Oral Microbiology Immunology 2008: 23: 474–481
Printed in Singapore. All rights reserved

© 2008 The Authors.
Journal compilation © 2008 Blackwell Munksgaard

ORAL MICROBIOLOGY
AND IMMUNOLOGY

Treponema denticola does not induce production of common innate immune mediators from primary gingival epithelial cells

C. A. Brissette[1,2,†], T.-T. T. Pham[2,3], S. R. Coats[2,3], R. P. Darveau[2,3], S. A. Lukehart[1,2,4]

Departments of [1]Pathobiology, [2]Oral Biology, [3]Periodontics, [4]Medicine, University of Washington, Seattle, WA, USA

Brissette CA, Pham T-TT, Coats SR, Darveau RP, Lukehart SA. Treponema denticola *does not induce production of common innate immune mediators from primary gingival epithelial cells.*
Oral Microbiol Immunol 2008: 23: 474–481. © 2008 The Authors. Journal compilation © 2008 Blackwell Munksgaard.

It has been hypothesized that the neutrophil chemoattractant interleukin-8 (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 the 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, because a protease mutant that does not degrade IL-8 does not induce IL-8 production with these stimuli either. The lack of innate immune mediator production may be a more global phenomenon because *T. denticola* fails to induce IL-6 or intercellular adhesion molecule 1 production from PGEC. *T. denticola* also fails to induce transcription of IL-8 and human β-defensin-2 messenger RNA. The lack of immune mediator production is not explained by the failure of *T. denticola* to interact with Toll-like receptor 2 (TLR-2), as *T. denticola* stimulates nuclear factor-κB nuclear translocation in TLR-2-transfected HEK293 cells. Not only can *T. denticola* degrade the IL-8 present in the periodontal lesion, but this organism also fails to induce IL-8 production by PGEC. The lack of an epithelial cell response to *T. denticola* may contribute to the pathogenesis of periodontitis by failing to trigger chemotaxis of neutrophils into the periodontal pocket.

Key words: epithelial cells; IL-8; innate immunity; periodontitis; Treponema

Sheila A. Lukehart, Department of Medicine, Box 359779, Harborview Medical Center, 325 Ninth Ave, Seattle, WA 98104, USA
Tel.: +1 206 897 5362;
fax: +1 206 897 5363;
e-mail: lukehart@u.washington.edu

Accepted for publication February 28, 2008

[†]Present address: Department of Microbiology, Immunology, and Molecular Genetics, University of Kentucky, Lexington, KY, USA

Periodontal disease is remarkably widespread, afflicting 50% of adults in the USA (36, 55), with severe disease affecting 6 million Americans. The ability of primary gingival epithelial cells (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 produce chemical mediators that recruit phagocytes and antigen-presenting cells to sites of bacterial attachment. 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 messenger RNA (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 production 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 (50). 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 the 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 the host's innate immune defenses, including the ability to inhibit cytokine production, evasion of complement, and modified outer surface molecules such as lipopolysaccharide (LPS) that are poorly recognized by 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 *Treponema pallidum* subsp. *pallidum*, and *Borrelia burgdorferi* readily activate macrophages to produce IL-8 and other cytokines (32, 49). The major outer surface protein of the oral treponemes *Treponema maltophilum* and *Treponema lecithinolyticum* upregulates production of ICAM-1 and proinflammatory cytokines in monocytic cells (29). *T. denticola* also activates macrophages, but little is known about its effects 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 nuclear factor-κB (NF-κB) 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 from PGEC. In addition, *T. denticola* does not induce transcription of IL-8 and human β-defensin-2 (hβD-2). This lack of responsiveness is not limited to PGEC; 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 (a 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

PGEC were provided by the Comprehensive Center for Oral Health Research (University of Washington; Director, Beverly Dale). Cells were isolated from excess tissue taken from retromolar extractions of consenting healthy adult donors, as approved by the 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 were obtained from Cambrex (Walkersville, MD). All epithelial cells were maintained in keratinocyte basal medium supplemented with bronchial epithelium growth supplements and 0.03 mM Ca^{2+} (Cambrex), and cells were used between passages 3 and 6. 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 whole blood was drawn into a syringe containing 5000 units heparin and leukocytes were isolated by Ficoll gradient centrifugation according to the manufacturer's instructions (GE Health Care, Piscataway, NJ). Cells were cultured in complete RPMI-1640 (Invitrogen, Carlsbad, CA) plus heat-inactivated, pooled AB human serum (Sigma Chemicals) and stimulated after 48 h as described below for PGEC.

Cell wall preparation

F. nucleatum ATCC 25586 was purchased from the American Type Culture Collection (ATCC, Manassas, VA). 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 min. Pellets were washed once and resuspended in phosphate-buffered saline (PBS). Cells were disrupted by three passages 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 min. Pellets were washed once and resuspended in PBS. Organisms were sonicated on ice six times, for 1 min each time, or until no intact treponemes were visible on dark-field microscopy. Sonicated material was collected by centrifugation at 4°C, 10,000 ***g*** for 20 min; supernatant was then centrifuged at 4°C, 40,000 ***g*** for 60 min and the pellet was resuspended in PBS. Protein concentration was determined as for the *F. nucleatum* cell walls.

Stimulation of PGEC

Cells at passage 3 to passage 6 were plated in 96-well plates (100 μl of 1×10^5 cells/ml) or 24-well plates (250 μl of 3×10^5 cells/ml) or six-well plates (1 ml of 5×10^5 cells/ml) and incubated overnight at 37°C in 5% CO_2. Cells were washed once with Hanks' balanced salt solution, and the medium was replaced with antibiotic-free keratinocyte basal medium supplemented with bronchial epithelium growth supplements plus 1% normal human serum. Four-day log-phase cultures of *T. denticola* (various strains) were pelleted at 10,000 ***g*** for 10 min at 20°C, and washed once in PBS. To determine the optimal multiplicity of infection (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). *F. nucleatum* cell wall protein (FNCW; described above) or the synthetic lipopeptide (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride (PAM; EMD Biosciences, San Diego, CA) was added at a final concentration of 10 μg/ml (lower concentrations of FNCW or PAM failed to induce IL-8 production from PGEC; data not shown). Cells were stimulated for

2–24 h; 4 h was determined to be optimal for collection of chemokine RNA, while 24 h 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 enzyme-linked immunosorbent assay (ELISA). The proteolytic capability of *T. denticola* cells was assessed by monitoring the 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 μg/ml rat tail collagen in 1 M acetic acid (Becton Dickinson, Franklin Lakes, NJ) for 1 h and then washed three times with PBS. Cells at passage 3 to 6 were plated in 96-well plates (100 μl of 1×10^5 cells/ml) or 24-well plates (250 μl of 3×10^5 cells/ml) and incubated overnight at 37°C in 5% CO_2 before treatment as described above for PGEC.

ELISA

IL-8 was detected using a standard capture ELISA (11). Briefly, 96-well Maxisorp plates (Nunc, Rochester, NY) were coated overnight at 4°C with 1 μg/ml monoclonal anti-human IL-8 (Pierce). Plates were blocked with 2% bovine serum albumin for 1 h at room temperature, and then washed once with PBS + 0.05% Tween-20. After the addition of test supernatants, a second monoclonal biotinylated anti-human IL-8 (0.5 μg/ml; Pierce) was added, followed by incubation at room temperature for 2 h with shaking. Plates were washed three times with PBS + 0.05% Tween-20. Following incubation with strepavidin-conjugated horseradish peroxidase (Vectastain, Vector Laboratories, Burlingame, CA) for 1 h at 37°C, plates were washed and IL-8 was indirectly detected after 3,3,5′5-tetramethylbenzidine 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 ref. (9). Briefly, stimulated PGEC (24 h) were fixed with 0.5% gluteraldehyde for 1 h at room temperature and blocked with PBS, 0.02 M ethylenediaminetetraacetic acid, 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 were incubated with shaking for 1 h at room temperature. Following three washes with PBS plus 0.05% Tween-20, ICAM-1 was detected indirectly after addition of anti-mouse immunoglobulin G–horseradish peroxidase for 1 h at 37°C (Jackson Immunochemicals, West Grove, PA) and 3,3,5′5-tetramethylbenzidine substrate as described above for the IL-8 ELISA.

Isolation and detection of PGEC mRNA

PGEC were incubated with a 100 : 1 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 Hanks' balanced salt solution and collected in Ultraspec (Biotecx Laboratories, Houston, TX) at 4 h for RNA isolation by the phenol/chloroform method (7). DNase-treated RNA was reverse transcribed into cDNA using Superscript First-Strand Synthesis System for reverse transcription–polymerase chain reaction (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′-CCC TGCTGGATTACATCAAAG; antisense: 5′-CGTCCAACACTTCGTGGGGTCCT; hβD-2 primers: sense: 5′-TCAGCCATGA GGGTCTTGTA; antisense: 5′-CTGATGA GGGAGCCCTTTCT.

Real-time PCR was performed in a total volume of 20 μl in LightCycler (Roche, Indianapolis, IN) glass capillaries. For the PCR, 5 μl cDNA was 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 10 mM $MgCl_2$), 0.2 μl PCR-grade H_2O, and 2.8 μl 25 mM $MgCl_2$. The PCR was initiated with a 10-min denaturation at 95°C and terminated with a 30-s 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, which was repeated for 45 cycles. Fluorescence detection was performed at the end of each annealing step.

For quantification, a calibration curve was obtained 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 were cloned into TOPO II TA cloning vector (Invitrogen) and transformed into One Shot chemically competent *Escherichia 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 6×10^{23} (copies/mol) × concentration (g/μl)/molecular weight of plasmid. Known amounts of plasmid (copies/μl) were used to generate optimal standard curves (mean squared error of ≤ 0.05 and a slope of about −3.32 to ensure a PCR efficiency near $2.0E = 10^{-1}$ slope) (30) for HPRT, IL-8, and hβD-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 × 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, UT). The NF-κB reporter construct (ELAM-1 firefly luciferase), the β-actin-*Renilla* luciferase reporter construct, the modified pDisplay expression vector, and the expression constructs for human TLR-2 (phuTLR-2), TLR-1 (phuTLR-1), and membrane-bound CD14 (phumCD14)

have been described previously (16, 17). HEK293 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 20–24 h posttransfection. Exposures to *T. denticola* cell walls (1–100 μg/ml) or peptidoglycan from *Staphylococcus aureus* (Sigma; 1–100 μg/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 PBS (Invitrogen) and lyzed with 50 μl passive lysis buffer (Promega, Madison, WI). Reporter gene expression in each lysate (10 μl) 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 of *T. denticola* (several strains) or 10 μg/ml FNCW in 1% normal human serum for up to 18 h. 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 supernatant after FNCW stimulation occurred 12–18 h post-stimulation (data not shown). Supernatants from stimulated cells were collected for analysis by ELISA. PGEC responded to FNCW by producing significant amounts of IL-8 ($P < 0.001$, Student's *t*-test, assuming unequal variances) compared to unstimulated cells; these data are represented in Fig. 1 as % basal levels of IL-8 where the baseline (unstimulated) value is 100%. In contrast, PGEC failed to produce IL-8 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 present in the epithelial cell supernatants (Fig. 1, $P > 0.1$, Student's *t*-test, assuming unequal variances). The

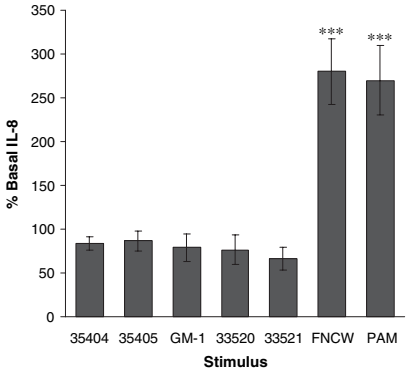

Fig. 1. Treponema denticola does not induce production of interleukin-8 (IL-8) from primary gingival epithelial cells (PGEC): 1×10^5 PGEC were incubated with 100 : 1 multiplicity of infection of *T. denticola*, 10 μg/ml *Fusobacterium nucleatum* cell wall protein (FNCW) or 10 μg/ml (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride (PAM) for 24 h at 37°C. Culture supernatants were assayed for IL-8 by enzyme-linked immunosorbent assay and % basal IL-8 was expressed as (amount IL-8 produced in response to stimulus/amount IL-8 produced in absence of stimulus) × 100. Data represent the mean and standard error from four or more experiments with six 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%.

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 passages 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 IL-8 production by human PGEC.

To determine whether the apparent lack of IL-8 production in response to *T. denticola* exposure is the result of proteolysis of secreted IL-8, we tested the K1 dentilisin-deficient mutant. As shown

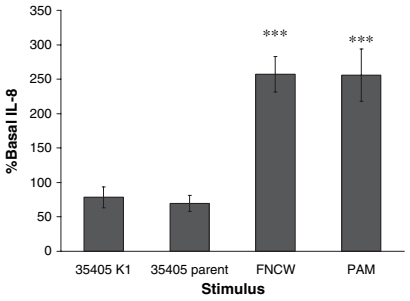

Fig. 2. The *Treponema denticola* 35405 dentilisin mutant does not induce production of interleukin-8 (IL-8): 1×10^5 primary gingival epithelial cells were incubated with 100 : 1 multiplicity of infection of *T. denticola*, 10 μg/ml *Fusobacterium nucleatum* cell wall protein (FNCW) or 10 μg/ml (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride (PAM) for 24 h at 37°C. Culture supernatants were assayed for IL-8 by enzyme-linked immunosorbent assay and % basal IL-8 was expressed as (amount IL-8 produced in response to stimulus/amount IL-8 produced in absence of stimulus) × 100. Data represent the mean and standard error from four or more experiments with six 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%.

in Fig. 2, K1, like its 35405 parent, failed to induce IL-8 production. K1 failed to demonstrate dentilisin activity in the SAAPFNA assay, and did not degrade exogenous IL-8 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 (Fig. 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 that the apparent failure of *T. denticola* to induce production of IL-8 (described above) is not the result of dentilisin-mediated IL-8 degradation.

To determine whether the observed unresponsiveness of PGEC to *T. denticola* is limited to IL-8 or represents a more generalized unresponsiveness, we tested the ability of PGEC to secrete IL-6 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 Fig. 4A, strain 35404 (other strains were not tested) did not induce production of IL-6 by PGEC, while a TLR-2 agonist, PAM, induced

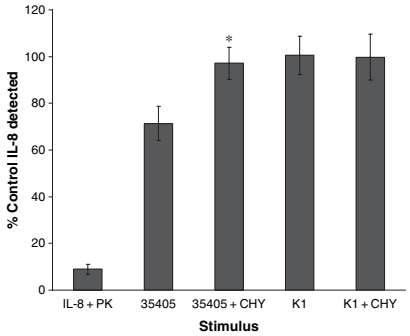

Fig. 3. Treponema denticola dentilisin mutant, K1, does not degrade interleukin-8 (IL-8): 25 ng/ml human recombinant IL-8 was incubated in preblocked polypropylene tubes with or without 3×10^7 *T. denticola* 35405 parent or K1 mutant or 5 μg/ml chymostatin (CHY) at 37°C overnight. Proteinase K (PK) was 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 the manufacturer's instructions. IL-8 was measured by capture enzyme-linked immunosorbent assay. Data represent the means and standard errors from five experiments with two replicates per experiment. $^*P < 0.05$ compared to 35405 parent strain IL-8 in the absence of chymostatin, Student's *t*-test assuming unequal variances.

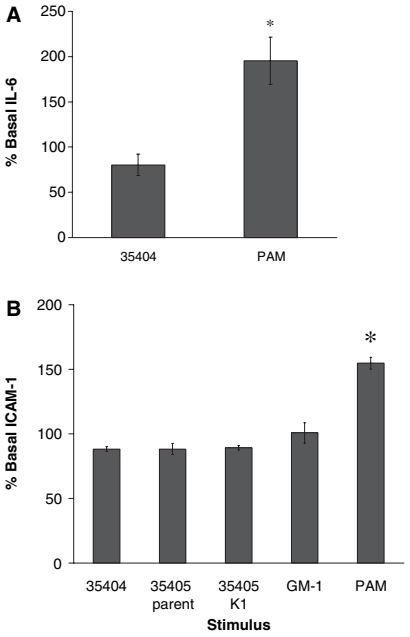

Fig. 4. Treponema denticola does not induce production of interleukin-6 (IL-6) or intercellular adhesion molecule 1 (ICAM-1) by primary gingival epithelial cells (PGEC): 1×10^5 PGEC were incubated with 100 : 1 multiplicity of infection of *T. denticola* or 10 μg/ml (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride (PAM)for 24 h at 37°C. Culture supernatants were assayed for IL-6 protein by enzyme-linked immunosorbent assay, 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) × 100. Data represent the mean and standard error from four or more experiments with six 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%.

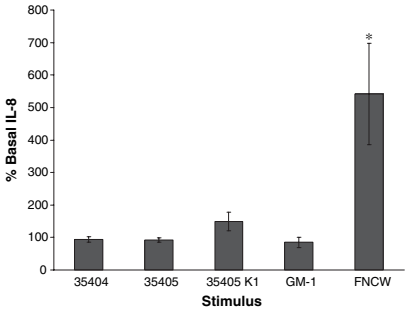

Fig. 5. Treponema denticola does not induce interleukin-8 (IL-8) from human umbilical vein endothelial cells (HUVEC): 5×10^5 HUVEC were incubated with 100 : 1 multiplicity of infection of *T. denticola* or 10 μg/ml *Fusobacterium nucleatum* cell wall protein (FNCW) for 24 h at 37°C. % Basal IL-8 is expressed as (amount IL-8 produced in response to stimulus/amount IL-8 produced in absence of stimulus) × 100. Data represent the mean and standard error from four or more experiments with six 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%.

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 by PGEC, while exposure to PAM resulted in a significant increase in ICAM-1 production (Fig. 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 h poststimulation, LPS induced IL-8 production 266% over the basal levels, and *T. denticola* induced IL-8 at 249% over the basal levels ($n = 5$). To determine whether the observed unresponsiveness was specific to primary epithelial cells, we tested the ability of HUVEC and PBMC to respond to *T. denticola* by producing IL-8. In general, these cell types were more responsive to bacterial stimulation, and produce more IL-8 than PGEC [compare FNCW stimulation, Fig. 1 (PGEC) with Fig. 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 (Fig. 5); although the production of IL-8 in response to K1 appeared to be higher than baseline, this difference was not statistically significant. PBMC respond to stimulation with spirochetal components by production of proinflammatory cytokines (32, 46, 49). The PBMC produced considerable IL-8 in response to *T. denticola*: the LPS-positive control induced IL-8 production that was 251% over background; *T. denticola* induced IL-8 production that was 218% over background.

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 h poststimulation, RNA was collected in Ultraspec and extracted using standard phenol–chloroform methods, followed by cDNA synthesis using random hexamers. In Fig. 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 stimulation and PAM stimulation induced transcription of IL-8 mRNA. We next tested the ability of PGEC to transcribe hβD-2 after *T. denticola* stimulation. As shown in Fig. 6B, *T. denticola* did not induce transcription of hβD-2, while PAM stimulated defensin transcription. These results suggest that *T. denticola* fails to induce IL-8 or hβD-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-κB translocation and resulting luciferase reporter activity. Peptidoglycan, a known TLR-2 ligand, served as a positive control. As shown in Fig. 7, *T. denticola* cell walls signaled through human TLR-2 and TLR-1 in a dose-dependent manner. hmCD14 was not necessary for activation but did

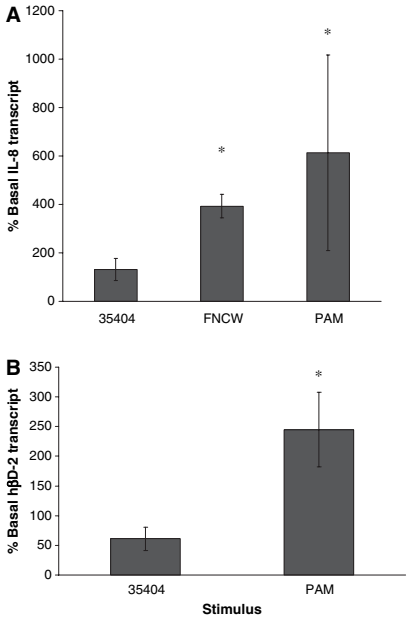

Fig. 6. Treponema denticola does not induce interleukin-8 (IL-8) or human β-defensin-2 (hβD-2) transcription in primary gingival epithelial cells (PGEC): 1×10^5 PGEC were incubated with 100 : 1 multiplicity of infection (MOI) *T. denticola*, 10 μg/ml *Fusobacterium nucleatum* cell wall protein (FNCW) or 10 μg/ml (*S*)-(2,3-bis(palmitoyloxy)-(2*RS*)-propyl)-*N*-palmitoyl-(*R*)-Cys-(*S*)-Ser(*S*)-Lys_4-OH, trihydrochloride (PAM), for 4 h at 37°C. RNA was isolated from PGEC, cDNA synthesized, and transcripts were detected by real-time reverse transcription–polymerase chain reaction (C). Data are expressed as % basal IL-8 transcription (A) or % 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%.

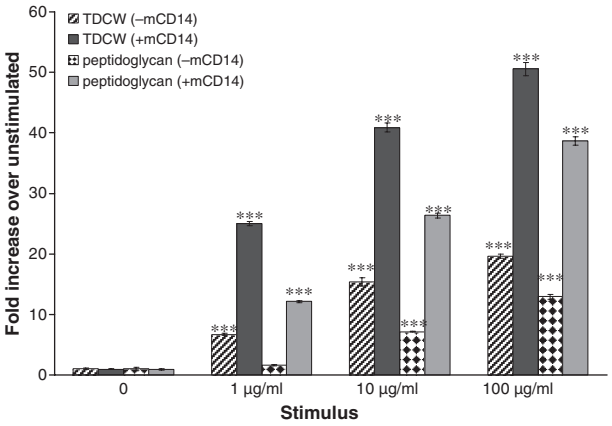

Fig. 7. Treponema denticola activation of Toll-like receptor-2 (TLR-2). HEK293 cells were transiently transfected with human TLR-2 + 1 with or without mCD14, and were exposed to varying concentration of *T. denticola* cell walls (TDCW) or peptidoglycan. Activation of TLR-2 and subsequent translocation of nuclear factor-κB was measured by luciferase activity. Results represent the means (fold increase over unstimulated) and standard errors from two experiments performed in triplicate; $***P < 0.01$, Student's *t*-test assuming unequal variances.

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 the failure of *T. denticola* 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 (51, 53).

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* (the 'red complex') (50). While periodontal pathogens like *Actinobacillus actinomycetemcomitans* induce IL-8 production by 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 production by 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 cells' response to microbial challenge. Two reports suggest that the failure of epithelial cells to respond to *T. denticola* by producing IL-8 is the result of 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 from PGEC, suggesting that another mechanism besides proteolytic degradation of IL-8 is involved. *T. denticola* has several proteases which could be involved in the degradation of host molecules; however, inactivation of dentilisin has no effect on other peptidase activities in the K1 dentilisin knockout strain (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 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 lipoteichoic acid and LPS do not elicit inflammatory mediator secretion (1, 20, 38, 54). However, PGEC used in this study responded to known TLR-2 agonists, and TLR-2 surface expression on PGEC has been demonstrated previously (31, 52). 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 disagree 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 production (43). In our studies, both K1 and its isogenic parent 35405 failed to induce IL-8 production (Fig. 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 hβD-2 transcription, while defensin transcripts are upregulated in response to PAM and other stimuli (8, 28, 29). Both IL-8 and hβD-2 transcription can be mediated by the transcription factor NF-κB (25, 45); it is possible that *T. denticola* fails to activate NF-κB and its translocation to the nucleus. However, TDCW are capable of activating TLR-2 and NF-κB, suggesting that transcription of IL-8 or hβD-2 in response to *T. denticola* stimulation is blocked downstream of NF-κB. Maximal IL-8 expression, for example, is dependent on the activation of multiple signaling pathways, transcription factors, and posttranscriptional controls (45). Activation of the mitogen-activated protein 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-κB that are necessary for maximal transcription of IL-8 or hβD-2. In addition, there are numerous examples of bacterial suppression of chemokine expression. Inhibition of IκB-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 they 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 PGEC. The ability of *T. denticola* to prevent the epithelium from producing chemotactic factors may be a necessary adaptation because 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 to explain this organism's prominence in periodontal disease.

Acknowledgments

The authors thank Heidi Pecoraro and Miko Robertson for manuscript preparation. C.A.B. was supported by an NIDCR Training Grant DE07023 and Public Health Service grant DE015354 (to S.A.L.) from the National Institutes of Dental and Craniofacial Research.

References

1. Abreu MT, Vora P, Faure E, Thomas LS, Arnold ET, Arditi M. Decreased expression of toll-like receptor 4 and MD-2 correlates with intestinal epithelial cell protection against dysregulated proinflammatory gene expression in response to bacterial lipopolysaccharide. J Immunol 2001: **167**: 1609–1616.
2. Asai Y, Jinno T, Ogawa T. Oral treponemes and their outer membrane extracts activate human gingival epithelial cells through toll-like receptor 2. Infect Immun 2003: **71**: 717–725.
3. Bainbridge B, Braham P, Darveau R. Characterization of *Tannerella forsythensis* lipopolysaccharide. Abstract 3484 in IADR/AADR/CADR 83rd General Session, Baltimore, MD: British Dental Association, 2005. http://iadr.confex.com/iadr/2005Balt/techprogram/abstract_61035.htm.
4. Blakemore RP, Canale-Parola E. Arginine catabolism by *Treponema denticola*. J Bacteriol 1976: **128**: 616–622.
5. Brissette CA, Lukehart SA. *Treponema denticola* is resistant to human beta-defensins. Infect Immun 2002: **70**: 3982–3984.
6. Brissette CA, Simonson LG, Lukehart SA. Resistance to human beta-defensins is common among oral treponemes. Oral Microbiol Immunol 2004: **19**: 403–407.
7. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal Biochem 1987: **162**: 156–159.
8. Chung WO, Dale BA. Innate immune response of oral and foreskin keratinocytes: utilization of different signaling pathways by various bacterial species. Infect Immun 2004: **72**: 352–358.
9. Coats SR, Reife RA, Bainbridge BW, Pham TT, Darveau RP. *Porphyromonas gingivalis* lipopolysaccharide antagonizes *Escherichia coli* lipopolysaccharide at toll-like receptor 4 in human endothelial cells. Infect Immun 2003: **71**: 6799–6807.
10. Correia FF, Plummer AR, Ellen RP et al. Two paralogous families of a two-gene subtilisin operon are widely distributed in oral treponemes. J Bacteriol 2003: **185**: 6860–6869.
11. Darveau RP, Belton CM, Reife RA, Lamont RJ. Local chemokine paralysis, a novel pathogenic mechanism for *Porphyromonas gingivalis*. Infect Immun 1998: **66**: 1660–1665.
12. DelMar EG, Largman C, Brodrick JW, Geokas MC. A sensitive new substrate for chymotrypsin. Anal Biochem 1979: **99**: 316–320.
13. Deng QD, Han Y, Xia X, Kuramitsu HK. Effects of the oral spirochete *Treponema denticola* on interleukin-8 expression from epithelial cells. Oral Microbiol Immunol 2001: **16**: 185–187.
14. Franzblau SG, Witzig RS, McLaughlin JC et al. Rapid, low-technology MIC determination with clinical *Mycobacterium tuberculosis* isolates by using the microplate Alamar Blue assay. J Clin Microbiol 1998: **36**: 362–366.
15. Guo L, Lim KB, Gunn JS et al. Regulation of lipid A modifications by *Salmonella typhimurium* virulence genes *phoP-phoQ*. Science 1997: **276**: 250–253.
16. Hajjar AM, O'Mahony DS, Ozinsky A et al. Cutting edge: functional interactions between toll-like receptor (TLR) 2 and TLR1 or TLR6 in response to phenol-soluble modulin. J Immunol 2001: **166**: 15–19.
17. Hajjar AM, Ernst RK, Tsai JH, Wilson CB, Miller SI. Human Toll-like receptor 4 recognizes host-specific LPS modifications. Nat Immunol 2002: **25**: 25.
18. Han YW, Shi W, Huang GT et al. Interactions between periodontal bacteria and human oral epithelial cells: *Fusobacterium nucleatum* adheres to and invades epithelial cells. Infect Immun 2000: **68**: 3140–3146.
19. Hashimoto M, Asai Y, Ogawa T. Treponemal phospholipids inhibit innate immune responses induced by pathogen-associated molecular patterns. J Biol Chem 2003: **278**: 44205–44213.
20. Hedlund M, Wachtler C, Johansson E et al. P fimbriae-dependent, lipopolysaccharide-independent activation of epithelial cytokine responses. Mol Microbiol 1999: **33**: 693–703.
21. Huang FC, Li Q, Cherayil BJ. A phosphatidyl-inositol-3-kinase-dependent anti-inflammatory pathway activated by *Salmonella* in epithelial cells. FEMS Microbiol Lett 2005: **243**: 265–270.
22. Huang GT, Haake SK, Kim JW, Park NH. Differential expression of interleukin-8 and intercellular adhesion molecule-1 by human gingival epithelial cells in response to *Actinobacillus actinomycetemcomitans* or *Porphyromonas gingivalis* infection. Oral Microbiol Immunol 1998: **13**: 301–309.
23. Huang GT, Kim D, Lee JK, Kuramitsu HK, Haake SK. Interleukin-8 and intercellular adhesion molecule 1 regulation in oral epithelial cells by selected periodontal bacteria: multiple effects of *Porphyromonas gingivalis* via antagonistic mechanisms. Infect Immun 2001: **69**: 1364–1372.
24. Ishihara K, Kuramitsu HK, Miura T, Okuda K. Dentilisin activity affects the organization of the outer sheath of *Treponema denticola*. J Bacteriol 1998: **180**: 3837–3844.
25. Kaiser V, Diamond G. Expression of mammalian defensin genes. J Leukoc Biol 2000: **68**: 779–784.
26. Kawasaki K, Ernst RK, Miller SI. Purification and characterization of deacylated and/or palmitoylated lipid A species unique to *Salmonella enterica* serovar Typhimurium. J Endotoxin Res 2005: **11**: 57–61.

27. Krisanaprakornkit S, Weinberg A, Perez CN, Dale BA. Expression of the peptide antibiotic human beta-defensin 1 in cultured gingival epithelial cells and gingival tissue. Infect Immun 1998: **66**: 4222–4228.
28. Krisanaprakornkit S, Kimball JR, Weinberg A, Darveau RP, Bainbridge BW, Dale BA. Inducible expression of human beta-defensin-2 by *Fusobacterium nucleatum* in oral epithelial cells: multiple signaling pathways and role of commensal bacteria in innate immunity and the epithelial barrier. Infect Immun 2000: **68**: 2907–2915.
29. Krisanaprakornkit S, Kimball JR, Dale BA. Regulation of human beta-defensin-2 in gingival epithelial cells: the involvement of mitogen-activated protein kinase pathways, but not the NF-kappa B transcription factor family. J Immunol 2002: **168**: 316–324.
30. Kuhne BS, Oschmann P. Quantitative real-time RT-PCR using hybridization probes and imported standard curves for cytokine gene expression analysis. Biotechniques 2002: **33**: 1078, 1080–1072, 1084 passim.
31. Kusumoto Y, Hirano H, Saitoh K et al. Human gingival epithelial cells produce chemotactic factors interleukin-8 and monocyte chemoattractant protein-1 after stimulation with *Porphyromonas gingivalis* via toll-like receptor 2. J Periodontol 2004: **75**: 370–379.
32. Lee SH, Kim KK, Choi BK. Upregulation of intercellular adhesion molecule 1 and proinflammatory cytokines by the major surface proteins of *Treponema maltophilum* and *Treponema lecithinolyticum*, the phylogenetic group IV oral spirochetes associated with periodontitis and endodontic infections. Infect Immun 2005: **73**: 268–276.
33. Lin M, Rikihisa Y. *Ehrlichia chaffeensis* downregulates surface Toll-like receptors 2/4, CD14 and transcription factors PU.1 and inhibits lipopolysaccharide activation of NF-kappa B, ERK 1/2 and p38 MAPK in host monocytes. Cell Microbiol 2004: **6**: 175–186.
34. Listgarten MA. Structure of the microbial flora associated with periodontal health and disease in man. A light and electron microscopic study. J Periodontol 1976: **47**: 1–18.
35. Loesche WJ. The role of spirochetes in periodontal disease. Adv Dent Res 1988: **2**: 275–283.
36. Loesche WJ, Grossman NS. Periodontal disease as a specific, albeit chronic, infection: diagnosis and treatment. Clin Microbiol Rev 2001: **14**: 727–752.
37. Maiden MF, Cohee P, Tanner AC. Proposal to conserve the adjectival form of the specific epithet in the reclassification of *Bacteroides forsythus* Tanner et al. 1986 to the genus *Tannerella* Sakamoto et al. 2002 as *Tannerella forsythia* corrig., gen. nov., comb. nov. Request for an Opinion. Int J Syst Evol Microbiol 2003: **53**: 2111–2112.
38. Melmed G, Thomas LS, Lee N et al. Human intestinal epithelial cells are broadly unresponsive to Toll-like receptor 2-dependent bacterial ligands: implications for host–microbial interactions in the gut. J Immunol 2003: **170**: 1406–1415.
39. Miyamoto M, Ishihara K, Okuda K. The *Treponema denticola* surface protease dentilisin degrades interleukin-1 beta (IL-1 beta), IL-6, and tumor necrosis factor alpha. Infect Immun 2006: **74**: 2462–2467.
40. Neish AS, Gewirtz AT, Zeng H et al. Prokaryotic regulation of epithelial responses by inhibition of IkappaB-alpha ubiquitination. Science 2000: **289**: 1560–1563.
41. Nixon CS, Steffen MJ, Ebersole JL. Cytokine responses to *Treponema pectinovorum* and *Treponema denticola* in human gingival fibroblasts. Infect Immun 2000: **68**: 5284–5292.
42. Oda D, Watson E. Human oral epithelial cell culture I. Improved conditions for reproducible culture in serum-free medium. In Vitro Cell Dev Biol 1990: **26**: 589–595.
43. Okuda T, Kimizuka R, Miyamoto M et al. *Treponema denticola* induces interleukin-8 and macrophage chemoattractant protein 1 production in human umbilical vein epithelial cells. Microbes Infect 2007: **9**: 907–913.
44. Paster BJ, Boches SK, Galvin JL et al. Bacterial diversity in human subgingival plaque. J Bacteriol 2001: **183**: 3770–3783.
45. Roebuck KA. Regulation of interleukin-8 gene expression. J Interferon Cytokine Res 1999: **19**: 429–438.
46. Rosen G, Sela MN, Naor R, Halabi A, Barak V, Shapira L. Activation of murine macrophages by lipoprotein and lipooligosaccharide of *Treponema denticola*. Infect Immun 1999: **67**: 1180–1186.
47. Saglie R, Newman MG, Carranza FA, Pattison GL. Bacterial invasion of gingiva in advanced periodontitis in humans. J Periodontol 1982: **53**: 217–222.
48. Sakamoto M, Suzuki M, Umeda M, Ishikawa L, Benno Y. Reclassification of *Bacteroides forsythus* (Tanner et al. 1986) as *Tannerella forsythensis* corrig., gen. nov., comb. nov. Int J Syst Evol Microbiol 2002: **52**: 841–849.
49. Sellati TJ, Bouis DA, Caimano MJ et al. Activation of human monocytic cells by *Borrelia burgdorferi* and *Treponema pallidum* is facilitated by CD14 and correlates with surface exposure of spirochetal lipoproteins. J Immunol 1999: **163**: 2049–2056.
50. Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol 1998: **25**: 134–144.
51. Suchett-Kaye G, Morrier JJ, Barsotti O. Interactions between non-immune host cells and the immune system during periodontal disease: role of the gingival keratinocyte. Crit Rev Oral Biol Med 1998: **9**: 292–305.
52. Sugawara Y, Uehara A, Fujimoto Y et al. Toll-like receptors, NOD1, and NOD2 in oral epithelial cells. J Dent Res 2006: **85**: 524–529.
53. Tonetti MS. Molecular factors associated with compartmentalization of gingival immune responses and transepithelial neutrophil migration. J Periodontal Res 1997: **32**: 104–109.
54. Uehara A, Yang S, Fujimoto Y et al. Muramyldipeptide and diaminopimelic acid-containing desmuramylpeptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. Cell Microbiol 2005: **7**: 53–61.
55. U.S. Department of Health and Human Services, National Institute of Dental and Craniofacial Research, National Institutes of Health, Oral health in America: a report of the Surgeon General, Rockville, 2000: 95–127.
56. van der Woude MW, Baumler AJ. Phase and antigenic variation in bacteria. Clin Microbiol Rev 2004: **17**: 581–611.
57. Winzen R, Gowrishankar G, Bollig F, Redich N, Resch K, Holtmann H. Distinct domains of AU-rich elements exert different functions in mRNA destabilization and stabilization by p38 mitogen-activated protein kinase or HuR. Mol Cell Biol 2004: **24**: 4835–4847.

This document is a scanned copy of a printed document. No warranty is given about the accuracy of the copy. Users should refer to the original published version of the material.