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Role of salivary epithelial toll-like receptors 2 and 4 in modulating innate immune responses in chronic periodontitis

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Background and Objective: Chronic periodontitis is initiated by sequential colonization with a broad array of bacteria and is perpetuated by an immune-inflammatory response to the changing biofilm. Host recognition of microbes is largely mediated by toll-like receptors (TLRs), which interact with conserved pathogen-associated molecular patterns. Based on ligand recognition, TLR-2 and TLR-4 interact with most periodontal pathogens. Extracrevicular bacterial reservoirs, such as the oral epithelial cells, contribute to the persistence of periodontitis. Human saliva is a rich source of oral epithelial cells that express functional TLRs. In this study we investigated the role of salivary epithelial cell (SEC) TLR-2 and TLR-4 in patients with generalized chronic periodontitis.

Material and Methods: Unstimulated whole saliva (UWS) was collected from patients with generalized chronic periodontitis and from healthy individuals after obtaining informed consent. Epithelial cells isolated from each UWS sample were assessed for TLR-2, TLR-4, peptidoglycan recognition protein (PGRP)-3 and PGRP-4 by quantitative real-time PCR. In addition, the SECs were stimulated *in vitro* with microbial products for up to 24 h. The culture supernatant was assessed for cytokines by ELISA.

Results: Stimulation with TLR-2- or TLR-4-specific ligands induced cytokine secretion with differential kinetics and up-regulated *TLR*2 and *TLR4* mRNAs, respectively, in cultures of SECs from patients with periodontitis. In addition, the SECs from patients with periodontitis exhibited reduced *PGRP3* and *PGRP4* mRNAs, the TLR-responsive genes with antibacterial properties.

Conclusion: SECs derived from the UWS of patients with chronic periodontitis are phenotypically distinct and could represent potential resources for assessing the epithelial responses to periodontal pathogens in the course of disease progression and persistence.

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Periodontitis is a significant global oral health problem, with over 80% of the adult population exhibiting some form of the disease and 2–5% suffering from severe disease (1,2). Within the periodontium, transition from health to disease is initiated by a change in the subgingival ecology from healthy biofilm to pathogenic biofilm (3,4). While treatment attenuates inflammation and reduces the pathogen burden in the subgingival plaque, recurrence is associated with re-establishment of the pathogenic biofilm, suggesting the presence of microbial reservoirs that allow re-infection or infection of new sites (5). Under normal conditions, oral microorganisms adhere to mucosal surfaces in the mouth for survival and multiplication (6,7). Exfoliating epithelial cells from the buccal mucosa have been shown to support a viable polymicrobial intracellular flora (7,8). Indeed, in patients with periodontitis, disease-associated bacteria, such as *Porphyromonas gingivalis and Aggregatibacter actinomycetemcomitans*, have been detected at high frequency in the surface epithelial cells of oral mucosa covering the cheek, tongue and palate (9,10). Significantly, it has been observed that periodontal treatment does not affect the prevalence of periodontopathic bacteria on oral mucosal epithelial cells (11,12). In fact, *A. actinomycetemcomitans* and *P. gingivalis* have been detected at the same prevalence rate in oral mucosal epithelial cells of the buccal mucosa and tongue before and 3 months after periodontal surgery (11,13). These observations substantiate the role of oral mucosal epithelial cells as potential sources for re-infection in periodontitis.

Host recognition of microbes is mediated largely by toll-like receptors (TLRs) that bind highly conserved pathogen-associated molecular patterns typically shared by groups of microorganisms. Ten mammalian TLRs, and many of their ligands, have been identified (14–16). Most periodontal pathogens possess molecular patterns recognized by TLR-2 and/or TLR-4 (17–19). While TLR-4 is associated with recognition of gram-negative bacterial lipopolysaccharides (LPS), TLR-2 has been shown to interact with the peptidoglycan of gram-positive bacteria as well as with the LPS of gram-negative *P. gingivalis* (20). Polymorphisms in CD14, a co-receptor for TLR-2 and TLR-4, have been variably associated with susceptibility to periodontitis (21,22). Gingival epithelial cells stimulated with *P. gingivalis*, *A. actinomycetemcomitans* and *Fusobacterium nucleatum* or their products have been shown to respond via TLR-2- and TLR-4-mediated signaling and to promote cytokine secretion, implicating a role for these receptors in the pathogenesis of periodontitis (23,24). TLR-2- and TLR-4-mediated signaling has also been shown to modulate the expressions of proteoglycan recognition protein (PGRP)-3 and PGRP-4, molecules with antibacterial properties, in oral epithelial cells (25).

Human saliva is a rich source of exfoliated epithelial cells derived from the mucosa covering various regions of the mouth, including the gingiva, cheek, tongue and palate (6,26). In healthy subjects, exfoliation of epithelial cells occurs as part of the process of maintaining mucosal homeostasis or as a result of mechanical separation (27). Previously, we and others have reported that over 40% of epithelial cells in unstimulated whole saliva (UWS) are viable (6,28–30). Bacterial invasion has been shown to increase exfoliation of epithelial cells from mucosal surfaces in humans and in experimental models (31,32). In chronic periodontitis, the degree of inflammation has been correlated with an elevated number of epithelial cells in the gingival crevicular fluid that is then admixed with the oral fluid (33).

Based on the observations of persistent periodontal pathogens in mucosal epithelial cells, increased exfoliation of oral epithelial cells in periodontitis and the viability of salivary epithelial cells (SECs), we hypothesized that the SECs in patients with chronic periodontitis will be phenotypically distinct from those in healthy saliva. We also postulated that SECs could represent an excellent tool with which to evaluate the status of the host response to periodontal pathogens in chronic periodontitis. Our results show that while the expression of TLR-2 is marginally higher, that of TLR-4 is significantly elevated in the SECs of patients with chronic periodontitis. Kinetic analysis suggested that stimulation with TLR-4-specific ligand induced an early up-regulation of interferon-gamma (IFN-γ) secretion and that, with TLR-2 ligand, mediated an initial increase in interleukin (IL)-8 secretion in cultures of SECs from patients with chronic periodontitis. Furthermore, in patients with chronic periodontitis the SECs exhibited reduced levels of expression of *PGRP3* and *PGRP4* mRNAs.

Material and methods

Study population, clinical measurements and treatment

The study cohort consisted of 20 systemically healthy nonsmokers, reporting to Indiana University School of Dentistry, who were diagnosed with severe chronic periodontitis, were not on antibiotics/anti-inflammatory therapy and were without any other oral pathological lesions. Twenty systemically healthy nonsmokers with no known oral or systemic conditions were recruited as control subjects. Informed consent was obtained from all participants in accordance with the Institutional Review Board at Indiana University Purdue University at Indianapolis, Indianapolis (IUPUI). Complete periodontal measurements, including clinical attachment loss, probing depth, plaque score and bleeding score, were recorded. Only patients exhibiting clinical attachment loss of $\geq$ 4 mm in $\geq$ 30% of sites (40–48 sites) were included in the group with severe chronic periodontitis, a stringent selection as opposed to the relatively common criteria of probing depth $\geq$ 4 mm and clinical attachment loss of $\geq$ 3 mm in four to 10 sites (34–36).

Isolation and culture of epithelial cells from UWS

UWS was collected by the drooling method, and epithelial cells were

isolated as described previously (28,30). Briefly, subjects were instructed to fast for 2 h before saliva collection. Seated with their head tilted towards one side, each subject was requested to provide a minimum of 2 mL of UWS by drooling passively for 5–10 min into a chilled centrifuge tube. All UWS samples were centrifuged at 250 ***g*** for 10 min at 4°C. The cellular sediment obtained was reconstituted in isotonic saline supplemented with two drops of Zap Oglobin (Stem Cell Technologies, Vancouver, BC, Canada) to lyse blood corpuscles and centrifuged at 1271.7 ***g*** for 10 min at 4°C. After washing in saline, the cell suspension was filtered through a membrane of 20 micron pore size. The epithelial cell-enriched cell preparation was then assessed by light microscope for morphology, reconstituted in RPMI-1640 (Catalog #10-040-CV; Mediatech Inc., Minnesota, MN, USA) supplemented with 5% fetal bovine serum (Hyclone fetal bovine serum, Catalog # SH30070; Thermo Scientific, Logan, UT, USA) and 5% dimethylsulfoxide (Catalog # 67-68-5; Sigma-Aldrich, St Louis, MO, USA), and stored at −80°C until further analysis. Each SEC sample preparation was stained for CD3 (Catalog #347347; BD Biosciences, San Jose, CA, USA) and CD45 (Catalog #555483; BD Biosciences) to screen for the absence of blood-derived cells.

After thawing, the SEC samples were washed twice each in serum-free RPMI-1640 and keratinocyte growth factor medium (Catalog # 131-500a; CELL Applications, Inc., San Diego, CA, USA). The SECs were then cultured in keratinocyte growth factor supplemented with 4 mM glutamine (Cataolog # ICN 19467880; MP Biomedicals, Solon, OH, USA) and 5% penicillin and streptomycin (Catalog # 1670049; MP Biomedicals) in an atmosphere of 5% CO_2 at 37°C. After three to four passages, the cells were rested in serum-free medium for 12 h. Approximately 1×10^5 cells were plated in 96-well plates and stimulated with 1 mg/mL of *Escherichia coli* LPS (Product # L-3880; Sigma-Aldrich), a TLR-4 ligand or 20 units/mL of protein peptide derivative (PPD) (Difco Laboratories, Detroit, MI, USA), a mycobacterial pathogen-associated molecular pattern recognized by TLR-2. Supernatants and cells collected 4, 8 and 24 h after stimulation were assessed for cytokines and TLR expression, respectively.

Flow cytometry

The cells isolated from the UWS samples were stained for cytokeratin-13, TLR-2 and TLR-4. For detection of cytokeratin, the SECs were permeabilized briefly with phosphate-buffered saline containing 1% Tween-20 and then incubated with goat polyclonal anti-(human cytokeratin-13) Ig (Catalog # GWB-Q00515; Genway Biotech Inc., San Diego, CA, USA) followed by fluorescein isothiocyanate-conjugated mouse anti-goat IgG (Catalog # Sc2356; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). For detecting TLR-2 and TLR-4, the SECs were incubated with phycoerythrin-conjugated mouse polyclonal anti-(human TLR-2) Ig (clone 38396)/anti-(human TLR-4) Ig (clone 610015) (R&D Systems, Minneapolis, MN, USA). Gingival epithelial cells obtained by swabs and stained with isotype-matched antibody were used as controls. The stained cells were fixed in 1% paraformaldehyde and analyzed on a FACS Calibur flow cytometer (BD Biosciences).

Quantitative real-time PCR

Total RNA isolated from epithelial cells using the RNeasy Mini-Kit (Qiagen Sciences, Valencia, CA, USA) was reverse transcribed using the iScript cDNA synthesis kit (BioRad, Hercules, CA, USA). Five-hundred nanograms of cDNA was used for real-time quantitative PCR as described. Message for small proline-rich protein 2a (*SPRR2a*), a gene abundantly expressed in stratified squamous epithelia and that of the housekeeping gene, beta-actin, were amplified as internal and endogenous controls, respectively. The primers were: TLR-2 forward: 5′-GGCCAGCAAATTACCTGTGT-3′ and TLR-2 reverse: 5′-TCTCCACC CAGTAGGCATC-3′; SPRR2a forward: 5′-AGTGCCAGCAGAAATAT CCTCC-3′ and SPRR2a reverse: 5′-GA ACGAGGTGAGCCAAATATCC-3′; PGRP-3 forward: 5′-CTGAAGGAG GCCCCACTCCCT-3′ and PGRP-3 reverse: 5′-GCTGAGCCAGCTTG-GCT GGTG-3′; PGRP-4 forward: 5′-TTTTGCCCTCCTCCC-CTGCCA-3′ and PGRP-4 reverse: 5′-ATGAGG-TTTGGAGG CCCTTGG-A-3′; and beta-actin forward: 5′-GCCAACCG-CGAGAAGATGA-3′ and beta-actin reverse: 5′-CATCACGATG CCAGT-GGTA-3′. Amplifications were performed in duplicate and normalized to beta-actin. At the end of the PCR cycles, specificities of the amplification products were controlled by dissociation curve analysis. The gene specific threshold cycle (C_t) for each sample was corrected by subtracting the C_t for *SPRR2a*. Untreated controls were chosen as reference samples and the C_t for all experimental samples was subtracted from the ΔC_t for the control samples ($\Delta\Delta C_t$) (37). The magnitude of change in mRNA was expressed as $2^{-\Delta\Delta C_t}$.

Statistical analysis

Statistical difference in the soluble and cellular molecules between the healthy and the diseased cohorts was determined using the pairwise *t*-test. $p < 0.05$ was considered significant. All assays were repeated three times.

Results

Demographic and clinical features

The general and demographic features of the healthy controls and the chronic periodontitis cohort are provided in Table 1. Periodontal measurements, including plaque index, bleeding on probing and the percentages of sites with pocket depth > 4 mm and mean clinical attachment loss of > 4 mm were significantly higher in the periodontitis cohort than in the healthy controls (Table 1). In the group with severe periodontitis almost 90% of sites exhibited clinical attachment loss of ≥ 4 mm, indicating that this cohort is a stringent representation of severe periodontal disease.

Table 1. Demographic details and clinical measurements

Patient profile	Healthy	Chronic periodontitis
Age (years)	28.60 ± 2.44	41.67 ± 10.07
Gender ratio (M:F)	10 : 8	9 : 9
Pocket depth (mm)	1.5 ± 0.11	3.49 ± 0.65
Clinical attachment loss (mm)	1 ± 0.11	4.05 ± 0.69
Bleeding on probing (%)	5.07 ± 2.21	54.84 ± 24.37
Plaque index (%)	24.86 ± 10.41	64.66 ± 15.28
Sites with pocket depth ≥ 4 mm (%)	NA	71.17 ± 34.12
Sites with clinical attachment loss ≥ 4 mm (%)	NA	89.39 ± 30.94

Values are given as mean or mean ± SD, unless indicated otherwise.
F, female; M, male.

SECs express increased levels of TLR-2 and TLR-4 in chronic periodontitis

The nature of the SECs was confirmed by staining with cytokeratin-13, a protein abundantly expressed in oral epithelial cells (38) (Fig. 1A). The percentage of viable SECs, determined by toluidine blue exclusion using an automated cell counter, was similar in the UWS samples from both the healthy controls (58.26 ± 22.7%) and the subjects with chronic periodontitis (54.05 ± 15.8%). This is in contrast to the previous observations of increased numbers of epithelial cells in the gingival crevicular fluid of subjects with severe periodontitis (33). The discrepancy could be largely attributed to the differences in the biospecimen – crevicular fluid vs. UWS. The SECs from both healthy controls and patients with chronic periodontitis exhibited considerable amounts of TLR-2 and TLR-4 proteins, as determined by flow cytometry (Fig. 1B and 1C). Quantitative PCR suggested that the levels of *TLR2* mRNA and *TLR4* mRNA were significantly higher in the SECs from patients with chronic periodontitis compared with those from healthy controls (Fig. 1D and 1E).

SECs exhibit elevated response to TLR-2 and TLR-4 ligands in chronic periodontitis

Exfoliating oral epithelial cells have been shown to host intracellular periodontopathic bacteria, suggesting that these cells may act as extracrevicular reservoirs and contribute to the persistence of chronic periodontitis (7,8). Here, we investigated the response of SECs to microbial products. When stimulated with LPS, a TLR-4 ligand, the epithelial cells from the UWS of patients with chronic periodontitis exhibited significantly increased secre-

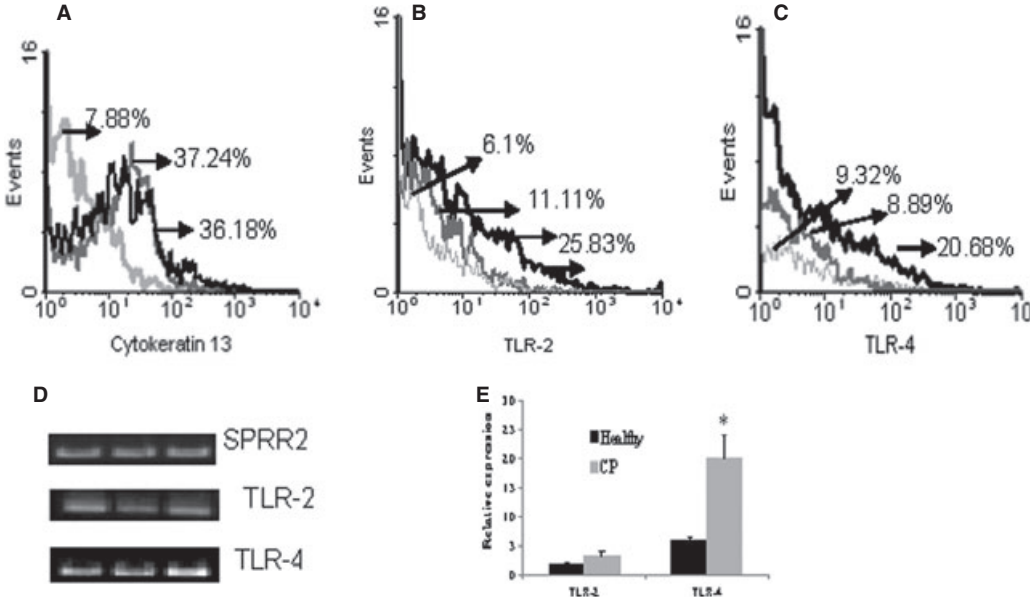

Fig. 1. Salivary epithelial cells (SECs) from patients with chronic periodontitis express elevated levels of toll-like receptor (TLR)-2 and TLR-4. Epithelial cells isolated from the unstimulated whole saliva (UWS) of patients with chronic periodontitis or healthy controls were assessed by flow cytometry for cell-surface expression of cytokeratin 13 (A), TLR-2 (B) and TLR-4 (C). Light gray line, isotype control; dark gray line, healthy controls; black line, patients with chronic periodontitis. Total RNA extracted from the epithelial cells was reverse transcribed and equal amounts of cDNA were used to amplify TLR-2 and TLR-4 by SYBR®Green real-time PCR. Amplification of small proline rich protein 2a (SPRR-2a) was performed as the internal control. (D) Relative quantities of *TLR2* and *TLR4* mRNAs with respect to *SPRR2a* mRNA were determined using the $2^{-\Delta\Delta C_t}$ method, as described in the Material and methods. (E) Gel electrophoresis of the PCR products SPRR-2a (112 bp), TLR-2 (148 bp) and TLR-4 (100 bp). CP, chronic periodontitis. $*p < 0.05$, compared with control saliva.

tion of the proinflammatory cytokines IFN-γ and IL-12, compared with the epithelial cells from the UWS of control subjects (Fig. 2A and 2B). No significant difference was observed in IL-6, IL-8, tumor necrosis factor-alpha and IL-4 secretion by the LPS-stimulated epithelial cells from the UWS of either group (Fig. 2A). Stimulation with PPD, a TLR-2 ligand, mediated marginally decreased IFN-γ secretion and dramatically lower IL-8 secretion in cultures of epithelial cells from UWS from patients with periodontitis compared with those from healthy controls (Fig. 2C). We next investigated the kinetics of cytokine secretion by the SECs. Following stimulation with LPS, IFN-γ secretion peaked at an earlier time point (4 h) in cultures of SECs from healthy controls compared with the 8-h peak in cultures of SECs from patients with periodontitis (Fig. 3A). Stimulation with PPD induced IFN-γ secretion that peaked at 24 h in cultures of SECs from both healthy subjects and patients with periodontitis (Fig. 3B). The IL-8 concentration was significantly higher at each time point in the supernatant of LPS-stimulated cultures of SECs from healthy controls compared with those from patients with periodontitis (Fig. 3C). Stimulation with PPD induced a significantly higher burst of IL-8 secretion, as early as 4 h after stimulation, in cultures of epithelial cells from the UWS of patients with periodontitis compared with the epithelial cells from healthy controls (Fig. 3D).

We next investigated the regulation of TLRs by microbial products. Quantitative real-time PCR showed that stimulation with PPD induced a linear up-regulation of *TLR2* mRNA in SECs from healthy controls as well as in those from patients with periodontitis, expression being significantly higher in the latter cohort than in the former at each time point (Fig. 3E). Twenty-four hours after stimulation with LPS, a dramatic up-regulation of *TLR4* mRNA was observed in epithelial cells from the UWS of patients with chronic periodontitis compared with those from healthy controls (Fig. 3F). Previously, stimulation of immortalized oral keratinocytes with microbial products has been shown to modulate the expression of antibacterial factors, such as PGRPs, via TLR-2/TLR-4-mediated signaling (33,39). We observed that expression of PGRP-3 and PGRP-4 is significantly decreased in the epithelial cells derived from the UWS of patients with chronic periodontitis compared with the epithelial cells from control subjects (Fig. 4A and 4B).

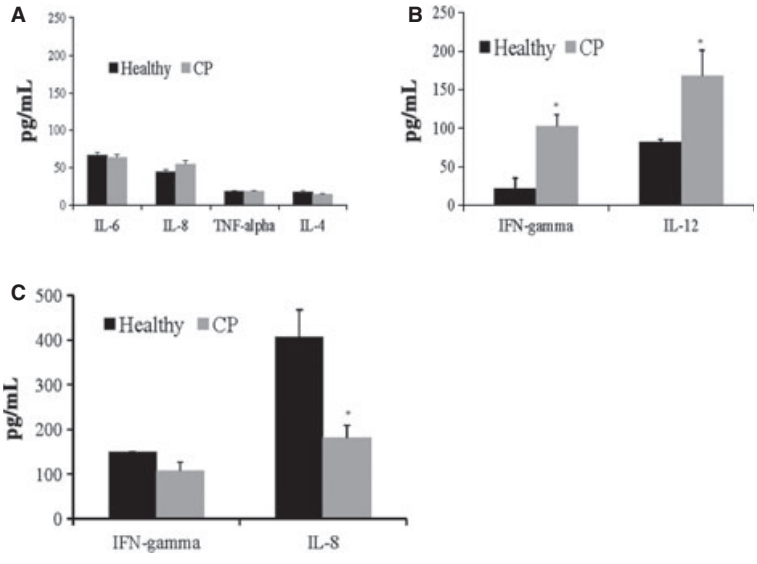

Fig. 2. Salivary epithelial cells (SECs) from patients with chronic periodontitis exhibit an enhanced response to lipopolysaccharide (LPS) and protein peptide derivative (PPD): 1×10^5 SECs isolated from patients with chronic periodontitis or from healthy controls were cultured in the presence of (A,B) *Escherichia coli* LPS (1 mg/mL) or (C) PPD (20 units/mL). Supernatant was collected at 24 h and the concentrations of interleukin (IL)-6, IL-8, tumor necrosis factor-alpha (TNF-α), IL-4, interferon-gamma (IFN-γ) and IL-12 were assessed by ELISA. CP, chronic periodontitis.*$p < 0.05$, compared with unstimulated cells.

Discussion

Cell exfoliation is a characteristic feature of epithelia covering the external surface and the internal cavities of the body. In physiological conditions, exfoliation is closely associated with the homeostatic control of the cell population and tissue size (40). Impaired physiological apoptosis, microbial invasion, disturbances in the intercellular adhesion systems and/or disturbances in the epithelial–mesenchymal interactions potentially contribute to increased exfoliation of epithelial cells (40–42). While the potential clinical benefits of cytologic and cytometric analyses of the exfoliated epithelial cells in the diagnosis of many pathological conditions, including oral cancer, precancer, type II diabetes and burning mouth syndrome (39,43,44), are well established, the clinical applications of epithelial cells obtained from body fluids are relatively unexplored (45,46). The origin (kidney, ureter and urinary tract mucosa) and the functional efficacy of epithelial cells isolated from urine have been recently documented (47,48). Primary cultures of epithelial cells from the urine of diabetic subjects exhibited increased expression and activity of the renal glucose transporter (45,48). Similarly, epithelial cells isolated from stools retain markers of anatomic location (proximal colon vs. distal colon), can be maintained in cultures and serve as potential research tools for biomarker investigations (49,50). Multiple mammary cell lines have been established from epithelial cells derived from breast milk (51). Here we report the functional efficacy of epithelial cells

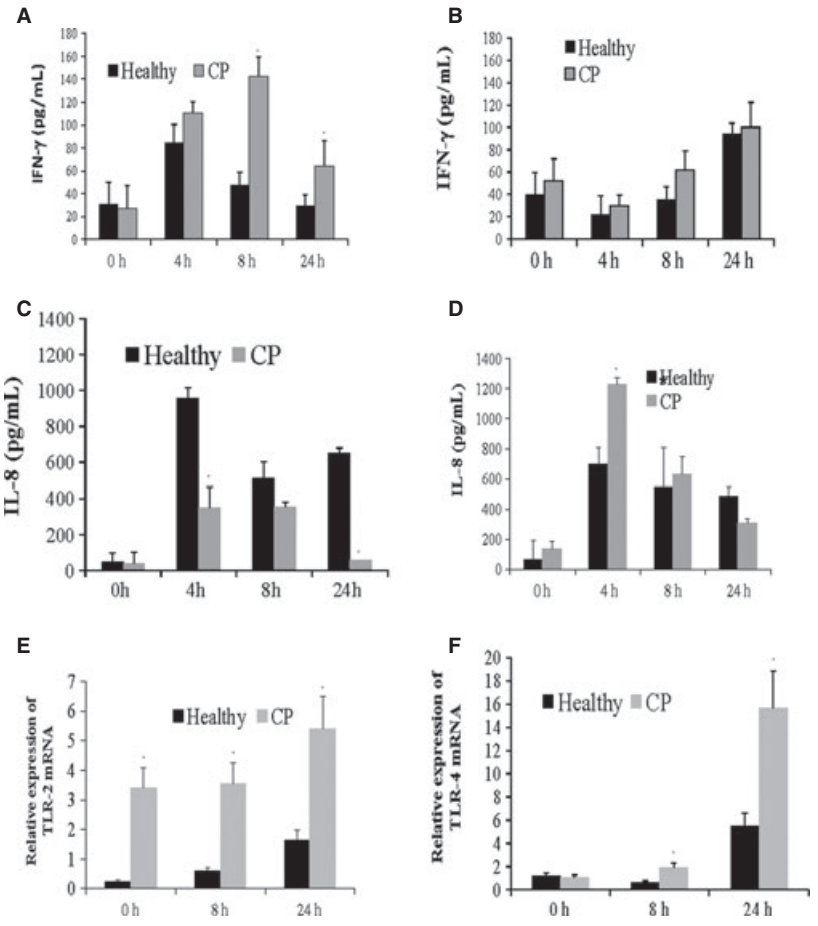

Fig. 3. Comparative analysis of the response of salivary epithelial cells (SECs) from patients with chronic periodontitis to toll-like receptor (TLR)-2 and TLR-4 ligands: 1×10^5 SECs from patients with chronic periodontitis or from healthy controls were cultured in the presence of (A, C, E) *Escherichia coli* lipopolysaccharide (LPS) (1 mg/mL) or (B, C, D) protein peptide derivative (PPD; 20 units/mL). Supernatant collected at the indicated time points was assessed for (A,B) interferon-gamma (IFN-γ) and (C,D) interleukin-8 (IL-8). Total RNA extracted from the epithelial cells at the end of 24 h was reverse transcribed and equal amounts of cDNA were used to amplify TLR-2 and TLR-4 by SYBR®Green real-time PCR. Amplification of small proline rich protein 2a (SPRR-2a) was performed as the internal control. The relative quantities of (E) *TLR2* and (F) *TLR4* mRNAs, with respect to *SPRR2a*, were determined using the $2^{-\Delta\Delta C_t}$ method, as described in the Material and methods. CP, chronic periodontitis. $*p < 0.05$, compared with unstimulated cells.

derived from human saliva in responding to antigenic stimulation and explore their applications in chronic periodontitis.

The epithelial cells in saliva are derived from different regions of the oral mucosa. The oral epithelium exhibits considerable variation in the degree of differentiation in different regions of the oral cavity. While the lining mucosa that covers approximately 60% of the oral cavity (area of buccal mucosa > labial mucosa > ventral tongue) is nonkeratinized, the masticatory mucosa that covers approximately 25% of the oral cavity (e.g. gingiva) is predominantly parakeratinized and the specialized mucosa that covers approximately 15% of the oral cavity (e.g. dorsum of the tongue) is orthokeratinized (52,53). The sulcular epithelium at the junction of the gingiva and the tooth is also nonkeratinized (5). In general, the nonkeratinized epithelium exhibits a higher rate of proliferation and a faster turnover than the keratinized epithelium (54). Thus, based on the area of distribution and the turnover time of the epithelium in different regions, it is highly likely that a greater proportion of the epithelial cells in saliva are derived from the nonkeratinized mucosal epithelium. Following exfoliation, while some epithelial cells undergo detachment-induced apoptosis (anoikis), others maintain their structural integrity and viability for extended periods of time, ranging from minutes to hours (40). A significant proportion of primary oral epithelial cells isolated from tissue explants have been shown to be resistant to anoikis (55).

A strong association of periodontal pathogens with exfoliating oral mucosal epithelial cells has been substantiated using different techniques, including DNA hybridization (12,56), culture-based study (57), advanced imaging techniques (such as fluorescence *in-situ* hybridization) and confocal microscopy with species-specific probes (7). Indeed, based on the propensity to culture *A. actinomycetemcomitans*, it has been suggested that oral mucosal epithelial cells exhibit a high diagnostic value to identify young adults colonized with this bacterium (57). Furthermore, it has been reported that the presence of *Prevotella intermedia, A. actinomycetemcomitans, P. gingivalis, Tannerella forsythia* and *Treponema denticola* in mucosal epithelial cells is not affected by treatment for chronic periodontitis, suggesting that recolonization may occur from the oral mucosa and contribute to recurrent or refractory disease (11,12). Thus, despite the turnover of the nonkeratinized oral mucosa, periodontal pathogens seem to persist in the epithelial cells of patients with chronic periodontitis. It is speculated that the cellular invasion with pathogens (7,58,59) and/or the ability to bind, release and transfer from one surface (mucosa) to another (tooth), as shown for *A. actinomycetemcomitans* (60), could contribute to the persistence of periodontopathic bacteria in oral epithelial cells.

The ability of oral epithelial cells to respond to microbial stimulation is well documented (12,56,58). Previously, epithelial cells from buccal

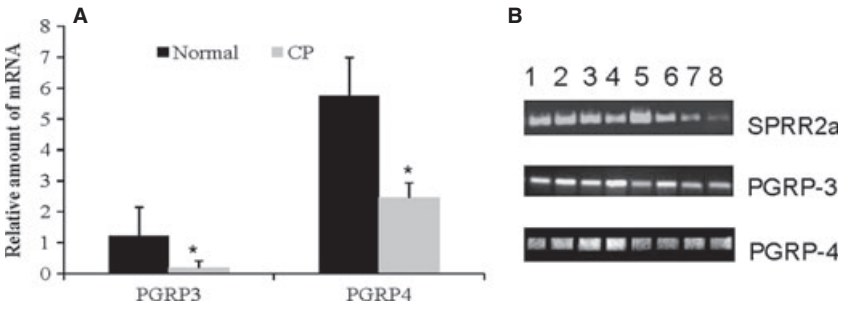

Fig. 4. Salivary epithelial cells (SECs) express decreased levels of peptidoglycan recognition protein (PGRP)-3 and PGRP-4 in chronic periodontitis. Epithelial cells were isolated from the unstimulated whole saliva (UWS) of patients with chronic periodontitis or healthy controls and assessed for PGRP-3 and PGRP-4 by real time-PCR. (A) Relative quantities of *PGRP3* and *PGRP4* mRNAs, with respect to small proline rich protein 2a (*SPRR2a*) mRNA, were determined using the $2^{-\Delta\Delta C_t}$ method, as described in the Material and methods. CP, chronic periodontitis. (B) Gel electrophoresis of the PCR products SPRR-2a (112 bp), PGRP-3 (106 bp) and PGRP-4 (78 bp). $*p < 0.05$, compared with cells from control saliva. The first four lanes are representative samples of SECs from healthy controls and lanes 5–8 represent SEC from patients with chronic periodontitis.

mucosal swabs of pediatric patients with Crohn's disease were shown to secrete elevated amounts of chemokines upon stimulation with LPS (61). More pertinent to our study, epithelial cells derived from saliva have been shown to secrete cytokines in response to *Candida albicans* (62). We observed that stimulation with TLR-2- or TLR-4-specific ligands induced differential responses in epithelial cells derived from healthy controls compared with those from saliva of patients with chronic periodontitis. While LPS stimulation significantly suppressed secretion of IL-8, stimulation with PPD induced an early increase of IL-8 secretion in cultures of SECs from patients with periodontitis. While LPS stimulation of SECs from patients with periodontitis mediated significant IFN-γ secretion at 8 h poststimulation, PPD stimulation of cultures of SECs from healthy subjects or from patients with periodontitis had equivocal effects. The expression of *TLR4* mRNA was significantly lower at earlier time points and increased dramatically at 24 h after LPS stimulation of epithelial cells from UWS of patients with periodontitis. The expression of *TLR2* mRNA was consistently higher in SECs from patients with chronic periodontitis. In contrast, although the SECs from healthy controls exhibited significantly lower levels of *TLR4* mRNA compared with SECs from patients with chronic periodontitis, stimulation with LPS up-regulated *TLR4* mRNA. Recently, the salivary concentrations of certain periodontal pathogens, namely *P. gingivalis, P. intermedia* and *T. forsythia*, have been shown to be higher in patients with periodontitis (63). Furthermore, the saliva of patients with periodontitis has been shown to contain increased amounts of TLR-2 and TLR-4 ligands or stimulants (64).

PGRPs are a class of innate immune-associated proteins that influence host–pathogen interactions through antibacterial, anti-inflammatory and peptidoglycan hydrolytic properties (65). High expression of PGRP-3 and of PGRP-4 has been observed in mature epithelial cells of the tongue and in the mucous cells of the submandibular salivary gland, respectively (66). Previously, Uhera *et al.* (25) showed that TLR-2- and TLR-4-mediated signaling modulated PGRP-3 and PGRP-4 expression in oral squamous cell carcinoma-derived epithelial cell lines. Our data showed that SECs from subjects with periodontitis exhibited significantly lower levels of expression of PGPR-3 and PGRP-4. In this context it is interesting to note that deficiency of PGRP-3 or PGRP-4 increases susceptibility to bacterial infections, whereas the presence of PGRP-3 or PGRP-4 protects against inflammation (67).

The reported association of periodontopathic bacteria with oral mucosal epithelial cells, and the differences in SEC phenotype between patients with periodontitis and healthy individuals, suggest that SECs could be valuable in investigating the pathogenesis of periodontitis. Taking together the elevated presence of TLR-2 and TLR-4 stimulants in the saliva (64) and our observations of differential responses of the SECs from patients with chronic periodontitis to TLR ligands, it is postulated that the TLR-2- and TLR-4-mediated signaling of SECs may contribute functionally to the persistence of periodontitis. However, it is cautioned that extraneous agents, such as medications and/or superimposed fungal infection, could potentially contribute to the modulation of epithelial cell responses to TLR stimulation *in vivo*.

Conclusions

Tissue destruction in periodontitis does not follow a predictable pattern either temporally or spatially (68,69). Assessment of innate immunologic parameter(s) that reflect modulation of the host response to microbial biofilm may provide information on the extent of the host response (19,70). Consistent with this, our data suggest that the TLR-2- and TLR-4-mediated signaling of epithelial cells derived from the saliva of patients with chronic periodontitis could provide an indication of the host–microbe interaction reflecting immune activity and hence that of the disease status in chronic periodontitis. Future studies that correlate the soluble and membrane-associated TLRs in saliva with concomitant microbial assessment will help to determine the mechanistic association with the pathogenic spectrum of periodontitis. Accumulating evidence implicating multiple innate immune-associated markers (including TLRs, nucleotide-binding oligomerization domains and PGPRs) in peri-

odontitis suggests that complementary or suppressive networks amongst these receptors may determine the disease outcome.

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References

1. Albandar JM, Brunelle JA, Kingman A. Destructive periodontal disease in adults 30 years of age and older in the United States, 1988–1994. *J Periodontol* 1999;**70**:13–29.
2. Burt B. Position paper: epidemiology of periodontal diseases. *J Periodontol* 2005;**76**:1406–1419.
3. Kornman KS. Mapping the pathogenesis of periodontitis: a new look. *J Periodontol* 2008;**79**:1560–1568.
4. Offenbacher S, Barros SP, Beck JD. Rethinking periodontal inflammation. *J Periodontol* 2008;**79**:1577–1584.
5. Offenbacher S, Barros SP, Singer RE, Moss K, Williams RC, Beck JD Periodontal disease at the biofilm-gingival interface. *J Periodontol* 2007;**78**:1911–1925.
6. Dawes C. Estimates, from salivary analyses, of the turnover time of the oral mucosal epithelium in humans and the number of bacteria in an edentulous mouth. *Arch Oral Biol* 2003;**48**: 329–336.
7. Rudney JD, Chen R, Sedgewick GJ. Actinobacillus actinomycetemcomitans, Porphyromonas gingivalis, and Tannerella forsythensis are components of a polymicrobial intracellular flora within human buccal cells. *J Dent Res* 2005;**84**:59–63.
8. Rudney JD, Chen R. The vital status of human buccal epithelial cells and the bacteria associated with them. *Arch Oral Biol* 2006;**51**:291–298.
9. Aas JA, Paster BJ, Stokes LN, Olsen I, Dewhirst FE. Defining the normal bacterial flora of the oral cavity. *J Clin Microbiol* 2005;**43**:5721–5732.
10. Socransky SS, Haffajee AD. Periodontal microbial ecology. *Periodontol 2000* 2005;**38**:135–187.
11. Danser MM, Timmerman MF, van Winkelhoff AJ, van der Velden U. The effect of periodontal treatment on periodontal bacteria on the oral mucous membranes. *J Periodontol* 1996;**67**:478–485.
12. Johnson JD, Chen R, Lenton PA, Zhang G, Hinrichs JE, Rudney JD. Persistence of extracrevicular bacterial reservoirs after treatment of aggressive periodontitis. *J Periodontol* 2008;**79**:2305–2312.
13. Riviere GR, Smith KS, Tzagaroulaki E *et al*. Periodontal status and detection frequency of bacteria at sites of periodontal health and gingivitis. *J Periodontol* 1996;**67**:109–115.
14. Imler JL, Hoffmann JA. Toll receptors in innate immunity. *Trends Cell Biol* 2001;**11**:304–311.
15. Pasare C, Medzhitov R. Toll-like receptors: linking innate and adaptive immunity. *Adv Exp Med Biol* 2005;**560**:11–18.
16. Underhill DM. Toll-like receptors: networking for success. *Eur J Immunol* 2003;**33**:1767–1775.
17. Beklen A, Hukkanen M, Richardson R, Konttinen YT. Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. *Oral Microbiol Immunol* 2008;**23**:425–431.
18. Hajishengallis G, Tapping RI, Harokopakis E *et al*. Differential interactions of fimbriae and lipopolysaccharide from Porphyromonas gingivalis with the Toll-like receptor 2-centred pattern recognition apparatus. *Cell Microbiol* 2006;**8**:1557–1570.
19. Kinane DF, Mark Bartold P. Clinical relevance of the host responses of periodontitis. *Periodontol 2000* 2007;**43**:278–293.
20. Kinane DF, Galicia JC, Gorr SU, Stathopoulou PG, Benakanakere MP. Gingivalis interactions with epithelial cells. *Front Biosci* 2008;**13**:966–984.
21. Brett PM, Zygogianni P, Griffiths GS. *et al*. Functional gene polymorphisms in aggressive and chronic periodontitis. *J Dent Res* 2005;**84**:1149–1153.
22. Tervonen T, Raunio T, Knuuttila M, Karttunen R. Polymorphisms in the CD14 and IL-6 genes associated with periodontal disease. *J Clin Periodontol* 2007;**34**:377–383.
23. Bainbridge BW, Darveau RP. Porphyromonas gingivalis lipopolysaccharide: an unusual pattern recognition receptor ligand for the innate host defense system. *Acta Odontol Scand* 2001;**59**:131–138.
24. Kikkert R, Laine ML, Aarden LA, van Winkelhoff AJ. Activation of toll-like receptors 2 and 4 by gram-negative periodontal bacteria. *Oral Microbiol Immunol* 2007;**22**:145–151.
25. Uehara A, Sugawara Y, Kurata S *et al*. Chemically synthesized pathogen-associated molecular patterns increase the expression of peptidoglycan recognition proteins via toll-like receptors, NOD1 and NOD2 in human oral epithelial cells. *Cell Microbiol* 2005;**7**:675–686.
26. Gallez F, Fadel M, Scruel O, Cantraine F, Courtois P. Salivary biomass assessed by bioluminescence ATP assay related to (bacterial and somatic) cell counts. *Cell Biochem Funct* 2000;**18**:103–108.
27. Buttler C. The pathology of dental substitutes. *Dental Practice* 1909; **VII**: 11–19.
28. Srinivasan M, Kodumudi KN, Zunt SL. Soluble CD14 and toll-like receptor-2 are potential salivary biomarkers for oral lichen planus and burning mouth syndrome. *Clin Immunol* 2008;**126**:31–37.
29. Watanabe T, Ohata N, Morishita M, Iwamoto Y. Correlation between the protease activities and the number of epithelial cells in human saliva. *J Dent Res* 1981;**60**:1039–1044.
30. Zunt SL, Burton LV, Goldblatt LI, Dobbins EE, Srinivasan M. Soluble forms of Toll-like receptor 4 are present in human saliva and modulate tumour necrosis factor-alpha secretion by macrophage-like cells. *Clin Exp Immunol* 2009;**156**: 285–293.
31. Schilling JD, Mulvey MA, Hultgren SJ. Dynamic interactions between host and pathogen during acute urinary tract infections. *Urology* 2001;**57**:56–61.
32. Sobel JD, Myers P, Levison ME, Kaye D. Comparison of bacterial and fungal adherence to vaginal exfoliated epithelial cells and human vaginal epithelial tissue culture cells. *Infect Immun* 1982;**35**: 697–701.
33. Bisson-Boutelliez C, Miller N, Demarch D, Bene MC. CD9 and HLA-DR expression by crevicular epithelial cells and polymorphonuclear neutrophils in periodontal disease. *J Clin Periodontol* 2001;**28**:650–656.
34. Al-Sabbagh M, Alladah A, Lin Y *et al*. Bone remodeling-associated salivary biomarker MIP-1alpha distinguishes periodontal disease from health. *J Periodontal Res* 2012;**47**:389–395.
35. Ramseier CA, Kinney JS, Herr AE *et al*. Identification of pathogen and host-response markers correlated with periodontal disease. *J Periodontol* 2009;**80**: 436–446.
36. Sexton WM, Lin Y, Kryscio RJ, Dawson DR 3rd, Ebersole JL, Miller CS *et al*. Salivary biomarkers of periodontal disease in response to treatment. *J Clin Periodontol* 2011;**38**:434–441.
37. Srinivasan M, Summerlin DJ. Modulation of the colonic epithelial cell responses and amelioration of inflammation by CD80 blockade in TNBS colitis. *Clin Immunol* 2009;**133**:411–421.
38. Kedjarune U, Pongprerachok S, Arpornmaeklong P, Ungkusonmongkhon K. Culturing primary human gingival epithelial cells: comparison of two isolation techniques. *J Craniomaxillofac Surg* 2001;**29**: 224–231.
39. Cheng B, Rhodus NL, Williams B, Griffin RJ. Detection of apoptotic cells in

whole saliva of patients with oral premalignant and malignant lesions: a preliminary study. *Oral Surg Oral Med Oral Pathol Oral Radiol Endod* 2004;**97**:465–470.
40. Loktionov A. Cell exfoliation in the human colon: myth, reality and implications for colorectal cancer screening. *Int J Cancer* 2007;**120**:2281–2289.
41. Stamm WE, Theodore E. Woodward Award: host-pathogen interactions in community-acquired urinary tract infections. *Trans Am Clin Climatol Assoc* 2006;**117**:75–83; discussion 83-74.
42. Wertz PW, Squier CA. Cellular and molecular basis of barrier function in oral epithelium. *Crit Rev Ther Drug Carrier Syst* 1991;**8**:237–269.
43. Jajarm HH, Mohtasham N, Moshaverinia M, Rangiani A. Evaluation of oral mucosa epithelium in type II diabetic patients by an exfoliative cytology method. *J Oral Sci* 2008;**50**:335–340.
44. Perez-Sayans M, Somoza-Martin JM, Barros-Angueira F *et al*. Exfoliative cytology for diagnosing oral cancer. *Biotech Histochem* 2009;**85**:177–187.
45. Rahmoune H *et al*. Glucose transporters in human renal proximal tubular cells isolated from the urine of patients with non-insulin-dependent diabetes. *Diabetes* 2005;**54**:3427–3434.
46. Stoker M, Perryman M. Cultures of exfoliated mammary epithelial cells: variation between donors. *Breast Cancer Res Treat* 1984;**4**:11–18.
47. Belik R, Follmann W, Degen GH *et al*. Improvements in culturing exfoliated urothelial cells in vitro from human urine. *J Toxicol Environ Health A* 2008;**71**:923–929.
48. Detrisac CJ, Mayfield RK, Colwell JA, Garvin AJ, Sens DA. In vitro culture of cells exfoliated in the urine by patients with diabetes mellitus. *J Clin Invest* 1983;**71**:170–173.
49. Albaugh GP, Iyengar V, Lohani A, Malayeri M, Bala S, Nair PP Isolation of exfoliated colonic epithelial cells, a novel, non-invasive approach to the study of cellular markers. *Int J Cancer* 1992;**52**:347–350.
50. Gireesh T, Sudhakaran PR. In vitro uptake of beta-carotene by human exfoliated colonic epithelial cells. *Int J Food Sci Nutr* 2009;**60**:109–118.
51. Boutinaud M, Jammes H. Potential uses of milk epithelial cells: a review. *Reprod Nutr Dev* 2002;**42**:133–147.
52. Collins LM, Dawes C. The surface area of the adult human mouth and thickness of the salivary film covering the teeth and oral mucosa. *J Dent Res* 1987;**66**:1300–1302.
53. Nagar P, Singh K, Chauhan I *et al*. Orally disintegrated tablets: formulation, preparation techniques and evaluation. *J Appl Pharm Sci* 2011;**1**:35–45.
54. Lavelle CLB. Oral Mucosa and Periodontium. *Applied Oral Physiology*, The University of, Michigan, J. Wright, 1975.
55. Hung PS *et al*. Insulin-like growth factor binding protein-5 enhances the migration and differentiation of gingival epithelial cells. *J Periodontal Res* 2008;**43**:673–680.
56. Mager DL, Haffajee AD, Socransky SS. Effects of periodontitis and smoking on the microbiota of oral mucous membranes and saliva in systemically healthy subjects. *J Clin Periodontol* 2003;**30**: 1031–1037.
57. Eger T, Zoller L, Muller HP, Hoffmann S, Lobinsky D. Potential diagnostic value of sampling oral mucosal surfaces for Actinobacillus actinomycetemcomitans in young adults. *Eur J Oral Sci* 1996;**104**:112–117.
58. Rudney JD, Chen R, Sedgewick GJ. Intracellular Actinobacillus actinomycetemcomitans and Porphyromonas gingivalis in buccal epithelial cells collected from human subjects. *Infect Immun* 2001;**69**:2700–2707.
59. Saglie FR, Carranza FA Jr, Newman MG, Cheng L, Lewin KJ. Identification of tissue-invading bacteria in human periodontal disease. *J Periodontal Res* 1982;**17**:452–455.
60. Fine DH, Markowitz K, Furgang D, Velliyagounder K. Aggregatibacter actinomycetemcomitans as an early colonizer of oral tissues: epithelium as a reservoir? *J Clin Microbiol* 2010;**48**:4464–4473.
61. Damen GM, Hol J, de Ruiter L *et al*. Chemokine production by buccal epithelium as a distinctive feature of pediatric Crohn disease. *J Pediatr Gastroenterol Nutr* 2006;**42**:142–149.
62. Lilly EA, Shetty KV, Leigh JE, Cheeks C, Fidel PL Jr. Oral epithelial cell antifungal activity: approaches to evaluate a broad range of clinical conditions. *Med Mycol* 2005;**43**:517–523.
63. Saygun I *et al*. Salivary infectious agents and periodontal disease status. *J Periodontal Res* 2011;**46**:235–239.
64. Lappin DF, Sherrabeh S, Erridge C. Stimulants of Toll-like receptors 2 and 4 are elevated in saliva of periodontitis patients compared with healthy subjects. *J Clin Periodontol* 2011;**38**:318–325.
65. Dziarski R, Gupta D. Review: Mammalian peptidoglycan recognition proteins (PGRPs) in innate immunity. *Innate Immun* 2010;**16**:168–174.
66. Lu X, Wang M, Qi J *et al*. Peptidoglycan recognition proteins are a new class of human bactericidal proteins. *J Biol Chem* 2006;**281**:5895–5907.
67. Saha S, Jing X, Park SY *et al*. Peptidoglycan recognition proteins protect mice from experimental colitis by promoting normal gut flora and preventing induction of interferon-gamma. *Cell Host Microbe* 2010;**8**:147–162.
68. Goodson JM. Diagnosis of periodontitis by physical measurement: interpretation from episodic disease hypothesis. *J Periodontol* 1992;**63**:373–382.
69. Haffajee AD, Socransky SS, Goodson JM. Clinical parameters as predictors of destructive periodontal disease activity. *J Clin Periodontol* 1983;**10**:257–265.
70. Kirkwood KL, Cirelli JA, Rogers JE, Giannobile WV. Novel host response therapeutic approaches to treat periodontal diseases. *Periodontol 2000* 2007;**43**:294–315.