molecular oral microbiology

molecular oral microbiology

DNA from *Porphyromonas gingivalis* and *Tannerella forsythia* induce cytokine production in human monocytic cell lines

S.E. Sahingur[1], X.-J. Xia[1], S. Alamgir[1], K. Honma[2], A. Sharma[2] and H.A. Schenkein[1]

1 Virginia Commonwealth University, School of Dentistry, Department of Periodontics, Richmond, VA, USA
2 State University of New York at Buffalo, School of Dentistry, Department of Oral Biology, Buffalo, NY, USA

Correspondence: Sinem E. Sahingur, Virginia Commonwealth University, School of Dentistry, Department of Periodontics, Richmond, VA, USA Tel.: +(804) 827-1710; fax: +(804) 828-0657; E-mail: ssahingur@vcu.edu

Keywords: cytokines; periodontitis; *P. gingivalis*; *T. forsythia*; TLR9
Accepted 22 September 2009

SUMMARY

Toll-like receptor 9 (TLR9) expression is increased in periodontally diseased tissues compared with healthy sites indicating a possible role of TLR9 and its ligand, bacterial DNA (bDNA), in periodontal disease pathology. Here, we determine the immunostimulatory effects of periodontal bDNA in human monocytic cells (THP-1). THP-1 cells were stimulated with DNA of two putative periodontal pathogens: *Porphyromonas gingivalis* and *Tannerella forsythia*. The role of TLR9 in periodontal bDNA-initiated cytokine production was determined either by blocking TLR9 signaling in THP-1 cells with chloroquine or by measuring IL-8 production and nuclear factor-κB (NF-κB) activation in HEK293 cells stably transfected with human TLR9. Cytokine production (IL-1β, IL-6, and TNF-α) was increased significantly in bDNA-stimulated cells compared with controls. Chloroquine treatment of THP-1 cells decreased cytokine production, suggesting that TLR9-mediated signaling pathways are operant in the recognition of DNA from periodontal pathogens. Compared with native HEK293 cells, TLR9-transfected cells demonstrated significantly increased IL-8 production ($P < 0.001$) and NF-κB activation in response to bDNA, further confirming the role of TLR9 in periodontal bDNA recognition. The results of PCR arrays demonstrated upregulation of proinflammatory cytokine and NF-κB genes in response to periodontal bDNA in THP-1 cells, suggesting that cytokine induction is through NF-κB activation. Hence, immune responses triggered by periodontal bacterial nucleic acids may contribute to periodontal disease pathology by inducing proinflammatory cytokine production through the TLR9 signaling pathway.

INTRODUCTION

Periodontal disease is the most common form of chronic inflammatory disease resulting in alveolar bone destruction and tooth loss (Taubman *et al.*, 2005; Cochran, 2008). While a bacterial biofilm composed of multiple species initiates periodontal disease, most of the tissue destruction in periodontal lesions is believed to occur as a result of host immune response-mediated destructive processes (Sahingur & Cohen, 2004; Taubman *et al.*, 2005; Schenkein, 2006; Cochran, 2008). *Porphyromonas gingivalis* and *Tannerella forsythia* are two periodontal pathogens that are frequently isolated from severe periodontal lesions (Haffajee & Socransky, 1994). The host immune system responds to invading pathogens by detecting conserved pathogen-associated molecular patterns (PAMPs) through an array of receptors,

called pattern recognition receptors, that includes Toll-like receptors (TLRs) (Janeway & Medzhitov, 2002; Akira, 2006; Akira *et al.*, 2006). PAMP-initiated, TLR-mediated immunoinflammatory responses play an important role in periodontal disease pathology mainly by promoting increased expression of inflammatory cytokines (Mahanonda & Pichyangkul, 2007; Gibson *et al.*, 2008; Hajishengallis *et al.*, 2008a,b, 2009; Onishi *et al.*, 2008; Uehara *et al.*, 2008). While various PAMPs and their associated receptors have been characterized and studied extensively in periodontal disease etiology, the possible role of bacterial nucleic acids in periodontal pathology has only recently started to receive attention (Takeshita *et al.*, 1999; Nonnenmacher *et al.*, 2003; Taubman *et al.*, 2007).

Bacterial nucleic acids are recognized mainly through TLR9 (Bauer *et al.*, 2001; Jurk *et al.*, 2006; Heeg *et al.*, 2008) and interactions between bacterial DNA (bDNA) and TLR9 have been proposed to be involved in inflammation-induced tissue destruction in various diseases, including sepsis (Plitas *et al.*, 2008; Sjolinder *et al.*, 2008), systemic lupus erythematosus (Komatsuda *et al.*, 2008; Wu *et al.*, 2009; Zorro *et al.*, 2009), and rheumatoid arthritis (Loos *et al.*, 2006; Nakano *et al.*, 2008; Rudnicka *et al.*, 2009), most of which share common pathophysiology with periodontal disease. The bDNA binding to the endosomally localized TLR9 leads to activation of nuclear factor-κB (NF-κB) and mitogen-activated protein kinase pathways, which stimulate not only potent proinflammatory activities but also the interferon regulatory factor pathway that induces anti-inflammatory activities (Kumagai *et al.*, 2008). Host cells distinguish pathogenic DNA from host DNA by the abundance of unmethylated/hypomethylated cytosine-phosphate-guanosine (CpG) dinucleotides (CpG motifs) in the bacterial genome (Bauer *et al.*, 2001). In eukaryotic DNA, these motifs are rare and mostly methylated. Most of the studies conducted so far used synthetic CpG oligonucleotides to investigate the receptor–ligand interactions in bDNA-mediated immune responses (Dalpke *et al.*, 2002; Krieg, 2006; Heeg *et al.*, 2008). The structures of naturally occurring TLR9 activators are more heterogeneous and different from the synthetic CpG oligonucleotides. Hence, although particular CpG oligonucleotides are undoubtedly useful in elucidating the pharmacology of TLR9, it is sometimes hard to predict the effects of TLR9 activation with natural DNA by extrapolating results obtained with synthetic oligonucleotides. Besides differences encountered between native DNA and oligonucleotides, the extent of the immune response to different bacterial nucleic acids also varies significantly among species and recognition of bacterial nucleic acids may show differences depending on cell type (Dalpke *et al.*, 2006; Mogensen *et al.*, 2006). Hence it is important to determine the immunostimulatory effect of native bDNA from different pathogens in different cell types to determine the contribution of these molecular structures to the pathology of specific diseases especially those involving multifactorial etiology like periodontal diseases.

Recent evidence suggests a possible role of bDNA-initiated immune responses in periodontal disease. Takeshita *et al.* (1999) reported interleukin-6 (IL-6) production in response to *P. gingivalis* DNA from gingival fibroblasts via NF-κB stimulation. Nonnenmacher *et al.* (2003) reported increased IL-6 production from gingival fibroblasts in response to *P. gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Peptostreptococcus micros* DNA in a CpG-dependent manner. Moreover, increased TLR9 expression in periodontally diseased tissues compared with healthy sites has been reported further, supporting a possible role of TLR9 and its ligands in periodontal disease pathology (Kajita *et al.*, 2007; Beklen *et al.*, 2008). Monocytes/macrophages are key players in immune response-mediated tissue destruction in periodontal disease. To date, there are no studies investigating the interactions between periodontal bDNA and human monocytes. Therefore the aim of this study is to investigate the role of periodontal bacterial nucleic acids in proinflammatory cytokine production (IL-1β, IL-6, and TNF-α) from human monocytic cells (THP-1) and to identify the receptors and the signaling pathways involved in this process.

METHODS

Reagents

Highly purified *Escherichia coli* lipopolysaccharide (LPS), calf DNA, DNAse I solution and 2-mercaptoethanol were purchased from Sigma-Aldrich (St. Louise, MO). Polymyxin B solution was purchased from Fluka (St. Louise, MO) and chloroquine

diphosphate was purchased from MP Biomedicals (Irvine, CA). Fetal bovine serum was purchased from Invitrogen (Grand Island, NY).

Bacterial growth and preparation of bDNA

The bacterial strains tested included *P. gingivalis* American Type Culture Collection (ATCC) 33277, *T. forsythia* ATCC 43037, and *E. coli* XL1Blue. All bacterial strains except *E. coli* were grown anaerobically (5% CO_2, 10%H_2, 85% N_2). Briefly, *T. forsythia* was grown in brain–heart infusion (Difco Laboratories, Detroit MI) broth containing 5 μg ml^{-1} hemin, 0.5 mg ml^{-1} menadione, 1 g l^{-1} l-cysteine, 5 g l^{-1} yeast extract, 0.001% *N*-acetyl muramic acid, and 5% fetal bovine serum (Life Technologies, Grand Island, NY). *P. gingivalis* was grown anaerobically in Trypticase soy broth supplemented with yeast extract (1 mg ml^{-1}), hemin (5 μg ml^{-1}), and menadione (1 μg ml^{-1}). *E. coli* was grown aerobically at 37°C in brain–heart infusion. Bacteria were harvested by centrifugation at 6000 ***g*** and 4°C for 10 min, washed in Dulbecco's phosphate buffered saline (Sigma, St Louis, MO), pH 7.2. The bDNA was isolated by repeated extraction with phenol–chloroform–isoamyl alcohol, and precipitated with sodium acetate and ethanol. All DNA preparations were suspended in DNAse-free, LPS-free distilled water and LPS contamination was determined by *Limulus* amebocyte assay (Cambrex, LONZA, Walkersville, MD). To inactivate any residual LPS, bDNA preparations were further treated with polymyxin sulfate (50 μg ml^{-1}) (Fluka) for 1 h before stimulation. In some experiments DNA preparations were exposed to DNAse (Sigma-Aldrich) before stimulation to further confirm that cytokine induction was the result of bDNA and not related to any protein or LPS contamination. DNAse activity was terminated using 5 mm ethylenediamine tetraacetic acid and incubating the preparation at 70°C for 10 min.

Cells and culture conditions

The human monocytic leukemia cell line (THP-1) cells and native human embryonic kidney (HEK) cells (HEK293), and HEK cells stably expressing human TLR9 were purchased from the ATTC (Manassas, VA) and Invivogen (Santa Cruz, CA), respectively. THP-1 cells were grown in RPMI-1640 (Invitrogen) medium supplemented with 2 mm l-glutamine, 10% fetal bovine serum (Invitrogen), 50 μm 2-mercaptoethanol, and 1% antibiotics (penicillin/streptomycin) (Invitrogen) and HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 4.5 g l^{-1} glucose (Sigma Aldrich), 10% fetal bovine serum, and 100 μg/ml Normocin™ (Invivogen) at 37°C in a humidified 5% CO_2 atmosphere. The cells were maintained in a logarithmic phase of growth (2×10^5 to 2×10^6) by passage every 3–4 days.

Stimulation of THP-1 and HEK293 cells and determination of cytokine levels

THP-1 cells, native HEK293 cells not expressing TLR9 (NHEK293), and stably transfected HEK293 cells expressing human TLR9 (HEK293/TLR9) were stimulated with 100 μg ml^{-1} bDNA from *P. gingivalis* ATCC 33277 and *T. forsythia* ATCC 43037 using 48-well plates (1×10^6 cells per well) at 37°C in 5% CO_2 for 12 h. DNA from *E. coli* (XL1 blue, Stratagene, La Jolla, CA) (100 μg ml^{-1}) and *E. coli* LPS (1 ng ml^{-1}) served as positive controls and eukaryotic DNA from calf thymus (100 μg ml^{-1}; Sigma-Aldrich) was used as negative control. Cell-free culture supernatants were analysed for specific cytokines using enzyme-linked immunosorbent assay (ELISA; R&D Systems, Minneapolis, MN). IL-1β, IL-6, and TNF-α levels were determined in THP-1 cell supernatants and IL-8 levels were determined in HEK293 cell supernatants.

Determination of TLR9 involvement in periodontal bDNA-induced inflammatory response

Two approaches have been used to determine the role of TLR9 in periodontal bDNA-induced cytokine production. The first approach included treatment of THP-1 cells with chloroquine (10 μg ml^{-1}) for 45 min before DNA stimulation. Chloroquine is an antimalarial agent and widely used to block TLR9 signaling by affecting endosomal acidification (El Kebir *et al.*, 2009). Cytokine levels (IL-1β, IL-6, and TNF-α) were determined in culture supernatants using ELISA (R&D Systems) following the manufacturer's instructions. The second approach included comparison of IL-8 production between native HEK293 cells not expressing TLR9 and HEK293 cells stably expressing

TLR9 in response to periodontal bDNA. Both transfected and untransfected cells were stimulated with DNA of *P. gingivalis* (Pg-DNA) and *T. forsythia* (Tf-DNA) (100 μg ml^{-1}) in 48-well plates (1×10^6 cells per well) at 37°C in 5% CO_2 for 12 h and culture supernatants were collected and assessed for IL-8 production by ELISA (R&D Systems).

Determination of NF-κB activation in HEK293 cells in response to Pg-DNA and Tf-DNA

E. coli GT110 transformed with pNiFty2 plasmid carrying a reporter gene encoding secreted alkaline phosphatase (SEAP), the expression of which is controlled by an NF-κB-inducible endothelial cell–leukocyte adhesion molecule (ELAM-1) composite promoter was purchased from Invivogen. The transformed *E. coli* cells were grown in Luria–Bertani agar plate supplemented with Zeocin™ at 37°C. Then, single bacterial colonies were isolated and grown in Terrils Broth (TB) supplemented with Zeocin™ overnight followed by purification of the plasmid DNA using PureYield™ binding columns (Promega, Madison, WA) following the manufacturer's instructions. The plasmid DNA was complexed with LyoVec™ (Invivogen) followed by transfection of native HEK293 cells (NHEK293) and HEK293 cells expressing human TLR9 (HEK293/TLR9) with the pNiFty2-SEAP plasmid-LyoVec™ complex in 48-well plates containing 1×10^5 cells per well at 37°C in 5% CO_2 for 24 h. The efficiency of transfection was determined by transfecting cells with LyoVec™-GFP/LacZ. The transfected cells were then stimulated with bDNA or left unstimulated as outlined in previous sections and NF-κB activity was determined in cell-free culture supernatants by quantification of SEAP activity using a colorimetric enzyme assay QUANTI-Blue™ (Invivogen) by reading the optical density at 620 nm with a microplate reader.

Determination of gene expression in human monocytic cells in response to Pg-DNA and Tf-DNA

Further experiments included polymerase chain reaction (PCR) arrays to determine the gene expression profile in response to periodontal bDNA in THP-1 cells. Briefly, cells grown in 24-well culture plates (2×10^6 per well) were stimulated with Pg-DNA or Tf-DNA (100 μg ml^{-1}) or left unstimulated for 24 h and collected at designated time points (15 min, 30 min, 1 h, 2 h, 4 h, and 6 h). RNA was isolated from stimulated and unstimulated cells by using the RNeasy kit (Qiagen, Valencia, CA) with on-column DNAse digestion and complementary DNA (cDNA) was generated using an RT^2 First Strand Kit (SABiosciences, Frederick, MD) following the manufacturer's instructions. Briefly, the amount of RNA was determined with a NaNoDrop (ND-1000) spectrophotometer (NaNo Drop Technologies, Wilmington, DE) and the quality of the RNA was determined by performing 1% agarose gel electrophoresis. A total of 0.5 μg RNA was incubated in genomic DNA elimination buffer at 42°C for 5 min and placed on ice for at least 1 min. Then the reverse transcription was performed using an RT^2 First Strand Kit (SABiosciences) using random primers by incubating at 42°C for 15 min followed by heating at 95°C for 5 min. The cDNA was held on ice before real-time analyses of gene expression or stored at −20°C until use. The quality of cDNA was determined by running an alkaline agarose gel electrophoresis. Gene expression was analysed with a total of 150 ng per well cDNA in 96-well plates coated with specific primers that reacted with probes labeled with SYBR green using RT^2 Profiler™ Human Toll-Like Receptor Signalling Pathway PCR Arrays (SABiosciences) with real-time PCR (Applied Biosystems 7500, Foster City, CA) following the manufacturer's instructions.

Statistical analyses

Cytokine levels were determined in supernatants using ELISA (R&D Systems). Data were analysed by analysis of variance, using the Tukey and Dunn method with GraphPad Prism (version 4.0) software and the results were presented as the mean ± SEM. The results represent at least three independent experiments which were run in triplicate. A value of $P \leq 0.05$ was considered significant. The results obtained from PCR arrays were analysed using a computer program available at the company's web site (SABiosciences). At least a four-fold change in gene expression compared with unstimulated controls was considered significant after adjusting baseline values to housekeeping genes.

RESULTS

Periodontal bacterial nucleic acids activate human monocytic cells to produce proinflammatory cytokines (IL-1β, IL-6, and TNF-α)

All bDNA preparations were tested for the presence of endotoxin by *Limulus amebocyte* assay (Cambrex, LONZA). Although the LPS concentration remained below stimulatory levels (< 0.05 ng ml^{-1}) throughout the study, to further control for the possible effect of LPS, we treated the bDNA preparations with polymyxin (50 μg ml^{-1}) before stimulation. The bDNA concentration that was used to stimulate the cells in our experiments was determined by stimulating THP-1 cells with different concentrations of DNA (50–400 μg ml^{-1}; data not shown). Consistent with previous reports (Takeshita *et al.*, 1999; Nonnenmacher *et al.*, 2003; Dalpke *et al.*, 2006), we also determined 100 μg ml^{-1} bDNA as the optimal stimulating concentration and therefore used this concentration in our assays. THP-1 cells were stimulated with periodontal bDNA (100 μg ml^{-1}) and *E. coli* bDNA (XL1 blue, Stratagene) (100 μg ml^{-1}) for 12 h. *E. coli* LPS (1 ng ml^{-1}) served as a positive control and DNA from calf thymus (100 μg ml^{-1}; Sigma-Aldrich) and DNAse-treated bDNA preparations were used as negative controls. The levels of all cytokines (IL-1β, IL-6, and TNF-α) were increased significantly in THP-1 cells treated with bDNA compared with untreated and calf DNA-treated controls ($P < 0.05$) (Fig. 1). DNAse treatment of bDNA preparations significantly diminished the cytokine response, further confirming that cytokine induction was the result of the bDNA ($P < 0.05$) (Fig. 1). There were no significant differences in the amount of cytokine induction among different bDNA. *E. coli* DNA, the positive control, and a known stimulant of cytokine production from THP-1 cells induced similar levels of cytokine production as periodontal bacterial nucleic acids.

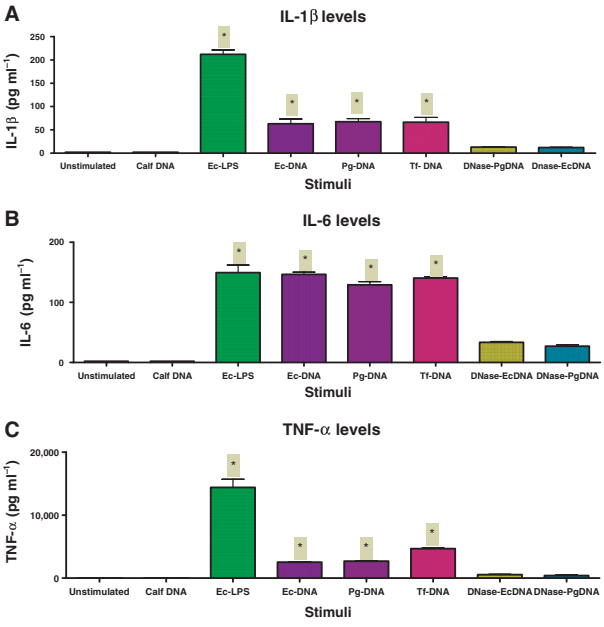

Figure 1 Interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) production was increased significantly in THP-1 cells treated with bacterial DNA compared with unstimulated and calf DNA-treated controls. DNAse treatment of bacterial DNA preparations (DNAse-EcDNA and DNAse-PgDNA) significantly diminished the cytokine response suggesting that cytokine production was linked to DNA. There were no significant differences in the amount of cytokine production among bacterial DNA preparations. *Escherichia coli* lipopolysaccharide (Ec-LPS) and DNA (Ec-DNA) were used as positive controls. *$P < 0.05$. Pg-DNA, *Porphyromonas gingivalis* DNA; Tf-DNA, *Tannerella forsythia* DNA.

TLR9 is involved in periodontal bDNA-mediated immune responses in monocytes

We next assessed the role of TLR-9 signaling in cytokine induction from monocytic cells in response to periodontal bDNA. Previous investigations demonstrated TLR9 expression in THP-1 cells and we confirmed these findings (data not shown). Chloroquine is an antimalarial agent and widely used to block TLR9 signaling by affecting endosomal acidification (Sacre *et al.*, 2008; Talati *et al.*, 2008; Abou Fakher *et al.*, 2009; El Kebir *et al.*, 2009). Chloroquine treatment (10 μg ml^{-1}) of THP-1 cells before bDNA stimulation significantly decreased the IL-1β, IL-6, and TNF-α production for all the bDNA preparations ($P < 0.05$) suggesting that TLR9-mediated signaling pathways are operant in the recognition of nucleic acids of periodontal pathogens (Fig. 2). Although IL-6 production was diminished in response to Tf-DNA after blocking TLR9 signaling (Fig. 2C), the reduction was not significant. Furthermore, even though the cytokine levels were diminished significantly in cells treated with chloroquine compared with the cells that did not receive the chloroquine, we were unable to abolish the cytokine production completely and the cytokine production was still significantly

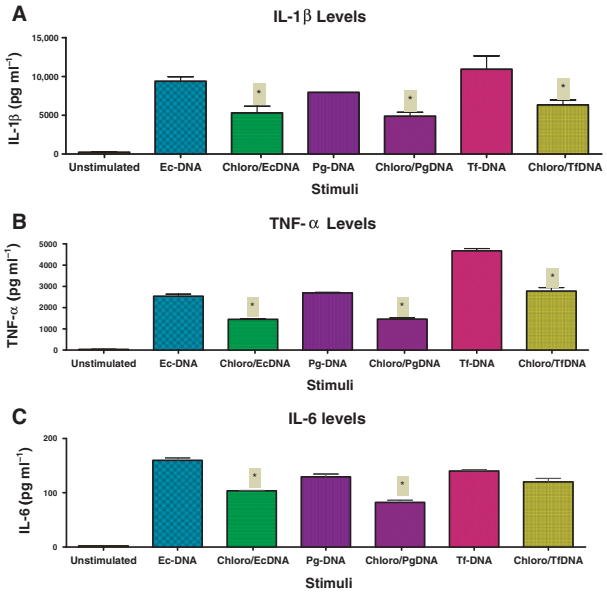

Figure 2 Chloroquine treatment of THP-1 cells before stimulation significantly decreased the cytokine production suggesting a role of Toll-like receptor 9 (TLR9) -mediated signalling in periodontal bacterial DNA-induced cytokine production in human monocytes. However, compared with unstimulated cells, cytokine production was still statistically elevated in stimulated cells. *$P < 0.05$.

elevated in response to bDNA compared with unstimulated cells. Therefore, to further confirm the involvement of TLR9 in periodontal bDNA-mediated immune responses, we tested cytokine induction (IL-8) and NF-κB activation in HEK293 cells stably transfected with human TLR9 in response to stimulation with *P. gingivalis* and *T. forsythia* DNA (100 ng/μl). Compared with native HEK293 (HEK293N) cells that are TLR9 deficient, HEK293 cells stably expressing human TLR9 (HEK293/TLR9) demonstrated significantly increased IL-8 production ($P < 0.001$) in response to Pg-DNA and Tf-DNA further confirming the role of TLR9 in periodontal bDNA recognition (Fig. 3). Moreover, NF-κB activation was also increased in response to periodontal bDNA in HEK cells expressing human TLR9 compared with unstimulated controls (Fig. 4). There was no significant difference in IL-8 production in response *E. coli* LPS between native HEK293 cells and cells expressing human TLR9 (Fig. 3). We also did not detect any NF-κB activity in response to *E. coli* LPS in HEK293 cells expressing TLR9 (Fig. 4). We were unable to detect NF-κB activity in HEK293 cells not expressing TLR9 (data not shown).

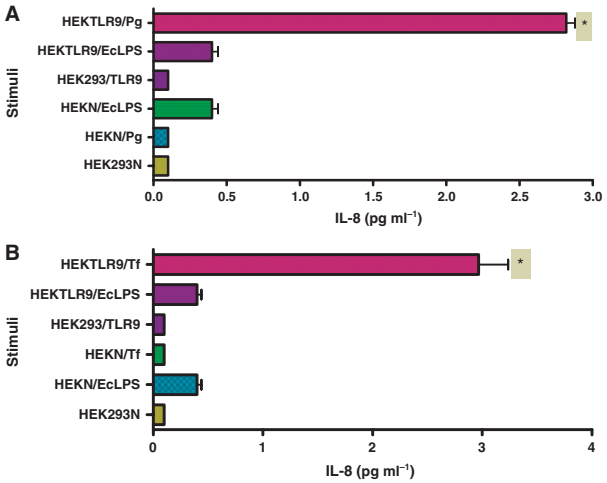

Figure 3 HEK293 cells stably transfected with human Toll-like receptor 9 (HEK/TLR9) showed significantly more interleukin-8 (IL-8) production in response to DNA from *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf) compared with wild-type cells (HEK293N) that do not express TLR9 (*$P < 0.001$). There was no significant difference in IL-8 production in response to *Escherichia coli* lipopolysaccharide (Ec LPS) in wild-type HEK293 cells compared with the cells expressing TLR9 (HEK/TLR9).

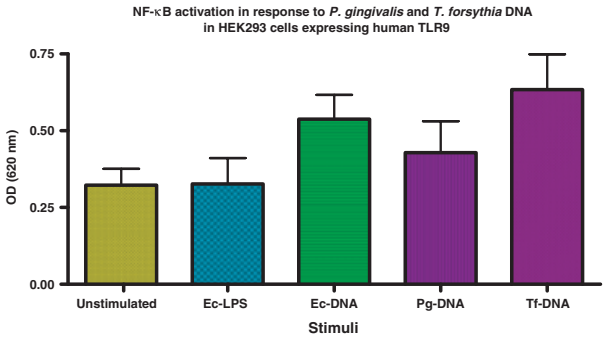

Figure 4 Nuclear factor-κB (NF-κB) activation was determined by measuring secreted alkaline phosphatase (SEAP) activity in HEK293 cells transfected with PNiFty2-SEAP. HEK293 cells expressing human Toll-like receptor 9 (HEK/TLR9) showed 1.5- to 2-fold more nuclear factor-κB (NF-κB) activation in response to DNA from *Porphyromonas gingivalis* (Pg) and *Tannerella forsythia* (Tf) compared with unstimulated cells. No NF-κB activity was determined in response to *Escherichia coli* LPS (Ec LPS).

Periodontal bDNA (Pg-DNA) induces gene expression of proinflammatory and anti-inflammatory cytokines and activates NF-κB signaling pathways

To determine the expression of cytokine genes and signaling molecules, Pg-DNA-treated THP-1 cells were collected at designated time points (15 min,

30 min, 1 h, 2 h, 4 h, and 6 h) and gene expression was analysed. Table 1 summarizes the gene expression profile of THP-1 cells stimulated with Pg-DNA compared with unstimulated control cells after adjusting the values to housekeeping genes. We have previously determined the constitutive expression of TLR9 in THP-1 cells (data not shown) and the results of PCR arrays further confirmed our findings (Table 1). The expression of proinflammatory cytokine genes (e.g. IL-1β, IL-6, IL-8, TNF-α) and NF-κB gene was upregulated in response to Pg-DNA suggesting that cytokine induction of Pg-DNA is mediated through NF-κB. The results of PCR arrays also demonstrated increased expression of an anti-inflammatory cytokine (IL-10) in response to Pg-DNA in monocytic cells. TLR9 expression was upregulated almost four-fold after 15 min of stimulation, but then the levels remained constant. Interestingly, TLR7 expression remained upregulated throughout the experiment.

Table 1 Gene expression profile of THP-1 cells stimulated with *Porphyromonas gingivalis* DNA[1,2]

	Fold Regulation (Comparing to unstimulated (Control) group)							Fold Regulation (Comparing to unstimulated (Control) group)					
Gene Symbol	16 min	30 min	1 h	2 h	4 h	6 h	Gene Symbol	16 min	30 min	1 h	2 h	4 h	6 h
BTK	1.15	1.16	1.11	−1.08	−1.71	−1.6	MAPK8	1.33	−1.02	1.08	−1.06	1.39	1.31
CASP8	1.25	1.39	1.5	−1.01	1.33	1.36	MAPK8IP3	1.69	−1.14	−1.12	−1.17	−1.57	−2.09
CCL2	−2	−1.49	−1.43	−2.0	−1.54	−1.25	MYD88	1.29	0.4	1.07	−1.18	1.21	−1.12
CD14	1.08	−1.05	−1.02	−1.75	**−3.99**	**−3.35**	NFKB1	−1	−1.13	1.19	**3.98**	**7.16**	**6.02**
CD80	1.38	−1.07	1.13	**3.26**	**13.58**	**8.79**	NFKB2	1.93	2.03	**4.44**	**3.53**	2.94	2.25
CD86	−1.6	−1.58	−1.14	−1.16	1.23	−1.17	NFKBIA	1.43	**6.7**	**18.75**	**11.34**	**6.43**	**4.61**
CHUK	1.07	−1.05	−1.05	−1.39	−1.16	−1.21	NFKBIL1	1.37	1.17	1.48	−1.07	1.24	1.15
CLEC4E	2.41	2.4	**5.88**	**5.8**	**11.58**	**7.89**	NFRKB	1.61	1	1.03	−1.31	−1.23	−1.31
CSF2	2.1	−1.62	−1.05	1.06	1.87	**4.45**	NR2C2	1.62	1.32	1.15	−1.2	1.14	−1.4
CSF3	2.1	−1.62	−1.05	2.39	4.36	**4.4**	PELI1	−1.11	1.18	2.2	2.38	**3.2**	2.14
CXCL10	−1.53	1.62	**3.27**	**3.96**	**9.74**	**7.69**	PPARA	1.46	1.24	1.26	−1.14	−1.16	−1.37
EIF2AK2	−1.02	1.03	−1.04	1.04	1.79	2.51	PRKRA	1.12	−1.04	1.03	−1.09	−1.05	−1.36
ELK1	2.34	1.45	1.23	1.65	2.59	2.86	PTGS2	**−8.4**	−1.02	2.48	−1.29	−1.64	−1.53
FADD	1.91	1.48	1.07	1.02	1.41	1.63	REL	1.22	1.11	1.98	**5.75**	**6.61**	**4.65**
FOS	2.64	**5.2**	2.71	−2.92	−2.4	−2.52	RELA	1.37	−1.09	1.09	1.03	1	−1.11
HMGB1	−1.07	−1.05	−1.15	−1.18	−1.05	−1.24	RIPK2	−1.05	1.28	**3.77**	**7.06**	**4.54**	2.44
HRAS	1.27	−1.18	−1.19	−1.38	−1.25	−1.6	SARM1	1.75	1.14	1.18	−1.11	−1.58	−1.81
HSPA1A	−1.11	−1.07	−1.73	1.46	**6.3**	**3.83**	SIGIRR	1.85	1.02	1.23	−1.15	−2.19	**−4.2**
HSPD1	−1.15	1.04	1.06	−1.1	−1.16	−1.49	ECSIT	1.39	−1.06	1.01	−1.41	−1.54	−1.62
IFNA1	2.1	−1.62	1.33	−1.41	−1.01	1.2	TBK1	−1.04	1.04	1.15	1.02	1.04	−1.16
IFNB1	−1.54	1.41	**4.96**	2.82	1.26	−1.52	TICAM2	1.31	1.3	1.05	1.19	**4.05**	**3.1**
IFNG	2.1	−1.62	−1.05	−1.41	1.01	1.2	TIRAP	1.42	1.08	1.05	−1.83	−1.27	−1.29
IKBKB	1.69	1.26	1.2	−1.02	1.44	1.08	TLR1	1.59	1.59	1.23	−1.79	−1.19	1.06
IL10	1.87	**4.04**	**5.45**	**6.32**	1.57	2.56	TLR10	2.15	−1.1	1.03	−1.68	−1.83	−1.25
IL12A	1.91	1.36	1.94	−1.76	**−3.29**	**−4.61**	TLR2	1.55	1.41	1.52	1.58	1.22	−1.92
IL1A	−1.42	2.76	**20.29**	**7.32**	**6.62**	**9.91**	TLR3	1.71	−1.48	1.14	−1.01	1.07	1.52
IL1B	**−3.33**	**3.06**	**18.43**	**16.65**	**23.14**	**29.75**	TLR4	1.2	−1.05	−1.06	−1.1	1.39	1.64
IL2	2.1	−1.62	1.41	−1.41	−1.01	1.2	TLR5	1.51	1.27	−1.06	−2.5	−1.84	−2.01
IL6	−1.82	2.23	2.44	−1.71	1.33	1.33	TLR6	−1.21	1.05	−1.23	−2.01	−1.15	1.42
IL8	−2.09	1.85	**4.68**	1.82	**3.08**	**3.65**	TLR7	1.32	1.46	2.37	**6.41**	**8.98**	**3.58**
IRAK1	1.65	−1.06	1.06	−1.07	−1.11	−1.2	TLR8	−1.18	1.32	1.53	1.02	1.67	1.7
IRAK2	1.27	1.21	1.76	**20.17**	**28.61**	**16.03**	TLR9	**3.98**	1.57	1.45	1.15	2.73	1.39
IRF1	1.87	1.69	2.97	2.19	1.89	1.94	TNF	1.4	**4.09**	**11.05**	**4.96**	1.15	−2.42
IRF3	1.63	−1.15	−1.02	−1.49	−1.49	−1.44	TNFRSF1A	1.15	1.08	−1.18	−2.18	−1.16	−1.32
JUN	−2.47	1.47	**4.07**	1.63	1.64	1.81	TOLLIP	1.79	1.33	1.2	−1.29	1.13	1.07
LTA	1.62	1.4	**5.51**	**21.49**	**34.76**	**20.42**	TRAF6	1.28	1.48	1.19	1.4	1.82	1.09
CD180	1.25	1.06	−1.22	−2.46	**−4.11**	−2.86	TICAM1	2.77	1.91	2.36	**5.15**	**6.92**	**3.6**
LY86	1.41	−1.13	−1.1	−1.34	−1.38	−1.47	UBE2N	1.27	−1.22	−1.15	−1.22	−1.46	−1.88

Table 1 (*Continued*)

	Fold Regulation (Comparing to unstimulated (Control) group)							Fold Regulation (Comparing to unstimulated (Control) group)					
Gene Symbol	16 min	30 min	1 h	2 h	4 h	6 h	Gene Symbol	16 min	30 min	1 h	2 h	4 h	6 h
LY96	1.15	−1.11	−1.03	1.68	−1.55	−1.37	UBE2V1	−1.05	−1.34	−1.15	−1.61	−1.47	−2.39
MAP2K3	−1.15	−1.17	1.11	2.2	2.64	1.85	B2M	−1.07	1.24	1.3	1.52	1.48	2.03
MAP3K4	−1.16	1.04	−1.01	−1.25	−1.02	−1.11	HPRT1	−1.2	1.09	−1.13	1.09	1.19	1.19
MAP3K1	1.01	1.24	1.18	−2.3	−1.81	−2.21	RPL13A	1.28	−1.02	1.01	−1.2	−1.22	−1.07
MAP3K7	1.14	−1.11	−1.12	−1.35	−1.13	−1.15	GADPH	1.47	−1.2	−1.17	−1.24	−1.06	−1.18
MAP3K7IP1	1.46	−1.03	1.05	−1.59	−1.39	−1.52	ACTB	−1.47	−1.11	1	−1.11	−1.37	−1.91
MAP4K4	1.16	1	1	1.02	1.88	1.76							

[1]Polymerase chain reaction arrays showing at least four-fold change in gene expression in THP-1 cells stimulated with *Porphyromonas gingivalis* DNA. The genes that are marked in bold represent at least four-fold downregulated (−) or upregulated (+) gene expression compared with unstimulated controls after adjusting the baseline values to housekeeping genes.

[2]The names and abbreviations of the genes included in polymerase chain reaction arrays are: BTK, bruton agammaglobulinemia tyrosine kinase; CASP8, caspase 8, apoptosis-related cysteine peptidase; CCL2, chemokine (C-C motif) ligand 2; CD14, CD14 molecule; CD80, CD80 molecule; CD86, CD86 molecule; CHUK, conserved helix-loop-helix ubiquitous kinase; CLEC4E, C-type lectin domain family 4, member E; CSF2, colony-stimulating factor 2 (granulocyte–macrophage);CSF3, colony-stimulating factor 3 (granulocyte); CXCL10, chemokine (C-X-C motif) ligand 10; EIF2AK2, eukaryotic translation initiation factor 2-α kinase 2; ELK1, ELK1, member of ETS oncogene family; FADD, Fas (TNFRSF6)-associated via death domain; FOS, V-fos FBJ murine osteosarcoma viral oncogene homologue; HMGB1, high-mobility group box 1; HRAS, V-Ha-ras Harvey rat sarcoma viral oncogene homologue; HSPA1A, heat-shock 70 kDa protein 1A; HSPD1, heat-shock 60 kDa protein 1 (chaperonin); IFNA1, interferon-α1; IFNB1, interferon-β1, fibroblast; IFNG, interferon-γ; IKBKB, inhibitor of κ light polypeptide gene enhancer in B-cells, kinase-β; IL10, interleukin-10; IL12A, interleukin-12A (natural killer cell stimulatory factor 1, cytotoxic lymphocyte maturation factor 1); IL1A, interleukin-1α; IL1B, interleukin-1β; IL2, interleukin-2; IL6, interleukin-6 (interferon-β2); IL8, interleukin-8; IRAK1, interleukin-1 receptor-associated kinase 1; IRAK2, interleukin-1 receptor-associated kinase 2; IRF1, interferon regulatory factor 1; IRF3, interferon regulatory factor 3; JUN, Jun oncogene; LTA, lymphotoxin-α (tumor necrosis factor superfamily, member 1); CD180, CD180 molecule; LY86, lymphocyte antigen 86; LY96, lymphocyte antigen 96; MAP2K3, mitogen-activated protein kinase kinase 3; MAP2K4, mitogen-activated protein kinase kinase 4; MAP3K1, mitogen-activated protein kinase kinase kinase 1; MAP3K7, mitogen-activated protein kinase kinase kinase 7; MAP3K7IP1, mitogen-activated protein kinase kinase kinase 7 interacting protein 1; MAP4K4, mitogen-activated protein kinase kinase kinase kinase 4; MAPK8, mitogen-activated protein kinase 8; MAPK8IP3, mitogen-activated protein kinase 8 interacting protein 3; MYD88, myeloid differentiation primary response gene 88; NFKB1, nuclear factor κ light polypeptide gene enhancer in B cells 1; NFKB2, nuclear factor of κ light polypeptide gene enhancer in B cells 2; NFKBIA, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor α; NFKBIL1, nuclear factor of κ light polypeptide gene enhancer in B cells inhibitor-like 1; NFRKB, nuclear factor related to κB-binding protein; NR2C2, nuclear receptor subfamily 2, group C, member 2; PELI1, Pellino homologue 1 (*Drosophila*); PPARA, peroxisome proliferator-activated receptor-α; PRKRA, protein kinase, interferon-inducible double-stranded RNA-dependent activator; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); RELA V-rel, reticuloendotheliosis viral oncogene homologue (avian); RELAV-rel, reticuloendotheliosis viral oncogene homologue A (avian); RIPK2, receptor-interacting serine-threonine kinase 2; SARM1, sterile α and Toll–interleukin receptor motif containing 1; SIGIRR, single immunoglobulin and toll–interleukin-1 receptor domain; ECSIT, ECSIT homologue (*Drosophila*); TBK1, TANK-binding kinase 1; TICAM2, Toll-like receptor adaptor molecule 2; TIRAP, Toll-interleukin 1 receptor domain containing adaptor protein; TLR1, Toll-like receptor 1; TLR10, Toll-like receptor 10; TLR2, Toll-like receptor 2; TLR3, Toll-like receptor 3; TLR4, Toll-like receptor 4; TLR5, Toll-like receptor 5; TLR6, Toll-like receptor 6; TLR7, Toll-like receptor 7; TLR8, Toll-like receptor 8; TLR9, Toll-like receptor 9; TNF, tumor necrosis factor (TNF superfamily, member 2); TNFRSF1A, tumor necrosis factor receptor superfamily, member 1A; TOLLIP, Toll interacting protein; TRAF6, TNF receptor-associated factor 6; TICAM1, Toll-like receptor adaptor molecule 1; UBE2N, ubiquitin-conjugating enzyme E2N (UBC13 homologue, yeast); UBE2V1, ubiquitin-conjugating enzyme E2 variant 1; B2M, β2-microglobulin; HPRT1, hypoxanthine phosphoribosyltransferase 1; RPL13A, ribosomal protein L13a); GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ACTB, actin-β.

DISCUSSION

The overall goal of this study was to determine periodontal bDNA-initiated inflammatory cytokine production from human monocytic cells and to identify receptors and the downstream signaling events that are triggered by periodontal bacterial nucleic acids. Our results showed that the DNA of *P. gingivalis* and *T. forsythia*, two putative periodontal pathogens, induce the production of proinflammatory cytokines (IL-1β, IL-6, and TNF-α) through the TLR9 signaling pathway and activate the transcription factor NF-κB.

Furthermore, the results of PCR arrays also demonstrated that in addition to the expression of proinflammatory cytokines, anti-inflammatory cytokine expression (IL-10) was also upregulated, suggesting the possible involvement of periodontal bDNA–TLR9 interaction with anti-inflammatory signaling pathways, which warrants further investigation.

Little is known about the role of bDNA-initiated and TLR9-mediated immune responses in periodontal disease whereas data in other disease models are accumulating that implicate this pathway with pathology associated with several immunoinflammatory conditions (Huang *et al.*, 2005; Loos *et al.*, 2006; Albiger *et al.*, 2007; Komatsuda *et al.*, 2008; Nakano *et al.*, 2008; Plitas *et al.*, 2008; Sjolinder *et al.*, 2008; Talati *et al.*, 2008; Ramaprakash *et al.*, 2009; Rudnicka *et al.*, 2009; Zorro *et al.*, 2009). Hall *et al.* (2008) reported that intestinal commensal bDNA affects intestinal homeostasis through TLR9. Mogensen *et al.* (2006) investigated the pattern of TLR activation by three live bacteria that cause meningitis, including *Streptococcus pneumoniae*, *Haemophilus influenza* type b and *Neisseria meningitidis* and demonstrated that *S. pneumoniae* activates TLR2 and TLR9, *H. influenza* uses TLR2 and TLR4, whereas *N. meningitidis* is able to activate TLR2, TLR4, and TLR9 and all through NF-κB. In the polymicrobial sepsis model, TLR9 knockout mice exhibited increased survival accompanied by lower serum cytokine levels and higher bacterial clearance compared with the wild-type strain (Plitas *et al.*, 2008). In contrast, in a murine model of meningococcal sepsis, TLR9 knockout mice displayed reduced survival and elevated levels of bacteremia compared with wild-type mice (Sjolinder *et al.*, 2008).

As all these studies indicate, TLR9 can either be protective or destructive depending on the disease model and the extent of TLR9-triggered immune responses varies significantly among different bacterial species as well as among hosts. A recent study by Klaschik *et al.* (2009) investigated the inductive and suppressive networks that regulate TLR9-dependent gene expression *in vivo* and reported increased expression of proinflammatory and anti-inflammatory cytokine genes. These results are consistent with our observations with native periodontal bDNA (Table 1) where we have demonstrated increased expression of both proinflammatory and anti-inflammatory genes.

TLR9 is considered to be the major receptor involved in bDNA recognition subsequently resulting in increased activation of NF-κB-regulated signaling pathways depending on the cell type and bacteria (Kumagai *et al.*, 2008). Using PCR arrays we also demonstrated increased expression of the NF-κB gene in response to Pg-DNA in THP-1 cells. Moreover, we have used two different approaches to investigate the role of TLR9 in periodontal bDNA-induced cytokine production. The first approach included using a TLR9 signaling inhibitor, chloroquine, before bDNA stimulation and determining cytokine production. Pretreatment of THP-1 cells with chloroquine significantly diminished the cytokine production in response to DNA from *P. gingivalis* and *T. forsythia* in THP-1 cells; however, we were unable to completely abolish the cytokine response (Fig. 2). It is important to note that chloroquine exerts its effect as a non-specific inhibitor of TLR9 signaling by affecting endosomal acidification. Hence, it is possible that chloroquine may not completely block the cytokine response. In addition, there are also cytosolic DNA sensors implicated in bDNA recognition, such as DNA-dependent activator of interferon-regulatory factors, inflammasome, and absent in melanoma-2 for inflammasome (Takaoka *et al.*, 2007; Muruve *et al.*, 2008; Burckstummer *et al.*, 2009; Fernandes-Alnemri *et al.*, 2009; Hornung *et al.*, 2009; Roberts *et al.*, 2009) that may take part in periodontal bDNA-induced responses in monocytic cells. Our second approach was assessing the cytokine production in HEK293 cells stably transfected with human TLR9. HEK293 cells are very useful tools to study TLR signaling pathways because they do not express these receptors naturally. Compared with native HEK293 (HEK293N) cells that are TLR9 deficient, HEK293 cells stably expressing human TLR9 (HEK293/TLR9) demonstrated significantly increased IL-8 production ($P < 0.001$) in response to Pg-DNA and Tf-DNA confirming the role of TLR9 in periodontal bDNA recognition (Fig. 3). We also showed increased NF-κB activity in HEK293 cells expressing TLR9 in response to bDNA compared with unstimulated cells (Fig. 4). Furthermore, the results of PCR arrays showed almost four-fold upregulation of TLR9 expression after 15 min of Pg-DNA stimulation in THP-1 cells, but then the levels remained constant (Table 1). Interestingly, TLR7 expression remained upregulated throughout the experiment. TLR9 and TLR7 were reported to have overlapping functions

(Zucchini *et al.*, 2008) and they are both thought to be involved in autoimmunity (Krieg & Vollmer, 2007). Increased levels of anti-cardiolipin antibodies in periodontitis patients were recently reported by our group and it has been suggested that the proteins of periodontal pathogens contain specific sequences that can cross-react with host proteins leading to autoantibody production (Schenkein *et al.*, 2007). The possible interaction between periodontal diseases with autoimmune disorders has also been proposed by other investigators (Scardina & Messina, 2007; Koutouzis *et al.*, 2009; Liao *et al.*, 2009; de Pablo *et al.*, 2009). Therefore, this finding may warrant further investigation.

Differences in the composition of bacterial nucleic acids and diversity of cells specialized in different effector functions can lead to differential immune responses. It is therefore important to determine the immunostimulatory effect of native bDNA in different cell types to determine the contribution of these molecular structures to the pathology of specific diseases, especially those involving multifactorial etiology like periodontal diseases. Previously, increased IL-6 production in response to *P. gingivalis*, *A. actinomycetemcomitans*, and *P. micros* DNA from human gingival fibroblasts has been reported (Nonnenmacher *et al.*, 2003). Moreover, increased expression of TLR9 in periodontally diseased tissues compared with healthy sites has been demonstrated (Kajita *et al.*, 2007; Beklen *et al.*, 2008). Our results combined with the results of previous investigations implicate a possible role of bDNA-initiated, TLR9-mediated immune responses in the periodontal disease process that warrants further investigation using primary cells.

Recent evidence suggests that periodontal diseases are not solely site-specific, but are associated with increased incidence of adverse pregnancy outcomes, cardiovascular and respiratory diseases, and diabetes (Mealey & Ocampo, 2007; Michalowicz & Durand, 2007; Paquette *et al.*, 2007; Raghavendran *et al.*, 2007). Identification of the immunostimulatory effects of bDNA opened a new area of research. Compared with TLR9 knockout mice, wild-type mice displayed increased proinflammatory cytokine production and NF-κB activation in response to bDNA and that bDNA caused myocardial dysfunction by reducing cardiomyocyte contractility (Erridge *et al.*, 2008). Another study reported that human umbilical vein endothelial cells express TLR9 and can respond to synthetic CpG oligonucleotides by upregulating cytokine production and promoting neutrophil recruitment (El Kebir *et al.*, 2009). Moreover, extracellular chromosomal bDNA plays an important role in the formation and composition of biofilms and can stimulate polymorphonuclear leukocytes (Petersen *et al.*, 2005; Fuxman Bass *et al.*, 2008a,b; Fredheim *et al.*, 2009; Vilain *et al.*, 2009). Altered biofilm formation and increased antibiotic efficiency after cleavage of extracellular DNA have been reported (Tetz *et al.*, 2009). Therefore, it is reasonable to speculate that the interaction of bDNA with host immune cells plays an important role in the host immune response to bacterial infections, particularly in those with a biofilm etiology like periodontal diseases. The presence of periodontal bDNA in atheromatous plaques has been reported in patients with cardiovascular disease (Haraszthy *et al.*, 2000). If bDNA is proinflammatory, it may not be sufficient to kill the bacteria to fully suppress the inflammatory response. The results obtained from this study provide further evidence for the interaction of DNA of periodontal pathogens with human monocytes and the role of TLR9 signaling in periodontal disease pathology. These findings may lead to the development of alternative therapeutics to control periodontal inflammation improving the overall well-being of the host.

ACKNOWLEDGEMENTS

This work was supported by A.D. Williams Research Grant from Virginia Commonwealth University (S.E.S.), DE014749 (A.S.), and DEO18125 (H.A.S.) from the National Institutes of Health.

REFERENCES

Abou Fakher, F.H., Rachinel, N., Klimczak, M., Louis, J. and Doyen, N. (2009) TLR9-dependent activation of dendritic cells by DNA from *Leishmania major* favors Th1 cell development and the resolution of lesions. *J Immunol* **182**: 1386–1396.

Akira, S. (2006) TLR signaling. *Curr Top Microbiol Immunol* **311**: 1–16.

Akira, S., Uematsu, S. and Takeuchi, O. (2006) Pathogen recognition and innate immunity. *Cell* **124**: 783–801.

Albiger, B., Dahlberg, S., Sandgren, A. *et al.* (2007) Toll-like receptor 9 acts at an early stage in host defence

against pneumococcal infection. *Cell Microbiol* **9**: 633–644.

Bauer, S., Kirschning, C.J., Hacker, H. *et al.* (2001) Human TLR9 confers responsiveness to bacterial DNA via species-specific CpG motif recognition. *Proc Natl Acad Sci U S A* **98**: 9237–9242.

Beklen, A., Hukkanen, M., Richardson, R. and Konttinen, Y.T. (2008) Immunohistochemical localization of Toll-like receptors 1-10 in periodontitis. *Oral Microbiol Immunol* **23**: 425–431.

Burckstummer, T., Baumann, C., Bluml, S. *et al.* (2009) An orthogonal proteomic–genomic screen identifies AIM2 as a cytoplasmic DNA sensor for the inflammasome. *Nat Immunol* **10**: 266–272.

Cochran, D.L. (2008) Inflammation and bone loss in periodontal disease. *J Periodontol* **79**: 1569–1576.

Dalpke, A., Zimmermann, S. and Heeg, K. (2002) Immunopharmacology of CpG DNA. *Biol Chem* **383**: 1491–1500.

Dalpke, A., Frank, J., Peter, M. and Heeg, K. (2006) Activation of toll-like receptor 9 by DNA from different bacterial species. *Infect Immun* **74**: 940–946.

El Kebir, D., Jozsef, L., Pan, W., Wang, L. and Filep, J.G. (2009) Bacterial DNA activates endothelial cells and promotes neutrophil adherence through TLR9 signaling. *J Immunol* **182**: 4386–4394.

Erridge, C., Burdess, A., Jackson, A.J. *et al.* (2008) Vascular cell responsiveness to Toll-like receptor ligands in carotid atheroma. *Eur J Clin Invest* **38**: 713–720.

Fernandes-Alnemri, T., Yu, J.W., Datta, P., Wu, J. and Alnemri, E.S. (2009) AIM2 activates the inflammasome and cell death in response to cytoplasmic DNA. *Nature* **458**: 509–513.

Fredheim, E.G., Klingenberg, C., Rohde, H. *et al.* (2009) Biofilm formation by *Staphylococcus haemolyticus*. *J Clin Microbiol* **47**: 1172–1180.

Fuxman Bass, J.I., Alvarez, M.E., Gabelloni, M.L. *et al.* (2008a) GM-CSF enhances a CpG-independent pathway of neutrophil activation triggered by bacterial DNA. *Mol Immunol* **46**: 37–44.

Fuxman Bass, J.I., Gabelloni, M.L., Alvarez, M.E. *et al.* (2008b) Characterization of bacterial DNA binding to human neutrophil surface. *Lab Invest* **88**: 926–937.

Gibson, F.C. III, Ukai, T. and Genco, C.A. (2008) Engagement of specific innate immune signaling pathways during *Porphyromonas gingivalis* induced chronic inflammation and atherosclerosis. *Front Biosci* **13**: 2041–2059.

Haffajee, A.D. and Socransky, S.S. (1994) Microbial etiological agents of destructive periodontal diseases. *Periodontol 2000* **5**: 78–111.

Hajishengallis, G., Wang, M., Bagby, G.J. and Nelson, S. (2008a) Importance of TLR2 in early innate immune response to acute pulmonary infection with *Porphyromonas gingivalis* in mice. *J Immunol* **181**: 4141–4149.

Hajishengallis, G., Wang, M., Liang, S., Triantafilou, M. and Triantafilou, K. (2008b) Pathogen induction of CXCR4/TLR2 cross-talk impairs host defense function. *Proc Natl Acad Sci U S A* **105**: 13532–13537.

Hajishengallis, G., Wang, M. and Liang, S. (2009) Induction of distinct TLR2-mediated proinflammatory and proadhesive signaling pathways in response to *Porphyromonas gingivalis* fimbriae. *J Immunol* **182**: 6690–6696.

Hall, J.A., Bouladoux, N., Sun, C.M. *et al.* (2008) Commensal DNA limits regulatory T cell conversion and is a natural adjuvant of intestinal immune responses. *Immunity* **29**: 637–649.

Haraszthy, V.I., Zambon, J.J., Trevisan, M., Zeid, M. and Genco, R.J. (2000) Identification of periodontal pathogens in atheromatous plaques. *J Periodontol* **71**: 1554–1560.

Heeg, K., Dalpke, A., Peter, M. and Zimmermann, S. (2008) Structural requirements for uptake and recognition of CpG oligonucleotides. *Int J Med Microbiol* **298**: 33–38.

Hornung, V., Ablasser, A., Charrel-Dennis, M. *et al.* (2009) AIM2 recognizes cytosolic dsDNA and forms a caspase-1-activating inflammasome with ASC. *Nature* **458**: 514–518.

Huang, X., Barrett, R.P., McClellan, S.A. and Hazlett, L.D. Silencing Toll-like receptor-9 in *Pseudomonas aeruginosa* keratitis (2005) *Invest Ophthalmol Vis Sci* **46**: 4209–4216.

Imai, K., Takeshita, A. and Hanazawa, S. (1999) TGF-beta inhibits lipopolysaccharide-stimulated activity of c-Jun N-terminal kinase in mouse macrophages. *FEBS Lett* **456**: 375–378.

Janeway, C.A. Jr. and Medzhitov, R. (2002) Innate immune recognition. *Annu Rev Immunol* **20**: 197–216.

Jurk, M., Kritzler, A., Debelak, H., Vollmer, J., Krieg, A.M. and Uhlmann, E. (2006) Structure–activity relationship studies on the immune stimulatory effects of base-modified CpG toll-like receptor 9 agonists. *ChemMedChem* **1**: 1007–1014.

Kajita, K., Honda, T., Amanuma, R. *et al.* (2007) Quantitative messenger RNA expression of Toll-like receptors and interferon-alpha1 in gingivitis and periodontitis. *Oral Microbiol Immunol* **22**: 398–402.

Klaschik, S., Tross, D. and Klinman, D.M. (2009) Inductive and suppressive networks regulate TLR9-

dependent gene expression *in vivo*. *J Leukoc Biol* **85**: 788–795.

Komatsuda, A., Wakui, H., Iwamoto, K. *et al.* (2008) Up-regulated expression of Toll-like receptors mRNAs in peripheral blood mononuclear cells from patients with systemic lupus erythematosus. *Clin Exp Immunol* **152**: 482–487.

Koutouzis, T., Haber, D., Shaddox, L., Aukhil, I. and Wallet, S.M. (2009) Autoreactivity of serum immunoglobulin to periodontal tissue components: a pilot study. *J Periodontol* **80**: 625–633.

Krieg, A.M. (2006) Therapeutic potential of Toll-like receptor 9 activation. *Nat Rev Drug Discov* **5**: 471–484.

Krieg, A.M. and Vollmer, J. (2007) Toll-like receptors 7, 8, and 9: linking innate immunity to autoimmunity. *Immunol Rev* **220**: 251–269.

Kumagai, Y., Takeuchi, O. and Akira, S. (2008) TLR9 as a key receptor for the recognition of DNA. *Adv Drug Deliv Rev* **60**: 795–804.

Liao, F., Li, Z., Wang, Y., Shi, B., Gong, Z. and Cheng, X. (2009) *Porphyromonas gingivalis* may play an important role in the pathogenesis of periodontitis-associated rheumatoid arthritis. *Med Hypotheses* **72**: 732–735.

Loos, T., Dekeyzer, L., Struyf, S. *et al.* (2006) TLR ligands and cytokines induce CXCR3 ligands in endothelial cells: enhanced CXCL9 in autoimmune arthritis. *Lab Invest* **86**: 902–916.

Mahanonda, R. and Pichyangkul, S. (2007) Toll-like receptors and their role in periodontal health and disease. *Periodontol 2000* **43**: 41–55.

Mealey, B.L. and Ocampo, G.L. (2007) Diabetes mellitus and periodontal disease. *Periodontol 2000* **44**: 127–153.

Michalowicz, B.S. and Durand, R. (2007) Maternal periodontal disease and spontaneous preterm birth. *Periodontol 2000* **44**: 103–112.

Mogensen, T.H., Paludan, S.R., Kilian, M. and Ostergaard, L. (2006) Live *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis* activate the inflammatory response through Toll-like receptors 2, 4, and 9 in species-specific patterns. *J Leukoc Biol* **80**: 267–277.

Muruve, D.A., Petrilli, V., Zaiss, A.K. *et al.* (2008) The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response. *Nature* **452**: 103–107.

Nakano, S., Morimoto, S., Suzuki, J., Nozawa, K., Amaro, H., Tokano, Y. and Takazaki, Y. (2008) Role of pathogenic auto-antibody production by Toll-like receptor 9 of B cells in active systemic lupus erythematosus. *Rheumatology (Oxford)* **47**: 145–149.

Nonnenmacher, C., Dalpke, A., Zimmermann, S., Flores-De-Jacoby, L., Mutters, R. and Heeg, K. (2003) DNA from periodontopathogenic bacteria is immunostimulatory for mouse and human immune cells. *Infect Immun* **71**: 850–856.

Onishi, S., Honma, K., Liang, S. *et al.* (2008) Toll-like receptor 2-mediated interleukin-8 expression in gingival epithelial cells by the *Tannerella forsythia* leucine-rich repeat protein BspA. *Infect Immun* **76**: 198–205.

de Pablo, P., Chapple, I.L., Buckley, C.D. and Dietrich, T. (2009) Periodontitis in systemic rheumatic diseases. *Nat Rev Rheumatol* **5**: 218–224.

Paquette, D.W., Brodala, N. and Nichols, T.C. (2007) Cardiovascular disease, inflammation, and periodontal infection. *Periodontol 2000* **44**: 113–126.

Petersen, F.C., Tao, L. and Scheie, A.A. (2005) DNA binding-uptake system: a link between cell-to-cell communication and biofilm formation. *J Bacteriol* **187**: 4392–4400.

Plitas, G., Burt, B.M., Nguyen, H.M., Bamboat, Z.M. and DeMatteo, R.P. (2008) Toll-like receptor 9 inhibition reduces mortality in polymicrobial sepsis. *J Exp Med* **205**: 1277–1283.

Raghavendran, K., Mylotte, J.M. and Scannapieco, F.A. (2007) Nursing home-associated pneumonia, hospital-acquired pneumonia and ventilator-associated pneumonia: the contribution of dental biofilms and periodontal inflammation. *Periodontol 2000* **44**: 164–177.

Ramaprakash, H., Ito, T., Standiford, T.J., Kunkel, S.L. and Hogaboam, C.M. (2009) Toll-like receptor 9 modulates immune responses to *Aspergillus fumigatus* conidia in immunodeficient and allergic mice. *Infect Immun* **77**: 108–119.

Roberts, T.L., Idris, A., Dunn, J.A. *et al.* (2009) HIN-200 proteins regulate caspase activation in response to foreign cytoplasmic DNA. *Science* **323**: 1057–1060.

Rudnicka, W., Burakowski, T., Warnawin, E. *et al.* (2009) Functional TLR9 modulates bone marrow B cells from rheumatoid arthritis patients. *Eur J Immunol* **39**: 1211–1220.

Sacre, S.M., Lo, A., Gregory, B. *et al.* (2008) Inhibitors of TLR8 reduce TNF production from human rheumatoid synovial membrane cultures. *J Immunol* **181**: 8002–8009.

Sahingur, S.E. and Cohen, R.E. (2004) Analysis of host responses and risk for disease progression. *Periodontol 2000* **34**: 57–83.

Scardina, G.A. and Messina, P. (2007) Microvascular periodontal alterations: a possible relationship between

periodontitis and rheumatoid arthritis. *Clin Hemorheol Microcirc* **37**: 229–235.

Schenkein, H.A. (2006) Host responses in maintaining periodontal health and determining periodontal disease. *Periodontol 2000* **40**: 77–93.

Schenkein, H.A., Best, A.M., Brooks, C.N. *et al.* (2007) Anti-cardiolipin and increased serum adhesion molecule levels in patients with aggressive periodontitis. *J Periodontol* **78**: 459–466.

Sjolinder, H., Mogensen, T.H., Kilian, M., Jonsson, A.B. and Paludan, S.R. (2008) Important role for Toll-like receptor 9 in host defense against meningococcal sepsis. *Infect Immun* **76**: 5421–5428.

Takaoka, A., Wang, Z., Choi, M.K. *et al.* (2007) DAI (DLM-1/ZBP1) is a cytosolic DNA sensor and an activator of innate immune response. *Nature* **448**: 501–505.

Takeshita, A., Imai, K. and Hanazawa, S. (1999) CpG motifs in *Porphyromonas gingivalis* DNA stimulate interleukin-6 expression in human gingival fibroblasts. *Infect Immun* **67**: 4340–4345.

Talati, A.J., Kim, H.J., Kim, Y.I., Yi, A.K. and English B.K. (2008) Role of bacterial DNA in macrophage activation by group B streptococci. *Microbes Infect* **10**: 1106–1113.

Taubman, M.A., Valverde, P., Han, X. and Kawai, T. (2005) Immune response: the key to bone resorption in periodontal disease. *J Periodontol* **76**: 2033–2041.

Taubman, M.A., Han, X., Larosa, K.B., Socransky, S.S. and Smith, D.J. (2007) Periodontal bacterial DNA suppresses the immune response to mutans streptococcal glucosyltransferase. *Infect Immun* **75**: 4088–4096.

Tetz, G.V., Artemenko, N.K. and Tetz, V.V. (2009) Effect of DNase and antibiotics on biofilm characteristics. *Antimicrob Agents Chemother* **53**: 1204–1209.

Uehara, A., Imamura, V., Potempa, J., Travis, J. and Takada, H. (2008) Gingipains from *Porphyromonas gingivalis* synergistically induce the production of proinflammatory cytokines through protease-activated receptors with Toll-like receptor and NOD1/2 ligands in human monocytic cells. *Cell Microbiol* **10**: 1181–1189.

Vilain, S., Pretorius, J.M., Theron, J. and Brozel, V.S. (2009) DNA as an adhesin: *Bacillus cereus* requires extracellular DNA to form biofilms. *Appl Environ Microbiol* **75**: 2861–2868.

Wu, O., Chen, G.P., Chen, H. *et al.* (2009) The expressions of Toll-like receptor 9 and T-bet in circulating B and T cells in newly diagnosed, untreated systemic lupus erythematosus and correlations with disease activity and laboratory data in a Chinese population. *Immunobiology* **214**: 392–402.

Zorro, S., Arias, M., Riano, F. *et al.* (2009) Response to ODN-CpG by B Cells from patients with systemic lupus erythematosus correlates with disease activity. *Lupus* **18**: 718–726.

Zucchini, N., Bessou, G., Traub, S. *et al.* (2008) Cutting edge: overlapping functions of TLR7 and TLR9 for innate defense against a herpesvirus infection. *J Immunol* **180**: 5799–5803.