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FREQUENCY OF TLR-2, 4, 9 AND CD14 POLYMORPHISMS IN AGGRESSIVE PERIODONTITIS POPULATION IN AFRICAN-AMERICANS

Melanie Chou
Virginia Commonwealth University

Virginia Commonwealth University
School of Dentistry
Department of Periodontics

This is to certify that the thesis prepared by Melanie Chou entitled Frequency of TLR-2, 4, 9 and CD 14 polymorphisms in Aggressive periodontitis population in African-Americans has been approved by his or her committee as satisfactory completion of the thesis requirement for the degree of MSD

Sinem Esra Sahingur, DDS MS PhD, VCU School of Dentistry Deparment of Periodontics

Thomas Waldrop, DDS MS, VCU School of Dentistry Deparment of Periodontics

John Gunsolley, DDS MS, VCU School of Dentistry Deparment of Periodontics

Harvey Schenkein, DDS PhD, VCU School of Dentistry Deparment of Periodontics

Harvey Schenkein, DDS PhD, Departmental Chair VCU School of Dentistry Deparment of Periodontics

Laurie Carter, DDS PhD, Director of Advanced Dental Education VCU School of Dentistry

Dr. F. Douglas Boudinot, Dean of the Graduate School

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FREQUENCY OF TLR-2, 4, 9 AND CD14 POLYMORPHISMS IN AGGRESSIVE PERIODONTITIS POPULATION IN AFRICAN-AMERICANS

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

by

MELANIE CHOU
DMD, University of Pennsylvania School of Dental Medicine, 2006

Director: ESRA SAHINGUR, DDS MS
ASSOCIATE PROFESSOR, DEPARTMENT OF PERIODONTICS

Virginia Commonwealth University
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Abstract

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By Melanie Chou, D.M.D.

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

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Major Director: Esra Sahingur, D.D.S., M.S.
Associate Professor, Department of Periodontics

Aim: The aim of this study is to determine the frequency of single nucleotide polymorphisms (SNPs) in various pattern recognition receptor (PRR) genes, including Toll like receptors (TLR) -2, -4, -9, and CD14 in chronic (CP), localized (LAP) and generalized aggressive (GAP) periodontitis and periodontally healthy (NP) patients in an African American population. Methods: A total of 205 subjects were involved in the study. The LAP group consists of 25 subjects, the GAP group 50 subjects, the CP group 73 subjects and the NP group 57subjects. Genotyping was performed in TLR2 (G2408A), TLR4 (A896G),TLR9 (T1486C) and CD14 (C260T) genes by TaqMan® allelic discrimination

using Assay-by-DesignSM SNP Genotyping Assays (Applied Biosystems). Accuracy of genotyping was confirmed by known DNA samples of each genotype and by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analyses on selected samples. Fisher's exact test and chi-square analyses were performed to compare genotype and allele frequencies. Within disease groups, we investigated whether SNPs were related to disease severity by step-wise logistic regression adjusted for age, gender, and smoking status. Results: There was a significant difference in the distribution of specific TLR9 (T1486C) genotypes between diseased-groups versus reference group. Expression of TT genotype was more prevelant in periodontally-diseased individuals compared to periodontally-healthy subjects ($p<0.0001$) whereas individuals expressing C allele of the TLR9 SNP (CC&CT) were more frequently found in healthy group after adjusting for age, gender, and smoking status ($p<0.0001$) There was no statistically significant difference in the distribution of genotypes between groups for any other TLRs or CD 14 polymorphism. Conclusion: Based on findings of this study, homozygocity for the T allele of TLR 9 polymorphism was related to the periodontal disease susceptibility in African Americans. Additionally, presence of C allele at TLR-9 appeared to confer resistance to periodontal destruction. Our results showed that specific SNPs in TLR-2, -4 and CD 14 genes are not related to periodontitis in African Americans. However, low copy number of certain alleles warrants further investigations with increased sample size to explore the role of SNPs in periodontal disease.

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CHAPTER 1 Introduction

The objective of this study is to determine the frequency of TLR-2, 4, and 9 and CD 14 polymorphisms in aggressive periodontitis and periodontally healthy patients in an African-American population

Aggressive periodontitis (AgP) is a rare but severe and progressive form of periodontal disease in otherwise healthy patients. It is characterized by a rapid destruction of periodontal tissue, and familial aggregation is considered.[1] There is considerable interest in studies aimed at understanding its etiology and pathogenesis although it is a relatively rare disorder (0.1-5%).[2] Literature has shown that AgP is caused by multiple potential risk factors. Previous studies implicated that the presence of pathogenic biofilm[3], disregulated cytokine and inflammatory mediator levels[4], quality and quantity of local inflammatory and immunological reactions to the bacterial challenge[5] and alterations in host defense cell functions[6] could attribute to the presentation of AgP. Several studies support the hypothesis that genetic variations in host response contribute to the multi-factorial background of AgP. [7,8,9] Numerous candidate genes of AgP that are involved in immunity and inflammation are under consideration. However, the immunopathogenic mechanism that surrounds this disorder is not thoroughly understood.

Recognition of invading pathogens by the innate immune system is an essential pre-requisite for triggering inflammatory response and developing acquired immunity. The immune system is traditionally divided into innate and adaptive components. The main distinction between the innate and adaptive immune systems lies in the mechanisms and receptors used for the immune recognition. The adaptive system is organized around two classes of specialized cells, T cells and B cells. Their receptors are generated somatically during development that endows each lymphocyte with a structurally unique receptor[10]. The innate immune recognition is mediated by germ-line-encoded receptors, and the specificity of each receptor is genetically predetermined. The innate immune cells detect invading pathogens primarily through an array of pattern-recognition receptor.[11]

Toll-like receptors (TLRs) are pattern-recognition receptors that mediate intracellular signaling and antimicrobial responses upon recognition of conserved pathogen associated molecular patterns (PAMP) of microorganisms, and play a major role in innate immunity.[12,13] TLRs form an important and potentially controllable checkpoint for a limited number of PAMPs derived from a large number of different bacterial species. Because the specificity of Toll-like receptors (and other innate immune receptors) cannot easily be changed in the course of evolution, these receptors recognize molecules that are constantly associated with threats (i.e. pathogen or cell stress) and are highly specific. PAMPs that meet this requirement are usually critical to the pathogen's function and cannot be eliminated or changed through mutation; they are said to be evolutionarily conserved. Some of the PAMPs associated with periodontal bacteria include bacterial

lipopolysaccharide (LPS), fimbriae, lipoproteins and bacterial DNA.[14] TLRs lack memory, which is characteristic to the innate immune system.

The Toll-like receptor cytoplasmic domains are homologous, but the extracellular domains differ and are characterized by the frequency of leucine-rich repeats.14 TLRs together with the Interleukin-1 receptors form a receptor superfamily, known as the "Interleukin-1 Receptor/Toll-Like Receptor Superfamily"; all members of this family have in common a so-called TIR (Toll-IL-1 receptor) domain. Three subgroups of TIR domains exist. Proteins with subgroup 1 TIR domains are receptors for interleukins that are produced by macrophages, monocytes and dendritic cells and all have extracellular Immunoglobulin (Ig) domains. Proteins with subgroup 2 TIR domains are classical TLRs, and bind directly or indirectly to molecules of microbial origin. A third subgroup of proteins containing TIR domains consists of adaptor proteins that are exclusively cytosolic and mediate signaling from proteins of subgroups 1 and 2.

TLRs are believed to function as dimers. Though most TLRs appear to function as homodimers, TLR2 forms heterodimers with TLR1 or TLR6, each dimer having a different ligand specificity. TLRs can be classified according to the types of ligands that they recognize. For example, TLR-1 recognizes triacyl lipopeptides. Lipid-based structures are recognized by TLR-2 and TLR-4. Viral and/or bacterial nucleic acids are recognized by TLR-3, TLR-7, TLR-8, and TLR-9; the flagellin and peptidoglycan ligands are recognized by TLR-5 and TLR-6, respectively.[15]

TLRs may also depend on other co-receptors for full ligand sensitivity, such as in the case of TLR4's recognition of LPS, which requires MD-2. CD14 and LPS Binding

Protein (LBP) are known to facilitate the presentation of LPS to MD-2. LPS acts as a potent stimulus to a variety of host cells via LPS-binding protein (LBP), and CD14 pathway, which subsequently results in the expression of proinflammatory cytokines and amplifies the related host immune response in periodontal diseases. Like TLRs, the CD14 receptor is considered as a key component of the innate immune system. Although TLRs are specific for bacterial species, the CD14 is a coreceptor of TLRs for gram-positive and gram-negative bacteria. Binding of bacterial cell wall component such as LPS to soluble CD14 causes activation of signalling via TLR4 in antigen-presenting cells, resulting in secretion of interleukin- 12 from these cells.

CD14, which is present in plasma as a soluble form or on the monocyte cell surface as a glycosylphosphatidylinositol (GPI)-anchored 50-KD protein, binds to LPS and facilitates its signaling. LPS-activated monocytes produce proinflammatory cytokines, such as tumor necrosis factor α, interleukin-1, interleukin-6, and growth factors[16]. Although LPS responses are detectable without CD14, CD14 is indispensable for the activation of the TRAM-TRIF pathway by TLR4/MD-2 (Fig. 1).[17]

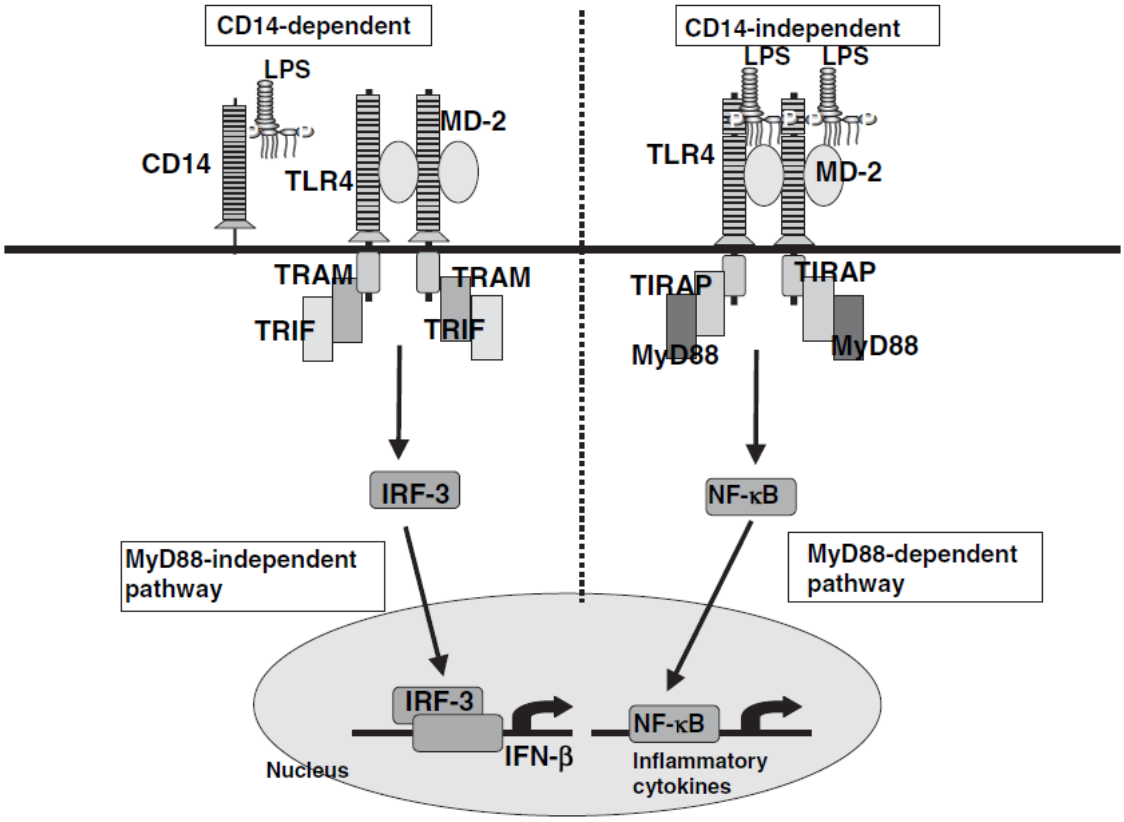

Fig 1. CD 14- dependent and independent pathways. CD 14 is required for the activation of the TRAM-TRIF pathway by TLR4/MD-218

In mammals, TLR activation results in the engagement of a set of adaptor molecules, which include myeloid differentiation factor-88 (MyD88), Toll- IL-1 receptor (TIR) Toll-interleukin 1 receptor domain-containing adaptor protein (TIRAP/MAL), Toll receptor-associated activator of interferon (TRIF), Toll receptor-associated molecule (TRAM), IL-1 receptor-associated kinase (IRAK), and TNF receptor-associated factor 6 (TRAF6) and factor3 (TRAF3). TLR signaling has two major pathways: MyD88-dependent and -independent/TRIF-dependent pathways. MyD88-dependent signaling is a nuclear factor (NF)-κB activation signal and is essential for proinflammatory cytokine

production through all TLRs but TLR3. On the other hand, the MyD88-independent/TRIFdependent signal induces delayed NF-κB activation and type-I interferon (IFN) production (Figs. 2,3).[18]

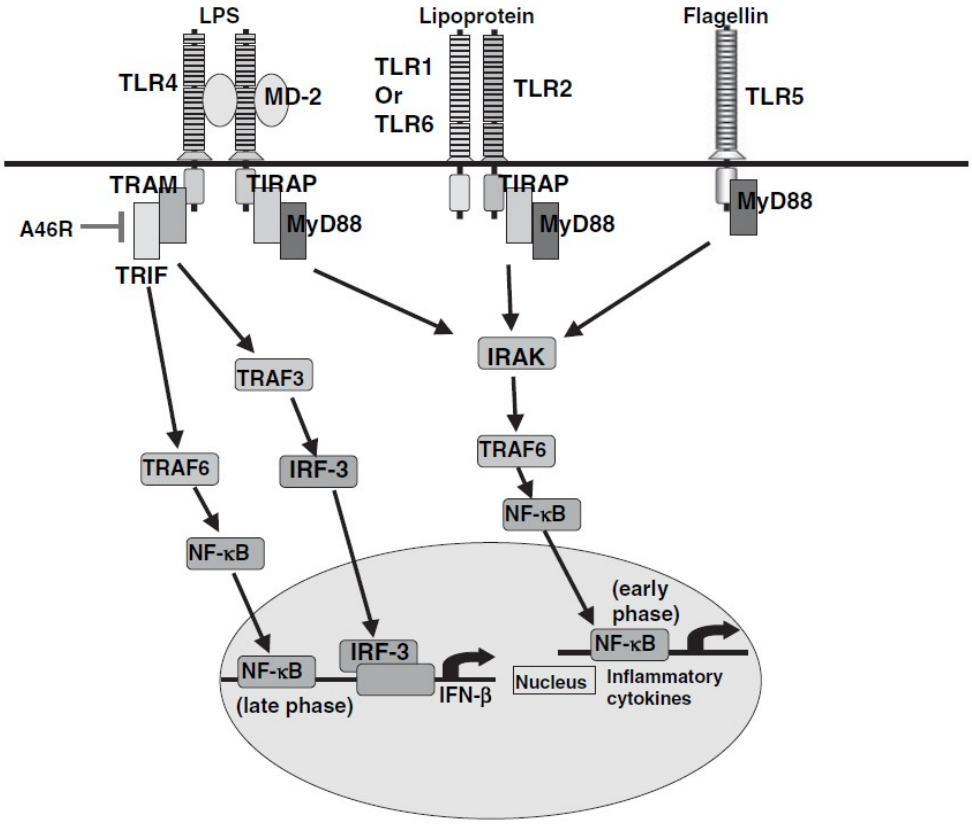

Fig. 2. Toll-like receptor 1 (TLR1), TLR2, TLR4, TLR5, and TLR6 signaling. TLR signaling has myeloid differentiation factor-88 (*MyD88*)- dependent and -independent/ (TRIF)-dependent pathways. The MyD88-dependent signal is a nuclear factor (*NF*)-κB activation signal and is essential for inflammatory cytokine production through all TLRs. On the other hand, the MyD88- independent/TRIF-dependent signal delays NF-κB activation and induces type-I interferon (IFN). *LPS*, lipopolysaccharide; *IRF3*; IF regulatory factor-3. A46R, which is a vaccinia virus protein, sequesters TRAM, TRIF, TIRAP, and MyD8818

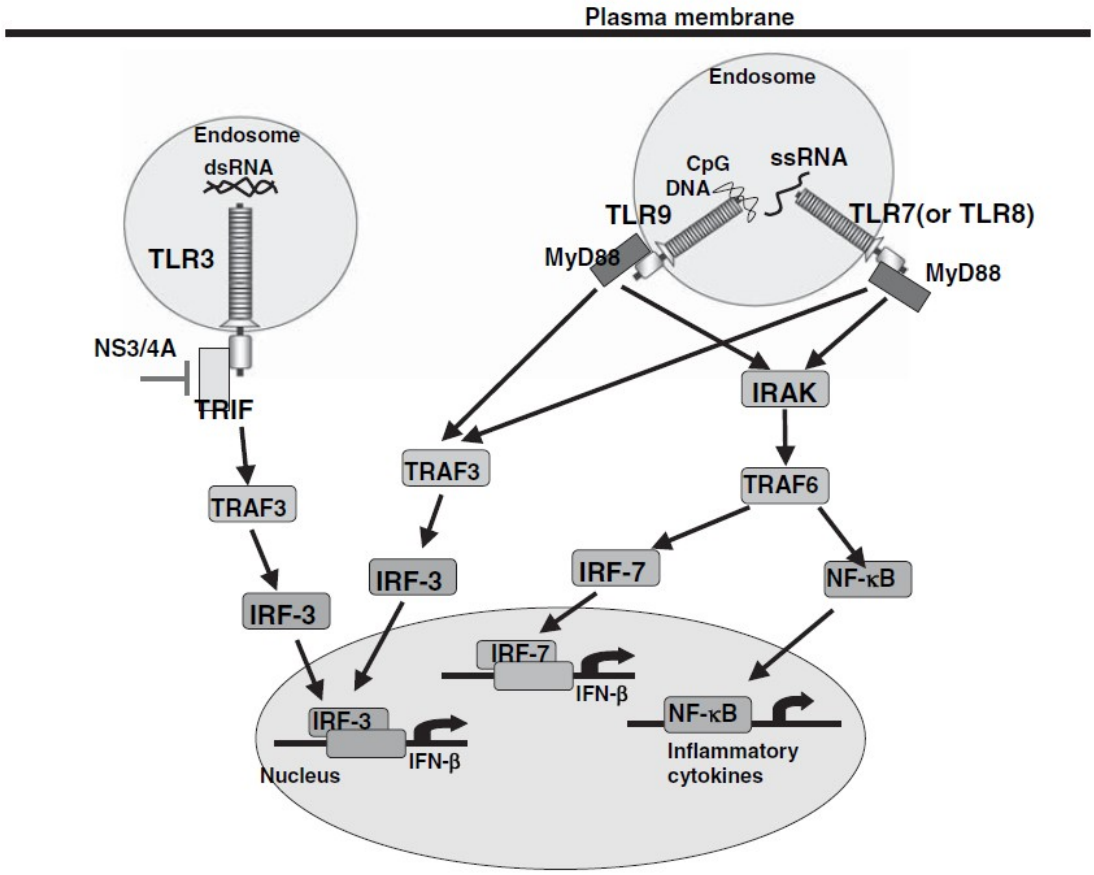

Fig. 3. TLR3, TLR7, and TLR8 or TLR9 signaling. TLR3 signals through TRIF. TLR7 and TLR9 signals induce type 1 IFN through IRF7. *dsRNA*, doublestranded RNA; *ssRNA*, singlestranded RNA. NS3/4A, which is a hepatitis C virus protein,degrades TRIF18

Through Recognition of pathogens and their products, TLRs activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) , which is a protein complex that acts as a transcription factor. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens. The activation results in synthesis and release of cytokines and chemokines, arachidonic acid molecules, and

generation of reactive oxygen metabolites that are associated with tissue destructive events in periodontal tissues.15 Cytokines that are produced in response to LPS-induced TLR activation play an important role in the regulation of host defence mechanisms against oral pathogens, as well as in host- mediated periodontal tissue destruction if inappropriately expressed. To date there are 10 Toll like receptors identified in humans that respond to distinctive PAMPs[19]. Table 1 lists the toll like receptors, their ligands, ligand location, adaptors, location and cell types.

Receptor	Ligands	Ligand Location	Adaptors	Location	Cell Type
TLR 1	Multiple triacyl lipopeptides	Bacteria	MyD88/MAL	Cell surface	• Monocytes/ macrophages • Dendritic cells • B lymphocytes
TLR 2	Multiple glycolipids Multiple lipopeptides Multiple lipoproteins Lipoteichoic acid	Bacteria Bacteria Bacteria Bacteria	MyD88/MAL	Cell surface	• Monocytes/ macrophages • Dendritic cells • Mast cells
TLR3	Double stranded RNA	Viruses	TRIF	Cell compartment	• Dendritic cells • B lymphocytes
TLR4	Lipopolysaccharide Heat shock proteins Fibrinogen Hyaluronic acid fragments Heparin sulfate fragments	Gram – bacteria Bacteria and host cells Host cells Host cells Host cells	MyD88/ MAL/ TRIF/ TRAM	Cell surface	• Monocytes/ macrophages • Dendritic cells • Mast cells • Intestinal epithelium
TLR5	Flagellin	Bacteria	MyD88	Cell surface	• Monocytes/ macrophages • Dendritic cells • Intestinal epithelium
TLR6	Multiple diacyl lipopeptides	Mycoplasma	MyD88/MAL	Cell surface	• Monocytes/ mcrophages • Mast cells • B lynphocytes
TLR7	Imidazoguinoline Loxoribine (guanosine analogue) Bropirimine Single stranded RNA	Small synthetic compounds	MyD88	Cell compartment	• Monocytes/macr ophages • Dendritic cells • B lymphocytes
TLR8	Small synthetic compounds; single stranded RNA		MyD88	Cell compartment	• Monocytes/ macrophages • Dendritic cells • Mast cells
TLR9	Unmethylated CpG DNA	Bacteria	MyD88	Cell compartment	• Monocytes/ macrophages • Dendritic cells • B lymphocytes
TLR 10	Unknown	Unknown	Unknown	Cell surface	• Monocytes/ macrophages • B lymphocytes

Table 1. Human Toll-Like Receptors 1-10. Lists of toll like receptors 1-10, their ligands, ligand location, adaptors, location and cell types. 15

For this study, we specifically looked at TLR 2, 4, 9 and CD 14. It should be noted that cell surface Toll like receptors (TLR 2,4) seem to recognize microbial products whereas intracellular Toll-like receptors (TLR 9) recognize nucleic acids.14

TLR 4 was the first Toll to be described in humans.[20] It recognizes lipopolysaccharides (LPS) derived from many pathogens including Aggregatibacter actinomycetemcomitans (A.a.) or Porphyromonas gingivalis (P.g.), the main pathogens in AgP. [21,22] Accordingly, it was shown that A.a. stimulates the TLR4-dependent production of several cytokines[23] and the optimal interferon-g (IFN-g) response induced by A.a.-stimulated dentridic cells was also TLR4 dependent.

Polymorphism in the coding region of the TLR4 (A896G) induces an amino acid substitutions of Asp 299 Gly which affect the extracellular domain of the TLR4 receptor. The TLR4(A896G) polymorphism results in reduction in cell surface expression of TLR4 and subsequent disruption of LPS mediated signaling and can modify response to endotoxin,[24] hence associated with increased susceptibility to gram-negative infections. The role of 299Gly in infectious diseases is explained by TLR4 hyporesponsiveness to its ligands, mainly LPS and respiratory syncytial virus (RSV), caused by these variants[25]. The replacement of the conserved Asp with Gly at position 299 has been reported to theoretically cause disruption of the α-helical protein structure, resulting in an extended β-strain, which is less functional[26]. Furthermore, epithelial airway cell lines transfected with 299Gly promoted a lack of surface TLR4 expression at the time of stimulation when compared with 299Asp transfected cells, and also, those variants were responsible for reduced nuclear factor κB (NF-κB) activity[27]. Activated TLR4 was demonstrated to induce

expression of proinflammatory cytokines (via NF-κB pathway), many of which have been implicated in Type II diabetes mellitus.[28] Therefore, evidence demonstrates that impairment of inflammatory signal transduction by 299Asp could increase the risk of acute bacterial infection but decrease risk of chronic diseases, type II diabetes.

Previous studies have shown that the Asp 299Gly variant not only impairs the inflammatory response and is associated with reduced plasma C-reactive protein levels but, in parallel, is linked with decreased angiographic coronary artery disease and diabetes prevalence.[29] Many studies have related the 299Gly variant polymorphism with an increased risk of septic shock[30], Gram-negative infections[31], severe malaria[32], respiratory syncytial virus (RSV) infection[33], expressed by tumor cells that promote tumor cell proliferation and survival, as well as invasion and metastasis, development of atopy and asthma[34] and premature birth associated with infection[35].

TLR2 is a membrane bound receptor that recognizes the widest range of PAMPs such as lipoproteins, glycopeptides, peptidoglycan, lipoteicoic acid, and lipoarabinomanan. It is also involved in *P.g.* fimbriae- and LPS-mediated signalling[36]. This large range is covered by the ability of TLR2 to build a heterodimer with TLR1 or TLR6. The receptor is expressed predominantly in hematopoietic system-derived cells that are involved in first-line host defense, including monocytes, macrophages, dendritic cells, polymorphonuclear leukocytes, B-cells, T-cells as well as regulatory T cells (CD4, CD25) and epithelial cells.

The SNP, TLR2 (G2408A), is located within the intracellular portion of the receptor resulting in an amino acid exchange, Arg753 to Gln. Previous work has suggested the association of TLR2 (G2408A) with other atopic disease and showed that all patients

with TLR2 (G2408A) allele have higher levels of total serum immunoglobulin E (IgE) and super-antigenspecific IgE[37].

Human alveolar epithelial cells type II and alveolar macrophages express both TLR2 mRNA and protein; this plays an essential role in TLR2 signaling during the inflammatory response of the alveolus to control the localized inflammatory reaction[38]. Thus, TLR2 is likely to play an important role in the etiology of inflammatory lung diseases. Studies have shown that Arg 753Gln impairs TLR 2 signaling and is associated with susceptibility to tuberculosis[39], Lyme's disease[40], but reduced susceptibility to asthma and allergy in farmer's children[41].

Toll-like receptor 9 recognizes unmethylated CpG (Cytosine phosphate guanine sites within DNA[42] molecules. They are regions of DNA where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases. It also recognizes DNAs of periodontal pathogens such as *A. actinomycetemcomitans*., *P. gingivalis* and *Peptostreptococcus micros*.[43,44] TLR9 is expressed by numerous cells of the immune system such as dendritic cells, B lymphocytes and natural killer (NK) cells. TLR9 is expressed intracellularly, within the endosomal compartments and functions to alert the immune system of viral and bacterial infections. In humans it is preferentially expressed by plasmacytoid dendritic cells (PDC) and B cells (reviewed in Ref. 21). TLR9 signals lead to activation of the cells initiating pro-inflammatory reactions that result in the production of cytokines such as type-I interferon, IL-1, IL-6, TNF-α and IL-12. The net effect of TLR9 activation is the differentiation of Th1 cells and the induction of IgG isotype switching and Ab secretion. Studies have shown that TLR9 stimulation promotes

the accumulation and activation of hepatic CD4 cells by Kupffer cell and that TLR9 to be involved in the pathogenesis of human hepatitis.[45]

SNP of TLR 9 (T1486C) is located in the promoter region of the gene, and previous studies showed that a potential binding site for transcription factor exists in that region which can affect transcription regulation and gene expression[46]. Indeed, the TT allelic variant sequence at C1486T showed a significantly higher promoter activity than the CC allelic variant sequence[47]. Single nucleotide polymorphism(SNP) in this region has been shown to be associated with atopic diseases such as eczema47 and systemic lupus[48]

The gene for CD14 has been localized to chromosome 5q31-33 and (C260T) polymorphism in this region has been shown to be associated with total and specific IgE level[49]. Upon binding with the soluble CD14 receptor, LPS also activates endothelial cells to express surface receptors for lymphocytes and macrophages, and stimulates smooth muscle cells to proliferate and migrate; the events representing atherogenesis[50]. Previous studies showed the TT genotype is correlated with a higher concentration of soluble CD14 in serum and a higher density of membrane-anchored CD14 on monocytes[51] and associated with atherosclerotic or macroangiopathic stroke. [52]

Gingival fibroblasts are the major constituents of periodontal connective tissue. They maintain gingival tissue integrity by regulating collagen and proteoglycan metabolism. Human gingival fibroblasts constituitively express mRNA of TLR 2, 4 and 9[53,54] and CD14. DNA microarray analysis demonstrated increased expression of Toll-like receptor 2, 4 and CD14 in human gingival fibroblasts in periodontitis patients compared to periodontally-healthy individuals.[55] Interestingly, upon in vitro stimulation by *P.gingivalis*

lipopolysaccharide, human gingival fibroblasts showed increased expression of Toll-like receptor 2, 4 and CD14.54 Another study showed that TLR-9 is expressed in significantly higher frequency in the epithelial cell layers in chronic periodontitis patients compared with healthy samples.[56] Based on these studies it can be suggested that TLR activation may have a critical role in amplifying the inflammatory reaction in periodontal tissues. The polymorphisms in toll-like receptor and CD14 genes are implicated in the host susceptibility to periodontal disease[57] and there are some suggestions that aggressive and chronic forms of the disease share the same genetic predisposition.69

The role of CD 14 in AgP as well as the function of TLR2, 4 and 9 in cytokine activation implies that genes coding for these proteins could be candidate genes for an increased periodontitis risk. We hypothesized a priori that the polymorphisms in these PRRs might modify the relative risk for development of AgP. Several studies on the association of single-nucleotide polymorphisms (SNPs) in the TLR2, 4, and CD 14 with periodontitis have been performed, with inconsistent results in different populations. [58,59,60,61,62,63] and no studies have investigated the association of TLR 9 SNP with periodontal disease to date. An individual's race plays a major role determining the genetic background of an individual, and discrepancies in studies regarding polymorphisms in PRRs and periodontal disease association can partly be explained by racial differences. Thus, the aim of this study was to evaluate the role of SNPs in TLR2 (G2408A), TLR4 (A896G), TLR9(T1486C) and CD 14 (C260T) in patients with AgP compared with healthy controls in African American population.

CHAPTER 2 Methods

Patient and control subjects were previously recruited to be participants in studies involving genetic analysis to elucidate the role of genetic background in aggressive periodontal disease. The protocol for genetic analysis was approved by an institutional review board and all participants provided written informed consent prior to their enrollment into the study.

60 African American aggressive periodontitis subjects are enrolled in this study. The LAP (25) group consists of subjects less than 30 years old, with attachment loss of at least 5mm affecting first molars and incisors. The GAP (50) group consists of the same criteria except the affected sites involve at least 3 permanent teeth other than first molars and incisors.

The periodontally healthy (NP) control group comprised a total of 70 unrelated, age matched, African American individuals without aggressive or any other form of periodontitis. The absence of periodontal disease was documented according to the following criteria: No sites with probing depth $\geq$ 3mm.

Individuals who had or have any systemic problems were excluded from the study. The smoking status was classified as current, former and non-smokers. Subjects who quit smoking for 5 years or more were assigned to non-smoker group and former smokers group included those who had quit smoking within 5 years.

Blood samples were collected from the antecubital vein of the forearm of each individual by a properly trained phlebotomist. The buffy coats were stored at -70ºC for future tests. Whole genomic DNA was isolated from 200µl of buffy coats using a DNA extraction Kit from QIAGEN following the manufacturer's instructions.

Genotyping were performed on the following genes: TLR 2, 4, 9, and CD 14. TLR2(G2408A) (NCBI SNP CLUSTER ID: rs5743708), TLR4(A896G) (NCBI SNP CLUSTER ID: rs4986790), TLR9 (T1486C) (NCBI SNP CLUSTER ID: rs187084) and CD14 (C260T) (NCBI SNP CLUSTER ID: rs2569190).

All screening methods were carried out with TaqMan SNP Assays (Applied Biosystems) using Real Time thermocycler AB 7500 (Applied Biosystems). Specific primers were determined using ABI PRISM Sequence Detection System (SDS) software following published protocols.[64,65,66,67,68] Positive controls with known genotypes and negative control without DNA template were included in each PCR run. To further validate the results obtained from real time analysis, PCR-RFLP analyses were performed on selected samples.

Thorough statistical power analysis was carried out in the program Power and precision version 2, based on the guidelines as follows: 1) 80% power, and alpha of 0.05. 2) Chi squared goodness of fit test. 3) Allele frequencies from published studies. The power analysis implies that noteworthy differences could be detected between the study samples in this pilot study and sample size is large enough for future power analysis. The chi-square test was performed to determine the significance of the difference in genotype frequency of TLR 2, 4, 9 and CD14 between healthy controls and patients with

periodontitis. Fisher's exact test was performed to compare allele carriage and frequency. Within diseased group, we investigated whether SNPs were related to disease severity by step-wise regression adjusted for age, gender, and smoking status. The difference between data sets with a probability of <0.05 was regarded as statistically significant.

CHAPTER 3 Results

The characteristics of the study population based on smoking status is shown in Table 2. There was a significant difference between diseased- versus healthy groups regarding smoking and age and therefore all statistical analyses were adjusted for these parameters.

Specific genotypes for TLR2 (G2408A), TLR4 (A896G), TLR9(T1486C) and CD 14 (C260T) are presented in Table 3. In the localized aggressive periodontitis group TLR 9(T1468C) is present in 16% of patients with the CC genotype, 32% with CT genotype and 52% with TT genotype. Generalized aggressive periodontitis group has TLR 9(T1468C) presence in 10% of the patients with the CC genotype, 42% with the CT genotype and 48% with the TT genotype. The control group has 7.02%, 75.44% and 17.54% of patients in the CC, CT and TT genotype groups respectively. The TT genotype of TLR9(T1486C) polymorphism was present significantly more frequently in aggressive periodontitis patients compared to control group. ($p<0.0001$) Distribution of genotypes was not statistically different among groups for TLR 2, 4, and CD 14 polymorphisms. (Table 3).

Further analyses demonstrated that individuals expressing C allele of the TLR9(T1468C) SNP (CC&CT) were more frequently found in healthy group (82.46%) compared to all the diseased groups (48.00% LAP, 52.00% GAP) after adjusting for age, gender, and smoking status ($p<0.0001$)(Table 4). Genotyping was performed on a real-time PCR and the results were further confirmed by RFLP analyses on selected samples and Fig 4 shows a representative agarose gel showing different genotypes in CD14 receptor. (Fig 4)

Smoking Status	LAP n (%)	NP n (%)	GAP n (%)
Former smoker	0 0.00	8 14.55	8 16.00
Never smoker	22 88.00	40 72.73	18 36.00
Undetermined	0 0.00	1 1.82	0 0.00
Current smoker	3 12.00	6 10.91	24 48.00

Table 2. Smoking data among different diseased groups. The distribution of smokers was significantly different between groups ($p<0.0001$)

Polymorphisms	Genotype	NP n (%)	GAP n (%)	LAP n (%)
TLR2(G2408A)	GG	51 (91.07)	49 (98)	25 (100)
	GA	3 (5.36)	1 (2)	0 (0)
	AA	2 (3.57)	0 (0)	0 (0)
TLR4(A896G)	AA	44 (83.02)	18 (75.00)	41 (89.13)
	AG	7 (13.21)	6 (25)	3 (6.52)
	GG	2 (3.77)	0 (0)	2 (4.35)
TLR9 (T1486C)	CC	4 (7.02)	5 (10)	4 (16)
	CT	43 (75.44)*	21 (42)	8 (32)
	TT	10 (17.54)*	24 (48)	13 (52)
CD14(C260T)	TT	4 (8.00)	5 (13.89)	3 (15)
	CT	36 (72.00)	22 (61.11)	15 (75)
	CC	10 (20.00)	9 (25)	2 (10)

Table 3. Distribution of TLR2, 4, 9, and CD14 Genotypes. There was no significant difference in genotype distribution between groups for TLR 2, 4 and CD 14. Frequency of genotypes were significantly different for TLR9 polymorphism between diseased versus control group (p<0.001).

Genotypes	NP n(%)	GAP n(%)	LAP n(%)
CC & CT	47 (82.46)*	26 (52.00)	12 (48.00)
TT	10 (17.54)*	24 (48.00)	13 (52.00)

Table 4. TLR9 (T1486C) polymorphism CC&CT versus TT. The frequency of individuals expressing the C allele of TLR9 gene polymorphism (CC&CT) was significantly higher in the control group (82.46%) compared to all the diseased groups (45.07% CP, 48.00% LAP, 52.00% GAP) (p<0.0001). *adjusted for age, gender, and smoking status

CHAPTER 4 Discussion

Periodontitis is a multifactorial polymicrobial infection initiated by the presence of Gram-negative bacteria, which accumulates in the gingival crevice region.[69] The presence of periodontopathogenic bacteria leads to the establishment of an inflammatory process in periodontal tissues, which leads to destruction of periodontal ligament and the adjacent supporting bone, causing tooth loss.[70] Periodontal destruction is considered as a result of the response of a susceptible host to bacterial challenge. The host's response to infection depends on the virulence of the pathogen, and is influenced by host response and genetic factors.[71] For this reason, genes involved in the individual immune response are considered as susceptibility genes for periodontitis. Recent studies on the pathogenesis of periodontitis have focused their attention on the topic of molecular events that lead to the inflammatory response through the TLR family. TLR polymorphisms can modify host susceptibility by altering the inflammatory and immune response to microbial infection. The objective of this study was to determine the frequency of TLR-2, 4, and 9 and CD 14 polymorphisms in aggressive periodontitis and periodontally healthy patients.

The genetic impact on the etiology of both aggressive and chronic periodontitis was demonstrated in previous studies. [72] Although, it could be shown that a similar amount of immune cells are available in patients with aggressive and chronic periodontitis compared with healthy controls, the functional activity of these cells is altered.[73] As a potential cause

of the observed alteration in immunological activity, genetic differences are discussed. Therefore, inflammatory genes are regarded as susceptibility genes for chronic and aggressive periodontitis. In this study, polymorphisms of TLR 2,4, 9 and CD14 are evaluated for susceptibility to aggressive periodontal disease.

From this current study using African American population, in the localized aggressive periodontitis group TLR 9(T1468C) was present in 16% of patients with the CC genotype, 32% with CT genotype and 52% with TT genotype. Generalized aggressive periodontitis group has TLR 9(T1468C) presence in 10% of the patients with the CC genotype, 42% with the CT genotype and 48% with the TT genotype. The control group has 7.02%, 75.44% and 17.54% of patients in the CC, CT and TT genotype groups respectively. The TT genotype of TLR9(T1486C) polymorphism was present significantly more frequently in aggressive periodontitis patients compared to control group ($p<0.0001$). Further analyses demonstrated that individuals expressing C allele of the TLR9(T1468C) SNP (CC&CT) were more frequently found in healthy group (82.46%) compared to all the diseased groups (48.00% LAP, 52.00% GAP) after adjusting for age, gender, and smoking status ($p<0.0001$) No statistically significant differences were noted aggressive periodontitis subjects and the control group when comparing genotype frequency for TLR2 (G2408A), TLR4 (A896G), and CD 14 (C260T).

Previous studies by Schulz 2008[74] evaluated links between genetic polymorphisms of CD14 and TLR4 and risk markers of periodontitis, they utilized one hundred and thirty-three periodontitis patients (chronic: n = 60, aggressive: n = 73) and 80 healthy controls. Polymorphisms in CD14 c.-159C>T and in TLR4 Asp299Gly, Thr399Ile were determined

by restriction fragment length polymorphism analyses. Subgingival bacterial colonization was analysed molecularbiologically using the micro-Ident test. The study found that Prevotella intermedia occurred less frequently in individuals positive for the TT genotype of CD14 in bivariate analysis (odds ratio = 0.36%, confidence interval: 0.14-0.91, P = 0.045). They also found no significant association with chronic and or aggressive periodontitis and polymorphisms in CD14 and TLR4 in German periodontitis patients, which is consistent with our results.

In another study by Noack 2008[75] looking at German population supports the findings of Schultz 2008. The study looked at one hundred and eleven patients with aggressive periodontitis and 80 periodontally healthy controls for their genotypes for four functional variants in the TLR4 gene (c.896A>G and c.1196C>T). The results found no statistical differences in genotype and allele frequencies within the four variants between the groups. All study subjects were further classified into carriers and non-carriers of at least one variant of both genes. The logistic regression analysis adjusted for gender and smoking showed no association between carrier status of at least one variant of both genes and periodontal status (OR=1.41, 95% CI: 0.43-4.70).

Zhu 2008[76] conducted a study to investigate the relationship between TLRs 2 and 4 gene polymorphisms and periodontitis in a Chinese population. Subjects include forty patients with generalized aggressive periodontitis, 50 patients with chronic periodontitis, and 100 periodontally healthy controls. TLRs 2 and 4 genes were analyzed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) with respective restriction endonucleases. The alleles were detected by polyacrylamide gel electrophoresis

and visualized with ethidium bromide. The result of the study finds heterozygosity for the TLR2 Arg677Trp polymorphism in all subjects. The TLR2 Arg753Gln mutant allele was not found in periodontitis patients, while a heterozygous frequency of 6% (6 of 100) was detected in the controls. The TLR4 Asp299Gly and Thr399Ile mutant alleles were not found in any of the subjects. From the date of this study, we can gather that the prevalence of TLR2 Arp677Trp polymorphism seemed to be higher than that of TLR4 Asp299Gly and Thr399Ile polymorphisms in the Chinese population, but neither TLR 2 or 4 polymorphism may not be associated with aggressive or chronic periodontitis.

Emingil 2007[77] again looked at TLR2 and TLR4 gene polymorphisms as they are related to susceptibility to generalized aggressive periodontitis (GAgP) in a Turkish population. 245 subjects were included in the present study with GAgP and 155 periodontally healthy subjects. The TLR2 gene Arg753Gln and Arg677Trp polymorphisms and TLR4 gene Asp299Gly and Thr399Ile polymorphisms were determined by the polymerase chain reaction-restriction fragment length polymorphism method. They found no significant difference in the distribution of TLR2 and TLR4 genotypes and allele frequencies between GAgP patients and healthy subjects ($P > 0.05$).

Some studies, however were able to show that TLR polymorphisms have an impact to the presence of aggressive periodontitis. James 2007[78] conducted a study to determine whether there is an association between the frequency of functional polymorphisms in the toll-like receptor 4 (TLR4) and cluster differentiation 14 (CD14) genes and periodontitis. Genotyping for the TLR4 single-nucleotide polymorphisms (SNPs) Asp299Gly, Thr399Ile and the CD14 SNPs -159 and -1359 was completed for 73 subjects with aggressive

periodontitis (AgP; 28 males, 45 females) and 95 males with chronic periodontitis (CP). The TLR4 and CD14 polymorphisms were determined using SNaPshot primer extension with capillary electrophoresis. The TLR4 Asp299Gly AG genotype was present in a significantly (p=0.026) lower proportion of AgP subjects (5.5%) compared with control subjects (16.3%). And AA genotype was significantly (p=0.026) higher in AgP subjects (94.5%) versus the control subjects (83.7%). The No differences were found in the prevalence of the TLR4 Asp299Gly genotype between the CP (18.9%) and control group (17%). In addition, there was no difference in the distribution of the CD14 polymorphisms in either the AgP or CP populations studied compared with controls. This study finds that the Asp299Gly TLR4 gene polymorphism is associated with a decreased risk of AgP but not CP in West European Caucasians. Promoter polymorphisms of the CD14 gene, however, did not influence susceptibility to inflammatory periodontitis in the population.

Another study by Schroder 2005[79] recruited 197 individuals suffering from generalized periodontitis, they were then screened for the presence of Asp299Gly and Thr399Ile of TLR-4 as well as Arg753Gln of TLR-2 in comparison to matched controls. Single-nucleotide polymorphisms (SNPs) of TLR-4 were elevated among patients with generalized chronic periodontitis (odd's ratio 3.650, 95% CI 1.573-8.467, P < or = 0.0001), while no difference was observed for TLR-2. TLR-4 SNPs were correlated with chronic periodontitis (odd's ratio 5.562, 95% CI 2.199-14.04, P < or = 0.0001), but not with aggressive periodontitis. These data demonstrate that genetic variants of TLR-4 may act as risk factors for the development of generalized chronic periodontitis in German population.

As the effect of various TLR polymorphisms on patient susceptibility and disease progression becomes better understood, genotypic profile will comprise an increasingly important factor in the consideration of various therapeutic options. Future studies will continue to improve our understanding of the relationships among TLR signaling, genetic polymorphism and the pathogenesis of aggressive periodontitis, revealing new approaches to modulate this system in ways that favorably affect disease mechanisms and clinical settings.

CHAPTER 5 Conclusion

Increasing number of reports indicate an association between toll-like receptors and disease processes, including periodontal disease. Our results indicated that genetic variations at the TLR-9 promoter region (T1486C) containing the C allele (CC & CT) appear to confer resistance to periodontal destruction, while individuals homozygous for the T allele were more susceptible to periodontal destruction when compared with a periodontally healthy control group in an African American-population. Additionally, the present results indicate that general susceptibility to periodontitis is not associated with the genetic background of the CD14, TLR 2, and TLR 4 polymorphisms. SNP of TLR 9 (T1486C) is located in the promoter region of the gene, and previous studies showed that a potential binding site for transcription factor exists in that region which can affect gene expression. Many reports have demonstrated increased susceptibility to periodontitis with TLR4 and CD14 polymorphisms in different patient ethnicities. Conversely, there are also reports that found no association between polymorphisms in toll-like receptors and periodontal disease. An individual's race plays a major role determining the genetic background of an individual, and discrepancies in studies regarding polymorphisms in PRRs and periodontal disease association can partly be explained by racial differences. This is the first study that investigated a potential role of specific polymorphisms in periodontal disease susceptibility in an African-American population. Additionally, this is the first study that investigated the role of TLR9 polymorphism in periodontal disease and

showed a possible association of specific alleles (TT) of TRL9 SNP with disease susceptibility. For verification of the presented results, prospective studies investigating larger cohorts should be conducted.

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VITA

Melanie Chou was born on September 13th 1980 in Taipei Taiwan. She moved to Singapore in 1989 with her family and remains a citizen of Singapore until today. She moved to the United States in 1996 and received her BS degree from the University of South Carolina with a major in Biology. She then attended the University of Pennsylvania School of Dental Medicine where she received her D.M.D.. She has a planned graduation date of July 2009 from the Virginia Commonwealth University with a M.S.D. and certificate in Periodontics. She has also received numerous honors and awards during her academic pursuits and is a member of numerous professional organization including the American Academy of Periodontology.