2013

MICROBIAL DNA RECEPTOR EXPRESSION IN CHRONIC PERIODONTITIS

Stephanie Voth
Virginia Commonwealth University

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A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

by

STEPHANIE C. VOTH
DDS, Virginia Commonwealth University School of Dentistry, 2009

Director: S. ESRA SAHINGUR, DDS, MS, PhD
Associate Professor, Department of Periodontics

Virginia Commonwealth University
Richmond, Virginia
April 29, 2013

Acknowledgements

Special thanks to S. Esra Sahingur, DDS, PhD, Mrs. Kimberly Hollaway, Xia-Juan Xia, PhD, John C. Gunsolley, DDS, MS, Harvey Schenkein, DDS, PhD, Thomas C. Waldrop, DDS, MS and Andrew Yeudall, DDS, PhD for their contributions towards this study. This study was supported by Presidential Quest Funds (Virginia Commonwealth University) awarded to Dr. Sahingur and a fellowship awarded to Dr. Voth from the Alexander Fellowship, Richmond, VA.

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Abstract

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AIM: The aim of this study was to determine the expression of microbial nucleic acid receptors including Toll like receptor 9 "TLR-9", DNA-dependent activator of interferon-regulatory factors "DAI" and absent in melanoma "AIM-2" in chronic periodontitis (P) versus healthy (H) tissues.

METHODS: 33 chronic periodontitis (P) and 27 periodontally-healthy (H) gingival biopsies were included. The gene and protein expression for each receptor was determined using real-time quantitative PCR and immunohistochemistry.

RESULTS: Our results revealed statistically significant up-regulation of TLR-9 ($p<0.006$) and DAI ($p<0.001$) gene expression in P tissues compared to H sites. We were also able to demonstrate significant correlation among three DNA receptors ($p<0.05$). Immunohistochemistry further confirmed the expression of DNA sensors in gingival tissues.

CONCLUSION: This study highlights a possible role for nucleic acid sensing in periodontal inflammation. Further investigations will determine whether cytoplasmic receptors and their ligands can be targeted to improve clinical outcomes in periodontitis.

CHAPTER 1 Introduction

Periodontitis is a complex, multifactorial disease that is initiated by an interaction between periodontal pathogens and host immune cells.[1] Specific bacteria activate the host immune response that aim to control the pathogens while simultaneously causing inflammation and destruction of the periodontal tissues.[2,3] The pathogenesis of periodontal disease has evolved further with the knowledge that environmental and genetic factors influence the disease process via modifying the expression of genes activated by bacteria and their byproducts.[4] The dynamic relationship between bacteria, genetics and environmental elements supports a biologic systems approach to modeling periodontal disease with the ultimate goal of defining expression patterns in the tissues with respect to environmental and genetic conditions along with corresponding clinical parameters.[4]

The "biologic systems model" is comprised of different components which interact with one another to produce the clinical phenotype (i.e. the clinical presentation of disease).[5] The outer-realm of this model is the subject level which involves individual unique exposures such as the characteristics of the bacterial biofilm, health conditions (e.g. diabetes) and environmental factors (e.g. cigarette smoke). In this model, an individual's genetics interacts with the subject-level components thus impacting the host's response to the bacterial biofilm. The biologic phenotype is associated with the clinical phenotype via cellular and molecular responses that involve inflammatory biomarkers.[6] This model suggests that identifying diagnostic biomarkers is

important since individuals may have similar clinical presentations but dramatically different predisposing factors and thus may respond differently to the same treatment.[7] Thus, by using the biologic systems approach to defining periodontal disease, homogenous diagnostic categories can be established, diagnostic biomarkers can be measured, all leading to more accurate prognoses and expected therapy responses.[6]

Tanerella forsythensis, Porphyromonas gingivalis (P. gingivalis) and *Treponema denticola* are putative periodontal pathogens that have been highly implicated in periodontal disease. They are all members of the "red complex" and frequently occur together in subgingival plaque biolfilms.[8] Microbiologic identification has been deemed useful for disease monitoring and treatment guidance (i.e. antibiotic therapy) but does not help to predict future periodontal disease progression.[9] While particular periodontal bacteria are required to initiate disease, they are only sufficient to cause disease in a susceptible host.[10] The host immune system is typically protective against pathogens but can be simultaneously destructive or dysregulated when it is overwhelmed by a bacterial challenge in susceptible individuals. Certain microorganisms have the capacity to evade the host immune response by escaping recognition, phagocytosis and even exerting immunosuppressive effects. Identifying microbes, particularly the evading type, with certain biomarkers may enhance the ability to predict future disease progression.[11]

Genomics can be relevant in identifying those who are predisposed to periodontitis and samples for testing can be obtained via blood, tissue, saliva, gingival crevicular fluid or dental plaque biofilm. Single nucleotide polymorphisms in the IL-1 gene have been researched extensively and it has been found that individuals who have both the IL-1α +4845 and IL-1β +3954 are predisposed to severe chronic periodontitis via a hyperinflammatory response to

periodontal pathogens.[12] Socransky et al. studied IL-1 genotype-positive and negative patients and found that those who were IL-1 genotype-positive had higher levels of "red complex" bacteria.[13] Thus, genetic polymorphisms can help to determine if a patient has an increased susceptibility to chronic periodontal disease.

Traditional methods for diagnosing periodontal disease include measurements of probing depth, clinical attachment loss and bleeding on probing, plaque levels, and bone loss.[14] One of the drawbacks of such measurements to diagnose disease is that they account for disease history. New research focuses on identifying and quantifying periodontal disease and risk with biomarkers at the cellular and molecular level.[15] Biomarkers that can be identified via plaque biofilm, gingival crevicular fluid (GCF) and saliva include microbial factors (i.e. periodontal pathogens), host response factors (i.e. IL-1β) and connective tissue breakdown products (i.e. collagen telopeptides). At the cellular level of biomarkers, inflammatory cell activation (i.e. neutrophils) of the innate immune system via bacteria or their byproducts ultimately leads to osteoclast type cell activation and alveolar bone loss**.** Pyridinoline cross-linked carboxyterminal telopeptide of type I collagen (ICTP) is released into the GCF during this process of bone resorption and is an example of a measureable biomarker of periodontal disease. On the other hand, at the molecular level, the activation of toll-like receptors (TLRs) can also be determined to identify susceptibility or disease activity. While TLRs represent possible targets as biomarkers of periodontal disease, more research is needed to determine such effects.[15]

It has been well documented in the literature that pathogen associated molecular pattern (PAMP) recognition via host pattern recognition receptors (PRRs) activates intracellular signaling cascades leading to periodontal inflammation through the up-regulation of cytokines, chemokines and costimulatory molecules. TLRs represent a type of host PRR and are the mammalian

homologues of *Toll* first discovered in Drosophilia as a gene that functions in immunity.[16, 17, 18] TLRs are comprised of an extracellular leucine-rich repeat (LRR) domain and an intracellular Toll/interleukin (IL)-1 receptor (TIR) domain.[19] The LRR can accommodate a variety of PAMP binding interactions and signal tranductions. While microbial products (i.e. bacterial LPS) comprise the majority of TLR ligands, other ligands include flagellin, bacterial DNA as well as viral RNA and DNA.[20] Specifically, *P. gingivalis* fimbrae signal through TLR2 or TLR4 which activates a signaling pathway that mediates production of proinflammatory cytokines such as IL-6 and TNF-α which is different than the signaling pattern stimulated by *P. gingivalis* LPS.[21] TLRs in humans are either extracellular (TLR -1, -2, -4, -5, -6, -10), mainly recognizing pathogenic structural components such as LPS, or intracellular (TLR -3, -7, -8, -9), specializing in the recognition of foreign or self RNA and DNA.[22, 23] While the extracellular receptors have been well studied, limited literature to date has examined intracellular PRR involvement in periodontal pathogenesis.

TLRs require co-receptors and adaptor molecules for pathogen recognition and downstream signaling. For example, TLR4 is influenced by co-receptor MD-2 which directly interacts with LPS[24] and in the CD14-dependent signaling pathway, CD14 is responsible for stimulating the TRAM-TRIP pathway which amplifies the production of TNF-α and Il-6 via NF-κB activation.[25] The engagement of adaptor molecules such as myeloid differentiation factor-99 (MyD88) is necessary for proinflammatory cytokine production for all TLRs except TLR3. Interestingly, MyD88-dependent signaling of the intracellular TLRs -7,-8, and -9 can either induce NF- κB or type-I interferon (IFN) production which are proinflammatory and anti-inflammatory responses respectively.[20]

Nucleic acids, such as DNA and RNA are typically sequestered within bacteria. During infection or inflammatory processes, however, they can be released and become detected by specific intracellular receptors.[26] Recognition of bacterial DNA by the host immune system leads to activation of distinct signaling pathways and immune responses through various receptors that may be operant and important in periodontal inflammation and pathology. Bacterial nucleic acids (bDNA) are recognized mainly through the intracellular TLR-9[27] thereby activating the NF-κB and MAPK signaling pathways that stimulate pro-inflammatory and anti-inflammatory activities depending on cell type.[28] Sahingur et al. demonstrated that, in response to periodontal bDNA, human monocytic cells (THP-1) produce pro-inflammatory cytokines via TLR-9 and the NF-κB signaling pathway.[29] Other investigators also determined similar responses in primary human gingival fibroblasts[30] and mouse macrophage cell lines.[31]

Supporting a role of TLR9 in periodontal disease, it has been shown that gene expression of this receptor is increased in periodontally-diseased tissues compared to healthy tissues.[32] Differential expression of TLR -1 through -9 between periodontitis patients and healthy controls amongst their gingival and connective tissues has also been demonstrated.[33] Two recent studies reported differences in the distribution of single nucleotide polymorphisms located in the TLR9 promoter region in subjects with chronic periodontitis versus periodontally healthy individuals.[34, 35] These polymorphisms may explain the differences in host susceptibility to periodontitis, hence further supporting a possible role of intracellular DNA receptors in periodontal disease pathogenesis.

Similar to TLR-3, 7, 8 and 9, absent in melanoma (AIM)2, is a recently identified intracellular receptor for cytosolic bacterial, viral and host double stranded DNA (dsDNA) that undergoes a series of reactions resulting in the formation of an inflammasome that generates the

pro-inflammatory cytokine IL-1β.[36, 37] Nucleotide binding oligomerization domain-like receptor (NLR) inflammasomes are intracellular PRRs that are activated by cell stress, bacteria or viruses and consist of the NLRP3 'sensor', the caspase-1 'effector' and the apoptotic speck protein containing a C-terminal caspase recruitment domain (APC) 'adaptor' which links NLRP3 and caspase-1.[38-42] AIM2 is a non-NLR inflammasome which recognizes different PAMPs than NLRP3, including cytosolic double-stranded DNA, but like NLRP3, AIM2 inflammasomes oligomerize with APC and caspase-1 leading to the activation of intracellularly stored Il-1β.[36] In a study by Bostanci et al. 2011, AIM2 expression was up-regulated by low concentrations and down-regulated at higher concentrations of *P. gingivalis.*[43] These pathogenic mechanisms of dampening the immune response highlight the covertness of *P. gingivalis* and its ability to evade the host innate immune system allowing for its continual survival.

DNA-dependent activator of IFN-regulatory factors (DAI), a cytosolic receptor for microbial, viral or host derived DNA, activates the innate immune system via the NF-κB pathway.[44, 45] DAI induces IRF3 and NF- κB transcription factors to produce type I IFN (IFNα and IFNβ).[45] The mechanism of DAI-mediated NF- κB activation involves adaptor receptor-interacting protein kinase (RIP)1 and RIP3.[46] The type 1 IFN cytokines have immunomodulatory, antiviral and antitumoral properties and their production initiates a positive feedback loop that amplifies the immune response.[47-49]

Several studies have linked periodontal disease with systemic inflammation and conditions such as adverse pregnancy outcomes[50], cardiovascular (CVD)[51], respiratory[52], autoimmune diseases[53], diabetes[54] and cancer[55]. The involvement of nucleic acid activated receptors and signaling pathways in the pathogenesis of several systemic conditions, including

viral and bacterial infections, autoimmune diseases and cancer, have been documented and are under active investigation.[56] Periodontal bDNA presence in atheromatous plaques[57, 58] as well as in synovial fluid and serum samples of refractory rheumatoid arthritis patients[59] may be the means linking periodontal disease with these systemic diseases. Hence, the knowledge gained from this proposal may not only lead to alternative treatment strategies to control periodontal inflammation, but also help to reveal new biological pathways linking periodontitis with systemic conditions. Furthermore, this will be the first study that will determine DAI and AIM-2 expression in periodontally diseased tissues as compared to healthy tissues

Considering the constant presence of nucleic acids in the periodontal tissues and within dental biofilms, bacterial DNA-initiated immune responses represent alterative pathways to periodontal inflammation that need further investigation using well defined clinical samples via in vivo studies. Findings from this study and similar research may lead to novel diagnostic tests that can determine and evaluate periodontal disease activity, chance for disease progression and response to therapy. It is hypothesized that the expression of intracellular receptors, specifically in this study TLR-9, DAI and AIM-2, will be increased in periodontally diseased sites. Therefore, the aim of this study is to determine and compare the expression of intracellular PRRs for DNA, specifically TLR9, DAI and AIM2 in the gingival tissues of chronic periodontitis patients and periodontally-healthy subjects.

CHAPTER 2 Methods

Patients and Tissue Samples

33 chronic periodontitis patients (Ps) and 27 healthy controls (Cs) who consented to participate in the study were included. The included subjects (age range 20-79 y.o.) were systemically healthy with at least 20 natural teeth present. The periodontitis subjects had pocket depths (PD) >4mm, clinical attachment loss (CAL) >2mm, BOP and radiographic evidence of alveolar bone loss of at least two teeth (excluding 3rd molars) per quadrant. These patients were treated with initial periodontal therapy including scaling and root planing and oral hygiene instruction. At the reevaluation appointment 4-6 weeks after initial therapy, Ps exhibiting sites with PD >4mm and persistent bleeding on probing (BOP) in at least one quadrant that needed further surgery as part of their definitive treatment plan were recruited for the study. Cs were recruited for the study if they had no sites with PD $\geq$ 4mm, CAL > 2mm nor radiographic evidence of alveolar bone loss and had teeth that were to undergo crown lengthening or extraction.

Subjects were excluded from the study if they were smokers (or reformed smoker if quit < 5 years ago), had a history of alcoholism, hepatitis, AIDS or HIV, recent radiation therapy, diabetes, uncontrolled hypertension, use of immunosuppressive medications, antibiotic or NSAID use within the past 6 months, or were pregnant. Patients were also excluded if they had a history of previous periodontal surgery or localized drug delivery at the site to be sampled.

One gingival biopsy was collected from each predetermined site, bisected half longitudinally and placed into two labeled vials containing either RNAlater® (Qiagen) or 10% Formalin to be used in gene expression and immunohistochemistry assays, respectively.

The experimental protocol was approved by the Institutional Review Board of Virginia Commonwealth University and informed consent was obtained from all patients.

RNA Isolation and Real-Time Quantitative Polymerase Chain Reaction

Isolation of total RNA from the gingival tissues was accomplished using the Qiagen RNeasy plus mini kit with on-column Dnase digestion according to manufacturer's instructions. The RNA concentration was measured using the Nano Drop (ND-1000) Spectrometer. Subsequently complementary DNA (cDNA) was generated from the isolated RNA using the High capacity cDNA Reverse Transcriptase kit (Applied Biosystems) according to the manufacturer's instructions. Quantitative real time PCR then amplified the synthesized cDNA to determine the gene expression of TLR-9, AIM2 and DAI receptors using specific primers (IDT Inc.) (Table 1) labeled with SYBR green (Qiagen SYBR Green Rox™). The specific thermal cycling conditions were 25 °C for 10 min followed by 37°C for 120 min, 85°C for 5 seconds and 4°C overnight. For an internal control, glyceraldehyde 3-phosphate-dehydrogenase (GAPDH) mRNA was used.

Immunohistochemistry

The expression of TLR-9, AIM-2 and DAI in the gingival tissues was further confirmed using immunohistochemistry (N=5 periodontitis and N=5 healthy samples). The deparaffinized tissue sections from periodontitis and healthy sites were first treated with primary polyclonal

antibodies (rabbit anti-human TLR-9, rabbit anti-human AIM-2 and rabbit anti-human DAI, (Abcam) and then the tissues were further treated with a secondary polyclonal anti-rabbit IgG antibody (Biotin-Avadin system, Vectastain Elite ABC Kit, Vector Laboratories). Slides were then stained using DAB solution (Vector Labs) and Hematoxylin Counterstain (Sigma) before dehydrating and permanent mounting. Negative controls were run in parallel with immunostaining using a secondary antibody only. For immunohistochemistry, the staining intensity was compared between healthy versus diseased tissue samples using a microscope (Nikon Eclipse E400).

Statistical Analysis

The difference in clinical parameters and the demographic variables between the two groups was compared using an unpaired student t-test and a chi-square test (Table 2). Gene expression levels of different receptors between healthy and diseased tissues were analyzed using the ddCT (Relative Quantitation) Study Program (7500 Fast System SDS Software, Applied Biosystems) after adjusting the values relative to the housekeeping gene (GAPDH). The gene expression level is represented as a fold change, and a minimum of a 2-fold difference between diseased and healthy tissues was required for further statistical analysis using ANCOVA which adjusted for the confounders of race, age and gender. The Spearman test was used to determine the correlations between each receptor (Table 3). The p value was set at <0.05.

CHAPTER 3 Results

Table 2 summarizes the demographic characteristics of the study population, including age, gender and race as well as the clinical parameters at the sampled sites. There was a significant difference between mean age (47.6 y.o. ± 2.70 in H versus 58.8 y.o. ± 2.44 in P) ($p<0.05$), gender distribution (7 males and 20 females in H versus 18 males and 15 females in P) ($p<0.05$) and the clinical parameters among groups. The statistical analyses were adjusted for all the confounders.

Gene Expression of Pattern Recognition Receptors in Gingival Tissues

33 periodontal and 27 healthy patients were included in the final analysis. Figure 1 shows the mRNA expression of pattern recognition receptors (TLR-9, AIM2 and DAI) in gingival tissues of subjects with and without periodontitis. Specifically, the mRNA expression of TLR9, DAI and AIM2 was increased 7.8-fold, 2-fold and 5.6-fold in periodontally-diseased gingival tissues compared to healthy sites, respectively. The relative expression levels of the receptors in periodontitis tissues compared to healthy sites are shown in Table 3. A statistically significant up-regulation of TLR9 ($p<0.006$) and DAI ($p<0.001$) in periodontally-diseased tissues compared to healthy sites was shown after adjusting for confounding factors. Although AIM2 did not show a significant change between diseased versus healthy tissues, our analysis indicates significant correlations between AIM2 and DAI as well as TLR9 (Table 4). This may suggest that multiple receptors interact with each other to mount an appropriate immune response.

Immunohistochemical Analyses of TLR-9, AIM2 and DAI in Gingival Tissues

To provide further substantiation of our gene expression data, immunohistochemistry was performed to confirm protein expression of DAI, AIM2 and TLR9 in the periodontally-diseased and healthy tissues. This was done by staining tissue sections prepared from 5 healthy and 5 periodontitis patients using antibodies that recognize these receptors. Figure 2 shows representative images from these tissue samples. While AIM2 and DAI had low constitutive expression in the gingival epithelium, they were both involved in the underlying connective tissue and elevated in the periodontally-diseased tissue samples. TLR9 staining however, while strong in the healthy epithelial cells, was elevated in tissues from periodontitis patients and was also prominent in the connective tissue. Thus, the immunohistochemical data confirm expression of AIM2, DAI and TLR9 in periodontally diseased tissues.

Table 1 – Pattern Recognition Receptors and Primers

Symbol	Gene name	Sequence (5'-3')
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	F: 5'-CAA TGA CCC CTT CAT TGA CC-3'
		R: 5'-TTG ATT TTG GAG GGA TCT CG-3'
AIM2	Human absent in melanoma 2	F: 5'-CAG AAA TGA TGT CGC AAA GCA A-3'
		R: 5'-TCA GTA CCA TAA CTG GCA AAC AG-3'
DAI	Human DNA-dependent activator of IFN-regulatory factor	F: 5'-AAC ATG CAG CTA CAA TTC CAG A-3'
		R: 5'-AGT CTC GGT TCA CAT CTT TTG C-3'
TLR9	Human toll-like receptor 9	F: 5'-CTG CCA CAT GAC CAT CGA G-3'
		R: 5'-GGA CAG GGA TAT GAG GGA TTT G-3'

Table 2 – Study Population Demographics

	Healthy (H)	Patient (P)	p-value
Gender (n) (Male/Female)	7/20	18/15	$p < 0.05$
Age (mean ± SD)	47.6 ± 2.70	68.8 ± 2.44	$p < 0.05$
Race (n) (Caucasian/Black/Asian/Hispanic)	22/4/0/1	21/9/3/0	$p > 0.05$
Periodontal Probing Depth (mm) (mean ± SD)	1.652 ± 0.714	7.121 ± 1.386	$p < 0.001$
Clinical Attachment Loss (mm) (mean ± SD)	0.682 ± 0.56	7.303 ± 1.928	$p < 0.001$

Table 3 - Relative Expression Levels of Pattern Recognition Receptors in Periodontitis

Pattern Recognition Receptors	Gene Expression (Fold increase in periodontitis)	p-value
AIM2	2.0-fold	N.S.*
DAI	5.6-fold	$p < 0.001$
TLR9	7.8-fold	$p < 0.006$

*N.S. (Non-significant)

Table 4 – Correlation between Pattern Recognition Receptors

Pattern Recognition Receptors	Spearman p	P-value
TLR9 & DAI	0.8517	<.0001
DAI & AIM2	0.6837	<.0001
TLR9 & AIM2	0.6086	<.0001

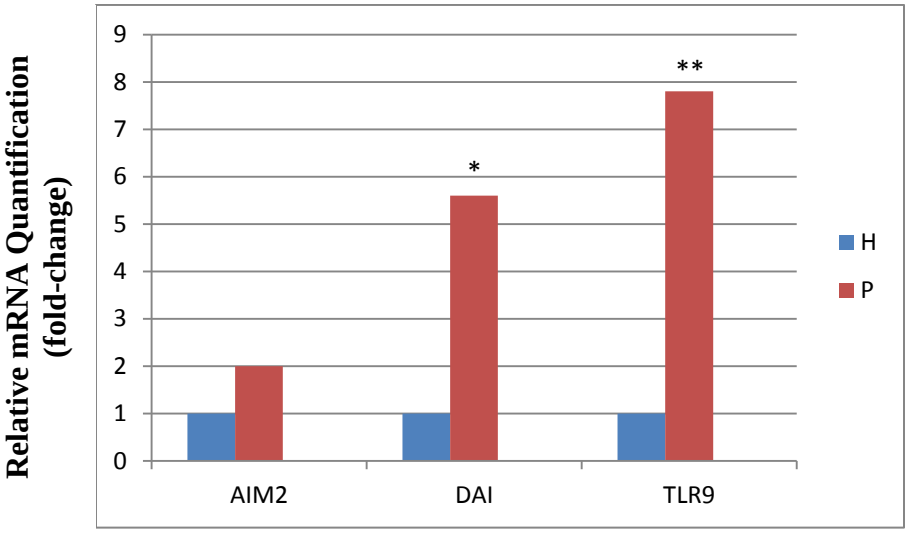

Figure 1 – **Pattern Recognition Receptor Expression -** Relative mRNA expression of pattern recognition receptors in healthy (H) and periodontally-diseased (P) tissues. The results are reported as fold-induction relative to H (=1) after normalization to GAPDH. Asterisks indicate statistically significant differences in receptor expression between H and P tissues. *p<0.001; **p<0.006

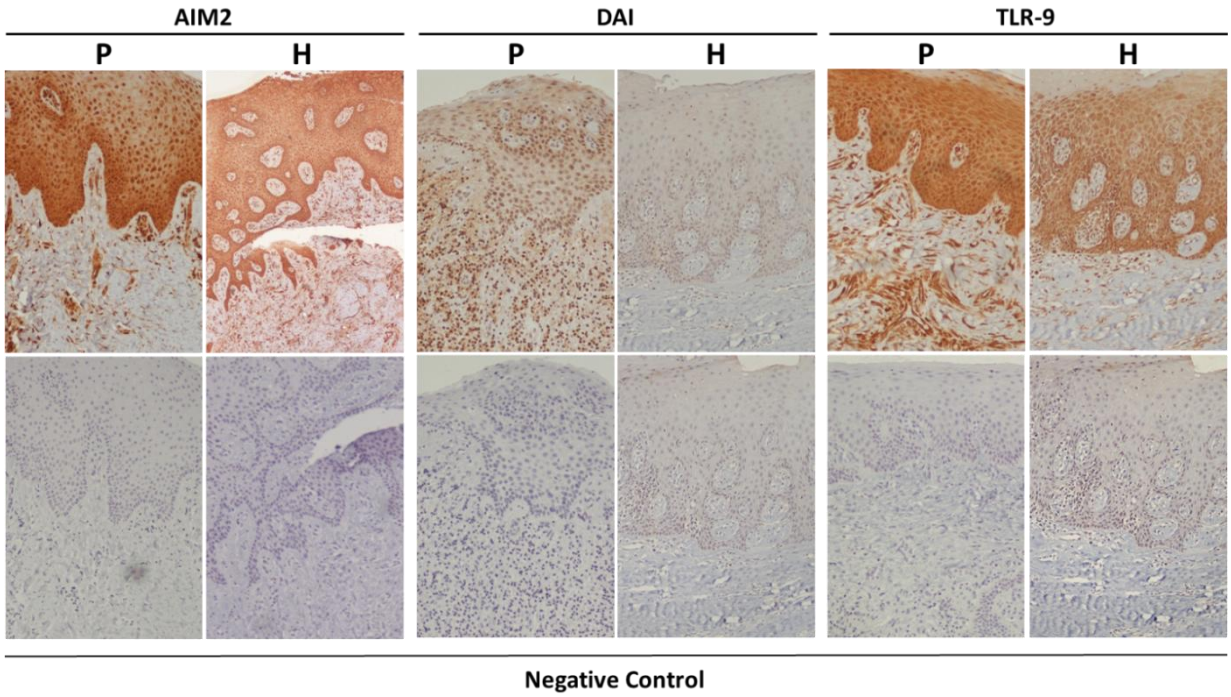

Figure 2 – Immunohistochemical detection of pattern recognition receptor expression. Tissue sections from healthy subjects (H) and periodontitis patients (P) were immunostained with the indicated antibodies (upper panels), as described in the Methods section. Lower panels: negative controls. Original magnification, x40.

CHAPTER 4 Discussion

This is the first study reporting increased DAI and AIM2 expression in periodontal tissues and also confirms and extends previous evidence that TLR-9 expression is enhanced in periodontally diseased tissues at both the mRNA and protein levels.[60] While many of the studies that investigate nucleic acid sensing in the context of periodontal inflammation are in vitro[28, 29, 31] in nature, there are only a few clinical reports that have addressed the role of TLR-9 in periodontitis.[32, 33] Similar to this study, Rojo-Botella et al. 2012 found the expression levels of TLR9 to be higher in all periodontal patients than in healthy individuals and that the expression of TLR9 is positively regulated with the severity of periodontal disease.[61] Recent studies have also shown that polymorphisms exist in TLR9 promoter regions in subjects with chronic periodontitis which may explain the differences in host susceptibility to periodontal disease.[34, 35] These studies together lend credence to TLR9 recognition of pathogen-derived nucleic acids having an essential role in triggering the immune mechanisms involved in periodontal disease.

In terms of immunohistochemistry, this study provides the first evidence regarding the expression of AIM2 and DAI proteins as well as extended evidence of the expression of TLR9 protein in periodontal tissues. This study is also in agreement with the study by Belken et al. in which the connective tissue had consistently higher TLR9 expression than the epithelial layer within the periodontitis group compared to the healthy group.[33] There was an overall increase in the staining intensity and distribution of TLR9 both in the epithelium and connective tissue in the periodontitis sites compared to healthy tissues. Since nucleic acid receptors have been found to

be expressed in gingival epithelial cells, gingival fibroblasts, monocytes/macrophages and dendritic cells, it would be important to design future clinical studies similar to this one that determine the specific cell types expressing each DNA receptor. This would allow for a better understanding of the pathogenesis of periodontitis and help to determine which cells to target therapeutically.

AIM2, while showing increased expression, was not statistically different between groups as was TLR9 and DAI. However, we showed in our study that AIM2 was significantly correlated to DAI and TLR9. This lends evidence to multiple receptors interacting with each other to mount an appropriate immune response as has been shown in a study by Brown et al.[62]

While bacterial etiology of periodontitis is well accepted, the contribution of viruses to periodontal disease pathology has also been supported.[63, 64] Since TLR9[65], DAI[65] and AIM2[36] all recognize both bacterial DNA as well as the DNA of viruses, it is possible that the increased expression of nucleic acid receptors in periodontitis patients may have been partly due to their ability to recognize viruses within the oral tissues as well. Future investigations are needed to differentiate the effect of viruses and bacteria on the upregulation of these receptors within gingival tissues of periodontally diseased versus healthy sites.

In conclusion, it was found that DAI and TLR9 were significantly upregulated in periodontally diseased tissues as compared to healthy tissue samples. In keeping with the multifactorial nature of periodontal disease pathogenesis these receptors' up-regulation may be a result of inflammation in response to pathogens that further augments the immune response or their higher expression may represent another biological pathway that directly initiates a response alone or in cooperation with other innate receptors leading to persistent inflammation. Further

studies are needed to fully understand the contribution of intracellular DNA sensors in periodontitis that may lead to alternative treatment strategies to control periodontal inflammation.

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Vita

Stephanie Chambers Voth was born on January 8, 1983 in Virginia Beach, Virginia and is an American citizen. She graduated from Cape Henry Collegiate School, Virginia Beach, Virginia in 2001. She received her Bachelor of Science degree from The College of William and Mary, Williamsburg, Virginia in 2005. She then received her Doctor of Dental Surgery Degree at Virginia Commonwealth University in 2009 followed by a 1 year Advanced Education in General Dentistry Residency also at Virginia Commonwealth University. After finishing her Master of Science Degree at Virginia Commonwealth University, along with a Certificate in Periodontics in June 2013, she plans to become a board certified Periodontist and practice in Richmond, Virginia.