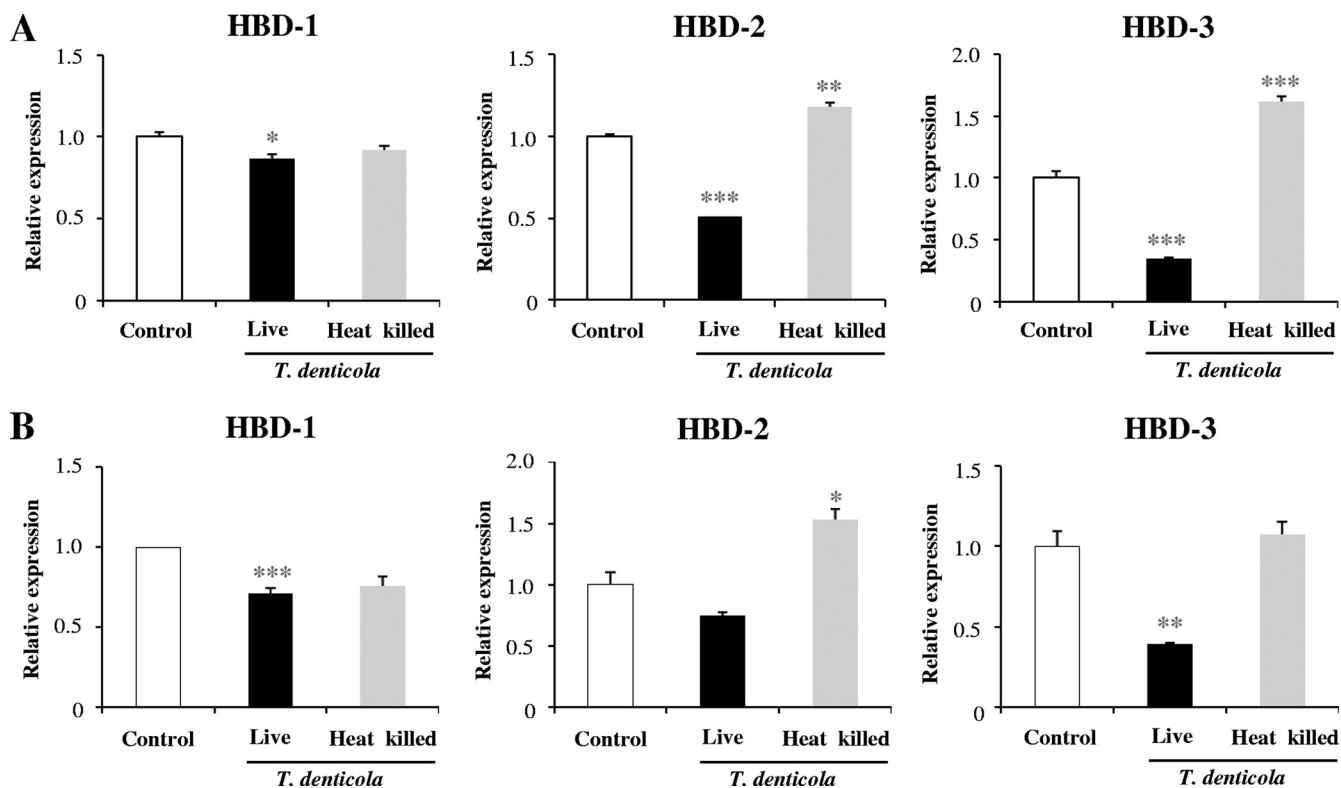


FIG. 1. Live but not heat-killed *T. denticola* suppressed the expression of HBDs in gingival epithelial cells. NGECs (A) or HOK-16B cells (B) were infected with live or heat-killed *T. denticola* at an MOI of 1,000 for 24 h. Expression of HBDs was examined by real-time RT-PCR. The means  $\pm$  the standard errors of the means (SEM) of the relative copy numbers are expressed as the level of induction (*n*-fold) compared to expression in the control culture without bacteria. These data are representative of findings from three independent experiments with similar results. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$  versus data for no infection.

#### MATERIALS AND METHODS

**Human materials.** The use of human materials was approved by the Institutional Review Board at the Seoul National University Dental Hospital. Normal human gingival tissue specimens were obtained with informed consent from healthy volunteers (aged 20 to 30 years) undergoing oral surgery.

**Bacterial culture.** *S. sanguinis* NCTC 10904, *S. gordonii* ATCC 10558, *V. atypica* ATCC 17744, *F. nucleatum* ATCC 25586, *P. intermedia* ATCC 25611, *P. gingivalis* ATCC 49417, *T. forsythia* ATCC 43407, and *T. denticola* ATCC 33521 were obtained from the ATCC (Bethesda, MD) and cultured in the appropriate medium as described previously (13). The bacteria were enumerated by flow cytometry. Heat-killed bacteria were prepared by being heated at 95°C for 1 h. Bacterial lysates were prepared by sonication with a Sonic Dismembrator 300 (Fisher Scientific, Fair Lawn, NJ) for 15 min on ice water, and then complete destruction of bacterial cells was confirmed using a microscope. Some heat-killed bacteria were fragmented, but most maintained bacterial morphology.

**Cell culture.** Normal human gingival epithelial cells (NGECs) were isolated from excised retromolar gingival tissue, and primary cultures were established in bullet kit medium comprising keratinocyte growth medium (KGM) containing 0.15 mM calcium and a supplementary growth factor bullet kit (Clonetics, San Diego, CA) as reported previously (19). Cells were subcultured when they reached 70% confluence, and second-passage cells were used for all experiments. Immortalized human gingival keratinocyte HOK-16B cells (20), a kind gift from N.-H. Park (University of California—Los Angeles, Los Angeles), were maintained in supplemented KGM. The reporter CHO cell line expressing human CD14 and human TLR2 that expresses surface CD25 as a result of TLR2-mediated NF- $\kappa$ B translocation (designated CHO/CD14/TLR2) (30) was kindly provided by D. Golenbock (University of Massachusetts Medical School, Worcester). The CHO/CD14/TLR2 cells were maintained in Ham's F-12 medium (Gibco, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), 1 mg/ml G418 (Invitrogen, Carlsbad, CA), 400 U/ml hygromycin (Amresco, San Francisco, CA), and 1 $\times$  penicillin-streptomycin (Gibco).

**Infection of epithelial cells with bacteria.** NGECs or HOK-16B cells were plated at 6  $\times$  10<sup>4</sup> cells/500  $\mu$ l/well in triplicate in 24-well plates 1 day before infection. At 80% confluence, the cells were infected with live or heat-killed bacteria at a multiplicity of infection (MOI) of 1,000 in KGM containing 2% heat-inactivated human serum (Sigma, St. Louis, MO). The cells were cultured at 37°C in a water-saturated atmosphere of 95% air and 5% CO<sub>2</sub> for 24 h or the time indicated below for a specific experiment. The antibiotics in the KGM were removed to prolong the survival of bacteria. Under these culture conditions, *T. denticola*, an absolute anaerobe, did not grow well but survived long enough to invade the cells. In addition, the viability of the HOK-16B cells was not affected. Total RNA was extracted from the cells of the combined triplicate wells using TRIzol (Invitrogen). Experiments were repeated three times.

**Real-time RT-PCR.** Total RNA (2  $\mu$ g) from gingival epithelial cells or CHO/CD14/TLR2 cells was subjected to reverse transcription (RT) using (dT)<sub>18</sub> and the SuperScript II enzyme (Invitrogen) in a 25- $\mu$ l reaction mixture at 42°C for 1 h. Real-time PCR was performed in a 20- $\mu$ l reaction mixture containing 1  $\mu$ l of template cDNA, SYBR Premix Ex *Taq*, ROX reference dye (Takara Bio, Otsu, Japan), and each primer (0.2  $\mu$ M), as reported previously (14). Primer sequences used to amplify the CD25 gene and the hamster glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene are as follows: 5'-GAATGCAAGA GAGGTTTCCG-3' and 5'-TGTGCATTGACATTGGTTGTC-3' for the CD25 gene and 5'-CATGTTCCAGTATGACTCCACTC-3' and 5'-GGCCTCACCCC ATTTGATGT-3' for the hamster GAPDH gene. Amplification was performed in a model 7500 real-time PCR fluorescence thermocycler (Applied Biosystems, Foster City, CA) under the following conditions: initial denaturation at 94°C for 1 min, followed by 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 15 s, and elongation at 72°C for 33 s. The specificity of the PCR product was verified by melting-curve analysis and by examination on a 3% agarose gel. The housekeeping GAPDH gene was amplified in parallel with the gene of interest. Copy numbers relative to those of the GAPDH gene were calculated using 2<sup>- $\Delta\Delta$</sup> . Real-time PCR was performed in triplicate for each RNA sample.

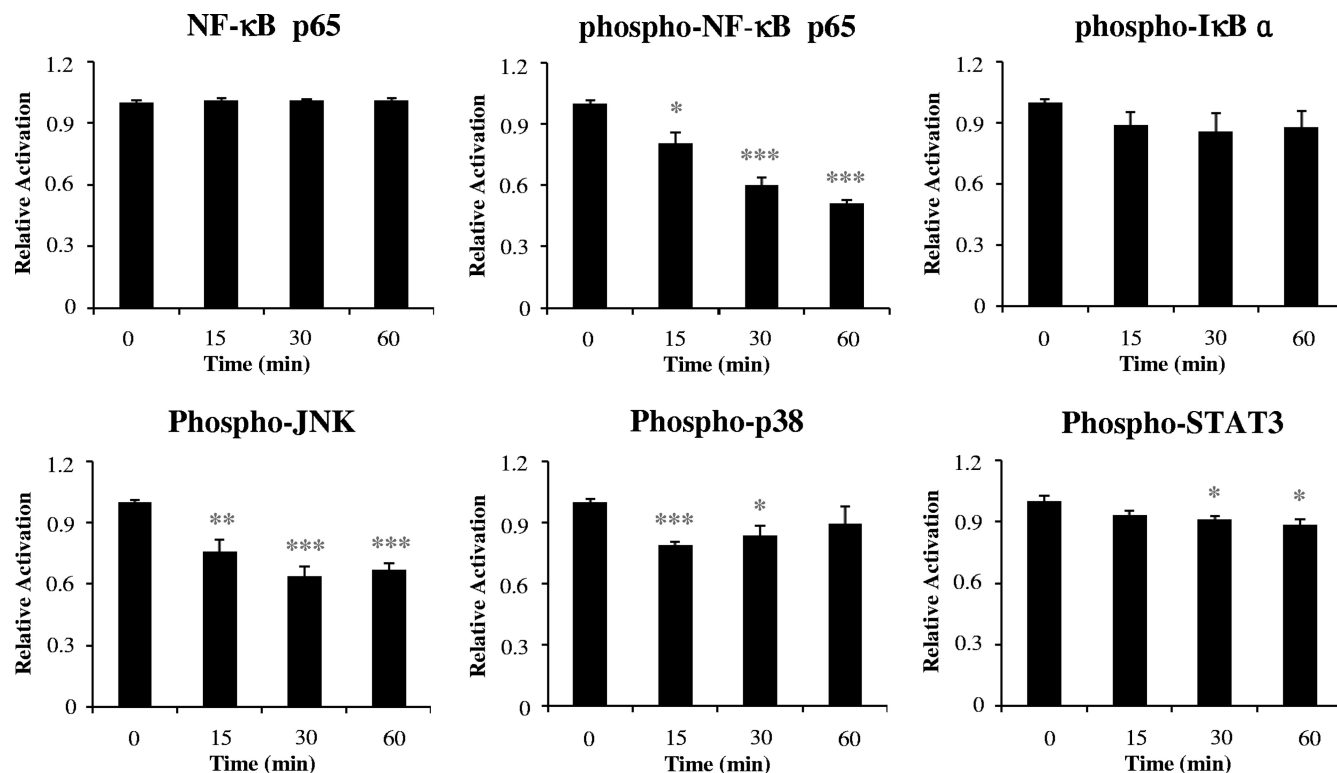


FIG. 2. *T. denticola* suppressed various signaling pathways. HOK-16B cells plated into a 100-mm dish were stimulated with live *T. denticola* at an MOI of 1,000 for 0, 15, 30, or 60 min. Protein lysates (50  $\mu$ g each) were prepared and subjected to ELISA analysis of NF- $\kappa$ B p65 and the phosphorylated forms of NF- $\kappa$ B p65, I $\kappa$ B, p38, JNK, and STAT3. The means  $\pm$  the SEM of the optical densities from four assays are expressed as the levels of activation relative to that in a control culture without bacteria. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; and \*\*\*,  $P < 0.001$  versus data for no infection.

**RNAi in HOK-16B cells and bacterial infection.** Stealth small interfering RNA (siRNA) duplex oligoribonucleotides against human TLR2 (corresponding to GenBank accession no. NM\_003264) were designed using the BLOCK-iT RNA interference (RNAi) designer program (<https://rnaidesigner.invitrogen.com/rnaexpress/>) and were synthesized by Invitrogen. The oligonucleotide sequences used for TLR2 were as follows: sense, 5'-UGAAGCAUCAACUCAAGUUCUCA-3', and antisense, 5'-UGAGGAACUUGAGAUUGAUGCUUCA-3'. The BLOCK-iT (Invitrogen) fluorescent oligonucleotide, which is not homologous to any known gene, was used as a transfection efficiency detector and as a negative control. HOK-16B cells were plated at  $4 \times 10^4$  cells/well in a 24-well plate. After overnight incubation, the cells were transfected with 20 nM siRNAs using a BLOCK-iT transfection kit (Invitrogen). After culture for 24 h, the cells were retransfected with siRNA and simultaneously infected with live *T. denticola* at an MOI of 1,000 for 24 h. Experiments were repeated three times. The *T. denticola*-induced suppression of HBD-3 expression was recorded as the percentage of expression relative to the expression in the control culture without infection.

**Flow cytometry.** HOK-16B cells were stained with fluorescein isothiocyanate (FITC)-conjugated mouse anti-human TLR2 monoclonal antibody (MAb) clone TL2.1 (BioLegend, San Diego, CA) and were analyzed with a FACSCalibur flow cytometer (BD Biosciences).

CHO/CD14/TLR2 cells were plated at  $2 \times 10^5$  cells/well in a 24-well plate. After overnight incubation, the cells were stimulated with various live oral bacteria (MOI, 10 or 100) for 16 h. The CHO cells were also stimulated with live or heat-killed *T. denticola* (MOI, 100) or with *T. denticola* lysate in the absence or presence of 0.3  $\mu$ g/ml Pam<sub>3</sub>CSK<sub>4</sub> for 16 h. After being stained with FITC-conjugated mouse anti-human CD25 MAb clone 2A3 (BD Biosciences, San Diego, CA) or FITC-conjugated mouse anti-human TLR2 MAb clone TL2.1 (BioLegend), the cells were analyzed with a FACSCalibur flow cytometer (BD Biosciences). To examine the levels of CD25 transcripts in CHO/CD14/TLR2 cells, total RNA was extracted using an RNeasy kit (Qiagen, Germantown, MD). Experiments were repeated three times.

**Examination of signaling pathways.** HOK-16B cells ( $1.2 \times 10^6$ ) plated into a 100-mm dish were stimulated with live *T. denticola* at an MOI of 1,000 for the

times indicated below. HOK-16B cells were also stimulated with live or heat-killed *T. denticola* at an MOI of 1,000 for 30 min. After the cells were washed with phosphate-buffered saline, protein lysates were prepared using the lysis buffer included in the PathScan inflammation multitarget enzyme-linked immunosorbent assay (ELISA) kit (Cell Signaling, Danvers, MA). The lysates were subjected to ELISA analysis for NF- $\kappa$ B p65 and the phosphorylated forms of NF- $\kappa$ B p65, I $\kappa$ B, p38, Jun N-terminal protein kinase (JNK), and STAT3 in duplicate by using the kit. The nonphosphorylated forms of JNK and NF- $\kappa$ B p65 in the lysates were detected by Western blotting using antibodies from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling, respectively. Experiments were repeated twice.

**Statistics.** The differences between two groups were analyzed with a two-tailed unpaired Student *t* test. Data were considered statistically significant at a *P* value of  $<0.05$ .

## RESULTS

**Live but not heat-killed *T. denticola* suppressed HBD expression in gingival epithelial cells.** We previously demonstrated that live *T. denticola* but not the bacterial lysate could suppress the expression of HBD-1 and HBD-3 in immortalized human gingival epithelial (HOK-16B) cells (13). To confirm the suppressive effect of *T. denticola* on HBD expression in normal cells, NGECs were infected with live or heat-killed *T. denticola* for 24 h and the expression of HBD-1, HBD-2, and HBD-3 was examined by real-time RT-PCR. Live *T. denticola* suppressed the expression of HBD-3 substantially and also suppressed the expression of HBD-1 and HBD-2. In contrast, heat-killed bacteria slightly upregulated the expression of HBD-2

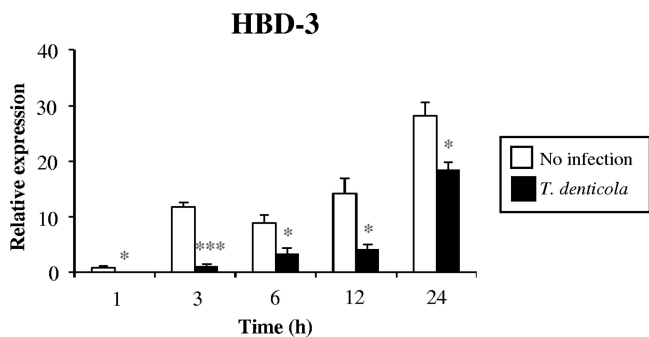


FIG. 3. *T. denticola* suppressed the expression of HBD-3 at early time points. HOK-16B cells were infected with live *T. denticola* at an MOI of 1,000 for 1, 3, 6, 12, and 24 h. The levels of HBD-3 transcripts were evaluated by real-time RT-PCR. The means  $\pm$  the SEM of the relative copy numbers are expressed as the levels of induction (*n*-fold) at 1 h compared to expression in the control culture without bacteria. These data are representative of findings from two independent experiments with similar results. \*,  $P < 0.05$ , and \*\*\*,  $P < 0.001$  versus data for no infection.

and HBD-3 (Fig. 1A). HOK-16B cells also experienced downregulation of HBDs only in response to live *T. denticola* infection (Fig. 1B). These results indicate that NGECS and HOK-16B cells respond to *T. denticola* similarly and

that live bacteria are required to suppress HBD expression in those cells. Thus, in the subsequent experiments, regulation of HBD-3 by *T. denticola* was studied using HOK-16B cells and live bacteria.

**Live *T. denticola* suppressed the NF- $\kappa$ B, MAPK, and STAT3 signaling pathways.** To understand the molecular basis for the suppression of HBD expression by *T. denticola*, the activation of several prominent signaling pathways involved in inflammation was examined. Infection with *T. denticola* significantly reduced the levels of the phosphorylated forms of NF- $\kappa$ B p65, JNK, p38, and STAT3 within 30 min (Fig. 2). These results suggest that *T. denticola* impedes various signaling pathways, including the NF- $\kappa$ B and MAPK pathways, that control HBD expression.

**Suppression of HBD-3 expression occurred at early time points.** Since *T. denticola* affected signaling through the NF- $\kappa$ B and MAPK pathways within 30 min of infection, time courses for the suppression of HBD-3 were examined. The level of HBD-3 transcripts in the control culture without infection increased with time, and this increase in transcripts was suppressed by infection with *T. denticola* starting at a very early time point (1 h) and continuing throughout the culture period (Fig. 3).

**Knockdown of TLR2 reversed the suppressive effect of *T. denticola* on HBD-3 expression.** Since TLR2 mediates the induc-

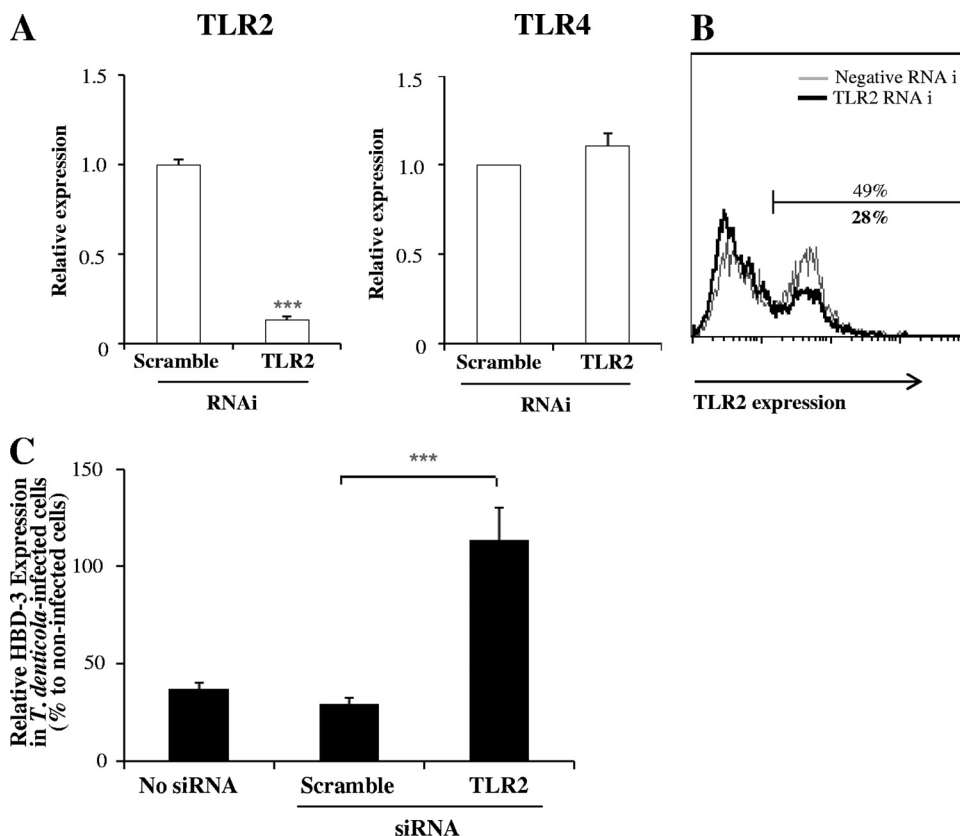


FIG. 4. Knockdown of TLR2 reversed the suppressive effect of *T. denticola* on HBD-3 expression. HOK-16B cells were transfected with control or TLR2-specific siRNA. After a 24-h incubation, the cells were infected with live *T. denticola* at an MOI of 1,000 for 24 h. (A and B) Gene silencing was assessed by real-time RT-PCR (A) and flow cytometry (B) 24 h after transfection. (C) The levels of HBD-3 transcripts were evaluated by real-time RT-PCR. The means  $\pm$  the SEM of results from nine real-time RT-PCR assays are given as the percentages of expression in *T. denticola*-infected cells compared to expression in the control culture without bacteria. \*\*\*,  $P < 0.001$  versus results for scramble RNAi.



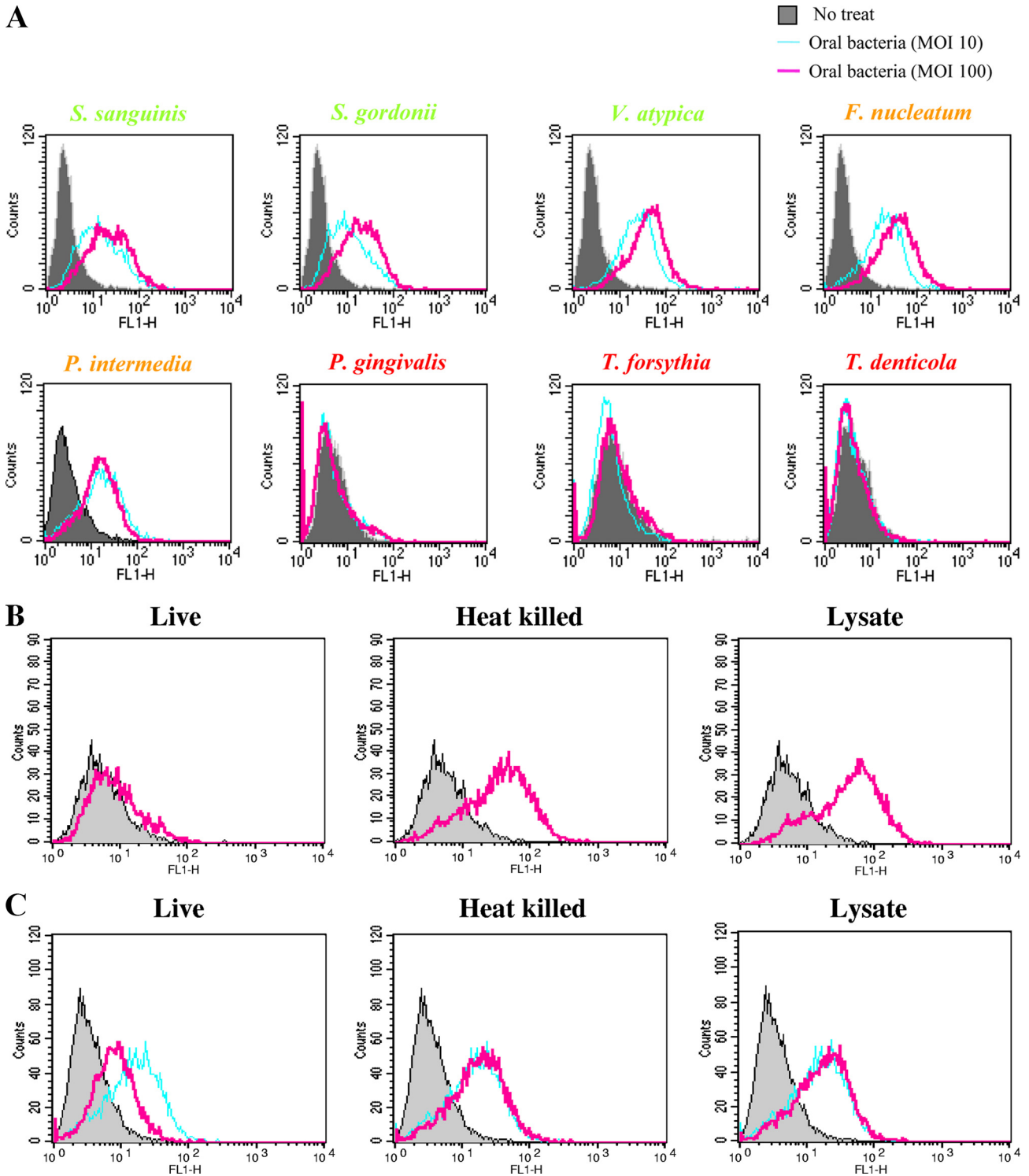


FIG. 5. *T. denticola* inhibited activation of TLR2 in CHO/CD14/TLR2 cells. (A) CHO/CD14/TLR2 cells were infected with various live oral bacterial species at an MOI of 10 or 100 for 16 h and were stained for surface CD25 expression. The plots representing the activation of CD25 expression by various oral bacteria are overlaid with that representing activation by medium alone (no treatment [no treat]). Color coding for the names of bacterial species is as follows: green, early colonizers; orange, orange complex members; and red, red complex members. (B) CHO/CD14/TLR2 cells were infected with live *T. denticola*, heat-killed *T. denticola*, or *T. denticola* lysate (all at an MOI of 100) for 16 h. The plots representing the activation of CD25 expression by *T. denticola* (pink lines) are overlaid with that representing activation by medium alone (gray shading). (C) CHO/CD14/TLR2 cells were infected with live *T. denticola*, heat-killed *T. denticola*, or *T. denticola* lysate (all at an MOI of 100) in the presence of Pam<sub>3</sub>CSK for 16 h. The plots representing the activation of CD25 expression by *T. denticola* (pink lines) are overlaid with those representing activation by medium alone (gray shading) and Pam<sub>3</sub>CSK alone (sky blue lines). (D) The expression of TLR2 on the surfaces of CHO/CD14/TLR2 cells after a 16-h incubation with medium alone (sky blue lines) or live *T. denticola* or *T. denticola* lysate (pink lines) was examined by flow cytometry. Staining with an isotype control antibody served as a negative control (gray shading). (E) The levels of CD25 transcripts in control CHO/CD14/TLR2 cells or cells stimulated with live *T. denticola*, heat-killed *T. denticola*, or *T. denticola* lysate for 16 h were examined by real-time RT-PCR. \*,  $P < 0.05$ , and \*\*,  $P < 0.01$  versus data for the control. All data (in panels A to E) are representative of results from three independent experiments.

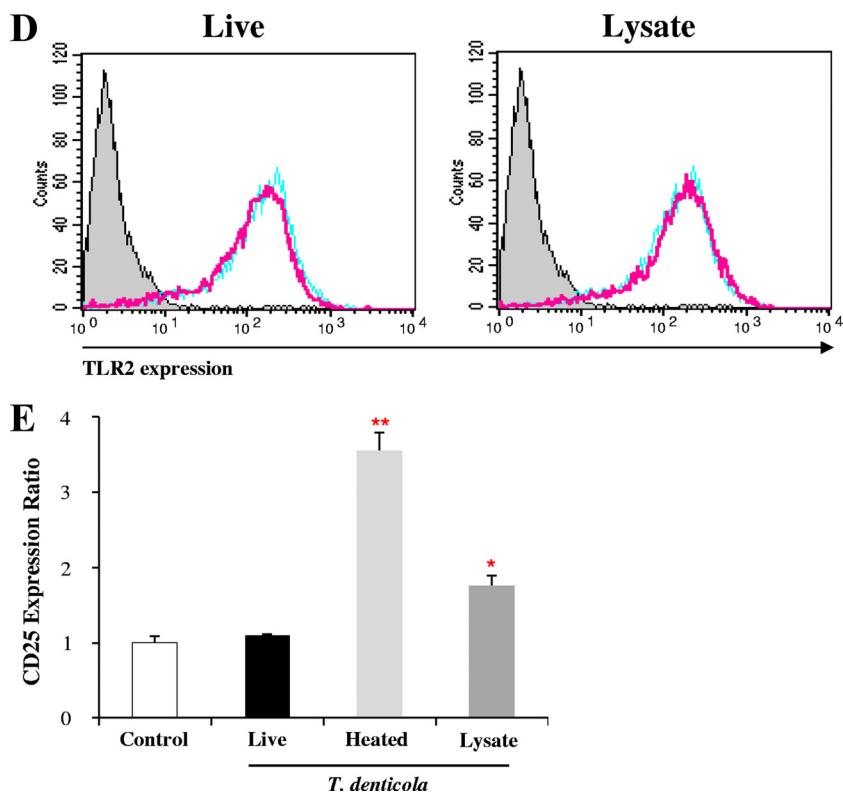


FIG. 5—Continued.

tion of HBD-2 and HBD-3 by *F. nucleatum* in gingival epithelial cells (14), we examined whether or not TLR2 is also involved in the modulation of HBD-3 by *T. denticola*. TLR2-specific RNAi decreased the level of TLR2 transcripts by 80% but had no effect on TLR4 expression (Fig. 4A). We previously reported very low levels of TLR2 protein expression on HOK-16B cells (14). With the use of an antibody from a source different from that used in the previous study, 49% of HOK-16B cells stained positive for expression of TLR2 on the cell surface. This level of expression was reduced to 28% by TLR2-specific RNAi (Fig. 4B). In non-transfected cells and cells transfected with scramble siRNA, *T. denticola* infection reduced the levels of HBD-3 expression to 37 and 29% of that in uninfected cells, respectively. Knockdown of TLR2 reversed the reduction of HBD-3 expression by *T. denticola* infection, changing the level of expression from 29 to 114% of that in uninfected cells (Fig. 4C).

***T. denticola* inhibited TLR2 activation.** To better understand the modulation of HBD expression via TLR2, the ability of *T. denticola* to activate TLR2 was compared with the TLR2-activating abilities of other oral bacteria by using the CHO/CD14/TLR2 reporter cell line. When the reporter cells were stimulated with various oral bacteria at an MOI of 10 or 100, three early-colonizing species and two species belonging to the orange complex substantially activated TLR2 in a dose-dependent manner. However, the red complex bacteria, including *T. denticola*, did not activate TLR2 (Fig. 5A). In contrast to live *T. denticola*, heat-killed bacteria and bacterial lysate were able to activate TLR2 (Fig. 5B). In addition, live *T. denticola* but not heat-killed bacteria or lysate suppressed TLR2 activation by a synthetic ligand, Pam<sub>3</sub>CSK<sub>4</sub> (Fig. 5C). Both live *T. denticola*

and *T. denticola* lysate, which are known to be rich in proteases (28), failed to degrade the TLR2 protein expressed on the cell surface (Fig. 5D). The possibility that proteases degraded the CD25 protein was further investigated by examining the levels of transcripts. Heat-killed bacteria and lysate, but not live bacteria, upregulated CD25 transcripts (Fig. 5E).

**Heat-killed *T. denticola* did not suppress signaling pathways.** The inability of heat-killed *T. denticola* to suppress HBD expression was further explored by examining the activation of NF- $\kappa$ B and JNK, which were the most substantially inhibited proteins of those listed in Fig. 2. In contrast to live bacteria, which inhibited both signaling pathways by more than 50%, heat-killed *T. denticola* did not show any inhibitory effect on the NF- $\kappa$ B and JNK signaling pathways (Fig. 6A). Uniform levels of the nonphosphorylated forms of NF- $\kappa$ B and JNK proteins validated the assay (Fig. 6B). These results indicate that heat-killed *T. denticola* has lost the ability to inhibit activation of TLR2 and downstream signaling pathways.

## DISCUSSION

In the present study, we have provided evidence that *T. denticola* suppresses the expression of HBD-3 in gingival epithelial cells through the inhibition of the TLR2 axis. First, *T. denticola* inhibited Pam<sub>3</sub>CSK-mediated activation of TLR2 in TLR2 reporter cells. Second, *T. denticola* significantly reduced the basal levels of activated NF- $\kappa$ B, JNK, and p38, which are involved in the regulation of HBD expression. Third, knockdown of TLR2 reversed the suppressive effect of *T. denticola* on HBD-3 expression. Brissette et al. have also shown that

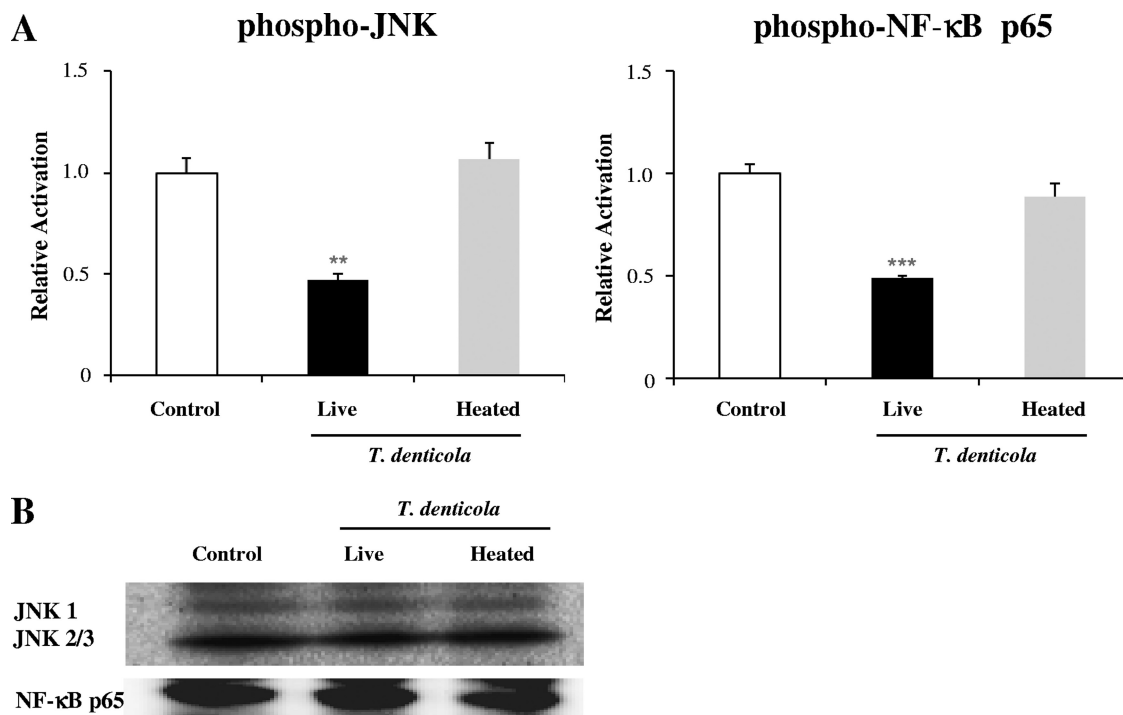


FIG. 6. Heat-killed *T. denticola* did not suppress signaling pathways. HOK-16B cells plated into a 100-mm dish were stimulated with live or heat-killed *T. denticola* at an MOI of 1,000 for 30 min, and protein lysates were prepared. (A) Protein lysates (50  $\mu$ g each) were subjected to ELISA analysis of the phosphorylated forms of JNK and NF- $\kappa$ B p65. The means  $\pm$  the SEM of the optical densities from four assays are expressed as the levels of activation compared to that in the control culture without bacteria. \*\*,  $P < 0.01$ , and \*\*\*,  $P < 0.001$  versus data for no infection. (B) Protein lysates (10  $\mu$ g each) were subjected to Western blotting to detect nonphosphorylated forms of JNK and NF- $\kappa$ B p65.

several strains of live *T. denticola* failed to induce various innate immune mediators in NGECS, including HBD-2 (3). However, Brissette et al. concluded that TLR2 does not have a role in the lack of immune modulator production by *T. denticola*, because they used bacterial cell wall, not live bacteria, in the testing of TLR2-stimulating ability.

The suppressive effects of *T. denticola* on HBD expression, TLR2 activation, and the activation of signaling pathways required live bacteria. Heat-killed *T. denticola* bacteria activated rather than suppressed TLR2 in the reporter cells, which explains the slight upregulation of HBD-2 and HBD-3 by these killed bacteria. Although activation of JNK and NF- $\kappa$ B by heat-killed *T. denticola* was not observed 30 min after infection, activation may occur at other time points. Activation of TLR2 by heat-killed but not by live *T. denticola* suggests that *T. denticola* contains a heat-labile inhibitor(s) of the TLR2 signaling axis in addition to ligands that activate TLR2. Since bacterial lysate suppressed neither HBD expression in gingival epithelial cells in a previous study (13) nor TLR2 activation in reporter cells in the present study, the suppressive effect of *T. denticola* is not likely to be attributable to the *T. denticola* proteases. Neither live *T. denticola* nor *T. denticola* lysate affected the level of TLR2 protein on the cell surface. The reason that live *T. denticola*, but not *T. denticola* lysate, produced a suppressive effect may be that the heat-labile inhibitor(s) targets intracellular events. *T. denticola* can invade gingival epithelial cells, and some of the internalized bacteria were examined in the cytosol 24 h after infection (our unpublished data). The dependence of immune evasiveness on viable

bacteria has also been shown previously for *Borrelia burgdorferi*. Whereas *B. burgdorferi* bacteria in viable culture medium preferentially enhanced production of an anti-inflammatory cytokine, IL-10, by macrophages, bacteria in nonviable culture medium or heat-killed bacteria preferentially induced a proinflammatory cytokine, tumor necrosis factor alpha (18).

Although the nature of the heat-labile inhibitor(s) in *T. denticola* is not clear, many bacterial virulence factors that subvert the host innate immune response are known. Toll/IL-1 receptor domain-containing proteins in *Brucella melitensis* and in virulent uropathogenic *Escherichia coli* (8) that impede signaling through multiple TLRs by binding to the MyD88 adaptor protein have been identified previously. The suppression of HBD-3 by *S. flexneri* depends on the MixE bacterial regulator (23). The MixE-dependent proteins include the type III secretion system effectors OspF and OspG, which modify the MAPK and NF- $\kappa$ B pathways and remodel chromatin structure to reduce the host inflammatory response (1, 16). *T. denticola* may employ a similar mechanism to suppress TLR2 activation, activation of the MAPK and NF- $\kappa$ B pathways, and the expression of HBDs.

The reversal of *T. denticola*'s suppressive effect by TLR2 RNAi suggests that there was an endogenous TLR2 ligand in the control culture without *T. denticola*. The function of HBD-3 as a ligand for TLR1/TLR2 has been reported previously (12). Thus, the interaction of HBD-3 and TLR2 in gingival epithelial cells is expected to form a positive feedback loop, unless there is something present to interfere with that loop. Indeed, the level of HBD-3 expression in the control cells

rapidly increased with time, and this increase was nearly halted by *T. denticola* for 12 h. In addition, the suppressive effect of *T. denticola* on HBD expression was more prominent in the presence of 2% human serum. It is noteworthy that the basal level of HBD-3 expression increased in the presence of 2% human serum (data not shown).

It is interesting that the members of the red complex triad commonly demonstrate poor abilities to activate TLR2. We observed previously that *T. forsythia* also suppresses HBD-3 expression (13). By suppressing HBD expression, *T. denticola* is expected to perturb the chemical barrier of the gingival epithelium. A breach in the epithelial barrier would facilitate the invasion of plaque bacteria, including *T. denticola*, thus contributing to the pathogenesis of periodontitis. Studies using sonicates or purified components of *T. denticola*, such as lipooligosaccharide, peptidoglycan, or a surface protein, have reported the induction of an inflammatory response in murine macrophages, human macrophage-like cells, and fibroblasts (4, 5, 15, 26, 27). However, our results suggest that live bacteria may suppress or paralyze the inflammatory responses of these cells. Infection of murine macrophage-like J774.1 cells with live *T. denticola* did not induce inflammatory cytokines (25). In contrast, a coculture model composed of epithelial cell-like transformed cells and macrophage-like cells induced some inflammatory mediators in response to *T. denticola* infection (2). If live *T. denticola* can reach the lamina propria after invading the epithelium, the evaluation of the immune responses of macrophages and fibroblasts to live bacteria will be important.

In conclusion, *T. denticola* suppresses the expression of HBD-3 in gingival epithelial cells by inhibiting TLR2 signaling, which may provide new insight into the pathogenesis of periodontitis caused by *T. denticola*.

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