TLR2 Promoter Hypermethylation Creates Innate Immune Dysbiosis

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M. Benakanakere¹, M. Abdolhosseini¹, K. Hosur², L.S. Finoti¹,
and D.F. Kinane^{1,3}

Abstract

Periodontitis is a common chronic inflammatory disease that is initiated by a complex microbial biofilm that poses significant health and financial burdens globally. *Porphyromonas gingivalis* is a predominant pathogen that maintains chronic inflammatory periodontitis. Toll-like receptors (TLRs) play an important role in periodontitis by recognizing pathogens and maintaining tissue homeostasis. Deficiencies in TLR expression and downstream signaling may reduce the host's innate defenses against pathogens, leading to bacterial persistence and exacerbated inflammation, which are now being better appreciated in disease pathologies. In the case of periodontitis, gingival epithelial cells form the first line of defense against pathogens. Innate immune dysregulation in these cells relates to severe disease pathology. We recently identified a blunted TLR2 expression in certain gingival epithelial cells expressing diminished cytokine signaling upon *P. gingivalis* stimulation. Upon detailed analysis of the TLR2 promoter CpG Island, we noted higher CpG methylation in this dysregulated cell type. When these cells were treated with DNA methyltransferase inhibitor, TLR2 mRNA and cytokine expression were significantly increased. If TLR2 expression plasmid was ectopically expressed in dysfunctional cells prior to *P. gingivalis* stimulation, the cytokine expression was increased, confirming the requirement of TLR2 in the *P. gingivalis*-mediated inflammatory response. We designed a chronic in vitro infection model to test if *P. gingivalis* can induce DNA methylation in normal gingival epithelial cells that express higher TLR2 upon agonist stimulation. Chronic treatment of normal epithelial cells with *P. gingivalis* introduced de novo DNA methylation within the cells. In addition, increased DNA methylation was observed in the gingiva of mice infected with *P. gingivalis* in a periodontitis oral gavage model. Moreover, tissues obtained from periodontitis patients also exhibited differential TLR2 promoter methylation, as revealed by bisulfite DNA sequencing. Taken together, DNA methylation of TLR2 can modulate host innate defense mechanisms that may confer increased disease susceptibility.

Keywords: gingival epithelial cells, *P. gingivalis*, DNA methylation, chronic in vitro infection, oral gavage, CpG island

Introduction

The oral mucosal surface is continuously exposed to pathogens and commensal microorganisms. In dysbiosis, pathogens elicit deleterious immune responses that affect oral tissue homeostasis. Oral gingival epithelial cells play a critical role in maintaining innate immune homeostasis (Kinane et al. 2008). These cells must differentiate pathogens from commensals and instigate inflammatory responses to the former but not the latter. Continual pathogen exposure evokes chronic inflammation, which may cause heritable changes in the epithelial cells such that they fail to respond to the bacteria; instead, the cells may turn off important pattern recognition receptor expression and downstream inflammatory signaling and become less responsive, thus losing their innate and antimicrobial defense systems. It is already well established that inflammation in the periodontium is initiated by microbial biofilm (Haffajee et al. 1984; Kinane et al. 2008; Teles et al. 2012).

Human gingival epithelial cells (HGEs) recognize *Porphyromonas gingivalis* through toll-like receptors TLR2 and TLR4 (Darveau et al. 2004). TLR2 is required in *P. gingivalis*-mediated innate immune responses (Burns et al. 2006). The TLR activation leads to elevated cytokine and antimicrobial peptide production following *P. gingivalis*

¹Department of Periodontics, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA

²Department of Microbiology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA

³Department of Pathology, School of Dental Medicine, University of Pennsylvania, Philadelphia, PA, USA

A supplemental appendix to this article is published electronically only at <http://jdr.sagepub.com/supplemental>.

Corresponding Author:

D.F. Kinane, Department of Pathology and Periodontics, School of Dental Medicine, University of Pennsylvania, 240 South 40th Street, Philadelphia, PA 19104, USA.
Email: dfkinane@upenn.edu

perturbation. Persistent perturbation of *P. gingivalis*, however, may lead to apoptosis and constant turnover of epithelial cells that can invoke specific epigenetic modifications that may result in a hyporesponsive phenotype. These epigenetic changes either at the level of DNA or histone can modify the cellular phenotype. For example, substantial work has been done with respect to epigenetics in cancer biology, epigenetic alterations with inflammation including age-related and airway inflammation, bronchial asthma, and autoimmune diseases such as lupus erythematosus and rheumatoid arthritis (Esteller 2008; Trenkmann et al. 2010).

DNA methylation is modified in a cell type-specific manner and is associated with distinct gene expression patterns (Issa 2011). In support of this, tissue-specific differentially methylated DNA regions have been reported in several types of cancers (Hansen et al. 2011). Epigenetic modifications have been implicated in periodontitis (Bobetsis et al. 2007; Offenbacher et al. 2008; Gomez et al. 2009). TLR2 and TLR4 promoter methylation was observed in gingival tissue cells of patients with chronic periodontitis (De Oliveira et al. 2011). Infrequent hypermethylation of the TLR2 promoter region was observed, although this was inconclusive as opposed to the hypomethylated TLR4 promoter region (De Oliveira et al. 2011). On the other hand, TLR4 promoter CpG methylation was shown to be unrelated to TLR4 expression in bronchial epithelial cells (Shuto et al. 2006). TLR2 CpG promoter hypermethylation was noted in non-cystic fibrosis bronchial epithelial cells in epigenetic control of TLR2 expression (Shuto et al. 2006). The tumor necrosis factor (TNF) promoter region in chronic periodontitis patients that modulated TNF mRNA expression had increased CpG methylation correlating to TNF mRNA expression (Zhang et al. 2013). The epithelial DNA methylation landscape showed wide regulation of inflammatory response by promoter DNA methylation (Barros and Offenbacher 2014). Susceptibility to *Salmonella enteritidis* in chickens has been linked to decreased TLR transcript levels due to CpG hypermethylation (Gou et al. 2012). While there are many signaling pathways that are affected in the periodontitis disease state, knowledge is limited regarding epigenetics in epithelium, which could be a source of variation in disease susceptibility. Here we report TLR2 CpG promoter methylation in periodontitis-affected human gingival tissues and in HGECS chronically stimulated with *P. gingivalis* that may instigate epithelial dysbiosis and create a pathogen niche in the gingival crevice and thus increase susceptibility to periodontitis.

Materials and Methods

Cell Isolation, Culture, Challenge Assays, and Transfection

The gingival tissue was obtained with signed informed consent approved by the Institutional Review Board (University

of Pennsylvania), and HGECS were cultured as previously described (Kinane et al. 2006). HGECS at the third passage were harvested and seeded at a density of 0.5×10^5 cells/well in 6-well culture plates. At ~95% confluence, the cells were stimulated with *P. gingivalis* (multiplicity of infection [MOI]: 10). Production of interleukin 1 β (IL-1 β) and TNF were measured using enzyme-linked immunosorbent assay kits (BD Biosciences, San Jose, CA) from supernatants according to the manufacturer's instructions. For the in vitro chronic infection model, *P. gingivalis* at MOI 10 was stimulated for 30 min and washed with plain medium, and the 30-min stimulation cycle was carried out at 4 h, 8 h, and 16 h. After 48 h from the first infection cycle, the cells were split and seeded equally. When these cells reached ~50% confluence, the cells were exposed to 1 more round of *P. gingivalis* stimulation either in the presence or absence of 1 μ M of 5-Aza-2'-deoxycytidine (decitabine) inhibitor. Forty-eight hours after the first stimulation, the DNA was extracted and purified using the QIAamp DNA isolation kit (Qiagen, Valencia, CA). The extracted DNA was subjected to methylation quantitative polymerase chain reaction (qPCR) using methylation-specific primers from SABiosciences (Valencia, CA) according to the manufacturer's instruction. Transfection in HGECS is carried out using GenMute transfection reagent. Please refer to the detailed protocol in the Appendix.

Real-Time PCR and Methylation qPCR

The TaqMan real-time PCR is carried out according to Benakanakere et al. (2009). Mean fold increase data were used to derive a heat map with 2-way hierarchical clustering using MeV v4.1 software. The genomic DNA samples from HGECS were subjected to restriction digestion using the EpiTect Methyl II DNA restriction kit (SABiosciences). The primers spanning the CpG Island (Appendix Fig. 3) was purchased from SABiosciences. The reaction mixture consisting of methylation-sensitive and methylation-dependent restriction enzymes was incubated for 6 h at 37 °C, after which the reaction was terminated and the enzyme was inactivated at 65 °C for 20 min. The DNA samples were subjected to real-time PCR using TLR2 promoter CpG primers from SABiosciences. The percentage methylation was calculated using data analysis software from Orion Genomics (St. Louis, MO).

Bisulfite Sequencing and Luciferase Activity

The bisulfite conversion of DNA from human gingival tissue samples was done using the EZ DNA methylation gold kit (Zymo Research, Irvine, CA) according to the manufacturer's instruction. Please refer to details of the bisulfite sequencing and luciferase activity in the Appendix.

Experimental Periodontitis, Oral Gavage Mouse Model

Experimental periodontitis was induced in 6- to 8-wk-old BALB/c mice by oral inoculation with *P. gingivalis* ATCC 33277 by means of an oral gavage model (Graves et al. 2008). Please refer to details in the Appendix.

Statistical Analysis

Statistical analyses for in vitro and in vivo experiments were performed using GraphPad InStat 3 software. One-way analysis of variance was used to compare the differences between groups using the Tukey post-test with a significance of $P < 0.05$.

Results

TLR2 Gene Expression Heterogeneity in HGEs

Having identified the importance of TLRs in gingival epithelial cells mounting innate immune responses correlated with gingivitis and periodontitis, we noted differential expression of TLR2 and their pro-inflammatory IL-1 β levels in certain cell types (Fig. 1A, B). We stimulated both cell types (3 in each group) with *P. gingivalis* (MOI: 10) for 4 h, and the total RNA was subjected to Taqman real-time PCR. The “normal” cell type up-regulated both proinflammatory cytokine and antimicrobial peptides; however, the “dysregulated” type cells with lower TLR2 expression in response to *P. gingivalis* exhibited blunted cytokine and antimicrobial peptide response (Fig. 1C). We observed heterogeneous TLR2 expression levels in normal cells (Fig. 1A). Further, we compared TLR2 promoter methylation of 4 normal and 4 dysregulated epithelial cells using EpiTect methyl II PCR primer and found that dysregulated cells had a significantly higher methylation percentage compared with normal cells (Appendix Fig. 1A). To verify that TLR2 is important in these cells, we knocked down TLR2 in high TLR2-expressing cells within the group. After stimulation with *P. gingivalis*, cells transfected with siTLR2 down-regulated TNF cytokine production (Appendix Fig. 1B).

Figure 1. Isolated human primary gingival epithelial cells were cultured and stimulated with *P. gingivalis* at multiplicity of infection (MOI): 10 for 4 h. The cDNA was subjected to real-time polymerase chain reaction (PCR) using TLR2 TaqMan probe. The relative expression of TLR2 was calculated using GAPDH as an endogenous control (A). The enzyme-linked immunosorbent assay results from the supernatant showed a significant difference in interleukin-1 β induction (B). The cDNA from 3 individuals from each group was subjected to gene expression analysis using TaqMan probes. The $\Delta\Delta CT$ values were used to generate a heat map based on 2-way hierarchical clustering with MeV v4.1 software (rows = genes, columns = sample). The color scale indicates relative expression: yellow, above mean (>3.0); blue, below mean (0.0); and black, unchanged (1.0) (C). The DNA from normal and dysregulated epithelial cells was subjected to TLR pathway DNA methylation PCR arrays from SABiosciences. The data were analyzed using the EpiTect methyl II PCR data analysis program, and methylation is represented as percentage compared with unmethylated DNA standard. TLR2 exhibited hypermethylation in dysregulated cells (indicated with a green circle) (D). The data are represented as mean $\pm$ standard error, with *** $P = 0.0001$ and ** $P = 0.001$.

Hence, we hypothesized that blunted TLR2 expression might be epigenetically regulated, leading to altered TLR2 mRNA expression and subsequently proinflammatory cytokines, consequently leading to an epithelial “dysregulated” phenotype in humans.

TLR2 Promoter Methylation in Dysregulated Gingival Epithelial Cell Type

Since we observed a blunted inflammatory response in dysregulated cells with *P. gingivalis* stimulation and because *P. gingivalis* is a known agonist for TLR2 and TLR4

Figure 2. Chronic *P. gingivalis* infection induces DNA methylation in vitro. Schematic representation of in vitro chronic infection model (A). The DNA was extracted at the end of the above experiment and subjected to TLR2 DNA methylation-sensitive quantitative polymerase chain reaction using TLR2 methylation primers (SABiosciences). The data are represented as percentage methylation compared with unmethylated standard DNA (B). Chronic *P. gingivalis* infection induced de novo methylation in human gingival epithelial cells. The TLR2 promoter region treated or untreated with M.sssI enzyme and cloned into pGL 4.20 luciferase vector. Renilla luciferase vector and pGL 4.20 containing the TLR2 promoter region was co-transfected to epithelial cells and stimulated with *P. gingivalis* (multiplicity of infection: 10) for 4 h, and luciferase activity was measured. M.sssI-treated luciferase construct down-regulated luciferase activity (C). The data are represented as mean $\pm$ standard error, with $^{**}P = 0.001$.

(Benakanakere et al. 2009), we carried out TLR pathway DNA methylation PCR (SABiosciences) to determine if there is specific epigenetic regulation at the promoter level in the TLR signaling network. The DNA sample was isolated from the representative dysregulated and normal cell type and subjected to methylation-specific enzyme digestion. After the digestion, the methylation-specific real-time PCR was carried out, and data were analyzed according to the manufacturer's instructions (SABiosciences). Twenty-six genes showed promoter methylation with TLR2 with higher promoter methylation in the dysregulated cell type compared with other gene promoters (Fig. 1D).

Chronic *P. gingivalis* Infection Model

In an attempt to mimic chronic infection status to assess a cause-and-effect relationship, and in our quest to understand

how bacteria change the methylation profile of epithelial cells, we developed a system called the "chronic in vitro infection model" (Fig. 2A). The time points for this chronic infection were carefully chosen based on our previously published results on *P. gingivalis*-induced inflammatory cytokine response in gingival epithelial cells (Eskan et al. 2008; Stathopoulou et al. 2009; Stathopoulou et al. 2010). By using this method, we successfully induced tolerance to *P. gingivalis* (live bacteria at MOI: 5) in normal epithelial cells. *P. gingivalis* (MOI: 5) stimulation was initially carried out for 30 min at 0-, 4-, 8-, and 16-h time intervals. Repeated stimulation with *P. gingivalis* induced reprogramming of the TLR2 region by inducing de novo methylation (Fig. 2B). By using decitabine, we were able to essentially eliminate the TLR2 promoter methylation that was induced by *P. gingivalis* (Fig. 2B). To verify whether DNA methylation in fact reduces TLR2 promoter activity, we PCR amplified the TLR2 promoter DNA from normal cells and then treated it with CpG methylase (M. SssI enzyme) following the method of Zhang et al. (2013). Following methylation, the methylated promoter DNA and normal DNA were cloned into pGL

4.20 vector (Promega, Madison, WI). After transfection, the luciferase activity was measured in a luminometer. The methylated TLR2 promoter DNA showed a significant reduction in luciferase activity when compared with unmethylated TLR2 promoter DNA (Fig. 2C). This suggests that DNA methylation within the promoter region of TLR2 can modify the gene expression pattern.

TLR2 Overexpression and DNA Methyltransferase Inhibitor Increase Inflammatory Response in Dysregulated Cells

As we noted blunted TLR2 expression in dysregulated epithelial cells, we wanted to overexpress TLR2 by transfecting the TLR2 overexpression vector in the dysregulated cell type to see whether blunted cytokine expression is due to

TLR2. TLR2 overexpression plasmid (Addgene, Cambridge, MA) and empty vector were transfected using GenMute transfection reagent (SigmaGen Laboratory, Rockville, MD), and the cells were incubated. After 24 h of transfection, the cells were stimulated with *P. gingivalis* (MOI: 10) for 4 h, and total RNA was subjected to real-time PCR against TLR2 and IL-1 β . The data showed that the overexpression of foreign TLR2 can increase the inflammatory response to *P. gingivalis* (Fig. 3A, B). We further wanted to test if the dysregulated cells can be modified to express TLR2; as a therapeutic approach, we sought whether DNA methyltransferase inhibitor can revert dysregulated epithelial cells back to the normal state in restoring inflammatory response. To confirm this, the dysregulated epithelial cells were cultured in the presence of 1 μ M 5-Aza-2'-deoxycytidine (Sigma-Aldrich, St. Louis, MO). When the cells reached 90% confluence, the cells were split and cultured on 6-well plates in the presence of the inhibitor. At 90% confluence, the cells were stimulated with *P. gingivalis* at MOI: 10 for 4 h. After stimulation, the total RNA was extracted and subjected to real-time PCR using TLR2 and TNF TaqMan probes (Life Technologies, Carlsbad, CA). The real-time PCR data showed that decitabine can restore TLR2 expression in dysregulated epithelial cells and induce an inflammatory response to *P. gingivalis* (Fig. 3B, C). This clearly shows that DNMT as a therapeutic target in restoring the inflammatory response to a pathogen in dysregulated epithelial cell types and DNMT inhibitor may serve as therapeutic agents against periodontitis.

TLR2 Promoter Methylation in the Gingiva In Vivo

To test the changes in TLR2 promoter methylation level following experimental periodontitis, a gavage experimental periodontitis model in mice was followed with slight modification (inoculation of *P. gingivalis* was done 5 times as opposed to 3 times) from the published work. After the experiment, the bone loss in millimeters was calculated according to Hajishengallis et al. (2009). The bone loss was evident in experimentally induced periodontitis, as shown in Figure 4A, B, and C. Next, the DNA was isolated from the gingival tissue of the sham and periodontitis group (Fig. 5D) and subjected to methylation qPCR (Qiagen) to compare the methylation percentages. The gingival tissue obtained from the periodontitis group showed higher TLR2 promoter methylation compared with the control group (Fig. 4E).

TLR2 Promoter Methylation in Periodontal Disease-Affected Tissue

Next, we wanted to examine whether these phenomena exist in patients with periodontitis. To confirm this, we

Figure 3. TLR2 overexpression up-regulates inflammatory cytokine in dysregulated cells. **(A)** The dysregulated cells were transfected with a plasmid overexpressing TLR2 (Addgene). Twenty-four hours posttransfection, the cells were stimulated with *P. gingivalis*, and cDNA was subjected to TLR2 and interleukin-1 β (IL-1 β) mRNA expression using TaqMan probes. TLR2 overexpression showed up-regulation of IL-1 β induction after *P. gingivalis* stimulation in dysregulated cells. DNA methyltransferase inhibitor rescued TLR2 expression in dysregulated epithelial cells. **(B)** The dysregulated epithelial cells were cultured in the presence of 1 μ M 5-Aza-2'-deoxycytidine (Sigma-Aldrich). When it reached 90% confluence, the cells were split and cultured on 6-well plates in the presence of 5-Aza-2'-deoxycytidine. At 90% confluence, the cells were stimulated with *P. gingivalis* at MOI: 10 for 4 h, and cDNA was subjected to real-time PCR using TLR2, TNF, and GAPDH probes. The data are represented as mean $\pm$ standard error from 3 independent experiments.

obtained gingival tissue from a periodontitis-affected site and a healthy site from patients who were undergoing flap surgery in the periodontics clinic. This tissue from 4 subjects was carefully separated from healthy and periodontitis-affected parts, and DNA was isolated. One microgram of DNA bisulfite converted and amplified the region within the TLR2 promoter region (Fig. 5A) with sequence-specific primers (Appendix Fig. 2) and was cloned to the TA vector and sequenced. The DNA sequence was exported to the BiQ program (Bock et al. 2005). The sequence analysis showed TLR2 promoter methylation in the disease tissue site (Fig. 5). This clearly indicates that there is host epigenetic regulation in the periodontitis disease-affected tissue.

Figure 4. Morphometric evaluation of periodontal bone loss in mice. Bone loss determination is based on cemento-enamel junction–alveolar bone crest measurements and represented as millimeter loss (A). Shown also are representative images of maxillae from sham and *P. gingivalis*-infected mice (B). The gingiva was excised from the maxillae (C), and the analysis of TLR2 promoter DNA methylation was done by methylation-specific quantitative polymerase chain reaction in *P. gingivalis*-infected mice and represented as mean percentage DNA methylation (D). The data are represented as mean $\pm$ standard error, with $P = 0.001$.

Discussion

Periodontitis is initiated by a microbial biofilm of ~700 different microorganisms, most of which are gram-negative anaerobic bacilli (Kinane et al. 2008a; Teles et al. 2012). Among these, an important putative pathogen is *P. gingivalis*, which is regarded as a keystone pathogen that instigates host innate immunity (Hajishengallis et al. 2012). The activated host innate immunity is characterized by elevated cytokine production following microbial perturbation. Successful triggering of cytokine production is considered homeostatic. Accordingly, the induction of inflammatory cytokines must be tightly regulated, and multiple regulatory mechanisms control the duration of TLR-induced inflammation (Medvedev et al. 2006). One such control could be at the level of TLR expression itself, in which blunted TLR expression could lead to a failure of the proper innate immune response.

We recently uncovered a blunted proinflammatory and antimicrobial response to *P. gingivalis* stimulation in the periodontitis-affected subject's gingival epithelial cells. In gingival epithelial cells, TLR2 expression is higher than that of TLR4 (Zhao et al. 2010). Hence, we sought to

understand the mechanism of TLR2 gene expression variation in these cells. Recent studies in understanding genetic variation implicated not only Mendelian inheritance but also non-Mendelian inheritance termed *epigenetics* (Adcock et al. 2007). There have been numerous studies in humans relating to variation caused by epigenetic changes with respect to aging (Issa 2003; Kwabi-Addo et al. 2007). Altered DNA methylation predicted Crohn's disease status where key host defense mechanisms including TH17 were dysregulated (Nimmo et al. 2011). Accumulating evidence points toward the association of aberrant DNA methylation in the development of various human diseases (Yost et al. 2011). With this background, we set out to test dysregulated and normal epithelial cells for changes in their CpG island methylation pattern on the promoter region of genes involved in the TLR inflammatory pathway. Importantly, we noted a highly methylated CpG pro-

motor region in the TLR2 gene in dysregulated cells. These cells induced diminished proinflammatory cytokines and antimicrobial peptides in response to *P. gingivalis*. In support of this finding, siRNA against TLR2 had a similar effect in normal cells in response to *P. gingivalis*. This innate immune compromise within epithelial cells may have a direct effect on antimicrobial defense as well as an indirect influence on adaptive immune responses such as inhibition of IL-12 from T cells that facilitate *P. gingivalis* persistence (Hajishengallis 2013).

The ENCODE project (www.genome.ucsc.edu/ENCODE) supports our data of differential TLR2 methylation status, in which one CpG island on the TLR2 gene was revealed with differential CpG methylation across different cell lines. Moreover, the CpG island hypermethylation in gene promoters has been shown to be an important mechanism in gene silencing (Jones and Baylin 2002). The changes pertaining to epigenetics may be brought about by age-related methylation (Issa et al. 1994) or changes that occur due to chronic inflammation, as in ulcerative colitis due to constant turnover of cells (Issa et al. 2001). Recently,

Figure 5. TLR2 promoter CpG targeted bisulfite DNA sequencing. The CpG island within the TLR2 promoter region was selected for DNA sequence analysis (A). Healthy and periodontitis-affected tissue was collected and subjected to bisulfite treatment (Zymo Research). After the treatment, the DNA was cloned to the TA vector before being subjected to bisulfite sequencing. The sequence data were analyzed using the BiQ software program. Filled (black) circles correspond to methylated Cs, unfilled (white) circles correspond to unmethylated Cs, and small vertical lines without a circle correspond to missing values that may be caused by sequencing errors. H1 to 4 represent healthy sites, and P1 to 4 represent disease sites from different patients (B).

bacteria-induced hypermethylation of the *Igf2* gene has been revealed (Bobetsis et al. 2007). The epigenetic changes can be induced by repeated bacterial ligand challenge (*Escherichia coli* LPS), as shown in murine macrophages, leading to tolerance in cells with blunted cytokine responses (Foster and Medzhitov 2009). It is possible that similar mechanisms exist for different pathogens. In humans, with chronic periodontitis, the hypermethylation pattern of the promoter of *PTGS2* is altered (Zhang et al. 2010). This study also showed that certain genes possess “hot spots” for epigenetic changes, leading to silencing of certain genes. Nevertheless, *P. gingivalis* inducing DNA methylation is relevant because of the nature of the bacteria and its pathogenic effect in periodontal disease pathology. By using a laser capture technique to isolate and evaluate DNA methylation in epithelial cells from periodontitis-affected gingival tissue, Barros and Offenbacher (2014) found wide epigenetic alterations in many chemokine and cytokine genes. The authors have reported the regulation of DNA methylation in the inflammatory response. The cytokine and chemokine genes’ DNA methylation observed by these authors appears to be downstream of receptor signaling and can affect the network of genes. It is also important to note that the primary signaling from the receptor is crucial in driving these gene activations. The approach used by these authors is indeed valid, but they compared the epithelial cells from healthy and periodontitis subjects in a population. Our approach is aimed at comparing the DNA methylation within the same subject as the genetics, age, gender, and habitual practices can account for differential DNA methylation pattern.

The identification of TLR2 DNA methylation status in periodontitis-affected tissue samples supported our *in vitro* data. However, this tissue was composed of not only

epithelial cells but also other types of cells. Nonetheless, the primary HGEs *in vitro* showed a significantly higher methylation status compared with normal cells. However, we also observed a different degree of methylation in normal cells that may be due to culture-induced alterations. It is important to note that further investigation may differentiate inherent and culture-induced changes. This will be dealt with in gingival tissue by using laser scanning microdissection to isolate epithelial cells and to determine their methylation status without having to culture the cells. Nonetheless, our data clearly indicate that epigenetic modification in gingival epithelial cells plays an important role in gene repression by pushing the cells to a hyporesponsive state, thereby failing to restrain harmful chronic inflammation. Taken together, our data strongly suggest that an epigenetic modification of the TLR2 promoter region plays an important role in inducing the hyporesponsive phenotype in dysregulated cells that can lead to failure of host defense mechanisms. The use of DNA methyltransferase inhibitor that restored dysregulated cells’ cytokine response shows therapeutic potential. Overall, it is plausible that the differences in epigenetic signatures on pattern recognition receptors may help explain periodontitis disease susceptibility.

Author Contributions

M. Benakanakere, contributed to conception, design, data acquisition, analysis, and interpretation, drafted and critically revised the manuscript; M. Abdolhosseini, K. Hosur, L.S. Finoti, contributed to design, data acquisition, analysis, and interpretation, critically revised the manuscript; D.F. Kinane, contributed to conception, design, data analysis, and interpretation, drafted and critically revised the manuscript. All authors gave final approval and agree to be accountable for all aspects of the work.

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