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***Treponema denticola* Does Not Induce Production of Common Innate Immune Mediators from Primary Gingival Epithelial Cells**

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Abstract

It has been hypothesized that the neutrophil chemoattractant IL-8 forms a gradient in the oral cavity, with the highest concentration of IL-8 produced closest to the bacterial biofilm. In periodontitis, this gradient is disrupted, impairing neutrophil chemotaxis to diseased sites. *Treponema denticola* is prominently associated with periodontal disease, yet little is known about its ability to modulate production of inflammatory mediators by epithelial cells. Others have shown that dentilisin, the major outer membrane protease of *T. denticola*, degrades IL-8 *in vitro*. We now provide evidence that *T. denticola* also fails to induce IL-8 production from primary gingival epithelial cells (PGEC). The lack of IL-8 production is not explained by IL-8 degradation, as a protease mutant that does not degrade IL-8 also does not induce IL-8 production by these stimuli. The lack of innate immune mediator production may be a more global phenomenon, as *T. denticola* fails to induce IL-6 or ICAM-1 production from PGEC. *T. denticola* also fails to induce transcription of IL-8 and hβD-2 mRNA. The lack of immune mediator production is not explained by failure of *T. denticola* to interact with TLR-2, as *T. denticola* stimulates NFκB nuclear translocation in TLR-2 transfected HEK293 cells. Not only can *T. denticola* degrade IL-8 present in the periodontal lesion, but this organism also fails to induce of IL-8 protein by PGEC. The lack of epithelial cell response to *T. denticola* may contribute to the pathogenesis of periodontitis by failing to trigger chemotaxis of neutrophils into the periodontal pocket.

INTRODUCTION

Periodontal disease is remarkably widespread, afflicting 50% of adults in the United States (36, 49), with severe disease affecting 6 million Americans. The ability of PGEC to respond to microorganisms is crucial, as homeostasis of the epithelial barrier depends on low-level inflammation. Epithelial cells can be major participants in the innate immune response to microorganisms. In addition to serving as an initial barrier to infection, epithelial cells

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produce chemical mediators that recruit phagocytes and antigen presenting cells to sites of bacterial attachment. Primary gingival epithelial cells (PGEC) express pattern recognition receptors such as Toll-like receptor 2 (TLR-2), display cell adhesion proteins on their surface, secrete chemokines, and produce antibacterial peptides (27, 28, 31). PGEC are known to upregulate mRNA and protein for several of these innate immune mediators in response to bacterial components (27, 28). Live oral bacteria such as *Fusobacterium nucleatum* induce production of these mediators, including interleukin-8 (IL-8), intercellular adhesion molecule-1 (ICAM-1), and β -defensins by PGEC (8, 18, 22).

Many oral bacteria, commensals and pathogens alike, induce IL-8 by PGEC (18, 22, 28). Three organisms (*Porphyromonas gingivalis*, *Bacteriodes forsythus*, [now *Tannerella forsythia*] (37, 48) and *Treponema denticola*) have been identified as the “red complex” of bacteria that are highly associated with severe periodontal disease (51). The chronic nature of periodontitis dictates that these bacteria must have well-developed mechanisms for evading or impeding the host’s innate immune response. Indeed, *P. gingivalis* degrades IL-8 protein, fails to induce IL-8 transcription by epithelial cells and decreases the amount of IL-8 produced by these cells in response to other stimuli (11, 23) a phenomenon called local chemokine paralysis (11).

Many bacterial species have evolved strategies to evade host innate immune defenses, including the ability to inhibit cytokine production, evasion of complement, and modified outer surface molecules such as LPS that are poorly recognized by Toll-like receptors (TLRs) (9, 15, 26, 56). In this work, we demonstrate that *T. denticola* is such a pathogen, in that it does not induce IL-8 production by the epithelial barrier.

Spirochetal lipoproteins from *T. pallidum* subsp. *pallidum*, and *Borrelia burgdorferi* readily activate macrophages to produce IL-8 and other cytokines (32, 50). The major outer surface protein of oral treponemes *T. maltophilum* and *T. lecithinolyticum* upregulates ICAM-1 and proinflammatory cytokine production in monocytic cells (29). *T. denticola* also activates macrophages, but little is known about the effects of *T. denticola* on gingival epithelial cells, with respect to cytokine or antimicrobial peptide production (46). Published studies indicate that gingival fibroblasts and an oral epithelial cell line fail to respond to *T. denticola* by producing IL-8 protein (13, 41). Another study demonstrated *T. denticola* induction of IL-8 mRNA and NF κ B activation in a different oral epithelial cell line (2). To more closely mimic the *in vivo* situation, we examined the effects of *T. denticola* on IL-8 production in low passage primary gingival epithelial cells. In this paper, we demonstrate that *T. denticola* fails to induce IL-8, IL-6, or ICAM-1 protein from PGEC. In addition, *T. denticola* also does not induce transcription of IL-8 and h β D-2. This lack of responsiveness is not limited to PGEC, as human umbilical vein endothelial cells (HUVEC) also fail to produce IL-8 protein in response to *T. denticola*. These data suggest that *T. denticola* is a pathogen able to exist in the periodontal pocket without triggering a response from epithelial and endothelial cells.

MATERIALS AND METHODS

Bacterial strains and culture

T. denticola strains 35404, 33520, 33521 and GM-1 were a gift from Pamela Braham (University of Washington, Seattle, WA). Strain K1 (dentilisin mutant) and its 35405 parent were a gift from Kazuyuki Ishihara (Tokyo Dental College, Chiba, Japan) (24). *T. denticola* was maintained in GM-1 medium (4) in an anaerobic jar at 37°C. K1 cultures were supplemented with 40 µg/ml erythromycin (Sigma Chemicals, St. Louis, MO).

Tissue culture

Primary gingival epithelial cells (PGEC) were provided by the Comprehensive Center for Oral Health Research (University of Washington; Director, Beverly Dale). Cells were isolated from excess tissue from retromolar extractions of consenting healthy adult donors, as approved by University of Washington Human Subjects Division (42). Epithelial cells from at least 10 individuals were used for these studies. Pooled primary normal human dermal epithelial keratinocytes (NHEK) were obtained from Cambrex, Walkersville, MD. All epithelial cells were maintained in keratinocyte basal medium (KBM) supplemented with Bronchial Epithelium Growth Supplements (BEGM) and 0.03mM Ca²⁺ (Cambrex), and cells were used between passages 3–6. Human umbilical vein endothelial cells (HUVEC) were obtained from Clonetics (San Diego, CA) and cultured as previously described (9). Peripheral blood mononuclear cells (PBMC) were isolated from a consenting normal donor. Briefly, 50 ml of whole blood was drawn into a syringe containing 5,000 units of heparin and leukocytes isolated by Ficoll gradient centrifugation according to manufacturer's instructions (GE Health Care, Piscataway, NJ). Cells were cultured in complete RPMI-1640 (Invitrogen, Carlsbad, CA) + heat inactivated, pooled AB human serum (Sigma Chemicals) and stimulated 48 hours later as described below for PGEC.

Cell wall preparation

F. nucleatum ATCC 25586 was purchased from ATCC. Frozen stocks were streaked on blood agar and grown anaerobically for 5–7 days at 37°C. *F. nucleatum* was transferred to liquid culture (Mycoplasma Broth Base, supplemented with 1 mg/ml menadione and 5 mg/ml hemin) for cell wall preparation. Log phase organisms were collected by centrifugation at 4°C at 10,000 × g for 20 minutes. Pellets were washed once and resuspended in phosphate buffered saline (PBS). Cells were disrupted by passage 3× through a French pressure cell at 1000 psi. The cell walls were centrifuged and washed to remove debris. Protein concentration was determined by bicinchoninic acid assay (Pierce, Rockford, IL). *T. denticola* cell walls were prepared as follows. Log phase organisms were collected by centrifugation at 4°C, 10,000 × g for 20 minutes. Pellets were washed once and resuspended in phosphate buffered saline (PBS). Organisms were sonicated on ice 6×, 1 minute each, or until no intact treponemes were visible by darkfield microscopy. Sonicated material was collected by centrifugation at 4°C, 10,000 × g for 20 minutes; supernatant was then centrifuged at 4°C, 40,000 × g for 60 minutes and the pellet resuspended in PBS. Protein concentration was determined as for *F. nucleatum* cell walls.

Stimulation of PGEC

Cells at passage 3–6 were plated in 96 well plates (100 μ l of 1×10^5 cells/ml) or 24 well plates (250 μ l of 3×10^5 cells/ml) or 6 well plates (1 ml of 5×10^5 cells/ml) and incubated overnight at 37°C, 5% CO₂. Cells were washed once with Hank's balanced salt solution, and medium replaced with antibiotic-free KBM + BEGM supplements plus 1% normal human serum. Four-day log phase cultures of *T. denticola* (various strains) were pelleted at 10,000 $\times$ g for 10 minutes, 20°C, and washed once in PBS. To determine the optimal MOI of *T. denticola* to PGEC, dose responses were performed; an MOI of 100:1 was optimal (1000:1 resulted in PGEC toxicity; data not shown). FNCW protein (described above) or the synthetic lipopeptide (S)-(2,3-bis(palmitoyloxy)-(2RS)-propyl)-N-palmitoyl-(R)-Cys-(S)-Ser(S)-Lys₄-OH, trihydrochloride (PAM; EMD Biosciences, San Diego, CA) was added at a final concentration of 10 μ g per ml (lower concentrations of FNCW or PAM failed to induce IL-8 production from PGEC; data not shown). Cells were stimulated for 2–24 hours; 4 hours was determined to be optimal for collection of chemokine RNA, while 24 hours of stimulation was optimal for IL-8 protein detection (data not shown). Supernatants were removed and stored in low-binding polypropylene plates at –20°C for later analysis by ELISA. The proteolytic capability of *T. denticola* cells was assessed by monitoring degradation of recombinant IL-8 protein (R&D Systems, Minneapolis, MN) by ELISA. Dentilisin activity was confirmed by examining *T. denticola*-induced cleavage of a chromogenic target of chymotrypsin-like activity, succinyl-ala-ala-pro-phe-p-nitroanilide (SAAPFNA, Sigma Chemicals) (10, 12). The viability of treated epithelial cells was confirmed by trypan blue dye exclusion or Alamar Blue (Invitrogen) assay for metabolic activity (14).

Stimulation of HUVEC

Culture plates were coated with 50 μ g/ml rat tail collagen in 1 N acetic acid (Becton Dickinson, Franklin Lakes, NJ) for 1 hour and then washed 3 $\times$ with PBS. Cells at passage 3–6 were plated in 96 well plates (100 μ l of 1×10^5 cells/ml) or 24 well plates (250 μ l of 3×10^5 cells/ml) and incubated overnight at 37°C, 5% CO₂ before treatment as described above for PGEC.

ELISA

IL-8 was detected using a standard capture ELISA assay (11). Briefly, 96 well Maxisorp plates (Nunc, Rochester, NY) were coated overnight at 4°C with 1 μ g/ml monoclonal anti-human IL-8 (Pierce). Plates were blocked with 2% bovine serum albumin for 1 hour at RT, and then washed 1 $\times$ with PBS + 0.05% Tween 20. After addition of test supernatants, a second monoclonal biotinylated anti-human IL-8 (0.5 μ g/ml; Pierce) was added, followed by incubation at RT for 2 hours with shaking. Plates were washed 3 $\times$ with PBS + 0.05% Tween 20. Following incubation with streptavidin-conjugated horseradish peroxidase (Vectastain, Vector Laboratories, Burlingame, CA) for 1 hour at 37°C, plates were washed and IL-8 was indirectly detected after 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Sigma Chemicals). Concentration was determined by comparison to a standard curve (range: 2000 pg/ml to 31.3 pg/ml). IL-6 was detected using a capture ELISA kit (R&D Systems) according to the manufacturer's instructions. ICAM was detected using a capture ELISA

modified from (9). Briefly, stimulated PGEC (24 hours) were fixed with 0.5% glutaraldehyde for 1 hour at RT and blocked with PBS, 0.02M EDTA, and 3% goat serum for an additional hour. Monoclonal anti-human ICAM-1 (R&D Systems) was added at 0.25 µg/ml, and plates incubated with shaking for 1 hour at RT. Following washes (3 × with PBS + 0.05% Tween 20), ICAM-1 was detected indirectly after addition of anti-mouse IgG-horseradish peroxidase for 1 hour at 37°C (Jackson Immunochemicals, West Grove, PA) and TMB substrate as described above for the IL-8 ELISA.

Isolation and detection of PGEC mRNA

PGEC were incubated with a 100:1 multiplicity of infection (MOI) of intact *T. denticola* type strain 35404, FNCW (10 µg/ml protein), PAM (10 µg/ml protein), or no stimulus. PGEC were washed with Hank's balanced salt solution and collected in Ultraspec at 4 hours (Biotecx Laboratories, Houston, TX) for RNA isolation by the phenol/chloroform method (7). DNase-treated RNA was reverse transcribed into cDNA using Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). Constitutively transcribed hypoxanthine phosphoribosyltransferase (HPRT) message was used as a positive control for PCR. The primers used for amplification of HPRT, IL-8 and hβD-2 cDNA from stimulated and control cells were synthesized by Oligos, Etc. (Wilsonville, OR). IL-8 primers: sense: 5' CTCTCTTGGCAGCCTTCCT; antisense: 5' TGAATTCTCAGCCCTCTTCAA; HPRT primers: sense: 5' CCCTGCTGGATTACATCAAAG; antisense: 5' CGTCCAACACTTCGTGGGGTCCT; hβD-2 primers: sense: 5' TCAGCCATGAGGGTCTTGTA; antisense: 5' CTGATGAGGGAGCCCTTTCT. Real-time PCR was performed in a total volume of 20 µl in LightCycler (Roche, Indianapolis, IN) glass capillaries. For the PCR, 5 µl of cDNA were placed into a 20-µl reaction volume containing 5 µl of the sense primer (4 µM), 5 µl of the antisense primer (4 µM), 2.0 µl of the LightCycler Fast Start DNA Master Reaction Mix (10 × concentration of FastStart Taq DNA polymerase, reaction buffer, dNTP mix, SYBR Green I dye, and 10mM MgCl₂), 0.2 µl PCR-grade H₂O, and 2.8 µl 25mM MgCl₂. The PCR reaction was initiated with a 10-minute denaturation at 95°C and terminated with a 30 second cooling step at 40°C. The cycling protocol consisted of a denaturation step at 95°C for 10 s, annealing at 62°C for 5 s, and extension at 72°C for 10 s, and repeated for 45 cycles. Fluorescence detection was performed at the end of each annealing step.

For quantification, a calibration curve was obtained by using an external standard plasmid. To construct the plasmid, total RNA was prepared from FNCW stimulated PGEC, and cDNA synthesis was performed as described above. HPRT, IL-8 and hβD-2 were amplified using primers described above and the products cloned into TOPO II TA cloning vector (Invitrogen) and transformed into One Shot chemically competent *E. coli* (Invitrogen). Clones containing HPRT, IL-8 and hβD-2 inserts were identified by standard screening procedures and confirmed by restriction digestion and sequencing. Plasmid from 2 liters of *E. coli* containing the appropriate clone was isolated and purified using a Qiagen (Valencia, CA) plasmid mini-kit. Concentration of plasmid was determined spectrophotometrically, and copy number was determined using the formula $6 \times 10^{23} \text{ (copies/mol)} \times \text{concentration (g/µl)} / \text{MW of plasmid}$. Known amounts of plasmid (copies/µl) were used to generate optimal standard curves (mean squared error of 0.05 and a slope near -3.32 to ensure a

PCR efficiency near $2.0E= (10^{-1} \text{ slope})$ (30) for HPRT, IL-8, and h β D-2. An anchor point (10^3 copies) was run in triplicate in each LightCycler reaction as a reference. To adjust for differences in cDNA concentration between control and stimulated samples, copy number was normalized to HPRT by multiplication with a conversion factor, calculated as: control (no stimulus) HPRT copy number/stimulus HPRT copy number $\times 1000$.

Transfection of HEK293 cells and stimulation assay

Human embryonic kidney (HEK) 293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) with 10% heat-inactivated fetal calf serum (HyClone, Logan, Utah). The NF- κ B reporter construct (ELAM-1 firefly luciferase), the β -actin-*Renilla* luciferase reporter construct, the modified pDisplay expression vector, and the expression constructs for human TLR2 (phuTLR2), TLR1 (phuTLR1), and membrane-bound CD14 (phumCD14) have been described previously (16, 17). HEK 293 cells were transfected by calcium phosphate precipitation and exposed as described previously with the modifications reported for a 96-well plate assay format (16, 17). Cells were washed twice with medium 3 h after transfection and exposed 20 to 24 h posttransfection. Exposures to *T. denticola* cell walls (1–100 μ g/ml) or peptidoglycan from *Staphylococcus aureus* (Sigma; 1–100 μ g/ml) were performed in Dulbecco's modified Eagle's medium containing 10% human serum, for 4 h at 37°C. After exposure, cells were rinsed with phosphate-buffered saline (Invitrogen) and lysed with 50 μ l of passive lysis buffer (Promega, Madison, WI). Reporter gene expression in each lysate (10 μ l) was measured using the Dual Luciferase reporter assay system (Promega). Data are expressed as the fold increase in relative light units (which represents the ratio of ELAM-luciferase to β -actin *Renilla*-luciferase expression) relative to that of a no-stimulant medium control.

RESULTS

T. denticola does not induce IL-8 protein from PGEC

Many bacteria stimulate chemokine production, particularly IL-8, from epithelial cells. To establish whether *T. denticola* induces IL-8 production, PGEC were stimulated with 100:1 MOI *T. denticola* (several strains) or 10 μ g/ml FNCW in 1% normal human serum for up to 18 hours. Preliminary experiments suggested that higher inocula of *T. denticola* (e.g., 1000:1 MOI) were detrimental to epithelial cell viability, while lower inocula (e.g., 10:1, 100:1 MOI) did not affect epithelial cell viability. Maximum IL-8 accumulation in the culture supernate after FNCW stimulation occurred 12–18 hours post stimulation (data not shown). Supernatants from stimulated cells were collected for analysis by ELISA. PGEC responded to *F. nucleatum* cell walls by producing significant amounts of IL-8 protein ($p < 0.001$, Student's t-test, assuming unequal variances) compared to unstimulated cells; these data are represented in Figure 1 as % basal levels IL-8 where the baseline (unstimulated) value is 100%. In contrast, PGEC failed to produce IL-8 protein in response to *T. denticola* strains 35405, 35404, 33520, 33521, or GM-1. In addition, none of the tested strains of *T. denticola* significantly reduced the basal levels of IL-8 protein present in the epithelial cell supernatants (Figure 1, $p > 0.1$, Student's t-test, assuming unequal variances). The ability of PGEC to produce IL-8 in response to FNCW is not universally observed. In our experience, only 30% of PGEC isolates tested between passes 3 and 6 are responsive to FNCW. In

contrast, 70% of PGEC isolates produce IL-8 in response to PAM. Consequently, only those experiments in which the positive control (FNCW or PAM) resulted in IL-8 expression are included in our data. Epithelial cells were unresponsive to FNCW in the absence of serum, as epithelial cells lack surface expression of CD14. Epithelial cell viability during the stimulation period was not affected by incubation with *T. denticola*, as determined by trypan blue dye exclusion or Alamar Blue assay for metabolic activity (data not shown). Commercially available pooled primary normal human dermal epithelial keratinocytes (NHEK) showed a similar lack of IL-8 production in response to *T. denticola* (data not shown). Taken together, these results suggest that *T. denticola* does not induce production of IL-8 protein by primary human gingival epithelial cells.

To determine whether the apparent lack of IL-8 production in response to *T. denticola* exposure is due to proteolysis of secreted IL-8, we tested the K1 dentilisin-deficient mutant. As shown in Figure 2, K1, like its 35405 parent, failed to induce IL-8 protein. K1 failed to demonstrate dentilisin activity in the SAAPFNA assay, and did not degrade exogenous IL-8 protein in our assay, while the parent strain did demonstrate dentilisin activity in the SAAPFNA assay, did degrade IL-8, and this degradation could be inhibited by chymostatin (Figure 3, and data not shown). The inactivation of dentilisin does not affect the activities of other *T. denticola* peptidases, suggesting that dentilisin is solely responsible for IL-8 degradation by 35405 (36). These results suggest the apparent failure of *T. denticola* to induce production of IL-8 protein (described above) is not due to dentilisin-mediated IL-8 degradation.

To determine whether the observed unresponsiveness of PGEC to *T. denticola* is limited to IL-8 or represents more generalized unresponsiveness, we tested the ability of PGEC to secrete IL-6 protein or express surface-associated ICAM-1 following incubation with *T. denticola*. PGEC were stimulated as described previously, and supernatants (for IL-6) or fixed cells (for ICAM-1) were examined by ELISA. As shown in Figure 4A, strain 35404 (other strains were not tested) did not induce production of IL-6 by PGEC, while a TLR-2 agonist, PAM, induced modest but significant IL-6 secretion. In addition, *T. denticola* strains 35404, 35405 parent, 35405 K1, and GM-1 failed to induce production of ICAM-1 from PGEC, while exposure to PAM resulted in a significant increase in ICAM-1 (Figure 4B). To confirm that immortalized gingival epithelium responded to *T. denticola* as has been previously demonstrated (2, 11), we stimulated KB cells, a widely used oral epithelial cell line, with *T. denticola* strain 35405 (MOI 100:1) or *E. coli* LPS (1 µg/ml) in the presence of 1% human serum. At 24 hours post-stimulation, LPS induced IL-8 production 266% over the basal levels, and *T. denticola* induced IL-8 at 249% of the basal levels (N=5). To determine whether the observed unresponsiveness was specific to primary epithelial cells, we tested the ability of HUVEC and peripheral blood mononuclear cells (PBMC) to respond to *T. denticola* by producing IL-8. In general, these cell types are more responsive to bacterial stimulation, and produce more IL-8 than PGEC (compare FNCW stimulation, Figure 1 (PGEC) versus Figure 5 (HUVEC); (32). As seen with PGEC, HUVEC failed to produce IL-8 secretion in response to *T. denticola* strains 35404, 35405, GM-1 and 35405 K1 (Figure 5); although the production of IL-8 in response to K1 appears to be higher than baseline, this difference is not statistically significant. PBMC respond to stimulation with

spirochetal components by production of proinflammatory cytokines (32, 46, 50). PBMC produced considerable IL-8 in response to *T. denticola*: LPS positive control induced 251% over background IL-8 production; *T. denticola* induced 218% over background IL-8 production).

***T. denticola* does not induce IL-8 transcription from PGEC**

To ascertain whether *T. denticola* induces transcription of innate immune mediators, we examined the effects of stimulation by *T. denticola* on transcription of IL-8 mRNA. PGEC were stimulated with *T. denticola* 35404 or FNCW. At 4 hours post-stimulation, RNA was collected in Ultraspec and extracted by standard phenol-chloroform methods, followed by cDNA synthesis using random hexamers. In Figure 6A, quantitative real-time PCR was performed using an external standard plasmid containing the gene fragments of interest. *T. denticola* failed to induce IL-8 transcripts, while both FNCW- and PAM-stimulation induced transcription of IL-8 mRNA. We next tested the ability of PGEC to transcribe h β D-2 after *T. denticola* stimulation. As shown in Figure 6B, *T. denticola* did not induce transcription of h β D-2, while PAM stimulated defensin transcription. These results suggest that *T. denticola* fails to induce IL-8 or h β D-2 transcription in PGEC.

***T. denticola* engages TLR-2**

To ascertain whether *T. denticola* is recognized by the pattern recognition receptor TLR-2, HEK293 cells were transfected with human TLR-2 (hTLR-2), hTLR-1 and/or human, membrane-bound CD14 (hmCD14). Cells were subsequently stimulated with *T. denticola* cell walls at varying concentrations. Engagement of the TLR was detected by NF κ B translocation and resulting luciferase reporter activity. Peptidoglycan, a known TLR-2 ligand, served as a positive control. As shown in Figure 7, *T. denticola* cell walls signaled through hTLR2+1 in a dose dependent manner. hmCD14 was not necessary for activation but did enhance the response. Similar results were obtained with HEK293 cells transfected with murine TLR-2 (mTLR-2) and murine mCD14 (data not shown). These results suggest that TLR-2 is a pattern recognition receptor for *T. denticola*, and that inability to engage this receptor does not explain *T. denticola*'s failure to stimulate PGEC.

DISCUSSION

Dental plaque is an ecologically complex community consisting of over 400 species of microorganisms (44). The healthy dental plaque community is dominated by streptococci and other gram-positive organisms. If this community shifts to a mainly gram-negative anaerobic flora, an inflammatory response is initiated (36). The subsequent destruction of the epithelium, connective tissue, and resorption of bone can result in tooth loss. The gingival epithelium, by producing chemokines, antimicrobial agents, and leukocyte adhesion molecules, plays an important role in limiting the overgrowth of plaque organisms (52, 54).

T. denticola and other oral spirochetes make up a large percentage of the microflora in periodontal pockets (35). Although *T. denticola* lipoproteins and outer membrane extracts activate monocytic cells (19, 46), the success of *T. denticola* at colonizing inflamed sites in the mouth suggests an ability to escape immune surveillance at the level of the epithelial

cell. *T. denticola* are often found in close association with the periodontal pathogens *P. gingivalis* and *T. forsythia* ('red complex') (51). While periodontal pathogens like *Actinobacillus actinomycetemcomitans* induce IL-8 protein from epithelial cells, the two other members of the red complex (*T. forsythia* and *P. gingivalis*) fail to do so (3, 11, 23). We have now shown that *T. denticola* also fails to induce IL-8 protein from PGEC, and that this phenomenon is found across strains. An important distinction between our studies and previous investigations must be made: earlier studies employed cell lines rather than primary, low passage epithelial cells; changes in these epithelial cell lines during transformation and passage may alter the cell's response to microbial challenge. Two reports suggest the failure of epithelial cells to respond to *T. denticola* by producing IL-8 is due to degradation of IL-8 (2, 13). Our data with PGEC suggest that, for most strains of *T. denticola*, this effect is minor or irrelevant. Further, we have shown that the K1 protease mutant, which does not degrade IL-8 protein (13), also fails to induce IL-8 protein from PGEC, suggesting that another mechanism besides proteolytic degradation of IL-8 is involved. *T. denticola* has several proteases which could be involved in degradation of host molecules; however, inactivation of dentilisin has no effect on other peptidase activities in the K1 dentilisin knockout (39) and this mutant fails to degrade exogenous IL-8. However, there are other sources of IL-8 besides PGEC in the periodontal pocket; dentilisin is a potent protease and in vivo probably plays an important role in reducing IL-8 and subsequent neutrophil chemotaxis.

The lack of epithelial cell response to *T. denticola* is not limited to IL-8, as PGEC also fail to produce IL-6 or ICAM-1 protein following incubation with *T. denticola*. Epithelial cells obtained from sites in contact with commensal bacteria display a screening mechanism in that TLR-2 or TLR-4 ligands such as LTA and LPS do not elicit inflammatory mediator secretion (1, 20, 38, 55). However, PGEC used in this study responded to known TLR-2 agonists, and TLR-2 surface expression on PGEC has been demonstrated previously (31, 53). HUVEC also fail to respond to *T. denticola* by producing IL-8, which implies a common 'blindness' of non-immune cells to this bacterium. Our results with HUVECs are in disagreement with a recent report from Okuda et al. In that study, *T. denticola* strain 35405 induced IL-8 protein from endothelial cells, while the K1 mutant failed to induce IL-8 protein (43). In our studies, both K1 and its isogenic parent 35405 failed to induce IL-8 protein (Figure 5). While our experimental designs were similar, the sources of HUVECs and their culture media were different, which may explain the discrepancy.

T. denticola does not induce IL-8 transcription compared to FNCW and also fails to induce h β D-2 transcription, while defensin transcripts are upregulated in response to PAM and other stimuli (8, 28, 29). Both IL-8 and h β D-2 transcription can be mediated by the transcription factor NF κ B (25, 45); it is possible that *T. denticola* fails to activate NF κ B and its translocation to the nucleus. However, TDCW are capable of activating TLR-2 and NF κ B, suggesting that transcription of IL-8 or h β D-2 in response to *T. denticola* stimulation is blocked downstream of NF κ B. Maximal IL-8 expression, for example, is dependent on activation of multiple signaling pathways, transcription factors, and post-transcriptional controls (45). Activation of the MAP kinase p38 is necessary for stabilization of the IL-8 transcript, and blocking its activity results in rapid degradation of the mRNA (57). It is

possible that *T. denticola* either interferes with or fails to induce one of the signaling pathways other than TLR/NF κ B that are necessary for maximal transcription of IL-8 or h β D-2. In addition, there are numerous examples of bacterial suppression of chemokine expression. Inhibition of I κ B- α ubiquitination, downregulation of pattern-recognition receptors or activation of anti-inflammatory signaling pathways (21, 33, 40) are also mechanisms by which other bacteria affect cell responses and may be relevant to *T. denticola*. We used *T. denticola* cell walls to stimulate TLR-2 activation; it is possible that live *T. denticola* would fail to activate TLR-2, perhaps by degradation of TLR-2 or its co-receptors, although there is no direct evidence for this activity at present.

In summary, *T. denticola* does not induce production of common innate immune mediators from primary gingival epithelial cells. The ability of *T. denticola* to prevent the epithelium from producing chemotactic factors may be a necessary adaptation, as oral spirochetes are frequently found in close association with the gingival epithelium (34, 47). Interestingly, *T. denticola* and other oral treponemes are relatively resistant to killing by epithelium-produced β -defensins (5, 6). This insensitivity to these antimicrobial peptides, in addition to the unresponsiveness of the gingival epithelium to *T. denticola*, may help explain this organism's prominence in periodontal disease.

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Figure 1. *T. denticola* does not induce production of IL-8 protein from PGEC

1×10^5 PGEC were incubated with 100:1 MOI *T. denticola*, 10 $\mu\text{g/ml}$ FNCW or 10 $\mu\text{g/ml}$ PAM for 24 hours at 37°C. Culture supernates were assayed for IL-8 protein by ELISA. % Basal IL-8 is expressed as (amount IL-8 produced in response to stimulus/amount IL-8 produced in absence of stimulus) $\times 100$. Data represent the mean and standard error from 4 or more experiments with 6 replicates per experiment; *** = $p < 0.001$ compared to unstimulated, Student's t-test assuming unequal variances on the raw data. This figure shows the results as % basal (unstimulated) levels, with the basal IL-8 level adjusted to 100%.

Figure 2. *T. denticola* 35405 dentilisin mutant does not induce production of IL-8 protein
 1×10^5 PGEC were incubated with 100:1 MOI *T. denticola*, 10 $\mu\text{g/ml}$ FNCW or 10 $\mu\text{g/ml}$ PAM for 24 hours at 37°C. Culture supernates were assayed for IL-8 protein by ELISA. % Basal IL-8 is expressed as (amount IL-8 produced in response to stimulus/amount IL-8 produced in absence of stimulus) $\times 100$. Data represent the mean and standard error from 4 or more experiments with 6 replicates per experiment; *** = $p < 0.001$ compared to unstimulated, Student's t-test assuming unequal variances on the raw data. This figure shows the results as % basal (unstimulated) levels, with basal IL-8 level adjusted to 100%.

Figure 3. *T. denticola* dentilisin mutant, K1, does not degrade IL-8 protein

25 ng/ml human recombinant IL-8 was incubated in preblocked polypropylene tubes with or without 3×10^7 *T. denticola* 35405 parent or K1 mutant or 5 µg/ml chymostatin (CHY) at 37°C overnight. Proteinase K (PK) was added to separate tubes at 25 ng/ml as a positive control for IL-8 degradation. Proteases were inactivated with Roche Complete protease inhibitor cocktail according to manufacturer's instructions. IL-8 was measured by capture ELISA. Data represent the means and standard errors from 5 experiments with 2 replicates per experiment. * = $p < 0.05$ compared to 35405+ IL-8 in the absence of chymostatin, Student's t-test assuming unequal variances.

Figure 4. *T. denticola* does not induce production of IL-6 or ICAM-1 protein by PGEC
 1×10^5 PGEC were incubated with 100:1 MOI *T. denticola* or 10 μ g/ml PAM for 24 hours at 37°C. Culture supernates were assayed for IL-6 protein by ELISA, while intact PGEC monolayers were assayed for ICAM-1. % Basal protein is expressed as (amount mediator produced in response to stimulus/amount mediator produced in absence of stimulus) $\times$ 100. Data represent the mean and standard error from 4 or more experiments with 6 replicates per experiment; * = $p < 0.05$ compared to unstimulated, Student's t-test assuming unequal variances on the raw data. This figure shows the results as % basal (unstimulated) levels with basal IL-6 and ICAM-1 levels adjusted to 100%.

Figure 5. *T. denticola* does not induce IL-8 protein from HUVEC

5×10^5 HUVEC were incubated with 100:1 MOI *T. denticola* or 10 $\mu\text{g/ml}$ FNCW for 24 hours at 37°C. % Basal IL-8 is expressed as (amount IL-8 produced in response to stimulus/ amount IL-8 produced in absence of stimulus) $\times 100$. Data represent the mean and standard error from 4 or more experiments with 6 replicates per experiment; * = $p < 0.05$ compared to unstimulated, Student's t-test assuming unequal variances on the raw data. This figure shows the results as % basal (unstimulated) levels, with basal IL-8 level adjusted to 100%.

A.

B.

Figure 6. *T. denticola* does not induce IL-8 or hβD-2 transcription in PGEC

1×10^5 PGEC were incubated with 100:1 MOI *T. denticola*, 10 $\mu\text{g/ml}$ FNCW or 10 $\mu\text{g/ml}$ PAM, for 4 hours at 37°C. RNA was isolated from PGEC, cDNA synthesized, and transcripts were detected by real-time RT-PCR. Data are expressed as percent basal IL-8 transcription (A) or percent basal hβD-2 transcription (B) in the absence of stimulus and represent the mean and standard error from three experiments with three replicates. * = $p < 0.05$, Student's t-test assuming unequal variances on the raw data. This figure shows the results as % basal (unstimulated) levels, with basal levels adjusted to 100%.

Figure 7. *T. denticola* activation of TLR-2

HEK 293 cells were transiently transfected with human TLR-2 + 1 with or without mCD14, and exposed to varying concentration of *T. denticola* cell walls (TDCW) or peptidoglycan. Activation of TLR-2 and subsequent translocation of NFκB translocation was measured by luciferase activity. Results represent the means (fold increase over unstimulated) and standard errors from 2 experiments done in triplicate; *** = $p < 0.01$, Student's t-test assuming unequal variances.