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Epigenetic Changes in the Innate Response of Gingival Epithelium

Abstract

In this study we showed TLR2 CpG promoter methylation in periodontitis affected human gingival tissues and in primary human gingival epithelial cells chronically stimulated with *P. gingivalis* that may instigate epithelial dysbiosis that may create a unique pathogen niche in the gingival crevice and susceptibility to periodontitis.

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University of Pennsylvania School of Dental Medicine

**Epigenetic changes in the innate response
of gingival epithelium**

THESIS

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05/28/2014

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Introduction

Periodontitis

Innate immunity

TLR2 in *Porphyromonas gingivalis* recognition

Heterogeneity in periodontal disease susceptibility

Epigenetics of innate immunity

DNA Methylation

Infectious and inflammatory diseases affect millions of people in the United States. A large percentage of patients are either killed or debilitated by infectious diseases. Periodontitis is a chronic infectious, destructive disease of the gingival tissues supporting the teeth. It begins when the bacteria in plaque, the microbial biofilm, causes the gums to become inflamed. If untreated, this disease can lead to severe tooth loss and may interact with other systemic health problems increasing morbidity and mortality¹. It affects more than ~70% of the population, causing significant debilitation for the affected population and imposes a major economic burden to the United States². This disease is very complex in nature that affects not only soft gum tissue but also affects hard bone. A recent report from the American Dental Association claims \$100 billion in dental spending per year, estimated to reach nearly \$179 billion by 2021. Over the past three decades, research has shown, and experts comply, that there is an association between periodontal diseases and other chronic inflammatory conditions, such as diabetes, cardiovascular and Alzheimer's disease, stroke, osteoporosis, respiratory diseases and pregnancy problems including pre-term birth³⁻¹⁰. Therefore, treating periodontal inflammation may not only help manage periodontal

diseases but may also help with the management of other chronic inflammatory conditions in general.

There are two major forms of periodontitis, chronic and aggressive^{11,12}. Epidemiologic studies have concluded that chronic periodontitis is the most commonly occurring form of periodontal disease with most subjects experiencing moderate amounts of periodontal tissue destruction. A recent study in United state shows over 47%, American adults ≥ 30 years, have periodontitis, 8.7% mild, 30.0% moderate, and 8.5% severe periodontitis².

The periodontitis is initiated by microbial plaque, which accumulates in the gingival crevice and induces an inflammatory response¹³. Initial inflammation is reversible and could progress in susceptible individuals to a chronic destructive inflammatory condition, in which tooth supporting tissue are destroyed. For years identifying micro-organisms associated with periodontal disease or health were limited to those which could be cultured under laboratory conditions. Indeed, classification of the periodontal microbiota by culturing techniques in the late 1970s and early 1980s created a dramatic change in our understanding of bacterial composition of disease compared to health¹⁴⁻¹⁸. Socransky et al. classified bacteria into six groups based on different potential pathogenicity and their role in the development of plaque, categorized by colors. “red complex”, which is a group of three Gram-negative anaerobic bacteria, includes *Porphyromonas gingivalis*, *Treponema denticola*, and *Tannerella forsythia*. The “orange complex” comprised species members of the genus *Prevotella*, *Fusobacterium*, and *Campylobacter*, *Streptococcus constellatus* and *Eubacterium nodatum*. The other four complexes (“blue”, “yellow”, “green”, and “purple”) primarily consisted of early colonizers of the tooth surfaces¹⁹.

Recent developments in molecular-methodologies of bacterial identification, for example

16S rRNA amplification and high-throughput sequencing, have revolutionized the understanding of the composition of the periodontal microbiota²⁰. The study of thousands of plaque samples derived from multiple clinical periodontal conditions has demonstrated a more heterogeneous and diverse periodontal microbiota than previously thought. Newly recognized non or poorly cultivable organisms that increase in number in diseased sites include the Gram-positive *Filifactor alocis* and species in the genera *Prevotella*, *Megasphaera*, *Selenomonas*, and *Desulfobulbus*. Many of these organisms show similar correlation with disease as red complex^{21,22}.

Chronic periodontitis is the result of a poly-microbial induced breakdown of host homeostasis in susceptible individuals, not just a bacterial infection caused by a single or a limited number of pathogens²³. The host reacts to this poly-microbial infection by producing cytokines and several immune modulators. Cytokines stimulate inflammatory events that activate effector mechanisms. These cytokines may directly or indirectly modulate periodontal destruction that involves the stimulation of bone resorption and induction of tissue degrading proteinases²⁴. In fact microbial dental plaque starts periodontal disease but the form and severity of the disease is dependent on the environmental, genetic and host defenses to this challenge²⁵.

The role of the host response in periodontal bone loss is pivotal. There is evidence that an insufficient host response increases periodontal destruction and on the other hand a very strong response leads to periodontal disease²⁶. A critical aspect of the host response is the detection of bacteria by Toll-like receptors (TLRs). Activation of the innate immune response by the binding of various bacterial components to TLRs results in the production of cytokines and chemokines²⁷. The Toll-like receptors (TLRs) are a major class of

eukaryotic receptors for microbial pathogen associated molecular patterns (PAMPs)²⁸. They are expressed in a number of cell types, such as phagocytic cells, endothelial cells, fibroblasts, and inflamed periodontal tissues. When TLRs recognize PAMPs, alone or in homodimerization or heterodimerization with other TLR or non-TLR molecules, they induce signals responsible for the activation of genes relevant to the host defense including the inflammatory and adaptive immune-related cytokines²⁹.

Toll receptor was originally identified in *Drosophila* as an essential receptor for the establishment of the dorsoventral pattern in developing embryos³⁰. In 1996, Hoffmann's group showed that Toll-mutant flies were more susceptible to fungal infection³¹. Later, mammalian homologues of Toll receptor were identified one after another, and named as Toll-like receptors (TLRs). 13 members of the TLR family have been identified in mammals which 11 of them express at the protein level in humans. TLRs are type I transmembrane proteins characterized by an extracellular domain containing leucine-rich repeats (LRRs) and a cytoplasmic tail that contains a conserved region called the Toll/IL-1 receptor (TIR) domain. The structure of the extracellular domain of TLR3 was revealed by crystallography studies as a large horseshoe-shape³². TLR signaling consists of at least two distinct pathways, a MyD88-dependent pathway that leads to the production of inflammatory cytokines, and a MyD88-independent pathway associated with the stimulation of IFN- β and the maturation of dendritic cells. The MyD88-dependent pathway is common to all TLRs, except TLR3³³. TLRs responding to particular pathogens may activate complex networks of pathways and interactions, positive and negative feedback loops, and multifunctional transcriptional responses. Among the key downstream targets of these networks are NF-kB, mitogen-activated protein kinases, and members of the IRF family³⁴. TLRs that detect

mainly extracellular microbial structures are expressed on the host cell surface (TLR-1, -2, -4, -5, -6), and TLRs that detecting viral or bacterial nucleic acids are located intracellularly on endocytotic vesicles or organelles (TLR-3, -7, -8, -9)³⁵. Also, TLRs has ability to heterodimerize within the family (e.g. TLR2 uses either TLR1 or TLR6 as signaling partners), or outside of TLR s family (e.g. TLR2 co- associates with CD14 or CD36). This ability might help to discriminate better among the abundant and diverse microbial structures³⁶. Crosstalk between complement and TLR signaling pathways has been shown which suggests that the complement–TLR interplay reinforces innate immunity or regulates excessive inflammation, through synergistic or antagonistic interactions³⁷.

Epithelial cells function as a physical barrier and in immune surveillance through their ability to elicit an innate immune response. TLRs are expressed predominantly in cells which mediate the first line of defense such as neutrophils, dendritic cells and monocytes/macrophages and in cells that are directly exposed to the outer environment such as epithelial cells³⁸.

Toll-like receptor 2 is involved in the recognition of peptidoglycans and lipoteichonic acid of Gram-positive bacteria^{39,40}. Also, TLR2 is specifically involved in the recognition of the periodontopathogenic bacteria *Porphyromonas gingivalis*³⁸. It was previously suggested that TLR 2 activity upon stimulation by *P. gingivalis* is related to the lipopolysaccharide⁴¹. Later It has been shown that TLR2 recognizes unknown cell wall components of *P. gingivalis* rather than the lipopolysaccharide itself⁴². It has been reported that TLR2 expression is higher in the gingival tissue of subjects with chronic periodontitis than in the gingival tissue of subjects with gingivitis⁴³. Beklen et al. (2008), observed higher numbers of cells expressing TLR2 in the periodontitis tissue than in the healthy tissue⁴⁴. Harokopakis and

Hajishengallis showed the evidence for TLR2 activation in response to the fimbriae of *P. gingivalis* which function in transmodulating the adhesive activities of human monocytes⁴⁵.

For many years, it was noted that everybody is not equally affected by the accumulation of bacterial plaque. Some individuals might be very susceptible and might develop aggressive forms of periodontitis at a relatively young age, while others might be resistant and never develop periodontitis¹³. In some cases, the disease progression is slow, and the risk for loss of function of the teeth during a lifetime will be minimal, while in others it progress quickly. In addition, some gingival sites are more susceptible develop periodontitis than other gingival sites within the same subject. The findings that high levels of inflammatory mediators such as IL-1, TNF and PGE₂ are correlated with periodontal destruction^{46 47} and that these mediators are able to aggravate the inflammatory response⁴⁸ led to the hypothesis that some individuals may respond to periodontal infection with the production of high levels of inflammatory mediators, which in turn will result in attachment loss. Eskin et al. (2008), showed that Human gingival epithelial cells act differently in the expression of TLR4, and TLR4-normal HGECs produce four fold more IL-1 β compare to the group of TLR4-deficient HGECs which induction of IL-1 β plays an important role in mediating the release of other pro-inflammatory cytokines from primary human epithelial cells following challenge with *P. gingivalis*, and this process may be an inflammatory enhancement mechanism adopted by epithelial cells⁴⁹.

Experimental gingivitis studies show that there are variations among individuals in the rate of development of gingival inflammation. Wiedemann et al., (1979) reported that in a group of 62 who were subject to a period of withdrawal from oral hygiene, eight were ‘‘resistant’’

and did not develop gingivitis within 21 days, while 25 subjects were “susceptible” and exhibited substantial gingival inflammation within 14 days. The remaining subjects formed an “intermediate” group⁵⁰. Van der Velden showed that a group of subjects consistently exhibited greater than average gingival inflammation, while another group was always resistant⁵¹. Loe’s investigation in a Sri Lankan population without dental care and an absence of oral hygiene identified three sub-populations: a group with no progression of periodontal breakdown (11%), a group with moderate progression (81%), and a group with rapid progression (8%)⁵². Van der Velden’s study in a remote village on Western Java who did not receive regular dental care reported that 20% of the population developed severe periodontal breakdown, but the rest of the population developed only minor to moderate breakdown⁵³.

Complex human diseases (for example Alzheimer disease, Crohns disease and cardiovascular diseases) present mostly a relatively mild phenotype, are slowly progressive and chronic in nature⁵⁴. These diseases are associated with variations in multiple genes, each of which has a small overall contribution and relative risk for the disease process. Complex diseases are typically polygenic, i.e. multiple genes each play a limited role (low-penetrance genes), and the disease genes in these diseases considered as disease-modifying genes⁵⁵. Many studies have been done on the role of genes and their variants in host responses in periodontitis. The genetic polymorphisms may cause a change in the encoded protein, or its expression, and could alter innate and adaptive immunity, and may be deterministic in disease outcome⁵⁶. Evidence for the role of a genetic component in periodontitis has been investigated in family and twin studies. Van der Velden’s study on young Indonesian siblings who did not receive regular dental care suggested that there may

be a genetic basis for the less severe forms of periodontitis⁵⁷. Family studies may provide information on familiar aggregation, but they cannot distinguish between genetic and environmental influences. The twin model is probably the most powerful method to study genetic aspects of any disease, including periodontal disease. Michalowicz evaluated the periodontal condition of 110 adult twins and the results showed that between 38 and 82% of the population variance for these measures may be attributed to genetic factors⁵⁸. In a study on 117 adult twin pairs, the analysis included evaluation of environmental factors such as smoking and utilization of dental services. The results showed that chronic periodontitis was estimated to have approximately 50% heritability, and this was unaltered following adjustments for behavioral variables⁵⁹. Genetic variations linked to complex diseases are not easily identified in multifactorial traits. Single nucleotide polymorphisms (SNP) of the DNA are often used as genetic markers when they can be linked to a distinct phenotype. SNPs in receptors, antigen sensors in cell surfaces, and cytokines and chemokines have been shown to influence host immunity and inflammatory response^{60,61}. Periodontitis-related SNPs have been investigated in the Fc-gamma receptor, interleukin-1, IL-4, IL-6, IL-10, IL-18, TNF α , vitamin D receptor, cluster of differentiation-14, matrix metalloproteinase-1, TLR-2, TLR4 and COX-2⁶².

There is a growing body of literature addressing the role of the environment on gene expression, but very little is known about the epigenetic pathways involved in the modulation of inflammatory and anti-inflammatory genes. Epigenetics, a relatively new concept in periodontitis research, may uncover the missing link between genetics, disease and environment. Epigenetics is described as changes in patterns of gene expression, which do not involve changes in the DNA sequence⁶³. Epigenetics (as in “epigenetic landscape”)

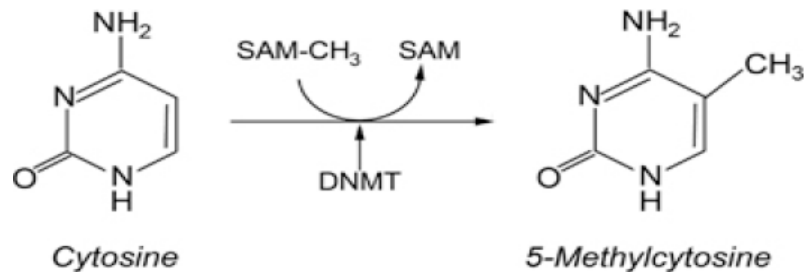
was coined by C. H. Waddington in 1942 in the development context. Waddington did not use a specific definition for epigenetics (“All those events which lead to the unfolding of the genetic program for development”). By the mid-1980s it was clear that there was a new type of inheritance, not based on changes in DNA sequence. In 1987, Robbin Holiday wrote a paper “The inheritance of epigenetic defects”. Holliday defined epigenetics as "the study of the mechanisms of temporal and spatial control of gene activity during the development of complex organisms⁶⁴⁻⁶⁶.

Later, Epigenetics has been defined by Riggs and colleagues as “ the study of mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence “⁶⁷. In 2008, an agreement on the definition of the epigenetic trait was made at a Cold Spring Harbor meeting, “stably heritable phenotype resulting from changes in a chromosome without alterations in the DNA sequence”⁶⁸. It has been founded that three important mechanisms provide the molecular basis of epigenetic regulation of gene expression. These are DNA methylation, histone modifications, and noncoding RNA (ncRNA)-mediated regulation. Also transvection, is also discussed as an epigenetic phenomena⁶⁹.

DNA methylation is a well-characterized epigenetic modification and it is also the most studied epigenetic mechanism in cancer⁷⁰. It has been shown to play an important role in numerous biological processes, such as transposable element silencing, genomic imprinting (epigenetic process that involves DNA methylation and histone methylation in order to achieve mono-allelic gene expression without altering the genetic sequence)⁷¹ , X-chromosome inactivation, and also in various disease processes including carcinogenesis.

DNA methylation is the addition of a methyl group to cytosines at the 5' position of a CpG dinucleotide by a covalent modification which results in the formation of 5-methylcytosine (5mC), a base that changes the interactions between protein(s) and DNA. In mammalian cells, DNA methylation is a replication-dependent reaction catalyzed by DNA methyl transferases (DNMTs) which are present at the replication fork during the S-phase. CpG dinucleotides are typically rare and scattered throughout the genome and are fully methylated. However, DNA methylation also involves CpG-rich regions called “CpG islands” (CGIs)⁷². Approximately, 50% of human genes contain CpG islands and most of these islands are unmethylated in normal tissue⁷³.

Figure 1



Conversion of cytosine to 5-methylcytosine by DNA methyltransferase (DNMT). DNMT catalyses the transfer of a methyl group (CH₃) from S-adenosylmethionine (SAM) to the 5-carbon position of cytosine.

Unmethylated CpG islands are related to transcriptionally active structure, but methylated DNA recruits methyl-binding proteins such as MeCP2, which promotes interaction with histone deacetylases (HDACs). HDAC remove acetyl groups of histones leading to chromatin compaction and preventing the binding of transcription factors⁷⁴, so gene

products are not expressed and many cellular functions might change by DNA methylation, including DNA repair, cell proliferation regulation and inflammatory gene expression⁷⁵⁻⁷⁷. Each tissue has a unique epigenetic profile, and changes occur as a result of developmental and regenerative processes. For example there is clear evidence that embryonic stem cells have a unique epigenetic pattern that changes upon differentiation cues⁷⁸.

Studies show that extrinsic factors, such as hormones, regulate differentiation, and infection cause epigenetic modifications⁷⁹. A similar effect can be achieved in the oral cavity, which is under the constant influence of extrinsic factors and foreign agents. Oral hygiene is naturally a contributing factor to oral health. Evidence shows that a lifestyle of smoking, food intake, lack of exercise, and use of drugs strongly influences the epigenetic pattern and predisposition to most conditions that lead to human disease⁸⁰. Although epigenetic studies on the oral epithelium are very new, several studies suggest that these cells have ability to respond to environmental factors. The inflammatory response involves up-regulation of transcription factors like NF-kB and STAT, and epigenetic chromatin changes similar to other inflammatory diseases⁸¹. Gingival tissues from periodontitis patients have been showed to have altered epigenetic patterns, particularly at inflammation-related genes. Alterations in the methylation pattern have been found in other oral diseases, such as squamous cell carcinoma⁸², tongue carcinoma⁸³ and odontogenic keratocyst⁸⁴. Olivera reported that the methylation pattern of the IL8 gene promoter in individuals with chronic periodontitis is altered when compared with healthy subjects⁸⁵.

The expression changes of some loci for example *IFNG*, occur as a result of the loss of methylation at their promoter⁸⁶. On the other hand, Hodge et al. (2001) showed that the

overexpression of IL-6 is not associated with DNA methylation at its promoter, but IL-6 upregulation may rather activate the DNMTs (DNA Methyltransferases), leading to methylation changes at the IL-6-induced target genes and development of a chronic inflammatory condition⁸⁷. Zhang et al. showed that the *TNFA* promoter was hypermethylated at two CpG sites, resulting in decreased expression. By reversing the methylation by treatment with a demethylating agent *in vitro*, it caused increased expression of *TNFA*, indicating that the methylation indeed regulated the expression⁸⁸.

The evidence of epigenetic changes associated with periodontitis also comes from data on COX-2, an enzyme governing the production of prostaglandins that promote inflammation and pain. It has been reported that COX-2 inhibitors were able to reduce the symptoms of periodontitis patients⁸⁹. On the other hand COX-2 expression in inflamed gingival tissues from chronic periodontitis patients was lower and its promoter was hypermethylated^{90,91}.

De Oliveria analyzed the status of DNA methylation in the promoter region of TLR2 and TLR4 genes in gingival tissue samples from healthy subjects, smokers and non-smokers affected by chronic periodontitis. The results showed major unmethylation of the TLR4 gene promoter in all groups, but the results for the TLR2 gene promoter are inconclusive; this gene was found as a mosaic of methylated and unmethylated DNA in the majority of samples of the three groups and they also observed a trend towards the DNA methylation of CpG sites recognized by the HhaI enzyme⁹².

Other epigenetic changes such as histone modifications also are involved in periodontitis. Treatment with HDAC inhibitors, such as 1179.4b and MS-275, on *P. gingivalis*-inoculated mice resulted in significantly reduced bone loss, indicating that maintenance of acetylation

is crucial to preventing bone loss⁹³.

While there are many signaling pathways that are affected in periodontitis disease state, there is limited knowledge of the involvement of epigenetics at the level of epithelium and this could shed light on heterogeneity in disease susceptibility. Characterization of TLR2 methylation status in gingival epithelial cells is highly significant as TLR2 is indispensable for TLR1 and TLR6 signaling and plays crucial role innate immune homeostasis.

In this study we showed TLR2 CpG promoter methylation in periodontitis affected human gingival tissues and in primary human gingival epithelial cells chronically stimulated with *P. gingivalis* that may instigate epithelial dysbiosis that may create a unique pathogen niche in the gingival crevice and susceptibility to periodontitis.

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Hypothesis

Epigenetic variation in the gingival epithelial cells lead to the variation in the periodontal susceptibility.

Materials and Methods

Cell isolation and culture:

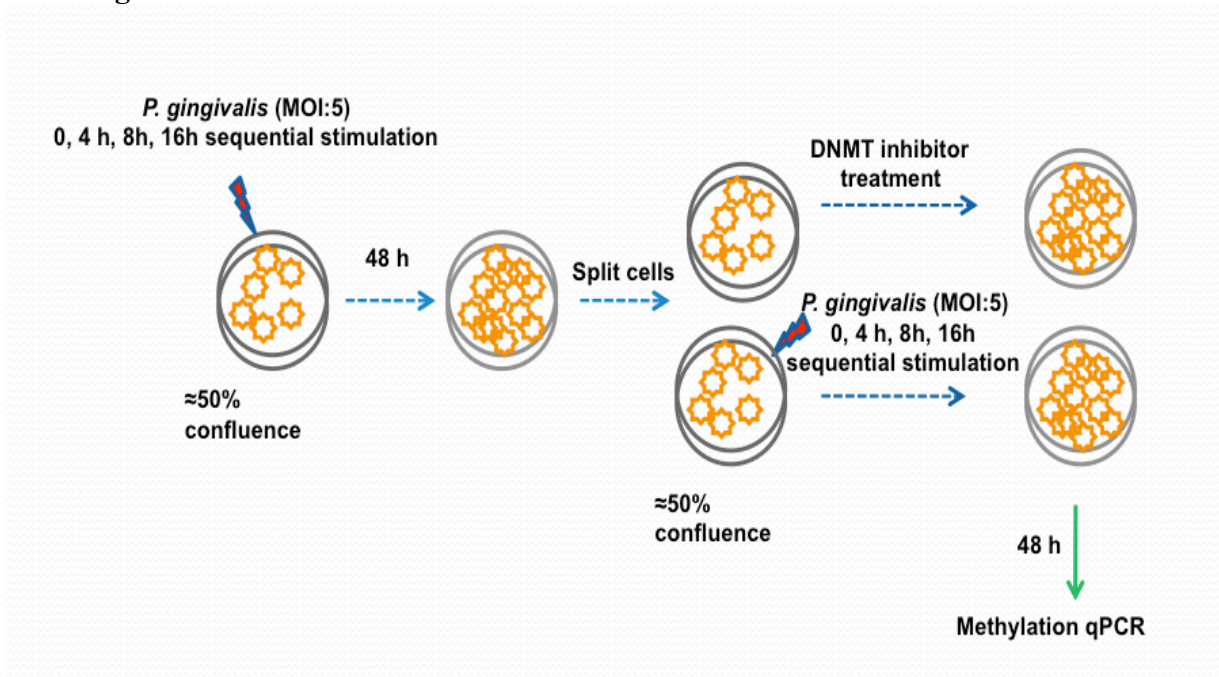
The gingival tissue was obtained with Institutional Review Board (University of Pennsylvania) approval from patients who are admitted to periodontal surgery at the School of Dental Medicine. The tissue was treated with 0.025% trypsin and 0.01% EDTA overnight at 4°C, and keratinocytes were isolated¹. Briefly, the cell suspension was centrifuged at 120 × g for 5 min, and the pellet was suspended in K-SFM medium (Invitrogen CA) containing 10 μg/ml insulin, 5 μg/ml transferrin, 10 μM 2-ME, 10 μM 2-aminoethanol, 10 mM sodium selenite, 50 μg/ml bovine pituitary extract, 100 U/ml penicillin/streptomycin, and 50 ng/ml fungizone (complete medium). The cells were seeded in 60-mm plastic tissue culture plates coated with type I collagen, and incubated in 5% CO₂ and 95% air at 37°C. When the cells reached sub-confluence, they were harvested and sub-cultured².

Cell challenge assays:

HGECs at the 3rd passage were harvested, seeded at a density of 0.5×10^5 cells/well in 6 well culture plate coated with type I collagen and maintained in 2 ml of complete medium as described above. When the cells reached ~95% confluence the cells were stimulated with *P. gingivalis* (MOI:10). Culture supernatants were collected at the end of the experiment and stored at -80°C until being assayed. Production of IL-1β and TNF were measured using

ELSA kits (BD Biosciences, CA). For *in vitro* chronic infection model (Figure 2), *P. gingivalis* at MOI:5 was stimulated for 30 minutes and washed with plain medium and 30 minutes 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 one more round of *P. gingivalis* stimulation either in the presence or absence of DNMT inhibitor. After 48 hours of first stimulation, the DNA was extracted and purified using QIAamp DNA isolation kit (Qiagen, CA), Lysis by Protease K followed by purification on spin column. The extracted DNA was subjected to methylation qPCR using methylation specific primers from SABiosciences, CA according to manufacturer's instruction.

Figure 2



Transfection:

Primary epithelial cultures at the fourth passage were harvested, seeded at a density of 0.5×10^5 cells/well in a 6 well culture plate coated with type-I collagen, and maintained in 2 ml of medium until they reached ~70% confluency. The epithelial cells were transfected with 100 pmol of pcDNA3-TLR2 and empty vector using GenMute transfection reagent according to manufacturer's instruction (SignaGen, MD). The plasmids were mixed with GenMute transfection reagent by pipetting up and down and incubated for 15 minutes at RT to let transfection complex form. The mixture was then added drop wise on to the cells and Gently rocked the plate back and forth. Transfection reaction was carried out for 24 h. After 24 h, the cells were challenged as mentioned above.

Real-time PCR:

Total RNA was extracted from cultured cells by using TRIzol reagent (Invitrogen, Carlsbad, CA). TRIzol reagent has been added directly to the culture dishes to lyse the cells and cell lysate has been passed through a pipette several times. The Homogenization phase has been followed by Phase Separation, RNA Precipitation, RNA Wash and re-dissolving the RNA. The isolated total RNA samples were used to perform first strand cDNA synthesis (Applied Biosystems, Foster City, CA). Real-time PCR was performed by using 50ng of cDNA, primers and probes and GAPDH as endogenous control on ABI 7500 Fast system (Applied Biosystems) in the presence of TaqMan DNA polymerase³. Quantitative TaqMan PCR-Array was custom designed based on previously published microarray data on keratinocytes⁴. The cDNA conversion and real time PCR were carried out as mentioned above. The fold increase was calculated as compared to untreated control sample according to $\Delta\Delta CT$ method⁵. Mean fold increase data was used to derive heatmap with two-way hierarchical clustering

using MeV v4.1 software.

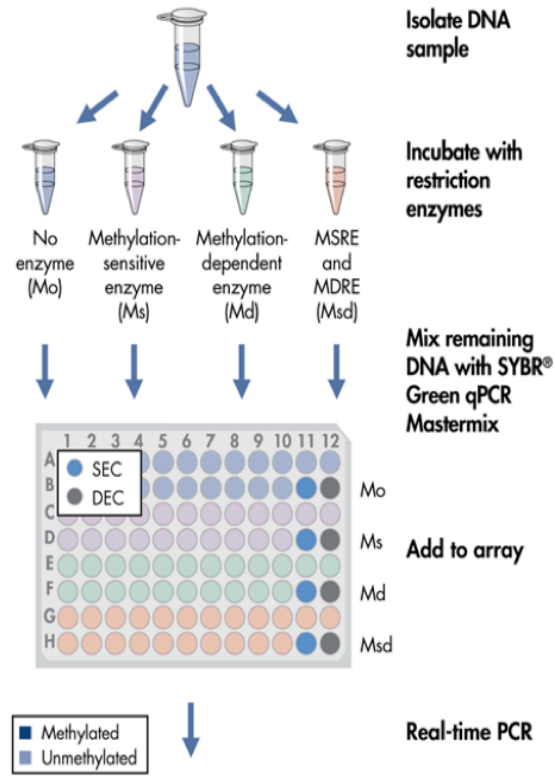
Methylation qPCR:

The genomic DNA was subjected restriction digestion using EpiTect Methyl II DNA restriction kit (SAbiosciences, CA. The reaction mixture consisting of methylation sensitive and methylation dependent restriction enzymes were incubated for 6 hours at 37°C. After which, the reaction was terminated and enzyme was inactivated at 65 °C for 20 minutes. The digested DNA samples were subjected to real time PCR using TLR2 promoter CpG discriminating primers from SAbiosciences, CA. The percent methylation was calculated using data analysis software from Orion Genomics, LLC.

The method employed by the EpiTect Methyl II PCR System is based on detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme. These enzymes will digest unmethylated and methylated DNA, respectively. Following digestion, the remaining DNA in each individual enzyme reaction is quantified by real-time PCR using primers that flank a promoter (gene) region of interest. The relative fractions of methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using a ΔC_T method. The reliability and simplicity of the procedure make this technology highly suited for semi-high-throughput DNA methylation profiling and biomarker development for various research fields, such as stem cell differentiation and development. Input genomic DNA is aliquoted into four equal portions and subjected to mock (no enzyme), methylation-sensitive (MSRE), methylation-dependent (MDRE), and double (MSRE and MDRE) restriction endonuclease digestion. After digestion, the enzyme

reactions are mixed directly with qPCR master mix and are dispensed into a PCR Array plate containing pre-aliquoted primer mixes. Real-time PCR is carried out using specified cycling conditions. Finally, the raw ΔC_T values are pasted into the data analysis spreadsheet, which automatically calculates the relative amount of methylated and unmethylated DNA fractions. The product of the mock (no enzyme) digestion represents the total amount of input DNA for real-time PCR detection. In the methylation-sensitive digestion (Ms) reaction, the MSRE will digest unmethylated and partially methylated DNA. The remaining hypermethylated DNA — DNA in which all CpG sites are methylated — will be detected by real-time PCR. In the methylation-dependent digestion (Md) reaction, the MDRE will preferentially digest methylated DNA. The remaining unmethylated DNA will be detected by real-time PCR. In the double digestion (Msd) reaction, both enzymes are present, and all DNA molecules (both methylated and unmethylated) will be digested. This reaction measures the background and the fraction of input DNA refractory to enzyme digestion (Figure 3).

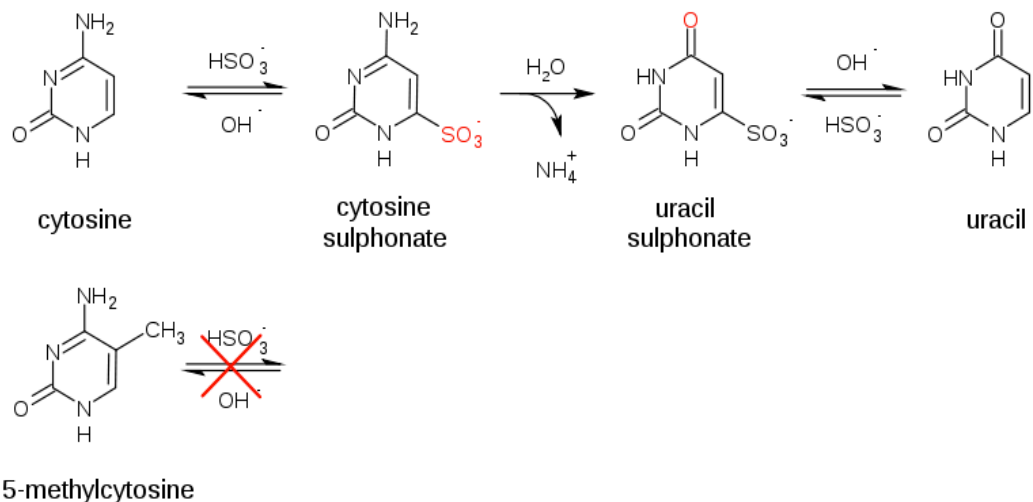
Figure 3



Bisulfite treatment and sequencing:

The methylation status of a DNA sequence can be determined using sodium bisulfite. Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA. The most critical step for correct determination of a methylation pattern is the complete conversion of unmethylated cytosines. This is achieved by incubating the DNA in high bisulfite salt concentrations at high temperature and low pH.

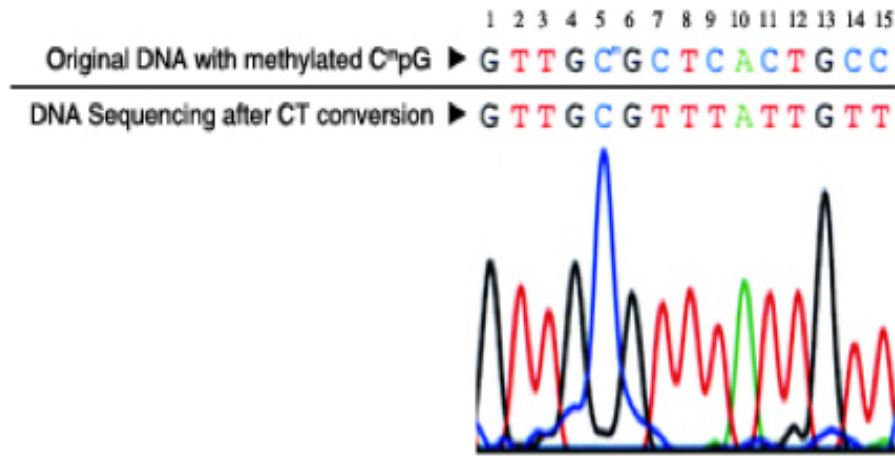
Figure 4



These harsh conditions usually lead to a high degree of DNA fragmentation and subsequent loss of DNA during purification. Purification is necessary to remove bisulfite salts and chemicals used in the conversion process that inhibit sequencing procedures. Common bisulfite procedures usually require high amounts of input DNA. However, due to DNA degradation during conversion and DNA loss during purification, such procedures often lead to low DNA yield, highly fragmented DNA, and irreproducible conversion rates.

In figure 5 as an example, DNA with methylated C^{m} pG at nucleotide position #5 was processed using the EZ DNA MethylationTM Kit. The recovered DNA was amplified by PCR and then sequenced directly. The methylated cytosine at position #5 remained intact while the unmethylated cytosines at positions #7, 9, 11, 14 and 15 were completely converted into uracil following bisulfite treatment and detected as thymine following PCR.

Figure 5



In our study the bisulfite conversion was done using EZ DNA methylation Gold kit from Zymoresearch Inc. Briefly, 500 ng of genomic DNA was mixed with 130 μ l of CT conversion reagent and the reaction was carried out on a thermal cycler (98°C for 10 minutes, 64° C for 2.5 hours). After the reaction, the product was washed using Zymo-spin column and desulphonated using M-Desulphonation buffer for 20 minutes at room temperature. The desulphonated product was purified according to manufacturer's instructions. The promoter sequence of TLR2 was obtained from Transcriptional Element Regulatory Database (Cold Spring Harbor Laboratory, USA). The CpG Island screening, bisulfite specific primer and methylation specific primers were designed using Mehtyl primer express v1.0 software (Lifetechnologies, CA). The primer sequences are as follows: methylation sensitive forward primer -TTTTGTACGGGGTAGTTGTC; reverse primer -ACTACGCTTTCTCGCTACC and non-methylation forward primer AGTTTTGTATGGGGTAGTTGTT; reverse primer -ACTACACTTTCTCACTACCTC. The bisulfite converted DNA was amplified using the above primer pairs and ZymoTaq hot start DNA polymerase (Zymoresearch Inc., CA). This generated product size of 295 bp

within promoter CpG Island. The PCR product was purified using DNA clean and concentrator kit and sequenced at Penn Genomic Core, University of Pennsylvania, PA. The sequence obtained was analyzed using BISMA program⁶.

Experimental Periodontitis, Oral gavage mouse model

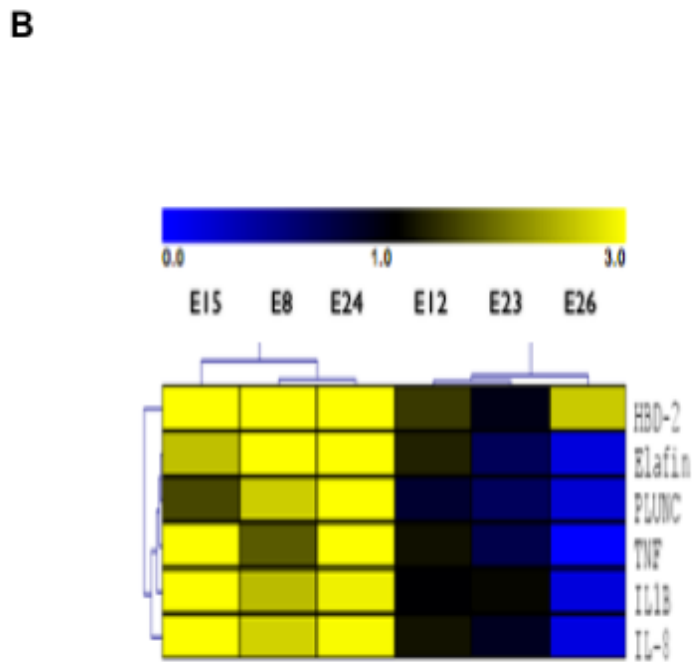
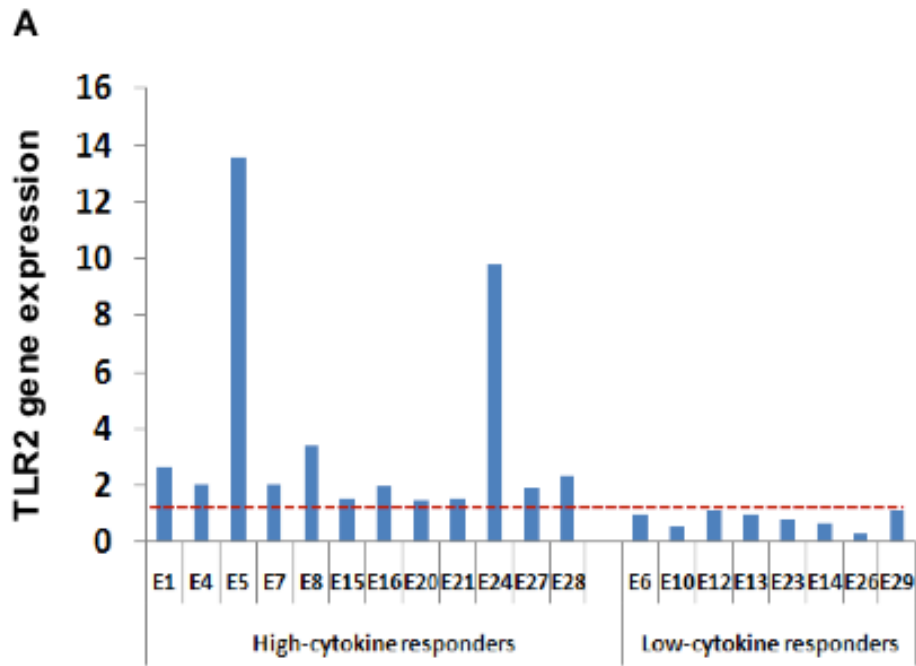
Periodontitis was induced in 6-8 week-old BALB/c mice by oral inoculation with *P. gingivalis* ATCC 33277 by means of a ball-ended feeding needle. Briefly, upon suppression of the normal oral flora with antibiotics (Mice were given sulfamethoxazoletrimethoprim at 10 ml per pint in deionized water ad libitum for 10 days. This was followed by a 3-day antibiotic-free period), mice were orally infected five times at 2-day intervals with 10^9 CFU *P. gingivalis* suspended in 2% carboxymethylcellulose/PBS. Sham-infected controls received 2% carboxymethylcellulose/PBS alone. The mice were euthanized one month after the last oral inoculation. Assessment of periodontal bone loss in defleshed maxillae was performed under a dissecting microscope (x40) fitted with a video image marker measurement system (VIA-170K; Boeckeler Instruments). Specifically, the distance from the cement enamel junction (CEJ) to the alveolar bone crest (ABC) was measured on 14 predetermined points on the buccal surfaces of the maxillary molars to sham controls. Gingival tissue samples has been excised and DNA has been extracted for TLR2 promoter methylation analysis.

Results

TLR2 gene expression heterogeneity in human gingival epithelial cells

Having identified the importance of TLRs in gingival epithelial cells mounting innate immune responses correlated to gingivitis and periodontitis, we noted that TLR2 gene expression in primary human gingival epithelial cells (HGECs) upon *P. gingivalis* stimulation showed variability within gingival epithelial cells isolated from different patients. We defined the group of HGECs which induced relatively higher level of TLR2 gene expression following challenge with *P. gingivalis* as TLR2-normal cells. The detected HGECs with reduced TLR2 expression at mRNA levels has been named TLR2-dysregulated cells (Fig. 6A). Epithelial cells with relatively lower TLR2 expression also secrete low levels of cytokines and antimicrobial peptides. The normal cell type upregulated both proinflammatory cytokine and antimicrobial peptides, however, the dysregulated type cells with lower TLR2 expression in response to *P. gingivalis* also exhibited blunted cytokine and antimicrobial peptide response (Fig.6 B, C). Hence, we hypothesized that blunted TLR2 expression might be epigenetically regulated leading to altered TLR2 mRNA expression and subsequently pro-inflammatory cytokines, consequently leading to epithelial “dysregualted” phenotype in humans.

Figure 6



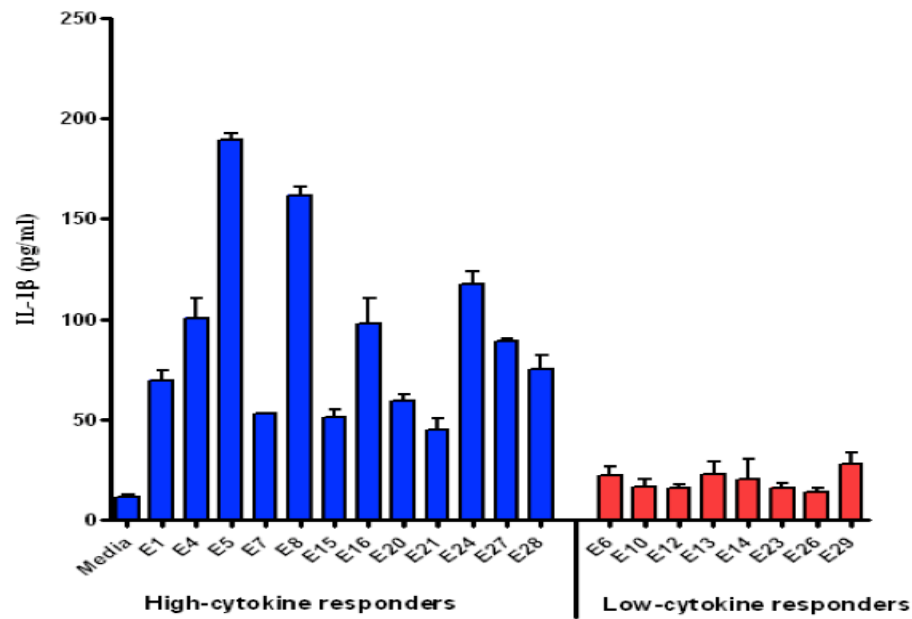
C

Fig 6: Isolated human primary gingival epithelial cells were cultured and stimulated with *P. gingivalis* at MOI:10 for 4 hours. The cDNA was subjected to realtime PCR using TLR2 TaqMan probe. The relative expression of TLR2 was calculated using GAPDH as endogenous control (A). The cDNA from three individuals from each group was subjected gene expression analysis using TaqMan probes. The $\Delta\Delta CT$ values were used to generate heatmap based on two-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) (B). The data is mean of two independent experiments. Production of IL-1B was assayed with ELISA(C)

TLR2 promoter methylation in dysregulated gingival epithelial cell type

Since we observed blunted inflammatory response in dysregulated cells with *P. gingivalis* stimulation and *P. gingivalis* is a known agonist for TLR2 and TLR4^{3,7,8}, we carried out TLR pathway DNA methylation PCR (SAbiosciences, CA) to determine if there is epigenetic deregulation at the promoter level in TLR signaling network. The DNA sample was isolated from representative dysregulated cell type and subjected to methylation specific enzyme digestion. After the digestion, the real time PCR was carried out and data was analyzed according to manufacturer's instruction (SAbiosciences, CA). 26 genes

showed promoter methylation with TLR2 higher promoter methylation in dysregulated cell type (Fig. 7). Dysregulated cells has been shown three fold more methylation of TLR2 promoter than normal cells group (Fig 8)

Figure 7

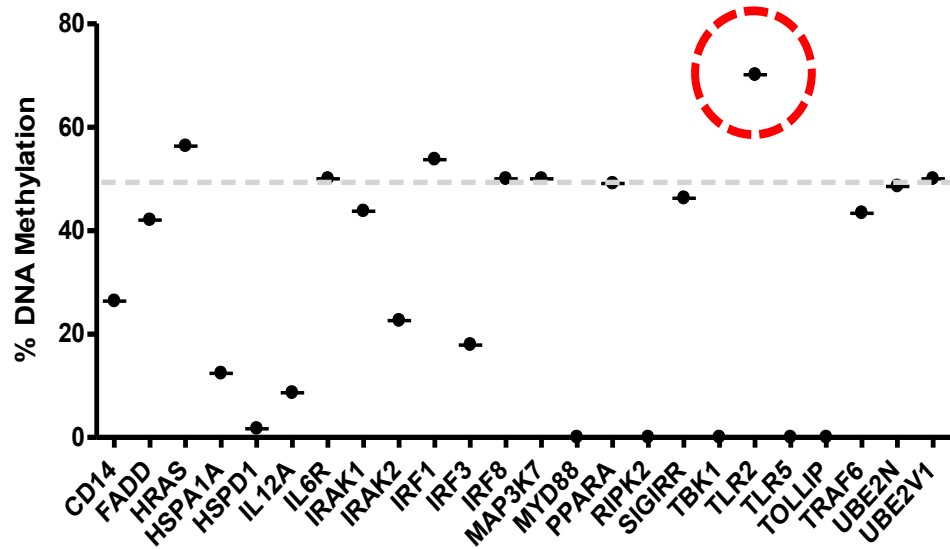


Fig 7: Promoter DNA methylation in dysregulated cells epithelial cells: The DNA from dysregulated epithelial cells were subjected to toll-like receptor signaling pathway DNA methylation PCR arrays from SABiosciences, CA. The data was analyzed using EpiTect methyl II PCR data analysis program and methylation is represented as percent compared to unmethylated DNA standard. TLR2 exhibited hypermethylation in dysregulated cells (dotted circle)

Chronic treatment of *P. gingivalis* induce *de novo* methylation in TLR2 promoter

In an attempt to mimic chronic infection status and in our quest to understand how the bacteria changes methylation profile of epithelial cells, we developed a system called “*chronic in vitro infection model*” (Fig. 2). The time points were chosen based on our Lab’s previously published results on *P. gingivalis* induced inflammatory cytokine response in

gingival epithelial cells^{2,7, 8,9,10}. By using this method, we successfully induced tolerance to *P. gingivalis* (live bacteria at MOI:5) in ‘normal’ epithelial cells. The chronic infection was done according to following method. *P. gingivalis* (MOI:5) stimulation was initially carried out for 30 minutes at 0, 4, 8 and 16 h time intervals. After 48 h from the last stimulation, the cells were split and re-exposed to the *P. gingivalis* stimulation cycle in the presence or absence of 1 μ M of 5-Aza-2'-deoxycytidine (decitabine) and evaluated for the TLR2 promoter methylation status. After which, DNA samples were subjected TLR2 promoter methylation PCR (SAbiosciences, CA) Repeated stimulation with *P. gingivalis* induced reprogramming of TLR2 region by inducing *de novo* methylation. By using decitabine we were able to essentially eliminate the TLR2 promoter methylation that was induced by *P. gingivalis* (Figure 8).

Figure 8

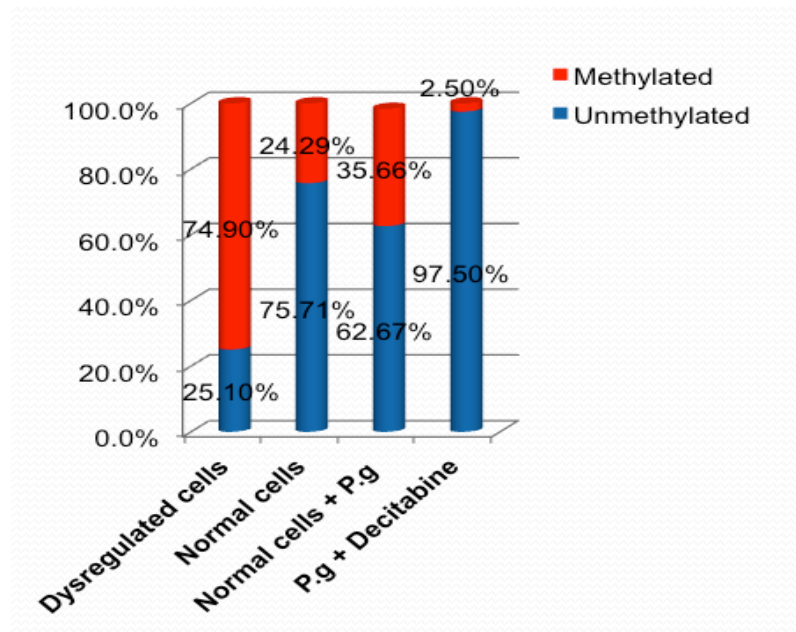


Fig 8: *In vitro* chronic infection model: The DNA was extracted at the end of above experiment and subjected to TLR2 DNA methylation sensitive qPCR using TLR2 methylation primers (SAbiosciences, CA). The data is represented as percent methylation compared to unmethylated standard DNA. Chronic *P. gingivalis* infection induced *de novo* methylation in HGECS.

Reconstitution of TLR2 rescue dysregulated epithelial cells

As we noted blunted TLR2 expression in dysregulated epithelial cells, we wanted to overexpress TLR2 by transfecting TLR2 overexpression vector in dysregulated cells type. TLR2 overexpression plasmid (Addgene, MA) and empty vector was transfected using GenMute transfection reagent (SignaGen Laboratory, MD) and incubated the cells. After 24 hours of transfection, the cells were stimulated with *P. gingivalis* (MOI:10) for 4 hours 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 rescue inflammatory response to *P. gingivalis* (Fig. 9).

Figure 9

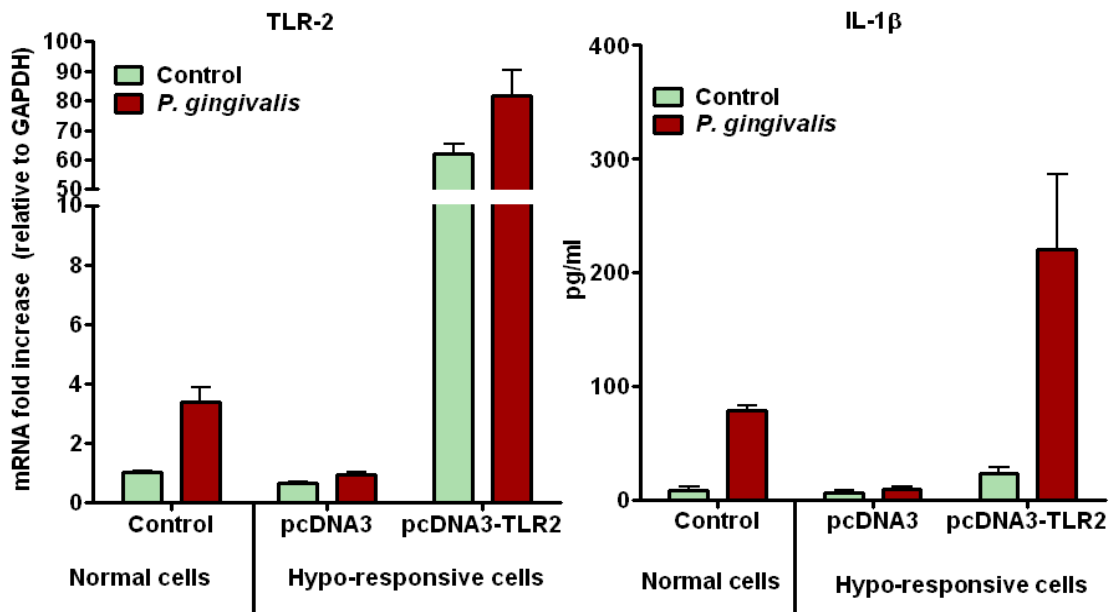


Fig 9: TLR2 overexpression up-regulates inflammatory cytokine in dysregulated cells: The dysregulated cells were transfected with a plasmid overexpressing TLR2 (Addgene, MA). After 24 h post transfection, the cells were stimulated with *P. gingivalis* and cDNA was subjected to TLR2 and IL-1 β mRNA expression using TaqMan probes. TLR2 overexpression showed up-regulation of IL-1 β induction after *P. gingivalis* stimulation in dysregulated cells.

DNA methyltransferase inhibitor rescue inflammatory response in dysregulated cells

As a therapeutic approach, we wanted to test if the dysregulated cells can be modified to express TLR2 by targeting DNA methyltransferase in HGECs. The dysregulated epithelial cells were cultured with DNA methyltransferase (DNMT) inhibitor at a concentration of 1 μ M before stimulating with *P. gingivalis* for 4 hours. After stimulation, the total RNA was extracted and subjected to real time PCR using TLR2 and TNF TaqMan probes (Lifetechnologies, CA). The real time PCR data showed that DNMT inhibitor can restore TLR2 expression in dysregulated epithelial cells and induce inflammatory response to *P. gingivalis*. This clearly shows that DNMT as a therapeutic target in restoring inflammatory response to a pathogen in dysregulated epithelial cell types and DNMT inhibitor may serve as therapeutic agent against periodontitis (Fig.10). DNA methyltransferase inhibitor could rescue TLR2 and TNF α expression in “dysreglated” epithelial cells.

Figure 10

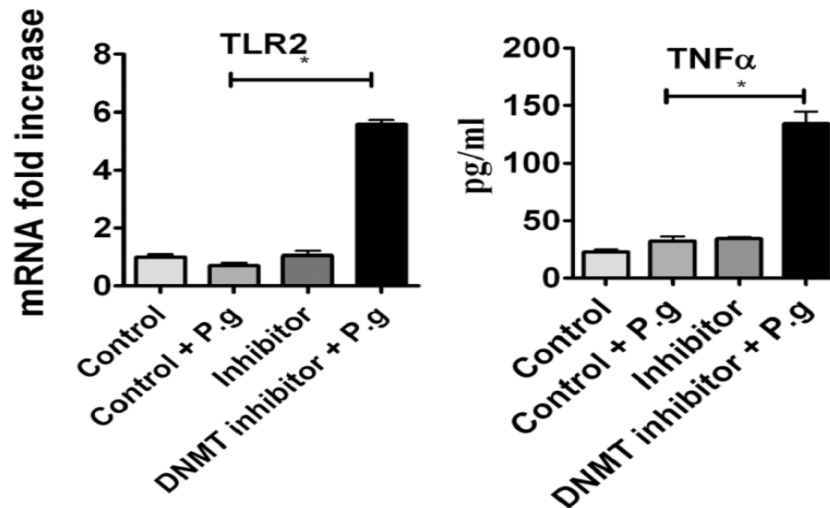


Fig 10: DNA methyltransferase inhibitor rescue TLR2 expression in dysregulated epithelial cells: The dysregulated epithelial cells were cultured in the presence of 1 μ M 5-Aza-2'-deoxycytidine (Sigma-Aldrich, USA). When it reached 90% confluence, the cells were split and cultured on 6 well plates in the presence of 5-Aza-2'-deoxycytidine. At 90% confluence, the cells were stimulated with *P. gingivalis* at MOI:10 for 4 hours and cDNA was subjected to real time PCR using TLR2, TNF and GAPDH probes. The data represented as mean standard error from three independent experiments.

TLR2 promoter methylation in periodontal disease affected tissue in mouse model

To test the change of TLR2 promoter methylation level following periodontitis, a gavage experimental periodontitis model in mouse has been designed. DNA isolated from the gingival tissue of sham and periodontitis group have been analyzed to compare the methylation percentage. The gingival tissue obtained from the periodontitis group showed higher percentage of methylated TLR 2 promoter than the control group.

Figure 11

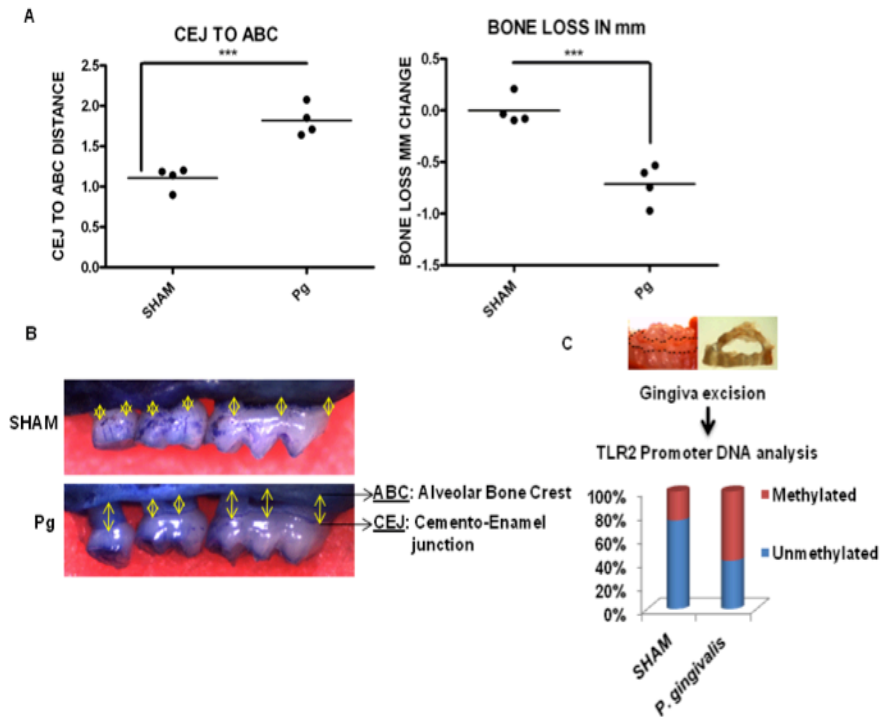


Fig11: TLR2 promoter methylation in periodontal disease affected tissue in mouse model: Periodontitis was induced in 6-8 week-old BALB/c mice by oral inoculation with *P. gingivalis* ATCC 33277. The gingival tissue obtained from the periodontitis group showed higher percentage of methylated TLR 2 promoter than the control group.

TLR2 promoter methylation in periodontal disease affected tissue

Next, we wanted to examine whether this phenomena exist in patients with periodontitis. To confirm this, we obtained gingival tissue from periodontitis affected site and healthy site from patients who are undergoing flap surgery from the periodontics clinic. This tissue was subjected to genomic DNA isolation and purification using QIAamp DNA kit (Qiagen, CA). 1 µg of isolated genomic DNA was subjected to bisulfite conversion using EZ DNA methylation gold kit (Zymo Research, CA). The converted samples were desulphonated and purified according to manufacturer's instructions. 2 µl of elute was used was then subjected

to PCR using bisulfite primers designed using Methyl Primer Express Software v1.0 (Lifetechnologies, CA). The amplified samples were sequenced using dye termination method. The DNA sequence obtained was exported to BISMA program¹². The sequence analysis showed TLR2 promoter methylation in the disease tissue site. This clearly indicates that there is host epigenetic regulation at the periodontitis disease affected tissue.

Figure 12

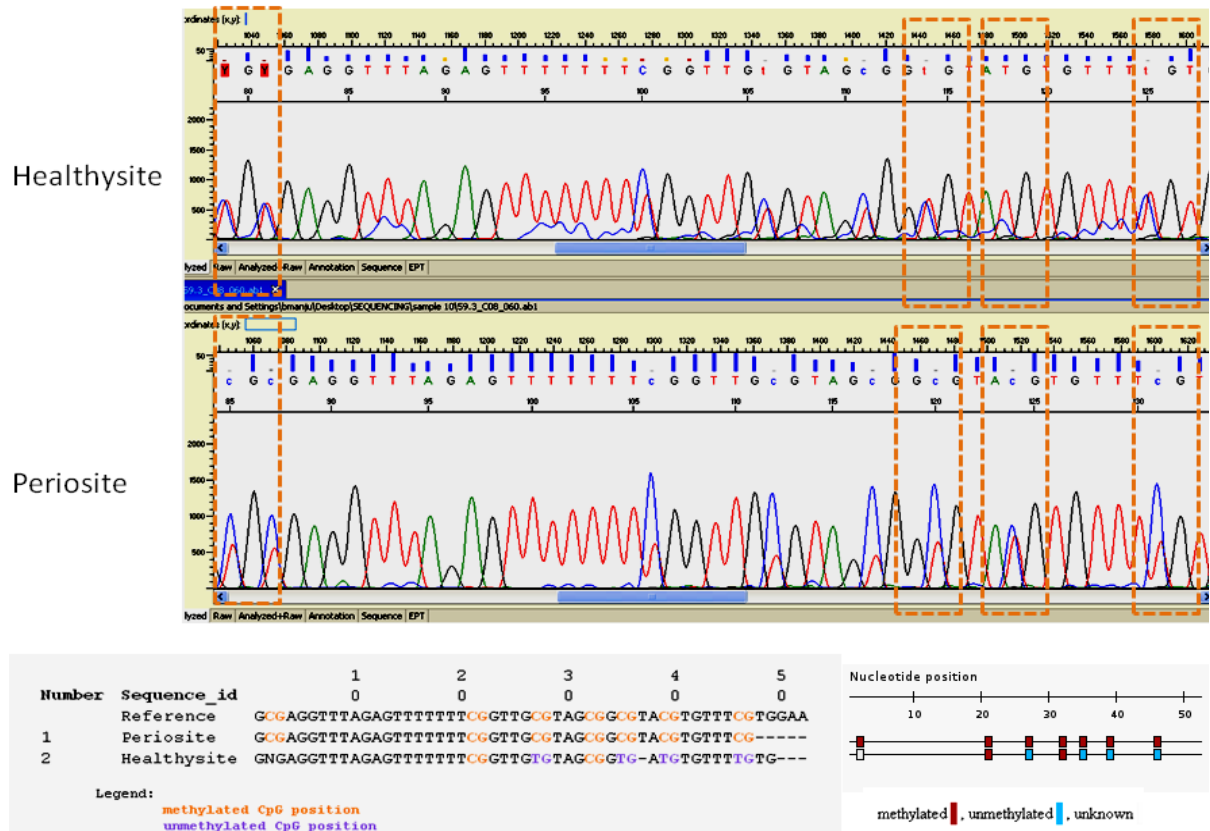


Figure 12: TLR2 promoter CpG targeted bisulfite DNA sequencing: Healthy and periodontitis-affected tissue was collected and subjected to bisulfite treatment (Zymo Research Inc., CA). After bisulfite treatment, the DNA was subjected to bisulfite sequencing. The sequence data was analyzed using BISMA software program. Disease site showing TLR2 promoter CpG methylation (dotted box).

Discussion

Periodontal disease is initiated by a microbial biofilm of ~700 different microorganisms most of which are Gram negative anaerobic bacilli^{13,14}. Among these, an important putative pathogen is *Porphyromonas gingivalis* and is now regarded as “key-stone pathogen”^{15,16}. *P. gingivalis* is recognized by toll-like receptors, TLR2 and TLR4¹⁷ and their activation is a crucial step in developing an innate immunity to pathogenic microorganisms. The activated host innate immune defense is characterized by elevated cytokine production following bacterial 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 inflammation¹⁸. One such control could be at the level of TLR expression itself where blunted TLR expression could lead failure of proper innate immune response.

We observed a blunted pro inflammatory and antimicrobial response to *P. gingivalis* stimulation in periodontitis affected persons’ gingival epithelial cells. In gingival epithelial cells, TLR2 is expression is higher than that of TLR4¹¹. Hence we sought to understand the mechanism of TLR2 gene expression variation in these cells.

It has been shown that genetic factors are very important in the development of periodontitis. Some Single nucleotide polymorphisms (SNPs) and single nucleotide variations (SNVs) alter gene expression levels that may influence host response levels to microbiological growth. For example, SNPs in receptors, antigen sensors in cell surfaces, and cytokines and chemokines have been shown to influence host immunity and inflammatory response^{19,20}. The most common polymorphism is a transition from a C to a T

nucleotide. These polymorphisms can affect numerous CpG sites in the genome, by altering a C in a CpG dinucleotide to another nucleotide that cannot be methylated²¹. Studies have shown that these SNPs can influence gene expression via effects on DNA methylation. The effect of SNPs on DNA methylation can either be direct, by changing a C (in a CpG dinucleotide) to a non-modifiable nucleotide, or indirect by altering transcription factor binding, which in turn independently affects gene expression and DNA methylation levels^{22,23}.

It is important to understand that the levels of transcripts, proteins and metabolites may reflect not only the genetic programming, but also the consequences of response to environmental factors and disease progression. There are layers of chemical modifications on the DNA and its associated proteins that regulate gene expression. Recent studies in understanding genetic variation implicated not only Mendelian inheritance but also non-Mendelian inheritance termed “epigenetics”^{24,25}. Epigenetics has been linked to many diseases including cancer and inflammation²⁶. There have been numerous studies in humans relating to variation caused by epigenetic changes with respect to aging²⁷⁻²⁹. DNA methylation and histone modification are the two major mechanisms of epigenetic alterations observed in human cells, and both mechanism block the transcriptional factors. studies have demonstrated that epigenetic events are able to influence the production of cytokines contributing to the development of diseases, such as airway inflammation and severe systemic inflammation^{24,30}.

The post-translational histone modifications, such as acetylation/de-acetylation, methylation and phosphorylation, could play its epigenetic role in the organization of chromatin domains

and the up or down regulation of gene expression. Recent studies have demonstrated that acetylation of core histone is associated with cardiovascular diseases³¹. Also accumulating evidences points towards association of aberrant DNA methylation in the development of various human diseases³¹. Altered DNA methylation predicted Crohn's disease status where key host defense mechanisms including TH17 were dysregulated³².

There is evidence of crosstalk between DNA methylation and histone modifications. It is not known how cross-talk between these two systems is mediated, but in at least some circumstances, changes to histone modifications may be induced prior to methylation changes that then serve as more stable epigenetic marks³³.

Accumulating evidences points towards association of aberrant chromatin methylation in the development of various human diseases³⁴. 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 promoter region in the TLR2 gene in dysregulated cells. These cells induced diminished pro-inflammatory cytokines and antimicrobial peptide in response to *P. gingivalis*. In support of blunted inflammatory response in dysregulated epithelial cells, siRNA against TLR2 had similar effect in normal cells in response to *P. gingivalis*. This innate immune compromise within epithelial cells may direct effect on antimicrobial defense as well as indirect influence on adaptive immune responses such as inhibition of IL-12 from T cells that facilitate *P. gingivalis* persistence³⁵.

The epigenetic changes can be induced by repeated bacterial ligand challenge (*E. coli* LPS) as shown in murine macrophages leading to tolerance in cells with blunted cytokine

responses^{36,37}. ENCODE project (www.genome.ucsc.edu/ENCODE) supports our data of differential TLR2 methylation status, where, one CpG Island on TLR2 gene was revealed with differential CpG methylation across different cells lines. Moreover, CpG island hypermethylation in gene promoters has been shown to be an important mechanism in gene silencing³⁷. The changes pertaining to epigenetics may be brought about by age related methylation³⁸ or changes that occur due to chronic inflammation as in ulcerative colitis due to constant turnover of cells³⁹. *Helicobacter pylori* is known to be associated with the accumulation of aberrant DNA methylation in gastric epithelial mucosa⁴⁰. LPS derived from *P. gingivalis* has been demonstrated to inhibit osteoblastic differentiation of osteoprogenitor cells derived from fetal rat calvaria⁴¹. Recently, bacterial induced hypermethylation of *Igf2* gene has been revealed⁴². It is possible that similar mechanisms exist for different pathogens. In humans, with chronic periodontitis, hypermethylation pattern of the promoter of Prostaglandin-endoperoxide synthase 2 (PTGS2) is altered⁴³. 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.

The identification of TLR2 DNA methylation status in periodontitis affected tissue samples supported our *in vitro* data. However, this tissue comprised not only of epithelial cells but also other type of cells. Further investigation with regard to gingival tissue is needed especially using laser scanning micro-dissection to isolate epithelial cells and to determine their methylation status. We are also aware of the fact that the data needs to be validated using *in vivo* experimental periodontitis model. Although we just tested one of the epigenetic alteration mechanism, Cytosine Hyper methylation, we know that other

Chromatin epigenetic modification like Histone changes might have effect on this phenomena. Nonetheless, our data clearly indicates that epigenetic modification in gingival epithelial cells plays an important role in gene silencing pushing the cells to a hypo-responsive state thereby failing to restrain harmful chronic inflammation.

Conclusion

Within the limitation of this study, our data suggest that epigenetic modifications of the TLR2 promoter region play an important role in inducing the hypo-responsive 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.

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