EXPRESSION OF INTERLEUKIN-37 (*IL37*) AND THE GENETIC VARIATIONS OF *IL37* IN RELATION TO CHRONIC PERIODONTITIS

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ABSTRACT

Shaoping Zhang: Expression of Interleukin-37 (*IL37*) and the Genetic Variations of *IL37* in Relation to Chronic Periodontitis
(Under the direction of Steven Offenbacher)

Objectives: To study the transcription of *IL37* in gingival biopsies and the association between transcription of *IL1B*, *IL6* and *IL37* and genotypes at the rs3811046 and rs3811047 *IL37* loci. **Materials and Methods**: Gingival biopsies were collected from chronic periodontitis patients and control subjects. Pyrosequencing was applied for genotyping. Real-time PCR was used to examine the transcription of genes from biopsies and cell culture models. **Results**: Transcription of *IL6* was significantly higher in periodontitis samples. Transcription of *IL37* was induced in oral epithelial and THP.1 cells by LPS. The presence of a minor allele at both loci in *IL37* was significantly related to a reduced *IL6* transcription in Caucasians, while the major allele was associated with a higher transcription of *IL6* in African Americans. **Conclusion**: Gingival epithelium and monocytic cells are the major source for IL-37. The genetic variants of *IL37* related to the altered transcription of *IL6* are race specific.

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BOP	Bleeding upon probing
CAL	Clinical attachmen tloss
CG	Cytosine-guanine dinucleotide
DSS	Dextran sodium sulfate
FcγR	Fc receptors for immunoglobulin G (IgG)
GCF	Gingvial crevicular fluid
GWAS	Genom wide association study
HGF	Human gingival fibroblast
Ig	Immunoglobulin
IFNG/IFN-γ	Interferon-γ
IL1	Interleukin-1
IL1A	Interleukin-1α
IL1B/IL-1β	Interleukin-1 β
IL1F7	Interleukin-1 family member 7
IL33	Interleukin-33
IL37/IL-37	Interleukin-37
IL6/IL-6	Interleukin-6
IL8	Interleukin-8
IL10	Inlerleukin-10
LPS	Lipopolysaccharide
NSAID	Non steroidal anti-inflammatory drug
MMP	Matrix metalloproteinase protein
PAI-I	Plasminogen activator inhibitor-1
PBMC	peripheral blood mononuclear cells

PI	Plaque index
PTGS2/Cox-2	Prostaglandin-endoperoxide synthase-2
RT-PCR	Real-time polymerase chain reaction
SNP	Single nucleotide polymorphisms
TGF-β	Transformation growth factor- β
TIMP	Tissue inhibitors of metalloproteinase
TLR	Toll-like receptor
TNFA/TNF-α	Tumor necrosis factor-α
Treg	Regulatory T cell

CHAPTER 1: REVIEW OF INTERLEUKIN-37 (*IL37*), A NOVEL ANTI-INFLAMMATORY IL-1FAMILY MEMBER

Introduction

Periodontal disease has been described as an abnormal inflammatory response to the pathogenic bacteria present in the bioflim. The uncontrolled inflammatory and immune responses initiated by the bacteria are the major etiology of periodontal destruction[1, 2] Activation of the innate immune system in periodontal disease leads to the rapid secretion of various chemokines and cytokines, such as interleukin-1β, (IL-1β), IL-6, IL-8, TNF-α, etc., most of which propagate inflammation and may potentially cause destruction in periodontium. For example, an elevated level of gingival crevicular fluid (GCF) IL-1β has previously been established as a robust biomarker for a hyper-inflammatory phenotype and for mediating severe inflammation, bone loss and periodontal disease progression[3]. Although feedback mechanisms might be present to dampen inflammation, only several secreted molecules have been described to possess anti-inflammatory activities, such as resolvins, IL-10 and transforming growth factor β (TGF-β) among more than 50 known cytokines[4]. Pathways to downregulate the extent and severity of inflammation in hosts may provide insight for the therapeutic potential to reduce the destruction caused by periodontal disease.

The course of inflammation is not only determined by the proinflammatory molecules but critically modified by resolution mechanisms that counteract the inflammation induced by pathogens. The disequilibrium of the pro- and anti- inflammatory pathways in periodontium results in the disease progression as evidenced by destruction of tooth

supporting apparatus. During the prolonged battle with pathogenic microorganisms that are present in the biofilm, the host may have developed dampening mechanisms that inhibit the uncontrolled inflammation. It has been suggested that periodontal inflammation resulting in the destruction of tooth-supporting tissue is partially due to the lack of adequate resolution activities [5].

Anti-inflammatory mechanisms involved in periodontal disease

A class of small lipid molecules, which are termed resolvins, has been elucidated to possess the capacity to promote the resolution of inflammation[5, 6]. Resolvins are derived from eicosapentaenoic acid and the production of resolvins shares the similar pathway of the metabolism of arachidonic acid. The activities of resolvins include reduction of neutrophile trafficking, control of cytokine production and reactive oxygen species, and mitigation of the magnitude of the inflammatory responses. Van Dyke et al. proved that the application of resolving E1 in a rabbit model at the same time when the experimental periodontitis was induced inhibited the onset and progression of periodontal destruction[5]. Interestingly, resolving E1 also demonstrated therapeutic efficacy because the application of this molecule in the established periodontitis in the rabbit model completely eliminated inflammation and the pocket depth was returned to pre-ligature condition[5].

Another molecule that possesses the inflammation resolving activity is transforming growth factor-β (TGF-β). TGF-β antagonizes IL-1 activity, and it also promotes both the synthesis of extracellular matrix proteins and proteinase inhibitors, such as tissue inhibitors of metalloproteinases (TIMPs) and plasminogen activator inhibitor-1 (PAI-I), which inhibit the synthesis of matrix metalloproteinase proteins (MMPs) [7]. Matsuda et al. reported that addition of TGF-β to the cell medium directly stimulated collagen synthesis in rat periodontal

ligament fibroblastic cells [8]. In addition, the stimulation with TGF-β progressively decreased the expression of procollagenase and increased both the mRNA and protein levels of TIMP [9]. TGF-β has also been identified to play a pivotal role in the mediation of the immunosuppressive activity of regulatory T (Treg) cells [10,11]. In gingival crevicular fluid, the level of TGF-β was elevated in both chronic and aggressive forms of periodontitis and such an increase was interpreted as a mechanism to act against detrimental immune and inflammatory responses and, therefore, may prevent further loss of periodontium [10].

Epigenetic modifications have recently been suggested as one of the most important regulatory mechanism for cytokine production in inflammatory disease including periodontitis [12, 13]. DNA methylation and histone modifications, which are two major types of epigenetic regulation, modulate host response at the chromatin level to environmental stressors, including smoking, nutrient deficiency, bacterial infection, inflammation, etc [13]. It is well known that stimuli as mentioned above can regulate cytokine gene expression through various signaling pathways. However, the enrichment of transcriptional factors in the nuclei induced by signal transduction may not account for the only driving force for gene expression. The state of chromatin confirmation, to which mammalian genes are packed, determines the transcriptional readiness of genes when the orchestration of signal transduction is not impaired and transcriptional factors are available[14, 15]. Therefore epigenetic regulation also plays a fundamental role in the control of gene expression. DNA methylation, which almost always occurs to the cytokine nucleotide in the cytokine-guanine (CG) dinucleotides, is a more stable epigenetic mark and less likely to be reversed as compared to post-translational histone modifications [16].

We have proposed that the persistence challenge from the pathogenic microflora in the biofilm may modify the methylation level of the promoter regions of certain host inflammatory genes that are involved in the pathogenesis of chronic periodontitis. Employing clinical gingival biopsies, we have discovered a hypermethylation pattern within the promoter region of prostaglandin-endoperoxide synthase-2 (*PTGS2* or *COX2*) and tumor necrosis factor α (*TNFA*) [17, 18]. Using the same biological samples, we also found that the transcriptional level of both *PTGS2* and *TNFA* genes was dampened and failed to elevate in comparison to the control samples that exhibited periodontal health [17]. Such a hypermethylation pattern in the promoter region and an inhibited transcriptional profile also confirm the generally accepted paradigm that the degree of promoter methylation is inversely related to the transcriptional levels [19]. Since TNF-α and PGE2, in which *PTGS2* is a key regulatory gate for its production, are among the most critical pro-inflammatory cytokines in periodontal disease, the dysfunction of the regulatory mechanism for those molecules leads to disease progression. The increased methylation level change in the promoter regions of those genes may reflect a metastable mechanism that can create a new "set-point" of the inflammatory homeostasis in the disease state [18]. Chronic periodontitis is a slow-progressing inflammatory/infectious disease featured by episodic periodontium destruction with long intervals of quiescence. In the chronic battle with the periodontal pathogens the host may develop a dampening compensatory mechanism through DNA methylation that helps to control the pronounced inflammatory response and therefore serves to prevent the unrestricted further tissue destruction.

Genetic variation of genes involved in inflammatory response and periodontal disease

Periodontal disease, similar to most other complex inflammatory diseases, is a polygenic disorder involving gene to gene interaction. The susceptibility to periodontal disease, which is an inflammatory disease exhibiting a complex genetic trait, has been explored through association studies aiming to target genetic markers and candidate disease-modifying genes by identification of single nucleotide polymorphisms (SNPs). Many previous studies have discovered important SNPs encoding molecules of the host defense system in chronic periodontitis [20, 21]. Kornman et al first described a composite *IL1* polymorphism including -889bp (C to T transition) in *IL1A* and +3953bp (C to T transition) in *IL1B* that was significantly related to the severity of periodontitis in non-smoking Caucasians [22]. Additionally, monocytes from individuals with homozygous *IL1B* +3953T allele were shown to produce 4-fold more IL-1β, and monocytes with heterozygous IL1B +3935T allele exhibited two-fold more IL-1β than cells without this SNP at the +3953 locus [22, 23]. Several later studies relating this composite *IL1* polymorphism to either susceptibility to or severity of periodontal disease showed diverse outcomes. Papapanou et al in a cross-sectional study found that although this *IL1* genotype failed to distinguish control subjects from patients with periodontitis, it was related to the severity of periodontal disease [24]. Walker and colleagues reported from a cross-sectional study that *IL1* composite polymorphism is not associated with the susceptibility to localized aggressive periodontitis in an African American population [25]. Armitage et al demonstrated that this composite was almost non-existent in a Chinese population and didn't show any association with the onset of periodontal disease [26]. McGuire and Nunn in a longitudinal study discovered that this composite polymorphism was related to the tooth loss [27]. From those studies investigating the association between this *IL1* composite polymorphism and periodontal disease it can be

concluded that *IL1* genotype might be regarded as a severity factor in certain racial groups, especially non-smoking Caucasians population[48].

Other studies examined the association of polymorphisms of several other inflammatory genes with periodontal disease. A recent meta-analysis showed that the T variant at -819bp locus of *IL10* seemed to be a risk factor among Caucasians for chronic periodontitis [28]. Similarly, the A allele at -592bp locus of the same gene was also associated with an increased risk for chronic periodontitis in Caucasian population. Another recent meta-analysis concluded that -308bp A/G variations in the promoter region of *TNFA* is significantly related to the susceptibility to periodontitis in Brazilian, Asian and Turkish population [29]. The same meta-analysis also found that the G/C polymorphism at -174bp *IL6* may increase the susceptibility to periodontitis in Brazilians, while the G/C variation at -572bp locus of the same gene is more prevalent in European decedents with chronic periodontitis[29]. It is evident that the identified association of certain SNPs present in genes that are critically involved in immune responses in periodontal disease is usually population-based and usually cannot be generalized across different racial groups.

In addition to cytokine genes, polymorphisms of other genes linking cellular and humoral immune responses to bacterial infection in periodontium have also been investigated extensively. Fc receptors for immunoglobulin G (IgG) or FcγRs play a pivotal role in phagocytosis of macrophages, neutrophil activation, and antibody-dependent cell-mediated cytotoxicity of natural killer cells. The phagocytic activity of macrophages is triggered by opsonization, a process that the FcγR on the macrophage surface binds Fc portion of antibodies that coat the invading bacteria. The presence of polymorphisms in the FcγR gene structure may induce functional alterations that affect the susceptibility of the host to various

inflammatory diseases such as rheumatoid arthritis, systemic lupus erythematosus and periodontitis [30]. So far several studies have targeted the association between various polymorphisms of different FcγR genes and periodontal disease. Yamatomo et al reported that the polymorphism in the FcγRIIa gene resulting in a homozygous H/H at the 131 residue was associated with chronic periodontitis in a Caucasian population [31]. However, Sugita and colleagues found that a G to T transition at nucleotide 559 within FcγRIIIa that results in an amino acid substitution at 158 from valine to phenylalanine was significantly related to the recurrence of chronic periodontitis in a Japanese population[32] . A study by Yasuda et al demonstrated that rather than polymorphism that resulting in the H allele in FcγRIIa, the A allele at the nucleotide 646 -184 in the intron 4 of FcγRIIb gene was over represented in chronic periodontitis patients as compared to healthy control in a Japanese population[33]. Similar to studies of *IL1* composite polymorphism, the association between a specific allele or genotype in the FcγR genes and periodontal disease is affected by the racial groups.

Structure, expression profile, activity and genetic variation of Interleukin-37 (*IL37*)

We have recently completed a genome-wide association study (GWAS) of 4910 Caucasians with known levels of GCF IL-1β to identify candidate genes whose changes due to polymorphism are associated with elevated secretion of GCF IL-1β. We have identified two novel quantitative trait loci with missense mutations within the coding region of *IL37* that are both inversely and significantly associated with high GCF-IL-1β levels. *IL37* was previously designated as IL-1 family member 7 (*IL1F7*) [34]. *IL37* is mapped to the cluster of genes on human chromosome 2, which contains other IL-1 family members except *IL18* and *IL33*. *IL37* May be exclusively expressed in human, since the mouse homolog hasn't been identified yet [4]. Sequencing analyses have shown that *IL1F7* gene structure contains 6 exons

and five splicing variants present (*IL1F7a-e*). *IL1F7b* has been found to be the largest and include five of those six exons. The protein structure of *IL37* comprises a 12 β-barrel strand and shares the common pattern of other IL-1 family particularly that of IL-18 [4, 35]. Although none of the variants of IL-37 contains a typical signal peptide, both b-isoform and c-isoform encode a putative caspase-1 cleavage site. Many cell types or tissues have been reported to express IL-37 [35]. The constitutive *IL37* mRNA has been found in the testis, thymus, uterus and colon[35]. Upon induction, peripheral blood mononuclear cells (PBMCs), dendritic cells and colon epithelial cells can express IL-37[36, 37]. It has been reported that microbial products such as lipopolysaccharide (LPS) that bind to toll-like receptors (TLRs) constitute the strong stimulation for IL-37 expression [36]. In addition, this molecule can be also detected in monocytes, plasma cells and breast carcinoma cells at the protein level [35]. Similar to *IL18*, the control of *IL37* expression is mainly at the post-transcriptional level by an instability element encoded in exon 5 in its coding region. Although it is structurally similar to other IL-1 family members, IL-37 has recently been reported to possess a unique and potent anti-inflammatory activity, which is in sharp contrast to the pro-inflammatory activity of IL-1β and IL-18 [36]. Because mice don't have known structural homolog of *IL37*, transgenic mice carrying human *IL37* offers an excellent tool for the studying the role of *IL37* in inflammation[38]. McNamee and colleagues compared the susceptibility of transgenic mice carrying human *IL37* with control mice to colitis induced by dextran sodium sulfate (DSS) in drinking water [38]. DSS-mediated colonic inflammation is significantly reduced in the transgenic mice, and such amelioration is associated with a reduced leukocyte infiltration and dampened expression of IL-1β, and TNF-α. Nold et al. observed a significant attenuation of the effects induced by non-lethal endotoxic shock through LPS intraperitoneal injection in

IL37 transgenic mice [36]. In addition to weaker hypothermia, transgenic mice achieved respiratory compensation of the metabolic acidosis, protection against a rise in blood potassium concentration, and prevention of liver damage in the LPS induced endotoxic model. Those physiological findings in *IL37* transgenic mice were paralleling a reduction in various cytokines, such as IL-6, IL-1β, IFN-γ in different compartments as compared with control wild-type mice. Those protected effects observed in *IL37* transgenic mice showed dosage response because homozygotes always demonstrated more pronounced protection from LPS induced endotoxic shock and less pro-inflammatory cytokine production than heterozygotes [36].

The anti-inflammatory activity of *IL37* can be further demonstrated *in vitro* cell culture experiment. THP-1 cells, a human monocytic cell line, transfected with *IL37* exhibited reduced expression of several inflammatory molecules such as IL-1β, TNF and IL-8 upon challenge by LPS [36]. Similar pattern of reduction in those pro-inflammatory cytokines upon IL-1β challenge was also observed in epithelial cell line A549 transfected with *IL37* [36]. Further mechanistic studies have shown that the anti-inflammatory activity of IL-37 is mediated by enhancing TGF-β signals through a direct association with Smad3, a transcriptional factor that constitutes the major effector of signaling downstream of TGF-β [4, 36]. From both *in vivo* and *in vitro* experimental models, it has been clearly indicated that IL-37 is a natural suppressor of innate inflammatory and immune responses that reduces the extent and severity of inflammatory insult.

Other than colitis, hepatitis, dermatitis and rheumatoid arthritis, the activity of IL-37 hasn't been explored in other diseases [39, 38]. The knowledge gap of how IL-37 is involved in periodontal disease pathogenesis remains to be filled. Our GWAS Caucasian study has

provided some clues of the involvement of IL-37 in the inflammatory pathogenesis of periodontal disease. The first trait locus contains two non-synonymous, coding single nucleotide polymorphisms (SNPs) within exon 2 [rs3811046 T/G and rs3811047 G/A] that result in a predicted altered IL-37 protein structure [(A34V) and (A42T)], respectively. These two SNPs demonstrate linkage disequilibrium because they are almost always present in the same minor allele. This variant 1 [*IL37v1*] is relatively common with a minor allelic frequency of 30% and is found to be significantly associated with high levels of IL-1β within the GCF [OR=1.70, (1.53-1.89), $p=6.8\ X10^{-21}$]. This p value is statistically significant, as it readily exceeds the strict genome-wide significance threshold value of $p<5X10^{-8}$, which is required by Bonferroni correction for multiple comparisons. This *IL37* variant is also associated with more severe periodontal disease [e.g. greater inflammation with higher gingival index scores (GI, p=0.016), greater extent probing depth>4mm (PD4+mm, p=0.026) and greater bone loss as reflected in attachment loss >4mm (AL4+mm, p=0.04)]. A second, less common locus within *IL37* (8.5% frequency of minor allele) has 5 non-synonymous, coding SNPs [rs2708943(exon 4), rs2723183(exon 4) , rs2723187(exon 5) , rs2708947(exon 6) and rs2723192 (exon 6), OR=1.52 (1.31-1.84), $p<4.2X10^{-7}$]. The association between this trait locus with IL-1β within the GCF is approaching to the significance threshold. The predicted protein sequence for this variant [*IL37V2*] is estimated to have a high probability of inducing structural alterations in IL-37 (p=0.04). The data from this unpublished study suggest a similar anti-inflammatory role of *IL37* in periodontal disease because the predicted alteration of protein structure due to the missense mutations present in both trait loci may disrupt the normal activities of this cytokine in periodontium. The altered protein structure of *IL37* may possibly intervene with the homeostasis between host and microflora present in the

biofilm in such a way that skews the inflammatory responses to favor the destruction of attachment and bone resportion.

According to literature and data from our GWAS study, we hypothesize that **genetic *IL37* variants cause a functional defect in IL-37 that is associated with an excessive pro-inflammatory innate immune response in chronic periodontitis.** Epidemiological studies have shown that approximately 90% of the US population exhibits gingivitis, with 48% of the US adult population having periodontitis[40, 41]. More importantly, evidence suggests that periodontal disease carries potential risks for worsening various systemic conditions including atherosclerosis, stroke, diabetes, and etc[42, 43, 44]. Genetics plays a role in the progression of periodontal disease. Studies by Michalowicz in monozygotic and dizygotic twins provided the first estimates of heritability and suggested that about half (48%) of the variance in disease could be attributable to genetics [45]. Identification of important genetic risk variants that contribute to periodontal disease is the approach to study diseases with complex trait, such as periodontal disease. Several genetic polymorphisms of several pro-inflammatory molecules, such as *IL1*, *FcγRs*, *TNFA*, have been associated with periodontitis [46, 47]. However, no association linking *IL37* and periodontal disease has been reported so far. Based on our GWAS study, *IL37* genetic variation may confer the increased susceptibility to chronic periodontitis due to functional alteration of this molecule.

One of the scientific merits of this study is to understand how a genetic basis of an anti-inflammatory gene, *IL37*, is involved in the presentation of chronic periodontitis. Unraveling the genetic basis of pathogenesis will lead to improved diagnosis and prognoses to identify individuals at risk for disease to optimize prevention strategies. In addition, exploration of molecular mechanism of how *IL37* is involved in periodontal inflammation

helps to develop effective and novel therapies to target this anti-inflammatory molecule to restore periodontal health.

Statements of purpose, hypothesis and specific Aims:

The role of IL-37 hasn't been explored in periodontal disease. Our GWAS study suggested an anti-inflammatory activity of *IL37* in the pathogenesis of periodontal disease. We are seeking to understand the expression pattern of *IL37* in gingival biopsies and compare the transcriptional level of *IL37* in biological samples from chronic periodontitis patients with control subjects of periodontal health. We are also aiming to study the association of SNPs in the first *IL37* trait locus identified from our GWAS data with the transcriptional expression of inflammatory genes including *IL37*, *IL1B* and *IL6*. Further, we will use *in vitro* cell culture models representing different cell populations present in human gingiva to study the expression profile of *IL37* upon stimulation by *E. coli* LPS. Two specific aims are developed to test our hypothesis that **genetic *IL37* variants are associated with an altered pro-inflammatory innate immune response in chronic periodontitis.**

Aim 1: to compare the transcriptional expression levels of *IL37, IL1B and IL6* in biopsies collected from chronic periodontitis patients (case) and subjects with periodontal health (control); and characterize the *IL37* genotypes (rs3811046 T/G and rs3811047 G/A in the first trait locus) and correlate different *IL37* genotypes with the transcriptional profiles of those genes;

In this aim, we will first to identify the expression pattern of IL-37 in biopsied gingival tissues by immuno- fluorescence technique in frozen sections and then compare the transcriptional level of *IL37* in gingival biopsies collected from periodontitis patients with controls by real-time PCR. We will also genotype *IL37* based on the first identified locus

carrying two SNPs (rs3811046 T/G and rs3811047 G/A) by extracting DNA from biopsies and pyro-sequencing technique. We will also associate the presence of minor allele and different genotypes of *IL37* with periodontal disease status and transcriptional profiles of those inflammatory genes including *IL1B* and *IL6*, in addition to *IL37*.

Aim2: to investigate the kinetic of *IL37* transcriptional expression in gingival fibroblasts, THP.1 Cells and oral keratinized cells in the response to *E. Coli* LPS challenge

We will analyze the transcription of *IL37*, *IL1B* and *IL6* by real-time PCR *in vitro* in three different cell cultures that represent the major cell populations present in human gingiva in response to the challenge by *E.Coli* PLS. This information will help to map the cellular sources for *IL37* expression in periodontium.

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CHAPTER 2: EXPRESSION OF INTERLUKIN-37 (*IL37*) AND THE GENETIC VARIATIONS OF *IL37* IN RELATION TO CHRONIC PERIODONTITIS

Introduction

Periodontitis is caused by the abnormal inflammatory and immune responses initiated by the bacteria [1, 2]. Activation of the innate immune system in periodontal disease results in the synthesis and secretion of various chemokines and cytokines, such as interleukin-1β, (IL-1β), IL-6, IL-8, tumor necrosis factor-α (TNF-α), etc., most of which propagate inflammation and may potentially cause destruction in periodontium. However, the periodontal destruction caused by inflammation is usually episodic and slow-progressing in chronic periodontitis patients. The homeostasis of inflammation, though altered in periodontal disease, must be maintained by anti-inflammatory mechanisms in the long intervals between breakdowns of periodontal supporting apparatus. So far several molecules have been described to possess anti-inflammatory activities, such as resolvins, IL-10 and transforming growth factor β (TGF-β) [3, 4, 5]. Pathways to down regulate the extent and severity of inflammation in hosts may provide insight for the novel therapeutic potential to reduce the destruction caused by periodontal disease.

Periodontal disease, similar to most other complex inflammatory diseases, is a polygenic disorder involving gene to gene interaction and exhibits complex genetic traits. The susceptibility to periodontal disease has been explored by association studies aiming for targeting genetic markers and candidate disease-modifying genes by identification of single nucleotide polymorphisms (SNPs). Many previous studies have discovered important SNPs

in genes encoding molecules of the host defense system in chronic periodontitis [6, 7]. Kornman et al first described a composite *IL1* polymorphism including -889bp (C to T transition) in *IL1A* and +3953bp (C to T transition) in *IL1B* that was significantly related to the severity of periodontitis in non-smoking Caucasians [8]. Additionally, monocytes from individuals with homozygous *IL1B* +3953T allele were shown to produce 4-fold more IL-1β, and monocytes with heterozygous IL1B +3935T allele exhibited two-fold more IL-1β than cells without this SNP at the +3953 locus [8, 9]. It has been concluded that *IL1* genotypes might be considered as a risk factor for the severity of periodontitis in certain racial groups, especially non-smoking Caucasians population (Lindhe J clinical Periodontology 4th edition). Other **association** studies have identified certain SNPs of other genes involved in immune response including *IL10*, *TNFA*, Fc receptor for immunoglobulin G (*FcRs*), etc [10, 11, 12]. All those studies have suggested that the association of certain SNPs with periodontitis is usually race specific and cannot be generalized across all racial groups.

From a genome-wide association study (GWAS) including 4910 Caucasians with known levels of gingival crevicular fluid (GCF) IL-1β, we have identified two novel quantitative trait loci with missense mutations within the coding region of *IL37* that are both inversely and significantly associated with GCF-IL-1β levels. *IL37*, which was previously designated as IL-1 family member 7 (*IL1F7*) [13], is mapped to the cluster of genes on human chromosome 2, which contains other IL-1 family members except *IL18* and *IL33*. Sequencing analyses have shown that *IL1F7* gene structure contains 6 exons and five splicing variants present (*IL1F7*a-e) with *IL1F7b* to be the best characterized. Upon induction with bacterial endotoxins such as lipopolysaccharine (LPS), peripheral blood mononuclear cells (PBMCs), dendritic cells and colon epithelial cells can express IL-37[14, 15].

Although it is structurally similar to other IL-1 family members, IL-37 has been reported to possess a unique anti-inflammatory activity [14]. McNamee and colleagues [16] discovered that the dextran sodium sulfate (DSS)-mediated colonic inflammation is significantly reduced in the *IL37* transgenic mice, and such amelioration is associated with a reduced leukocyte infiltration and dampened expression of IL-1β, and TNF-α. Nold et al. observed a significant attenuation of the effects induced by non-lethal endotoxic shock through LPS intraperitoneal injection in *IL37* transgenic mice [14]. Those physiological findings in *IL37* transgenic mice paralleled a reduction of a panel of pro-inflammatory cytokines, such as IL-6, IL-1β, IFN-γ in different compartments as compared with control wild-type mice. [14].

The anti-inflammatory activity of *IL37* was further demonstrated *in vitro* cell cultures. THP-1 cells transfected with *IL37* exhibited reduced expression of several inflammatory molecules such as IL-1β, TNF-α and IL-8 upon challenge by LPS [14]. Similar pattern of reduction in those pro-inflammatory cytokines upon IL-1β challenge was also observed in epithelial cell line A549 transfected with *IL37* [14].

The activity of IL-37 hasn't been explored in diseases other than colitis, hepatitis, dermatitis and rheumatoid arthritis [17, 16] yet. The knowledge gap of how IL-37 is involved in periodontal disease pathogenesis remains to be filled. Our GWAS Caucasian study has provided some clues of the involvement of IL-37 in the pathogenesis of periodontal disease. The identified novel trait locus contains two non-synonymous, coding single nucleotide polymorphisms (SNPs) within exon 2 [rs3811046 T/G and rs3811047 G/A] that result in a predicted alteration of IL-37 protein structure [(A34V) and (A42T)], respectively. These two SNPs demonstrate linkage disequilibrium because they are almost always present in the same allele. This variant is relatively common with a minor allelic frequency of 30% and is found

to be significantly associated with high levels of IL-1β within the GCF [OR=1.70, (1.53-1.89), p=6.8 $X10^{-21}$].

In this study, we are seeking to understand the expression pattern of *IL37* in gingival biopsies and compare the transcriptional level of *IL37* in biological samples collected from chronic periodontitis patients with control subjects with periodontal health. We are also aiming to study the association of both SNPs in this identified *IL37* trait locus (rs3811046 and rs3811047) with the transcriptional expression of inflammatory genes including *IL37*, *IL1B* and *IL6*. Further, we will use *in vitro* cell culture models representing different cell populations present in human gingiva to study the expression profile of *IL37* upon stimulation by *E. coli* LPS. By employing both *in vivo* and *in vitro* approaches, we will test our hypothesis that genetic *IL37* variants are associated with an altered pro-inflammatory innate immune response in chronic periodontitis.

Materials and Methods

Participants and tissue collection

This study was approved by the Biomedical Institutional Review Board of the University of North Carolina at Chapel Hill (UNC). Written informed consent was obtained from all study participants. All subjects are aged between 18-70 years of different races including Caucasians, African Americans, Hispanics, Asians and others.

In this cross-sectional clinical study, a total of 61 subjects were enrolled. 24 of the subjects were patients diagnosed with chronic periodontitis, exhibiting probing depth (PD) $\geq$4mm and clinical attachment loss $\geq$3mm. Another 37 subjects served as control subjects with periodontal health, which is defined by no site of PD greater than 3mm or attachment loss greater than 2mm. Periodontitis patients were recruited from the Department

of Periodontics, University of North Carolina at Chapel Hill, School of Dentistry, while control subjects were enrolled either from the General and Oral Health Clinical Center at the School of Dentistry who volunteered for this study after screening or from the Department of Periodontics and presented for clinical crown lengthening procedure. Major exclusion criteria are: 1) use of either antibiotics or non-steroidal anti-inflammatory drugs (NSAIDs) within 1 month before enrollment; 2) medical treatment for systemic diseases 3 months prior to the gingival biopsy or periodontal surgery; 3) active infectious diseases, pregnancy, cancer or systemic diseases with oral manifestations; 4) severe unrestored caries or aggressive periodontitis. Periodontal clinical measurements, including probing depth (PD), clinical attachment loss (CAL), bleeding on probing (BOP), and plaque index (PI) will be obtained prior to the biopsies. Biopsies obtained from periodontitis patients were collected from the most severe site during the scheduled periodontal surgeries.

Upon removal, all biopsied gingival tissues were divided at least into two pieces. One piece, used for genotyping analysis, was kept in the −80°C freezer immediately, while the other half, used for real-time polymerase chain reaction (RT-PCR), was incubated with RNA-later (Applied Biosystems/Ambion, Austin, TX, USA) overnight at 4°C, and then transferred to −80°C freezer for storage. In addition, another piece of tissue from 5 chronic periodontitis biopsies and 5 control biopsies were immersed in Tissue-Tek® O.C.T™ compound (Sakura Finetek, Netherland) and snap frozen by dry ice and then stored in −80°C freezer for histological study.

DNA isolation and genotyping of *IL37*

Genomic DNA was extracted from the collected gingival biopsies using a DNeasy Mini kit (QIAgen, Valencia, CA, USA) according to the manufacturer's manual. The primers

and sequencers designed by Assay Design Software 2.0 (QIAgen) for SNP detection were listed in table 1. For each PCR amplicon generated by forward and reserve primers was directly genotyped in a real-time DNA synthesis approach by prosequencing technology. In brief, 6-8 ul of PCR product was mixed with 2ul of streptavidin-coated sepharose beads and 40ul of PyroMark binding buffer (QIAgen). After captured by the PyroMark vacuum prep filter tips (QIAgen), the beads to which biotin-labeled single strand PCR product attached were washed sequentially with 70% ethanol, denaturing buffer, and PyroMark washing buffer, and then released into a PyroMark Q96 HS plate (QIAgen) onto which 12ul of PyroMark annealing buffer mixed with 3.6pmol of sequencer corresponding to each amplicon has already been preloaded. After heated for 2 minutes at 86°C, the plate was then loaded onto a PSQ HS 96 Pyrosequencing system (QIAgen). The sequencing was determined by Pro Gold CDT reagents (QIAgen). The presence of variation or the copy of the minor allele (heterozygote or homozygote) was determined by the pyrogram pattern based on the peak height.

Table 1. Primers and sequencers used for *IL37* genotyping

SNP sites	Sequences	Nealing Temperature (°C)
Rs3811046 and Rs3811047	Forward: 5'-TGCTAACCTCACTGCGTCTGAC-3'	65
	Reverse: 5'-ATCACCTCACCCCGAGGC-3'	
	Sequencer: 5'-CCTTACTTGTGTGAACAAA-3'	63
Rs2708943 and Rs2723183	Forward: 5'-AAAGGGCTGAGTCTTCCATTT-3'	
	Reverse: 5'- TTGTGATCCTGGTCATGAATG-3'	
	Sequencer:5'- TCTGGTGATATTGATCTAGG-3'	

Cell cultures

A human monocytic cell line (THP.1), which was originally purchased from American Type Culture Collection (ATCC, Manassas, VA) , was maintained in RPMI 1640 culture medium (Invitrogen, Grand Island, NY) supplemented with 10% fetal bovine serum (FBS) (Mediatech, Manassas, VA), $5X10^{-5}$ M 2-mercaptoethanol (Sigma-Aldrich, St. Louise, MO), and 1% penicillin/streptomycin (Invitrogen). Cells with a density of $1X10^{6}$ were seeded into a 6-well plate just before use.

A primary human gingival fibroblast cell culture (HGF), which was originally purchased from ATCC, was maintained in DMEM culture medium supplemented with 10% fetal bovine serum (FBS, Mediatech) and 1% penicillin/streptomycin (Invitrogen). $0.4X10^{5}$ cells were seeded into a 6-well plate 48 hours prior to challenge.

A primary human gingival epithelial progenitor cell culture (HGEPp, CELLnTEC Advanced Cell Systems, Bern, Swizerland) was maintained in CnT basal medium (CnT-BM.1) supplemented with CnT-24.S (CELLnTEC Advanced Cell Systems). $0.5X10^{6}$ cells were seeded into a 24-well plate 24 hours prior to challenge.

E.coli Lipopolysaccharide (LPS) (Sigma-Aldrich) was diluted with each individual cell culture medium and added to each cell culture to achieve either 100ng/µl or 1µg/µl according to experimental protocols. After challenging for different intervals, cells were collected and RNA was isolated.

RNA isolation and quantitative Real-time RT-PCR

Total RNA was isolated from gingival biopsy samples and cells with a RNeasy Mini Kit (QIAgen, Valencia, CA). cDNA from 300-600ng isolated RNA was synthesized with a SuperScript VILO cDNA synthesis kit (Invitrogen). Quantitative Real-time RT-PCR of the messenger levels of *IL1B*, *IL6* and *IL37* were determined by an X20 assay on-demand gene-

expression assay mix (Hs01555410_m1, Hs00985639_m1, and Hs00367201_m1, respectively, Applied Biosystems/Ambion, Austin, TX) in a 7000 Sequence Detection System (Applied Biosystems/Ambion). A TaqMan® GAPDH control reagents kit was used as an internal control (Applied Biosystems/Ambion).

Immunofluorescence

The O.C.T embedded and frozen preserved biopsy samples were stored at -80°C freezer until use. After cryostat sectioning, the frozen gingival biopsies from periodontal health and chronic periodontitis were fixed with 70% ethanol for 30 seconds and then immersed in acetone for another 15 minutes. After fixation, the tissue slides were blocked for 1 hour at room temperature, and then incubated overnight at 4°C with monoclonal antibody specific for IL-37 (6.7µg/ml, Cat #1975-IL, R&D Systems, Minneapolis, MN) or vehicle. The blocking PBS buffer contains 8% bovine serum albumin, and 0.1% Triton-X-100, and the antibody was diluted with the same blocking buffer. After vigorous washing with 1X PBS containing 0.1% Triton-X-100 for, the slides were incubated for another hour at room temperature with a donkey anti-goat IgG secondary antibody labelled with northenlights 557 Fluorochrome (NL007, R & D Systems). Sections were then washed with PBS, mounted with ProLong Gold antifade reagent with DAPI (Invitrogen) and coverslipped. Immunofluorescence signals were observed using confocal microscopy (Carl Zeiss LSM 710 Confocal Microscope, Thornwood, NY).

Statistics:

Pearson's chi-square or exact Pearson chi square test was applied to test the null hypothesis that the distribution of genotypes at both rs3811046 and rs3811047 loci was not different among different racial groups. The genotypes are categorized by homozygous

populational major alleles and heterozygous alleles/homozygous for populational minor alleles. The same statistical analysis was also performed to test the gender difference between periodontitis patient group and control group of subjects with periodontal health.

Two sample student-t test was used to detect the difference of periodontal indices and age between diseased and control groups. To compare the transcriptional expression of cytokines between diseased and control subjects, we applied non-parametric Wilcoxon-Mann-Whitney rank test. Using the same statistical analysis, we compared the transcription of those cytokines between genotypes at both loci in Caucasian subjects and African-American subjects. The level of significance was set at $p=0.05$.

Results

Demographic information of the clinical subjects:

The demographic information, periodontal indices and racial status of the study participants are listed in Table 2. The mean age of patients with chronic periodontitis was significantly older than subjects with periodontal health (Table 2, $p=0.014$). The proportion of males in the chronic periodontitis group was significantly higher than the control group (56% vs. 30%, respectively, $p=0.04$, Table 2). The distribution of different racial groups was not significantly different between the two groups of participants. As expected, sites with chronic periodontitis exhibited significantly higher measurements for both probing depth and attachment loss when compared to the periodontally healthy sites ($p<0.0001$ for both, Table 2).

Genotypes of *IL37* in the clinical subjects:

21 out of 37 (56.8%) Caucasian subjects and all the participants from Hispanic, Asian and other races (100%) presented homozygous "T" allele at the rs3811046 locus with the TT

genotype (or 1.1), which is in contrast to only 2 out of 17 (11.8%) African Americans who were homozygous for "T" allele at this locus. The prevalence for 1.1 genotype at this locus was significantly higher in non-African American subjects than African American participants (p=0.0005, Table 3). However, the prevalence of 1.1 genotype and other genotypes TG/GG (or 1.2/2.2) in periodontitis group was not significantly different from that of periodontal health group (p=0.3, data not shown).

Similarly, 27 out of 44 (61.4%) non-African American subjects presented homozygous "G" genotype (GG or 1.1) at the rs3811047 locus, while only 3 out of 17 (17.6%) African Americans were homozygous for "G" allele. The distribution of 1.1 genotype was significantly different among different racial groups with higher percentage of GA/AA genotype (or 1.2/2.2) at this locus in African American subjects than in non-African American group (Table 3, p=0.001). When disease status was considered, again the prevalence of 1.1 genotype in periodontitis patients was not statistically different from the subjects with periodontal health (p=0.4, data not shown).

Changes of transcriptional levels of cytokines *IL37, IL1B* and *IL6* in gingival biopsies with periodontitis

The transcriptional level of *IL37* was not significantly different from gingival biopsies between periodontitis and periodontal health group (p=0.97, Figure 1A). The transcriptional level of *IL1B* from the gingival biopsies with periodontitis was higher than that in the periodontal healthy group, though the difference was also not statistically significant (p=0.11, Figure 1B). However, the messenger level of *IL6* was significantly elevated in samples collected from chronic periodontitis patients in comparison to biopsies with periodontal health (p=0.027, Figure 1C).

Transcriptional levels of cytokines *IL37, IL1B* and *IL6* in gingival biopsies in relation to genotypes of *IL37* at rs3811046 and rs3811047 loci

Because the presence of genetic variations in the loci of *IL37* may affect its transcriptional activity, messenger stability, and the transcription activity of other proinflammatory cytokine genes, we observed the messenger levels of *IL1B* and *IL6*, in addition to *IL37*, in relation to the genotypes of *IL37* at both rs3811046 and rs3811047 loci. The transcriptional levels of *IL37*, *IL1B* and *IL6* were not significantly different between the homozygous major T allele (TT or 1.1) and TG/GG genotypes (1.2/2.2) at rs3811046 locus (p=0.15, 0.97, and 0.23, respectively, Figure 2A, 2B and 2C).

Because the G (2) minor allele was more prevalent only in African American subjects, which is different from other racial groups, we next compared the transcriptional levels of those cytokine genes between different genotypes stratified by race. Since there were very few non-Caucasian and non-African American subjects, we only focused on Caucasian and African American participants. In Caucasian subjects, the transcriptional level of *IL37* in gingival samples positive for TT (1.1) genotype was not significantly different from samples with TG/GG (1.2/2.2) genotypes at the rs3811046 locus (p=0.12, Figure 3A). Nor was the transcriptional level of *IL1B* from samples with 1.1 genotype significantly different from biopsies with 1.2/2.2 genotypes (p=0.11, Figure 3B). However, a trend of higher level of *IL37* and *IL1B* was present in the 1.1 genotype. The messenger level of *IL6* in samples present GG (1.1) genotype was significantly higher than TG/TT (1.2/2.2) samples in Caucasian participants (p=0.02, Figure 3C).

In African American participants who exhibited a G (or 2) dominating allele, we compared the transcriptional levels of those cytokine genes in samples with homozygous G

allele genotype (GG or 2.2) with samples present for other allelic variations (GT/TT or 2.1/1.1). We found that the transcriptional expression of both *IL37* and *IL1B* from samples with 2.2 genotypes was not significantly different from samples present 1.1/1.2 genotypes (p=0.09 and p=0.45, respectively, figure 4A and 4B). However, the transcriptional level of *IL6* in samples with 2.2 was significantly higher than other genotypes in African American subjects (p=0.014, Figure 4C).

Due to the similar consideration that in African Americans the A allele is more dominating than G allele, while the G allele is more prevalent in non-African American population, we separated the Caucasian participants from the African-Americans for comparisons. Because rs3811047 presented strong linkage disequilibrium with rs3811046, the results for rs3811047 mirrors what we obtained from rs3811046. Briefly, the transcriptional levels of *IL37*, *IL1B* and *IL6* were not significantly different between the GG genotype (GG or 1.1) and GA/AA (1.2/2.2) genotypes at rs3811047 locus in overall participants (p=0.11, 0.71, and 0.38, respectively, data not shown). The expression of *IL37* and *IL1B* among Caucasians were not significantly different between 1.1 genotype and 1.2/2.2 genotypes (p=0.1 and p=0.14, respectively, data not shown), while the expression of IL6 was significantly higher in samples with genotype GG (1.1) than from samples with GA/AA (1.2/2.2) genotypes (p=0.03, Figure 5A).

In African American subjects, the transcriptional levels of *IL37* and *IL1B* in samples present AA (2.2) genotypes were not significantly different from samples with AG/GG (1.2/1.1) (p=0.2 and p=0.9, respectively, data not shown). Again, the messenger level of *IL6* was significantly elevated in samples with 2.2 genotype at this locus than 1.2/1.1 variations (p=0.04, Figure 5B).

Immunofluorescence staining of IL-37 in frozen sections of gingival biopsy samples:

IL-37 staining was prevalently present across the epithelial layer of the gingival biopsy samples (Figure 6A-C). The staining was primarily intracellular. Scattered staining of IL-37 can be also identified in the sub-epithelial layer to a milder extent. The staining pattern of IL-37 in frozen sections collected from the periodontitis site (Figure 6A and 6B) was not distinctly different from the sample collected from a control subject (Figure 6C), though the staining seemed stronger in the diseased sample.

Transcriptional levels of *IL1B, IL6* and *IL37* in gingival fibroblasts challenged with *E.coli* LPS:

Upon challenge by *E.coli* LPS (1 μg/ml) for 24 hours, the messenger level of *IL1B* in oral gingival fibroblasts was elevated about 287-fold as compared to the control cells challenged with cell culture medium (Figure 7A). Similarly, the transcriptional level of *IL6* was induced about 40-fold in LPS challenged cells in comparison to mock challenged cells at 24 hours (Figure 7A). However, the transcriptional level of *IL37* in fibroblasts was unchanged in response to LPS stimulation (Figure 7B). This may suggest that gingival fibroblast present in periodontium is not the major source for *IL37* production.

THP.1 cells, which are a monocytic cell line, exhibited elevated *IL1B* and *IL6* transcriptional expression by about 240-fold and 274-fold, respectively, in response to LPS challenge in comparison to mock challenge at 24 hours (figure 8A). Treatment with LPS increased *IL37* in a time-dependent manner as compared to control cells. After 4 hours of treatment messenger level of *IL37* was induced about 1.7-fold, while the transcription was induced about 2.3-fold at 24 hours in comparison to mock treated cells (figure 8B). However,

the magnitude of *IL37* transcriptional induction in THP.1 cells is not as dramatic as other two cytokines.

The gingival epithelial cells were challenged with a lower dose of *E.coli* LPS at the 100ng/ml and a higher dose at 1μg/ml at 6 hours, 24 hours and 48 hours. The increase of *IL1B* and *IL6* transcriptional levels in gingival epithelial cells demonstrated both dose- and time-dependent manner (figure 9A, 9B): the higher concentration of LPS in the cell culture resulted in more elevation of transcription for both molecules than the lower concentration, and the messenger levels of both cytokines increased as cells were treated longer by LPS. The *IL37* transcriptional expression pattern mimicked the kinetics of *IL1B* and *IL6* production by LPS treatment (Figure 9C). It was noticeable that the fold change of *IL37* upon challenge by LPS in gingival epithelial cells was higher than that in THP.1 cells.

Discussion

In this study, we used human gingival biopsy samples collected from periodontitis patients and subjects with periodontal health to analyze the genetic variations at rs3811046 and rs3811047 loci of *IL37* and related the genotypes at both loci to disease status and race. We also compared the transcriptional levels of several cytokine genes including *IL37*, *IL1B* and *IL6* from samples with periodontitis to control biopsies and genotypical variations of *IL37* stratified by race. We further observed the expression pattern of IL-37 in gingiva by immunofluorescence approach. At last, using different cell cultures that represent different cell populations in periodontium, we explored the readiness of *IL37* transcriptional activation in different cell types upon challenge by *E.coli* LPS.

IL37 was first identified by several independent groups in 2000 [18, 19, 20]. It has been designated as *IL1F7* previously. Many human tissues have been discovered to express IL-37

such as colon, tonsils, placental tissue, normal breast, testis, thymus, etc [19, 21]. Peripheral blood monocytic cells and dendritic cells are also the source of IL-37.In this study, we reported that gingival tissue is also the source for *IL37* expression. Immunofluorescnce staining clearly demonstrated that the epithelium layer of gingival biopsies taken from both diseased and control patients moderately expressed *IL37*. IL-37 staining seems to be in the cytoplasm of epithelial cells across the whole layer of epithelium. The production of IL-37 in the gingival epithelium has been verified by a gingival epithelial progenitor cell line unpon challenged by *E. Coli* LPS at the transcriptional level. This epithelial cell line exhibited a both time-dependent and LPS dose-dependent elevation of the *IL37* messenger level, which coincided with the transcriptional patterns of *IL1B* and *IL6*. The messenger level of *IL37* was saturated at 48 hours because increasing LPS concentration to 1μg/ml in the cell cuture medium didn't further elevate its transcription as compared to lower dose (100mg/ml) challenge. It seems that barrier epithelium separating commensal microbiota and pathogenic bacterial microorganisms from the inner tissue poccesses IL-37 production activitiy, since colon epithelium was also found to produce IL-37 in humans [14]. Therefore, it is possible that lining epithelium interfaced with commensal or invading microorganisms may synthesize and secret IL-37 to keep the homeostatic state of the mucosal defense to prevent tissue destruction induced by uncontrolled production of inflammotry molecules as repsonded to the constant microbial challenge from the bio-film. It is reasonable to argue that epithelial cells express IL-37, which possesses the anti-inflammatory activity, as a protective mechanism to tune down the inflammation mounted by various pro-inflammatory cytokines, enzymes or chemokines. However, the signaling pathway(s) that regulate *IL37* transcription in gingival epithelial cells remain to be elucidated. Recently, it has been reported that PI3K,

MAP kinase and NF-κB pathways are involved in the control of *IL37* transcriptional activation in intestinal epithelial cells challenged by TNF-α [15].

We also observed scattered staining of IL-37 in the sub-epithelial layer in the gingival biopsies. From the results of *in vitro* cell culture experiment, it is apparent that monocytic cells, but not gingival fibroblasts, respond to LPS stimulation by increasing *IL37* transcriptional activity. So far it hasn't been reported that fibroblasts from any source express IL-37. Although gingival fibroblasts share some innate immunological response with monocytic cells by producing inflammatory cytokines as reported in this study [22, 23], there is a divergent regulatory mechanism for IL-37 production between those two types of cells. Although from the immunofluorescence staining it is difficult to differentiate the cell types that exhibit positive IL-37 staining in the connective compartment, cells such as macrophage, dendritic cells, et al. may constitute the major cell population that produce IL-37 in the subepithelial layer of gingival tissue.

Employing real-time PCR, we observed that the transcriptional expression of *IL37* from biopsies of chronic periodontitis was not significantly different from the samples collected from controls with periodontal health. One explanation is that the transcription of *IL37* is cell type specific, which has been proved in the immunofluorescence and cell culture experiments, and the heterogeneity of cell populations in those biopsy samples may mask the difference of *IL37* production between periodontally diseased and healthy samples. The vast amount of fibroblasts present in the gingiva that do not alter transcriptional level of *IL37* upon inflammatory stimulation outweighed the epithelial cells or macrophage/dendritic cells that are competent for *IL37* production. In addition, we lack of the knowledge of *IL37* transcriptional activation from other cell populations present in gingiva, such as endothelium.

Another explanation for the insignificant results is possibly due to the limited sample size in this pilot study. The power may be not strong enough to detect the significance.

In biopsied gingival tissues we observed that the transcriptional level of *IL6* in samples collected from periodontitis patients was significantly elevated compared to biopsies from subjects with periodontal health. However, the *IL1B* level between those groups was not significantly different, though a trend of elevation was present in diseased samples. This result is similar to a study performed by Muthukuru et al, who found a non-significant 3-fold increase of *IL1B* transcripts in gingival biopsies from chronic periodontitis patients as compared to healthy controls [24]. Previous studies have demonstrated that the level of several inflammatory molecules, such as IL-6, IL-8, MMPs, *etc*, was enhanced in the gingival crevicular fluid (GCF) samples or tissue biopsied from chronic periodontitis patients as compared to controls [25, 26]. However, the extent of *IL1B* elevation in chronic periodontitis sites was not significant. Several explanations can be applied. First, the expression of IL-1β can be inhibited in the tissue due to the constant challenge by endotoxins of pathogens that are omnipresent in the biofilm. This tolerated expression of *IL1B* has been reported by several studies [27, 28]. Second, *IL1B* expression is subject to the regulation at transcriptional, post-transcriptional, and post-translational levels [29, 30]. Therefore, the transcripts of *IL1B* do not necessarily correlate to the protein level. In addition, all the periodontitis patients had undergone initial non-surgical periodontal therapy such as scaling and root planning several weeks before biopsy collection. The treatment rendered before sample collection may reduce the tissue level of *IL1B* transcripts due to the resolution of inflammation as suggested by other studies [31, 32].

We confirmed through pyrosequencing that the major allele at rs3811046 and rs3811047 in *IL37* locus is "T" and "G" in non-black populations, while populational "G" and "A" minor allele are more prevalent in African Americans at both loci, respectively . The genotype frequency of cytokine genes varies among different racial groups. For example, the GG genotype at the -330 of *IL2* didn't exist among studied African American subjects while this genotype was present in 6% White, 14% Asian and 4% Hispanic subjects [33]. We further demonstrated that the genotypes of *IL37* at those two loci were not related to the periodontal disease status (p=0.32, data no shown). Nor was the transcriptional expression of *IL37* from the biopsied samples correlated with its genotypes (data not shown). This may indicate that the polymorphisms at those two loci do not seem to predict the susceptibility to periodontal disease. This speculation was supported by a recent study reporting that the genotype of rs3811047 was not related to rheumatoid arthritis in Chinese Han population [34]. The frequency of GG genotype is not significantly different from other genotypes (GA/AA) at this locus in patients with rheumatoid arthritis as compared to matched non-rheumatoid arthritis control subjects [34]. The substitutions at rs3811046 and rs3811047 loci result in a predicted altered IL-37 protein structure [(A34V) and (A42T)], respectively. However, those non-synonymous SNPs present in the coding region may affect more of its function rather than the transcriptional activity.

In this study we also compared the transcriptional expression of different inflammatory cytokines to the genotype of *IL37* stratified by race because the dominating alleles at both rs3811046 and rs3811047 loci are different in African Americans and Caucasians. However, the genotypes at both loci were not related to the transcriptional levels of *IL1B* either in Caucasian or African American populations, though the presence of at least

one copy of minor allele specific to either racial group was related to a trend of decreased transcription. It seems that the presence of SNPs at those loci may not necessarily cause the change of the transcription efficiency or the stability of *IL37* transcripts. This result seems contradictory to our GWAS study, which identified that the 1.2 and 2.2 genotypes at both loci were associated with a higher IL-1β in GCF from the Caucasian subjects. As mentioned above, the discrepancy can be explained by the fact that the transcription of *IL1B* doesn't necessarily reflect the protein level of IL-1β in the GCF due to its multi-layered complex regulation. In addition, the reduction of inflammation in the gingival tissue after initial therapy in periodontitis patients in this study may not represent the association between genotypes and expression of the molecule as observed in an un-intervened general population.

Nevertheless, we observed that the presence of minor allele in the genotype (1.2/2.2) at both loci in Caucasian participants was related to a significant lower transcriptional level of *IL6*, while in African Americans the presence of a major allele in the genotype (1.2/1.1) was associated with a significant reduced messenger level of *IL6* in the gingival tissue. Previous studies have shown that *IL6* transcription can be induced by TGF-β in several cell cultures including human bronchial epithelium and airway smooth muscle cells, and this induction was very likely mediated through Smad 3 activation [35, 36]. It has been known that in lung tissue of *IL37* transgenic mice the production of *IL6* was significantly reduced if Smad3 was silenced through inhalation of Smad3 specific siRNA [14]. Therefore, the anti IL-6 activity of IL-37 is mediated through TGF-β signaling pathway specifically involving Smad3. It is speculated that the presence of a minor allele at both loci in the exon 2 of *IL37* in Caucasians may increase the activity of Smad3. It hasn't been known how IL-37 is wired to TGF-β signaling. However, the structural alteration of IL-37 induced by those two miss sense SNPs

at both loci seems to enhance Smad3 activity resulting in reduction of *IL6* transcription in gingival tissue.

In African American subjects, we found a paradoxical reverse relationship between genotypes and the transcriptional level of *IL6* in gingival biopsies. It is possible that the mechanistic link between genotypes of *IL37* at those two loci and the transcriptional regulation of *IL6* is different from that found in Caucasians. Since genetic variations are more diverse in African Americans than other racial groups, the association of certain SPNs with disease state and gene expression profile might be modified by the presence of a far more complex network of genetic variations. The detailed biological mechanism of how the structural changes of IL-37 induced by the allelic variations uniquely affect the transcription of *IL6* in African Americans remains to be elucidated.

This study has several limitations. First the sample size is limited due to the nature of a pilot study, especially when it is stratified by race. The power to detect the difference of genetic variations related to disease among difference racial groups is reduced. We intended to increase our participants to repeat the association analysis between the genotypes and transcriptional levels of cytokines. Second, the biopsies we collected from the diseased sites do not genuinely reflect the inflammatory nature of the periodontitis due to prior treatment rendered before sample collection. In this study we used convenient samples collected from a previous study, and all the periodontitis patients recruited in the study had previous non-surgical therapy. The transcriptional levels of inflammatory molecules are not congruent to their protein levels. Therefore, the protein levels of those molecules need to be evaluated by collecting GCF samples from untreated participants. In addition, we are also seeking to

investigate the regulatory control of IL-6 in monocytic cell lines transfected with *IL37* fragment carrying different genotypes upon inflammatory stimulation.

Table 2. Demographic information and periodontal indices

Demographic/Clinical parameters	Periodontally Healthy (n=37)	Chronic Periodontitis (n=23)
Age (mean± SD, years)*	40.3± 13.2	48.6± 11.2
Sex (males/females)#	11/26	13/10
PD (mean±SD, mm)&	2.25± 0.8	6.1± 1.0
AL (Mean± SD, mm)&	0.89± 0.6	5.7 ± 1.4
Alveolar bone loss	No	Yes
Caucasians	24	14
African-Americans	10	6
Hispanics, Asians and Others	3	3

* p=0.014 by Student t-test; # p=0.04 by Chi-square (χ^2) test; & p<0.0001 by student-t test

Table 3. Genotypes at rs3811046 locus of *IL37* in the study participants

Race	rs3811046 Genotypes		Total (%)	p
	TT (%)	TG/GG (%)		
Caucasians	21 (35.0)	16 (26.7)	37 (61.7)	
Hispanics, Asians, and Others	6 (10)	0 (0)	6 (10)	
African-Americans	2 (3.3)	15 (25.0)	17 (28.3)	
Total	29 (48.3)	31 (51.7)	60 (100)	0.0005*

* Chi-square (χ^2) test

Table 4. Genotypes at rs3811047 locus of *IL37* in the study participants

Race	rs3811047 Genotypes		Total (%)	p
	GG (%)	GA/AA (%)		
Non African-Americans	21 (35)	16 (26.7)	37 (61.7)	
Hispanics, Asians, and Others	6 (10)	0 (0)	6 (10)	
African-Americans	3 (5)	14 (23.3)	17 (28.3)	
Total	30 (50)	30 (50)	60 (100)	0.001*

* Chi-square (χ^2) test

Figure 1A:

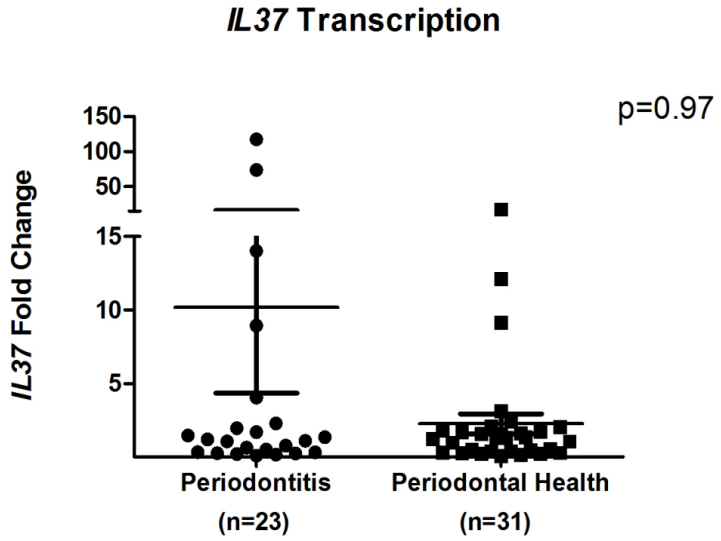

Figure 1B

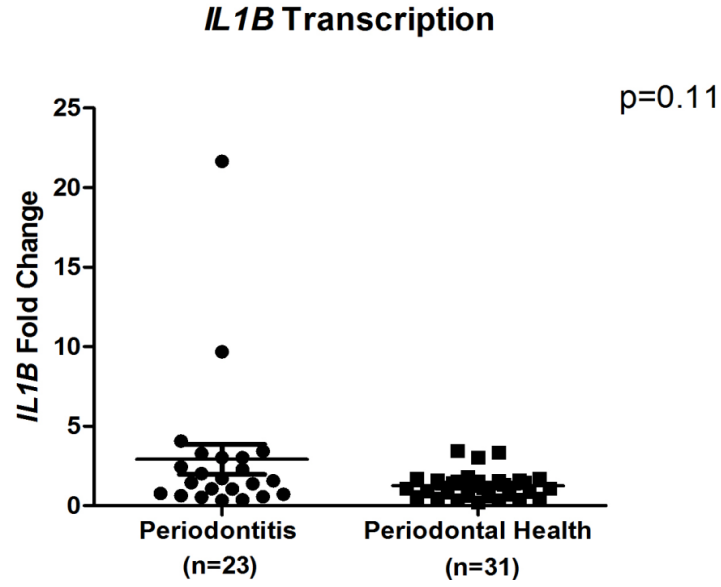

Figure 1C:

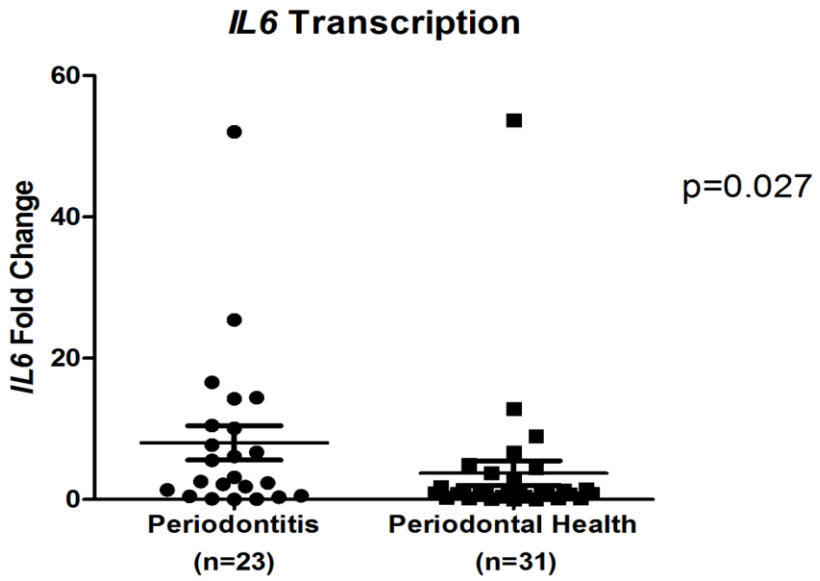

Figure 1. Transcriptional levels of *IL37*, *IL1B* and *IL6* in gingival biopsy samples from sites with periodontitis and with periodontal health. The transcriptional levels of *IL37* (Figure 1A) and *IL1B* (Figure 1B) from periodontitis gingival samples were not significantly different from control samples with gingival health (p=0.97 and p=0.11, respectively), while the messenger level of IL6 was significantly higher in periodontitis samples than control samples (p=0.027, Figure 1C). Wilcoxon-Mann-Whitney rank test was used.

Figure 2A:

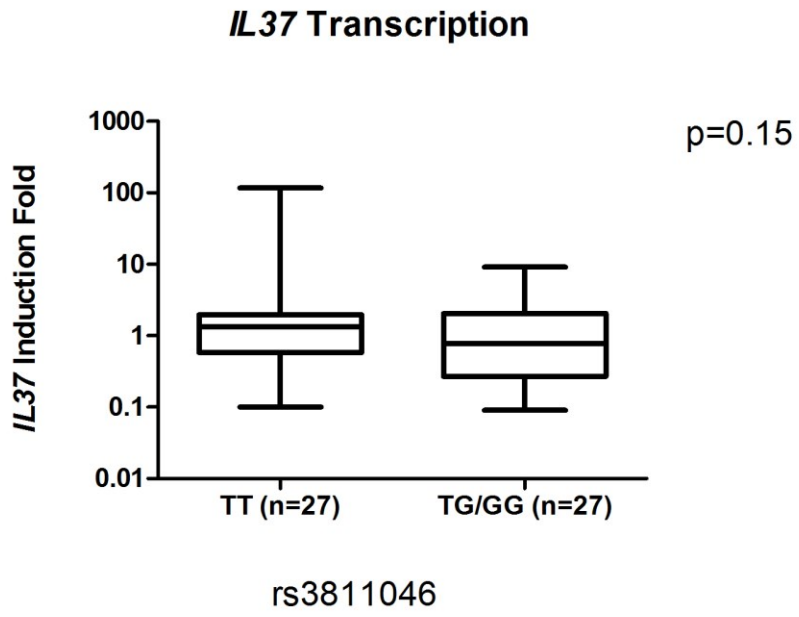

Figure 2B:

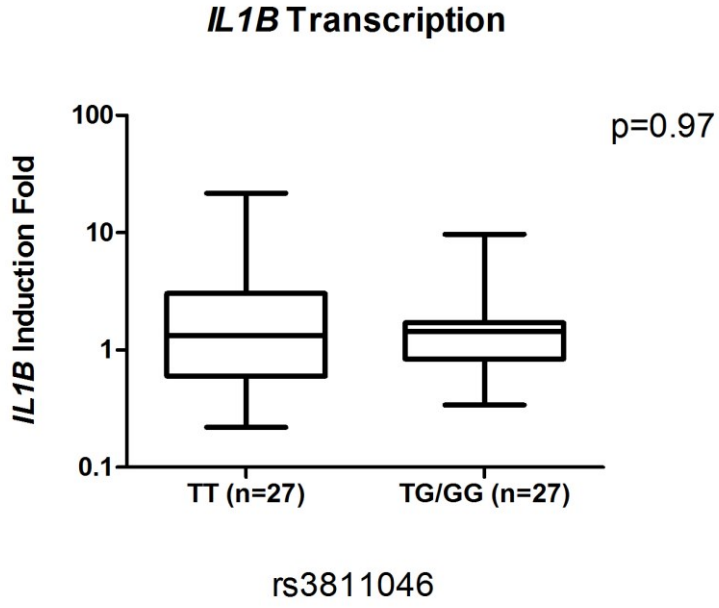

Figure 2C:

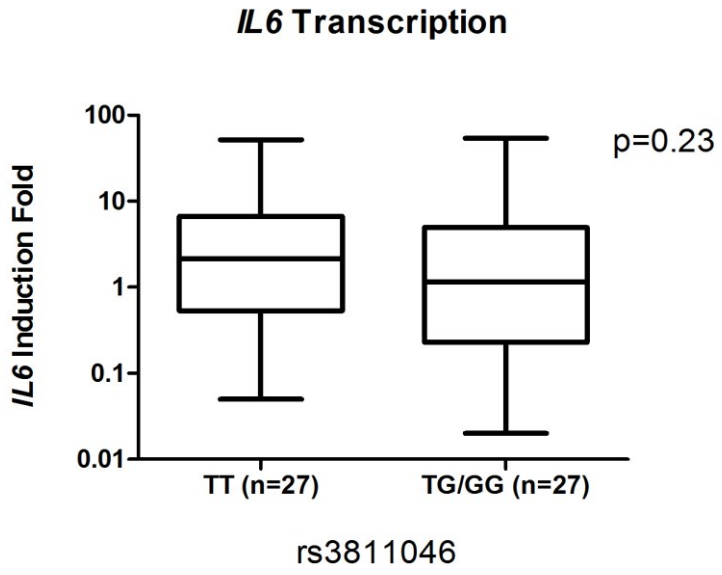

Figure 3. Transcription of *IL37*, *IL1B*, and *IL6* in samples with different genotypes at the rs3811046 locus of *IL37*. The transcriptional levels of *IL37* (Figure 2A), *IL1B* (Figure 2B), and *IL6* (Figure 2C) in samples with TT (1.1) genotype were not significantly different from samples with TG/GG (1.2/2.2) genotypes (p=0.15, p=0.97, and p=0.23, respectively). Wilcoxon-Mann-Whitney rank test was used.

Figure 3A:

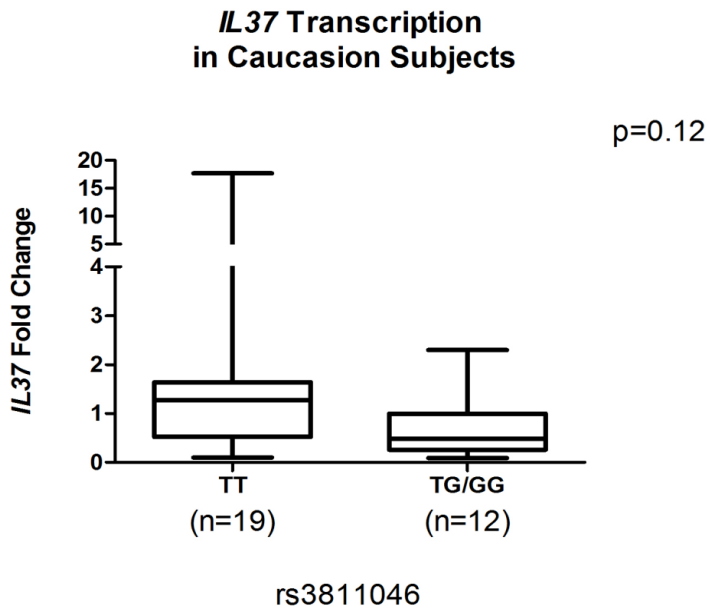

Figure 3B:

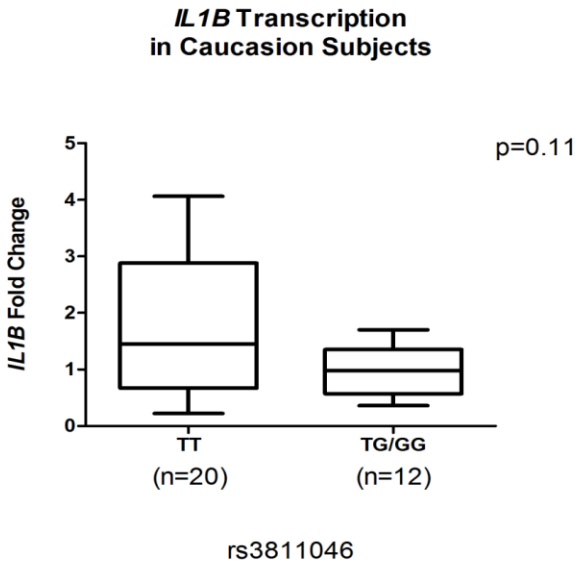

Figure 3C:

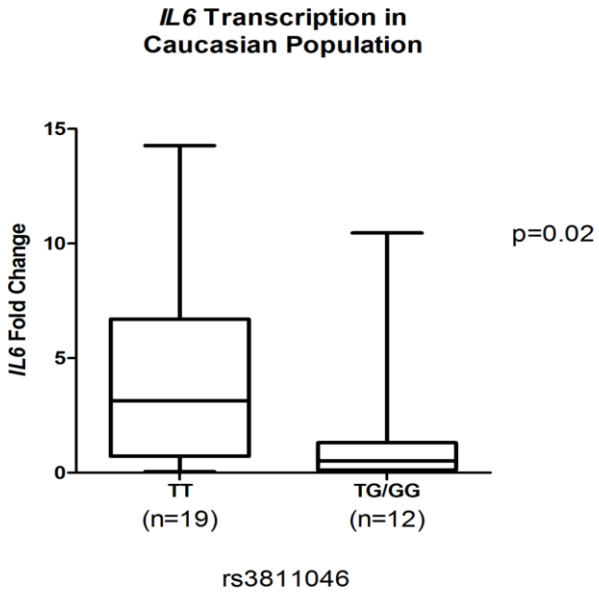

Figure 3. Transcription of *IL37*, *IL1B*, and *IL6* in samples with different genotypes at the rs3811046 locus of *IL37* from Caucasian participants. The transcriptional levels of *IL37* (Figure 3A) and *IL1B* (Figure 3B) in samples with TT (1.1) genotype were not significantly different from samples with TG/GG (1.2/2.2) genotypes (p=0.12 and p=0.11, respectively), while the messenger level of *IL6* in samples with TT (1.1) genotype was significantly higher than samples with TG/GG genotypes (p=0.02, Figure 3C). The sample size included for each cytokine analysis was slightly different in chronic periodontitis group due to the exclusion of a failed sample in the real-time PCR experiment, which resulted in a "non detected" CT value. Wilcoxon-Mann-Whitney rank test was used.

Figure 4A:

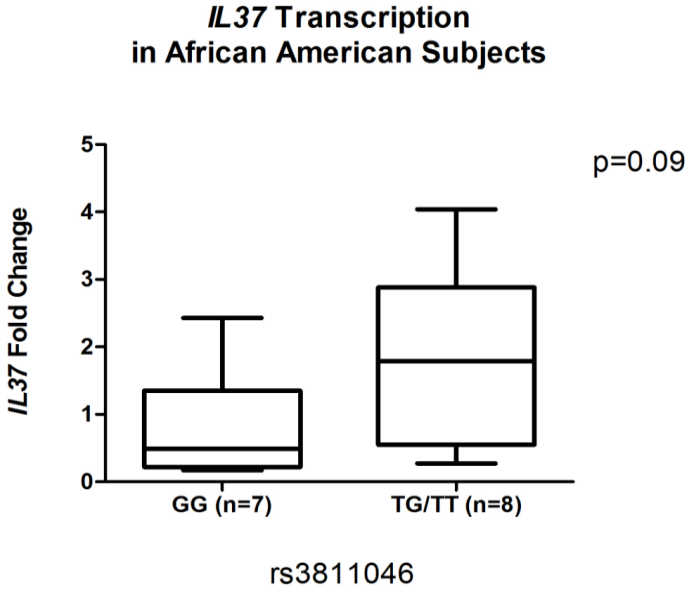

Figure 4B:

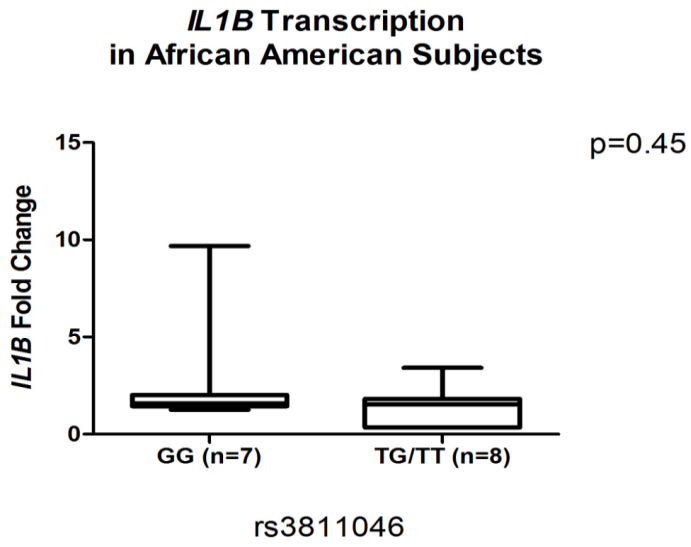

Figure 4C:

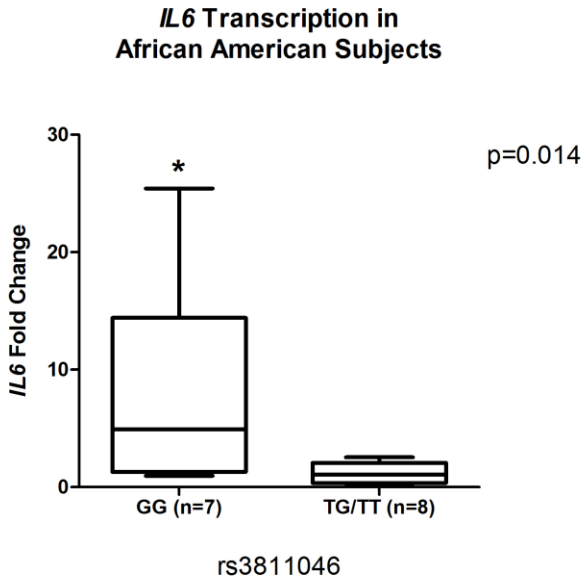

Figure 4. Transcription of *IL37*, *IL1B*, and *IL6* in samples with different genotypes at the rs3811046 locus of *IL37* from African American participants. The transcriptional levels of *IL37* (Figure 4A) and *IL1B* (Figure 4B) in samples with GG (2.2) genotype were not significantly different from samples with TG/TT (1.2/1.1) genotypes (p=0.09 and p=0.45, respectively), while the messenger level of *IL6* in samples with GG (2.2) genotype was significantly higher than samples with TG/TT (1.2/1.1) genotypes (p=0.0014, Figure 4C). Wilcoxon-Mann-Whitney rank test was used.

Figure 5A:

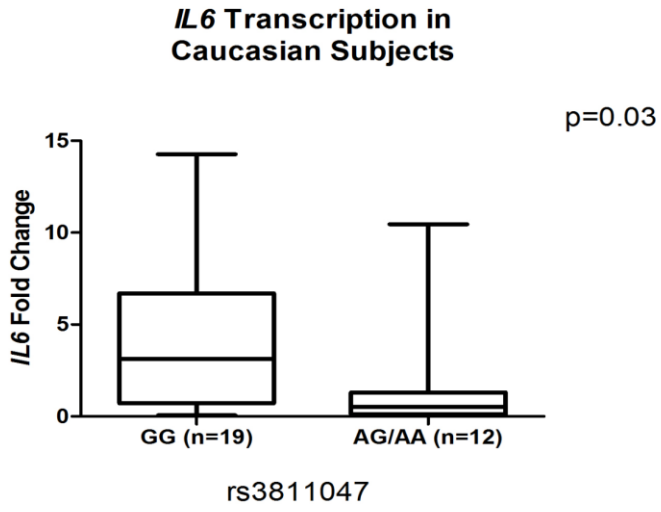

Figure 5B:

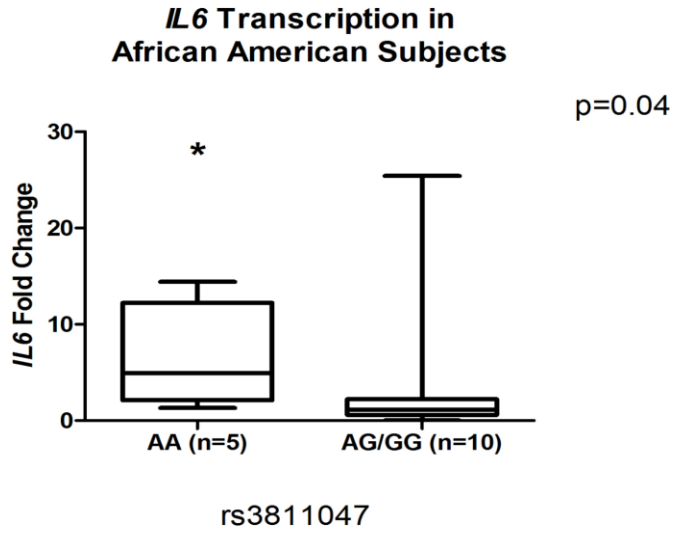

Figure 5. Transcription of *IL6* in samples with different genotypes at the rs3811047 locus of *IL37* from Caucasian and African American participants. The transcriptional level of *IL6* in samples with GG (1.1) genotype was significantly higher than samples with GA/AA (1.2/2.2) genotypes in Caucasian participants (p=0.03, figure 5A); the messenger level of *IL6* in samples with AA (2.2) genotype was significantly higher than samples with AG/GG (2.1/1.1) genotypes in African American participants (p=0.04, Figure 5B). Wilcoxon-Mann-Whitney rank test was used.

Figure 6A:

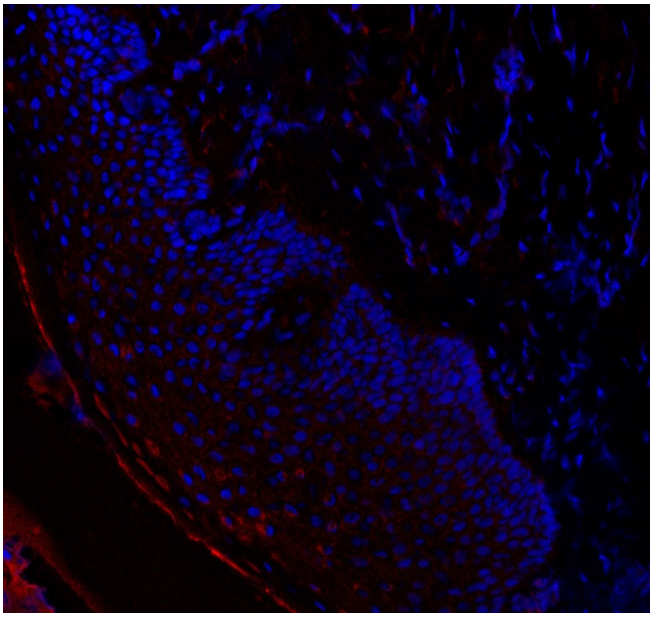

Figure 6B:

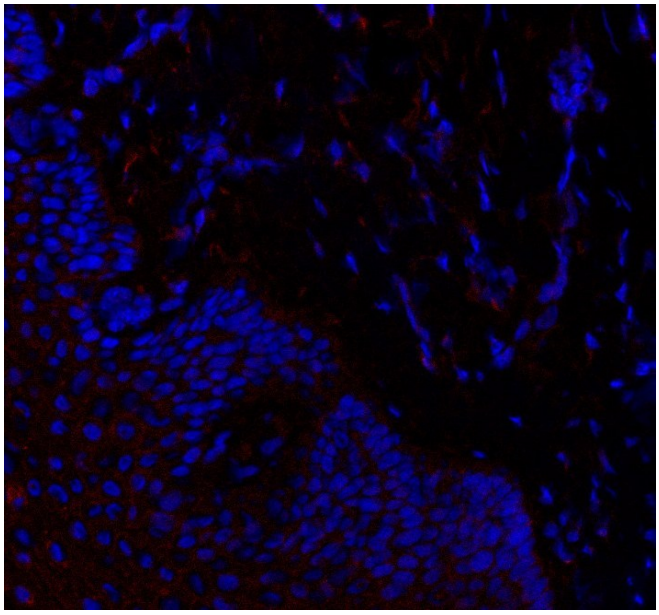

Figure 6C:

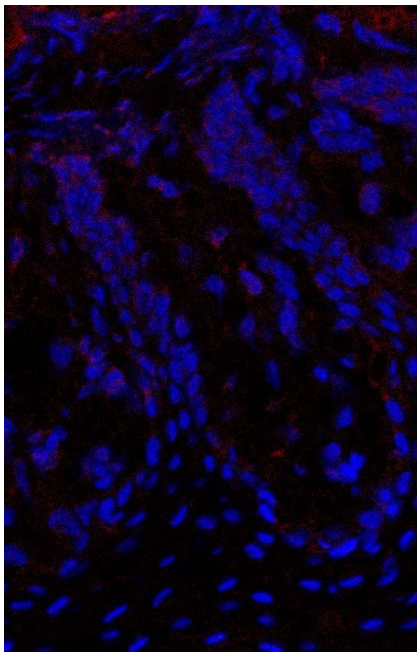

Figure 6. Immunofluorescence staining of IL-37 in gingival biopsies. One sample collected from is chronic periodontitis patient is presented with different magnifications (Figure 6A and 6B); another sample collected from periodontal health is presented in Figure 6C. In both samples, staining of IL-37 was more prevalent in the cytoplasm of epithelial cells than the connective tissue in the sub-epithelial compartment.

Figure 7A:

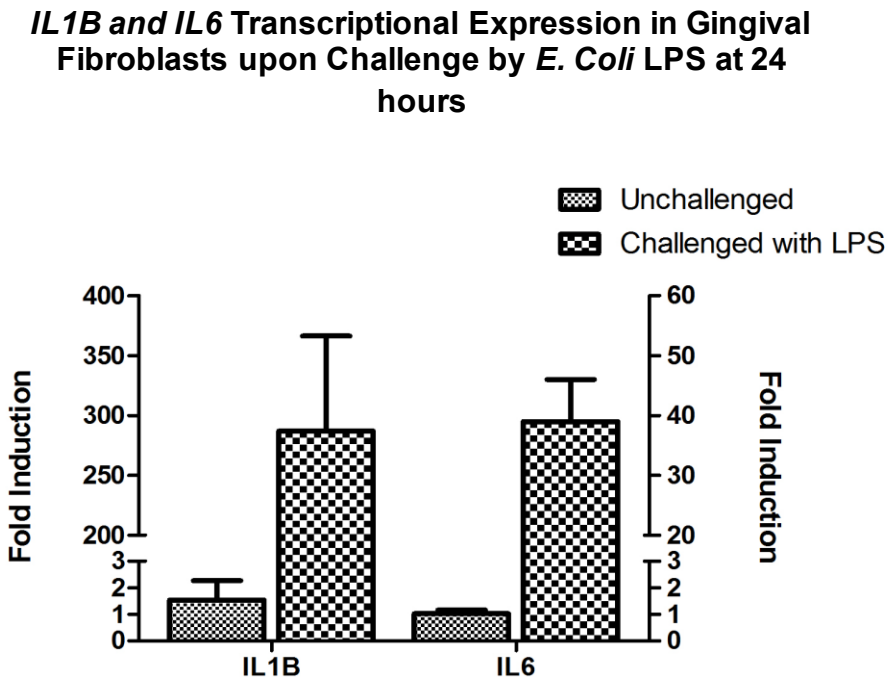

Figure 7B:

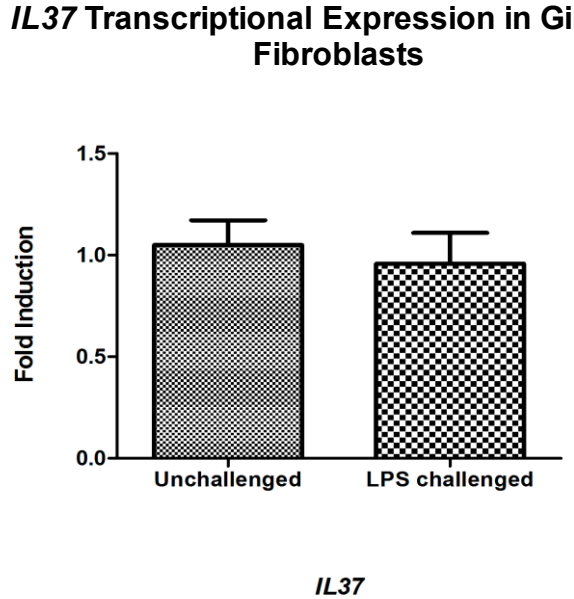

Figure 7. Transcription of *IL1B*, *IL6* and *IL37* in gingival fibroblasts upon challenge with *E.Coli* LPS (1µg/ml) at different time points. The induction of *IL1B* and *IL6* transcription was 278 fold and 40 fold, respectively, 24 hours after stimulation of LPS (Figure 7A). The transcription of *IL37* was unchanged in fibroblasts 24 hours after challenge (Figure 7B).

Figure 8A:

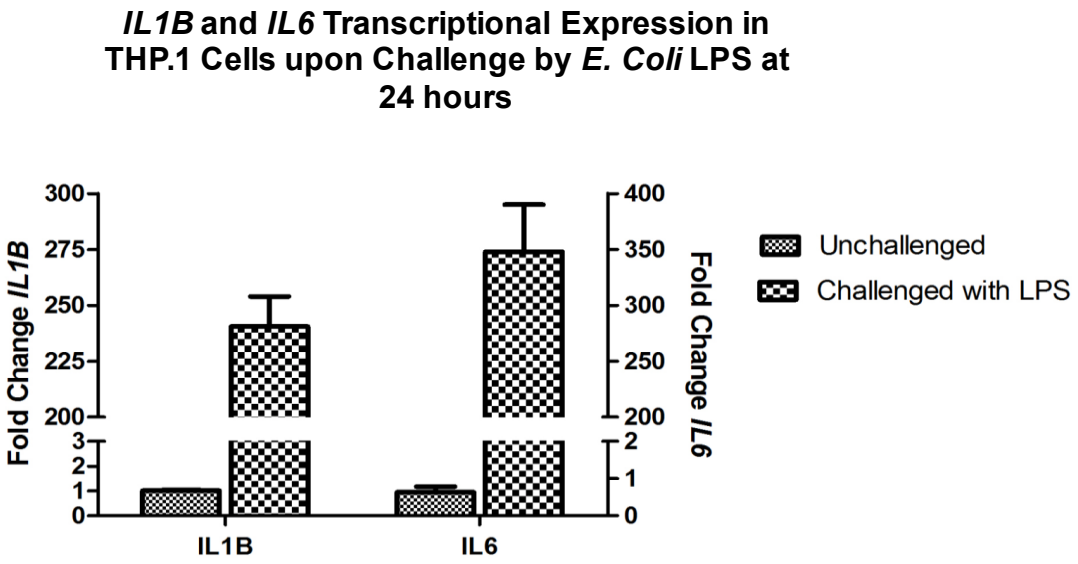

Figure 8B:

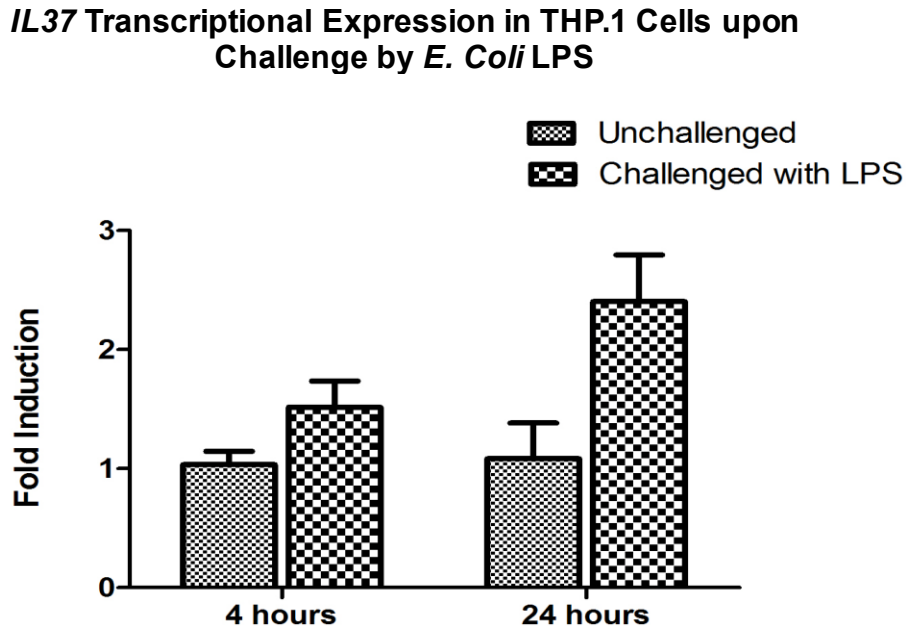

Figure 8. Transcription of *IL1B*, *IL6* and *IL37* in THP.1 cells upon challenge with *E.Coli* LPS (1µg/ml) at different time points. The induction of *IL1B* and *IL6* transcription was 240 fold and 274 fold, respectively, 24 hours after stimulation of LPS (Figure 8A). The transcription of *IL37* was induced about 1.7-fold at 4 hours and 2.3-fold at 24 hours after challenge with LPS in THP.1 cells (Figure 8B).

Figure 9A:

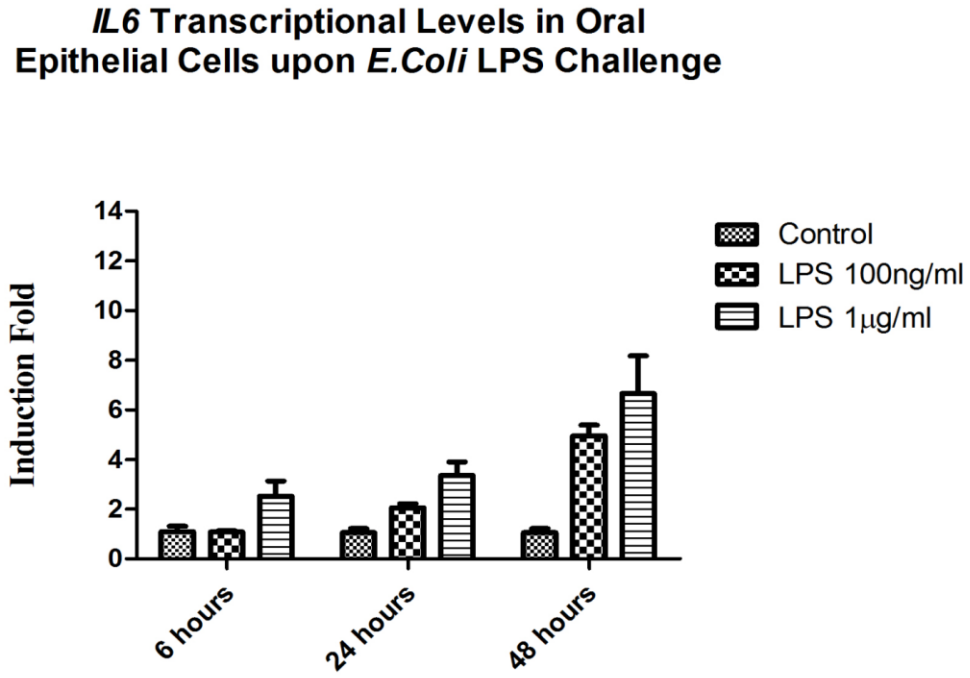

Figure 9B:

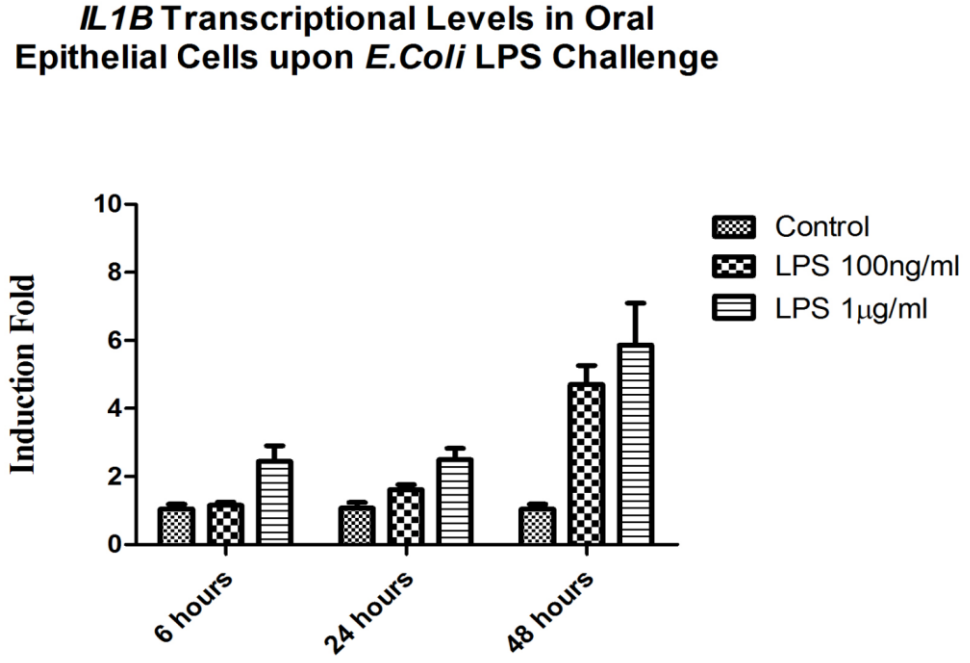

Figure 9C:

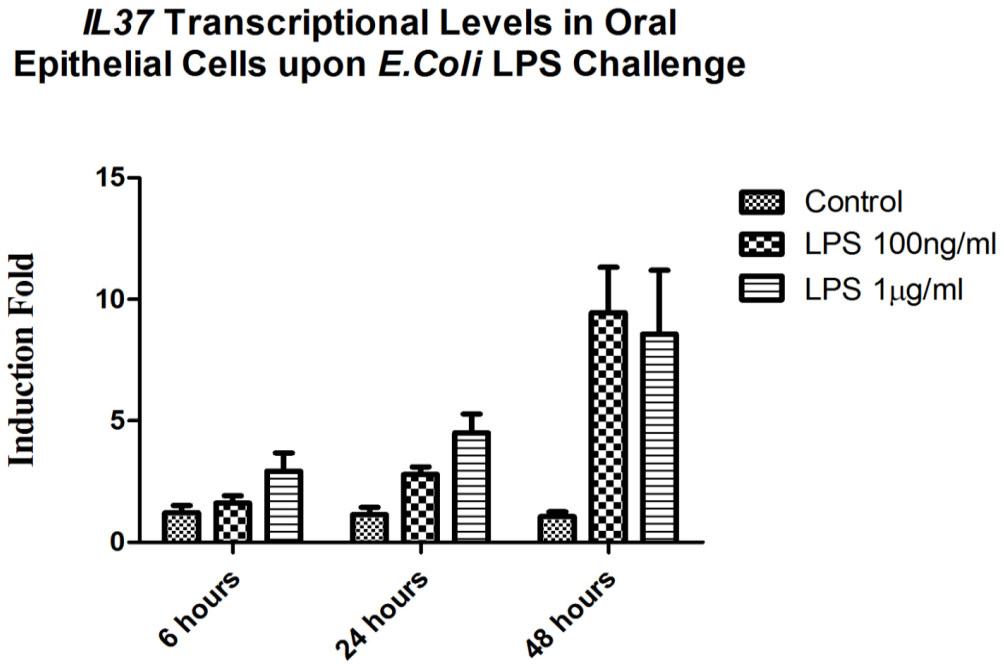

Figure 9. Transcriptional expression of *IL6*, *IL1B* and *IL37* in an oral epithelial cell line (HGEPp) challenged by *E. Coli* LPS (100mg/μl and 1μg/μl) at different time points. Transcription of both *IL6* and *IL1B* exhibited a time- and dose- dependent induction upon challenge by LPS (Figure 9A, and 9B). The transcription pattern of *IL37* presented similar kinetics as *IL1B* and *IL6*.

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