THE ROLE of MORN2 AND TRAF3IP2 GENETIC VARIANTS IN MODULATING INNATE IMMUNE RESPONSE TO PERIODONTAL PATHOGENS

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ABSTRACT

Lu Sun: The role of MORN2 and TRAF3IP2 genetic variants in modulating innate immune response to periodontal pathogens
(Under the direction of Steven Offenbacher)

Periodontal disease is a polygenic disease that is associated with inflammatory response to the oral biofilm. Although it is believed that microbial pathogens are necessary to the causal pathway, a key factor to determine whether individuals will develop periodontitis is the way how the hosts respond to the microflora resides in their periodontium. Genetic polymorphisms that will cause a change in the encoded protein or its expression, which may alter host innate or adaptive immunity, such as host barrier function and inflammatory responses, to microorganism and determine susceptibility to inflammatory disease. Thus it is necessary to seek candidate genetic function to explain the differences in susceptibility to periodontal disease.

Our recent genome-wide association study (GWAS) identified a missense single nucleotide polymorphism SNP (rs3099950) in the gene *MORN2* that codes for a key membrane protein. We have also identified a missense SNP (rs13190932) in the gene *TRAF3IP2* locus which is involved IL-17 signaling. These two genetic variants are associated with a *P.gingivalis* (*P.g*) dominant and *A. actinomycetemcomitans* (*A.a*) dominant periodontal disease subtypes. To date, only one study has described the function of MORN2 that serves as a macrophage protein that promotes the phagocytosis and killing of organisms via LC3-asscociated phagocytosis. TRAF3IP2 is involved in IL-17 signaling and mucosal immunity serving as an adaptor protein for the IL-17 receptor. However, the role of MORN2 and TRAF3IP2 in periodontal disease is unknown.

In Chapter 1, the background of periodontal disease and how the SNPs were identified by GWAS were described. In Chapter 2, we investigated the role of MORN2 in modulating innate immune response to periodontal pathogens and our studies suggest that MORN2 plays a critical role in the LC3-associated

phagocytosis – mediated killing of periodontal pathogen (*P.g*) as well as cytokine/chemokine response through Ca^{2+} flux and NF-kB activation. In Chapter 3, we explored the role of TRAF3IP2 mediated IL-17 pathway in periodontal disease. Our study suggests that TRAF3IP2 engaged homeostatic IL-17 pathway plays a protective role in a *P.g* induced alveolar bone loss and colonization through neutrophil recruitment, maintenance epithelial barrier and induction of antimicrobial peptides. Defective TRAF3IP2 shifts the oral commensal communities. Finally in Chapter 4, we discuss the significance and future study directions. In summary, these findings clarify the molecular mechanism of *MORN2* and *TRAF3IP2* function and provide insight into the genetic basis of *MORN2* and *TRAF3IP2* in periodontal disease susceptibility.

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I was fortunate enough to be accepted into the Oral and Craniofacial Biomedicine program at UNC-CH and had a chance to work with Dr. Offenbacher. The first time I knew him is from the term periodontal medicine, as first suggested by Dr. Offenbacher, when I was a dental student. I would like to give my ardent gratitude to my great mentor – Dr. Steven Offenbacher. I feel extremely lucky to be your Ph.D. student for the past four years. Your enthusiasm for science, foresight of research, great diligence for work never stop to inspire and enlighten me. Your smile, your hello, your email and even your view of back is always great encouragement to me to move forward. It is my cherished experience during discussing our studies and exchanging ideas or thoughts. Thank you Dr. Offenbacher for your guidance, patience and countless support and I really appreciate that you accompany with me during this journey. I am grateful to my committee members, Dr. Toni Darville, Dr. Jennifer Webster-Cyriaque, Dr. Mitsuo Yamauchi, Dr. Sompop Bencharit, who afford constructive comments and encouragement for my dissertation, which always keeping me on the right track.

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At last, I want to thank my parents for their endless love and you are my strength and energy for trusting my heart, fighting against obstacles, winning the battle and pursuing my dream.

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A.a	*Aggregatibacter actinomycetemcomitans*
Act1	NF-κB activator 1
AMPs	Antimicrobial peptides
AP-1	Activator protein 1
APC	Antigen presenting cell
ARIC	Atherosclerosis Risk in Communities
BOP	Bleeding on probing
BSA	Bovine serum albumin
CAL	Clinical attachment loss
CCL6	Chemokine (C-C motif) ligand 6
CCR2	C-C chemokine receptor type 2
CD68	Cluster of differentiation 68
CIKS	Connection to IKK and SAPK/JNK
CMC	Carboxymethyl cellulose
C.r	*Campylobacter rectus*
CXCL1	C-X-C motif ligand 1
CXCL2	C-X-C motif ligand 2, MIP2-alpha
CXCL3	C-X-C motif ligand 3, MIP2-beta
CXCL5	C-X-C motif ligand 5
DAMP	Damage associated molecular signal

DARIC Dental Atherosclerosis Risk in Community Study

DEFB1 Defensin beta 1

DEFB4 Defensin beta 4

DSC-1 Desmocollin-1

DSG-1 Desmoglein-1

FBS Fetus bovine serum

F.n *Fusobacterium nucleatum*

FILAGGRIN Filament aggregating protein

GAPDH Glyceraldehyde-3 phosphate dehydrogenase

GCF Gingival crevicular fluid

GCP-2 Granulocyte chemotactic protein, CXCL6

G-CSF Granulocyte colony-stimulating factor

GM-CSF Granulocyte macrophage colony-stimulating factor

GWAS Genome-wide association study

HGEs Human gingival epithelial cells

IFN-γ Interferon-γ

IL-1 Interleukin-1

IL-1β Interleukin 1 beta

IL-4 Interleukin 4

IL-6 Interleukin 6

IL-8 Interleukin 8

IL-17 Interleukin 17

IL-17R	Interleukin-17 receptor
IL-23	Interleukin 23
IL-36G	Interleukin-36 gamma
IP3	Inositol trisphosphate 3
JNK	Jun amino-terminal kinases
KLF4	Kruppel-like factor 4
KRT1	Keratin 1
KRT10	Keratin 10
LAP	LC3-associated phagocytosis
LCN2	Lipocalin 2
LPS	Lipopolysaccride
MAF	Minor allele frequency
MAPK	Mitogen-activated protein kinases
MCP-1	Monocyte chemoattractant protein 1, CCL2
MIP-1α	Macrophage inflammatory protein 1 alpha, CCL3
MMP3	Matrix metallopeptidase 3
MMP9	Matrix metallopeptidase 9
MORN2	Membrane Occupation and Recognition Nexus repeat-containing-2
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NLRs	Nod like receptors
OCLN	Occludin
PAMP	Pathogen-associated molecular pattern

PCR Polymerase chain reaction

PCT Principle component trait

PD Probing depth

P.g *Porphyromonas gingivalis*

P.i *Prevotella intermedia*

P.n *Prevotella nigrescens*

PIP2 Phosphatidylinositol 4,5-bisphosphate

PRR Pattern recognition receptors

qRT-PCR Quantitative reverse transcription PCR

RANKL Receptor activator of nuclear factor kappa-B ligand

RANTES Regulated on activation, normal T cell expressed and secreted, CCL5

RORγ RAR-related orphan receptor gamma

RUNX2 Runt-related transcription factor 2

S100A8 S100 calcium binding protein A8

S100A9 S100 calcium binding protein A9

SEFIR SEF/IL-17R signaling motif

SNP Single nucleotide polymorphism

STAT3 Signal transducer and activator of transcription 3

T.d *Treponema denticola*

T.f *Tannerella forsythia*

TLR Toll-like receptor

TNF-α Tumor necrosis factor α

TRAF3IP2 Tumor necrosis factor-Receptor Associated Factor 3 Interacting Protein 2

Treg Regulatory T cell

Th1 Type 1 T helper cells

Th2 Type 2 T helper cells

Th17 T helper 17 cells

ZNF750 Zinc Finger Protein 750

CHAPTER 1: INTRODUCTION

Periodontitis is an inflammatory response to the oral bacterial flora and represents one of the most prevalent infectious disease in human worldwide. Approximately 11% of the world population (Richards 2014) exhibits severe periodontitis and 46% United States adults, representing 64.7 million people, had periodontitis, with 8.9% having severe periodontitis (Eke, Dye et al. 2015). Periodontitis is well characterized by local tissue destruction that involves loss of alveolar bone and supporting ligament around the teeth, and if left untreated, can lead to loss of teeth. Increasing evidence suggests that the chronic inflammation of periodontitis represents potential risk factor for association with various systemic disease including adverse pregnancy, cardiovascular disease, rheumatoid arthritis, stroke as well as diabetes (Offenbacher and Beck 2014, Hasturk and Kantarci 2015, Duda-Sobczak, Zozulinska-Ziolkiewicz et al. 2018, Sen, Giamberardino et al. 2018).

Although pathogenic bacteria are assumed to be essential to the destruction in host tissues, they are not sufficient serve as a strong predictor of periodontal disease, for the reason that specific microorganisms, in most cases, are not sufficient to cause disease. Thus, periodontitis is considered as a multifactorial oral disease that involves a susceptible host influenced by genetic factors, dysbiotic oral microbial shift and other factors such as smoking(Haffajee and Socransky 2001, Merchant, Pitiphat et al. 2002). One of key factors to determine whether individuals will develop periodontitis is the way how the hosts respond to the microflora resides in their periodontium (Taba, Souza et al. 2012). Genetic polymorphisms that will cause a change in the encoded protein or its expression that may alter host innate or adaptive immunity and/or microbial colonization patterns, which results in the manifestation as the clinical phenotype of disease. For example, genetic mutations within the NLRP6-inflammasome complex results in alteration of the colonic microbial ecology and the inflammatory pathways associated with colitis (Elinav, Strowig et al. 2011). Thus, it is understood that genetic variants in inflammatory pathways can induce dysbiotic shifts in the microbiome that combine to create chronic inflammatory conditions at mucosal surfaces including periodontal disease(Walker, Sanderson et al. 2011, Craven,

Egan et al. 2012, Darveau, Hajishengallis et al. 2012, Hajishengallis, Darveau et al. 2012, Hajishengallis and Lamont 2012, Henao-Mejia, Elinav et al. 2012). However, we cannot identify these implicit variants based on clustering of clinical signs (probing depth, attachment loss, bleeding index) alone. There are also several lines gained from family studies to support the genetic role in determining the predisposition of periodontitis. Familial aggregation of aggressive periodontitis is not unusual in clinic and a pilot study of the aggregation within families also suggests a genetic predisposition(Nibali, Donos et al. 2008). Case-control (Schaefer, Jochens et al. 2014, Guedes, Planello et al. 2015) and cross-sectional study (Casado, Aguiar et al. 2015) exploring candidate genes have also demonstrated association of gene polymorphic variants with periodontitis. These candidate genes include Interleukin-1 (Trevilatto, de Souza Pardo et al. 2011, Braosi, de Souza et al. 2012, Armingohar, Jorgensen et al. 2014), Matrix Metalloproteinase (Song, Kim et al. 2013, Emingil, Han et al. 2014), Fc receptor (Hans, Mehta et al. 2015), TNF-α (Ozer Yucel, Berker et al. 2015) and pattern recognition receptor (Han, Ding et al. 2015) in periodontitis could be chosen based on previously association study with other types of chronic inflammatory disease, such as inflammatory bowel disease and rheumatoid arthritis, or their relationship with immune response. However, these studies explored only one locus or several loci and some candidate gene association studies performed with contradictory results (Laine, Loos et al. 2010, Zhang, Sun et al. 2011, Laine, Crielaard et al. 2012) because of small cohort size of subjects involved, limited number of loci tested and the lack of power and replication. Thus, the identification of considerable genetic risk variants that contributes to periodontitis needs a large population that can detect small effects given by genetic variant.

Genome wide associate study (GWAS) is a powerful tool to uncover millions of variations in genomic DNA at one time to determine whether any genetic locus is associated with disease phenotype (Loos, Papantonopoulos et al. 2015). Unlike candidate gene association study, GWAS is an open-ended method and not based on a prior knowledge of disease pathogenesis and phenotypes. Single nucleotide polymorphisms (SNPs) are important genetic markers that link DNA variants to phenotype changes. The SNP identified by GWAS covering the entire human genome including coding regions and regulatory regions. By testing all common variants, we could highlight key genes and shed light on the underlying mechanisms. Recently, GWAS of periodontitis have been performed by our group (Divaris, Monda et al. 2012, Divaris, Monda et al. 2013) and other groups (Teumer, Holtfreter et al. 2013, Lee and Kim 2015, Shimizu, Momozawa et al. 2015) and highlighted loci might be associated with this type of prevalent

disease. However, these association studies does not meet strict genome-wide significance criteria and could only illustrate a small portion of the total population variance, which indicates the need to explore the gene centric of this condition by using more distinct clinic parameters.

Recently, our group create distinct periodontal complex traits (PCT) by using principle component analysis to interrogate in GWAS analysis to identify novel potential candidate genetic loci related to the biological basis and pathogenesis of chronic periodontitis. Specifically, we have completed a genome-wide association study of 975 European American adult individuals [394 (40%) subjects with periodontal health, which includes subjects with gingivitis, 389 (39.9%) subjects with mild-moderate periodontitis and 192 (19.7%) subjects with severe periodontal disease] selected from Dental Atherosclerosis Risk in Communities (DARIC) cohort (Beck, Elter et al. 2001, Elter, Champagne et al. 2004) including eight periodontal pathogens [*P. gingivalis (P.g), Prevotella intermedia (P.i), T. denticola (T.d), T. forsythia (T.f), C. rectus (C.r), Fusobacterium nucleatum (F.n), A. actinomycetemcomitans (A.a), P. nigrescens (P.n)*], inflammatory mediator in gingival crevicular fluid (interleukin1-β) and clinical disease classification (CDC/AAP chronic periodontitis classification) as well as probing depth and interproximal attachment loss. The principle component profile illustrating correlations with the constitutive parameters was shown in Figure 1.1. The first PCT was referred as the Socransky Trait since the microbial community structure in PCT1 has a high correlation and positive loading with all eight periodontal organisms that associated with microbial clusters identified by Socransky et al (Socransky, Haffajee et al. 1998). PCT3 has a high positive loading of *A. actinomycetemcomitans* and GCF-IL-1β and is designated as *A.a* trait. PCT5 is characterized by the highest loading of *P. gingivalis* with an eigenvalue of 0.882 (*P.g* trait) across all PCTs. Significant gene-centric associations were found in the first six complex traits by using MAGENTA. Genes in loci associated with PCT1 (Socransky trait), PCT3 (*A.a* trait) and PCT5 (*P.g* trait) suggest a biological basis that controls the epithelial barrier function and innate immune response (Figure 1.2).

Interestingly, there are two genes with nonsynonymous substitutions emerge as potential candidates for regulating immune response in PCT3 and PCT5. The *MORN2* locus within PCT5 contains a missense SNP that is significantly associated with the highest level of *P.g* counts and severe periodontal disease clinical scores. MORN2 was identified as a phagosome protein of macrophage that is involved in the recruitment of LC3 in phagocytosis to *M. tuberculosis*-containing phagosomes and subsequent maturation to degradative phagolysosomes (Abnave, Mottola et al. 2014). Our structural

analysis of *MORN2* variant demonstrates that *MORN2* variant changes the charge distribution across a PIP3 binding region of *MORN2* structure that may result in impaired membrane interactions or phagolysosome fusion. The *A.a* trait (PCT3) showed one gene *TRAF3IP2* (*Act1*) contains a non-synonymous SNP that is significantly associated with the higher levels of *A.a* and severe periodontal disease scores, which point to potential abnormalities in the innate immune response. The paradigm that T helper 1 (Th1) cell and T helper 2 (Th2) cell characterized by the secretion of distinct cytokines was first postulated by Coffman, Mosmann and colleagues in 1986 (Mosmann, Cherwinski et al. 1986). Naïve $CD4^+$ T cells can differentiate into either interferon-γ (IFN-γ)-producing Th1 cells or IL-4-producing Th2 cells when they first encounter foreign antigens presented by antigen-presenting cells (APCs), which is largely controlled by various environmental factors, especially by signals coming directly from APCs (Glimcher and Murphy 2000). Uncontrolled T cell responses can drive the onset of allergy or autoimmunity such as inflammatory bowel disease (IBD), rheumatoid arthritis (RA) or psoriasis (Bouma and Strober 2003, Lowes, Bowcock et al. 2007). Substantial advances and efforts have been made to resolve some complicated pathological situations that cannot be explained by simple Th1/Th2 paradigm, which resulted in the expanded research and the discovery of a third subset of effector T cells that produce IL-17 and exhibit distinct functions from Th1 and Th2 cells. It was not until 2005 that investigators proposed that IL-17-producing T cell, a distinct $CD4^+$ T helper cell subset that are critically responsible for the production of IL-17, which was named Th17 cells (Harrington, Hatton et al. 2005, Park, Li et al. 2005). IL-17 have pro-inflammatory properties (Kolls and Linden 2004) and act on different cell types to induce the gene expression of cytokines, chemokines and antimicrobial peptides (Jovanovic, Di Battista et al. 1998, Kolls and Linden 2004, Abusleme and Moutsopoulos 2017). In 2000, ACT1 was first identified as a protein that activates both NF-κB and JNK constitutively, which indicates that it functions at a point before bifurcation of these two signal pathways (Li, Commane et al. 2000). In the same year, Leonardi's group identified CIKS (connection to IKK and SAPK/JNK) may represent a point of multiple points convergence in AP-1 and NF-κB pathways (Leonardi, Chariot et al. 2000). Later, Novatchkova's group identified ACT1 and CIKS were the same protein and a conserved sequence segment in transmembrane receptors and soluble factors (like CIKS/ACT1) in eukaryotes – SEFIR domain (Novatchkova, Leibbrandt et al. 2003). Bioinformatics analysis identified this conserved motif in the cytoplasmic domains of IL-17R that was homologous to the Toll/IL-1R (TIR) domain and named the conserved region as SEFIR domain (O'Quinn,

Palmer et al. 2008). Evidence that IL-17RA and ACT1 interact based on overexpression studies and co-immunoprecipitation studies with endogenous protein (Chang, Park et al. 2006, Maitra, Shen et al. 2007, Qian, Liu et al. 2007). Indeed, ACT1 deficiency by RNAi-mediated knockdown or in knockout mice impairs IL-17 induced activation of NF-κB (Linden 2007, Dong 2008). It has been shown that homozygous deletion of IL-17RA abrogates the increase in splenic neutrophil progenitors resulting from the overexpression of IL-17A (Ye, Rodriguez et al. 2001). IL-17RA$^{-/-}$ mouse fibroblasts fail to produce CXCL1 production in response to IL-17A and in human epithelial cells, a monoclonal antibody against IL-17RA effectively blocks both IL-17A and IL-17F induced expression of G-CSF and CXCL1 (McAllister, Henry et al. 2005). IL-17RA deficient mice are susceptible to many pathogens such as *Bacteroides fragilis*, *Candida albicans*, *Klebsiella pneumoniae* and *Toxoplasmosis gondii* (Chung, Kasper et al. 2003, Huang, Na et al. 2004, Kelly, Kolls et al. 2005, Ouyang, Kolls et al. 2008) and *P.g*-driven bone destruction is increased in IL-17RA knockout mice (Yu, Ruddy et al. 2007). Collectively, these data indicate that TRAF3IP2 is critical component involved in IL-17 signaling and mucosal immunity.

Thus, we hypothesize that *MORN2* and *TRAF3IP2* genetic variant may negatively modify the immune response to periodontal pathogen and influences dysbiotic shifts in the oral pathogen, which contributes to creation of chronic inflammatory conditions in periodontal disease. The objective of this study is to clarify the molecular mechanism of *MORN2* and *TRAF3IP2* function and to provide insight into the molecular and genetic basis of *MORN2* and *TRAF3IP2* in periodontal disease susceptibility.

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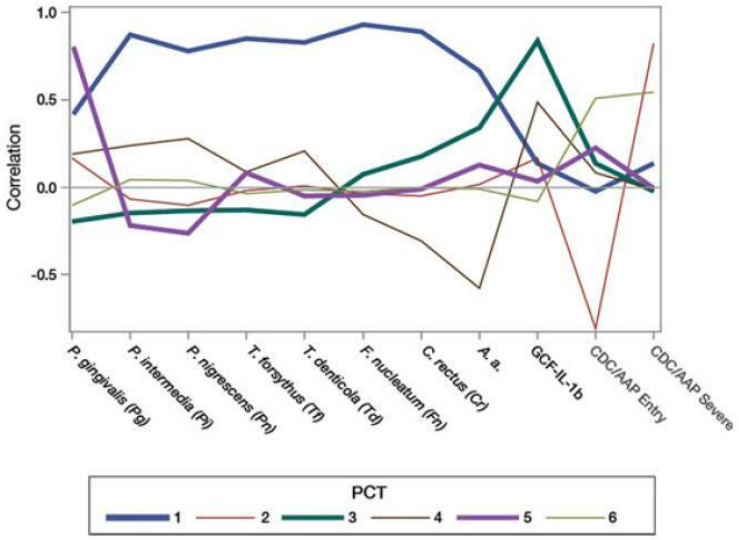


Figure 1.1. Principal component trait pattern profiles.

(Adapted from Offenbacher S, et al. Genome-wide association study of biologically informed periodontal complex traits offers novel insights into the genetic basis of periodontal disease. Hum Mol Genet. 2016 May 15;25(10): 2113-2129. Adapted with permission)

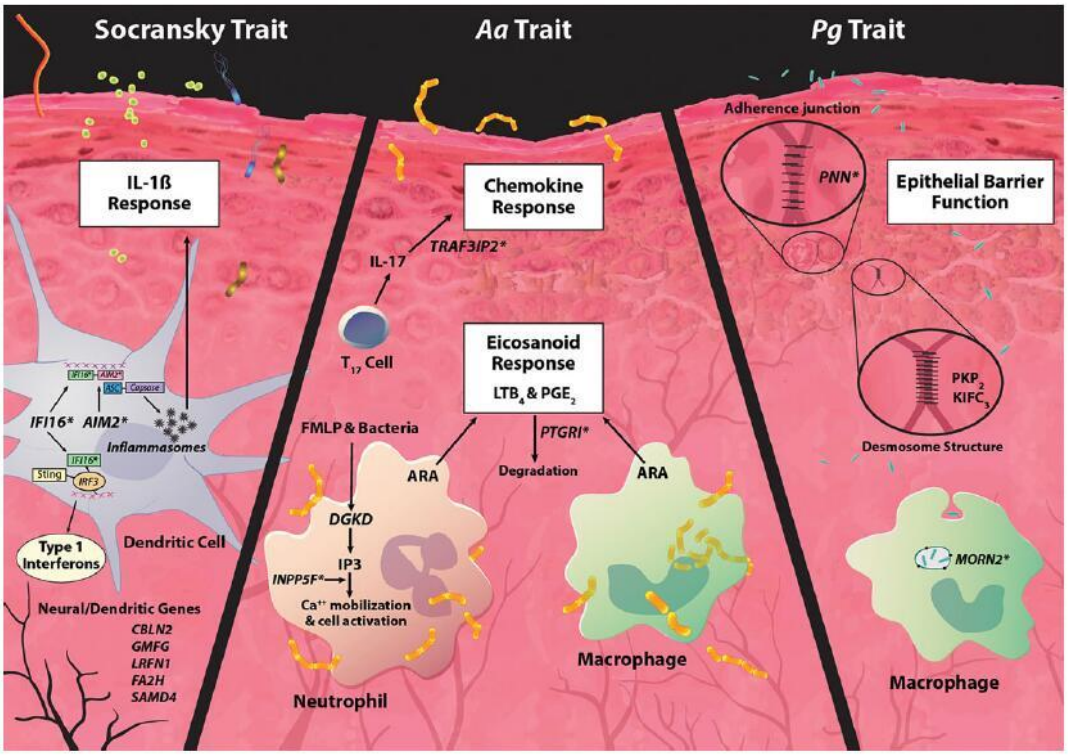


Figure 1.2. Model of genetic and microbial patterns of periodontal disease.

(Adapted from Offenbacher S, et al. Genome-wide association study of biologically informed periodontal complex traits offers novel insights into the genetic basis of periodontal disease. Hum Mol Genet. 2016 May 15;25(10): 2113-2129. Adapted with permission)

CHAPTER 2: THE ROLE of MORN2 GENETIC VARIANTS IN MODULATING INNATE IMMUNE RESPONSE TO PERIODONTAL PATHOGENS

2.1. Introduction

Periodontitis is a polygenic disease that represents one of the most prevalent infectious diseases worldwide that is associated with an exaggerated inflammatory response to the oral bacterial flora. Approximately 11% of the world population (Richards 2014) exhibit severe periodontitis and 46% of United States adults have periodontitis, with 8.9% having severe periodontitis (Eke, Dye et al. 2015). The disease is characterized by local tissue destruction that involves loss of alveolar bone and supporting ligament around the teeth, and if left untreated, can lead tooth loss. Although pathogenic bacteria are assumed to be essential to the destruction in host tissues, they are not a strong predictor of periodontal disease severity, suggesting that microbial challenge alone is not sufficient to cause disease. Thus, periodontitis is considered as a multifactorial oral disease with the host response playing a key factor to determine whether individuals will develop periodontitis, as well as the severity of the condition (Taba, Souza et al. 2012).

Genetic variants that will cause a functional change in the encoded protein may alter host barrier function and host innate or adaptive immunity that modifies inflammatory responses to microorganism to determine susceptibility and severity. There are several reported family studies that support the genetic role in determining the predisposition to periodontitis. Familial aggregation studies of aggressive periodontitis suggests a genetic predisposition (Nibali, Donos et al. 2008) and studies by Michalowicz provides the first estimates of heritability that suggested that about half (48%) of variance in disease expression in the population was attributable to genetics (Michalowicz, Diehl et al. 2000). Case-control (Schaefer, Jochens et al. 2014, Guedes, Planello et al. 2015) and cross-sectional (Casado, Aguiar et al. 2015) studies exploring candidate genes have also demonstrated association of gene polymorphic variants with periodontitis. Taken together, this evidence supports the concept that genetic alterations

controlling the immune response of the host can lead to alterations of microbial communities and predispose individuals to periodontal disease.

Genome-wide associate study (GWAS) of chronic periodontitis (CP) have been performed in an attempt to identify single nucleotide polymorphisms (SNPs) that either contribute to the pathogenesis and/or risk of developing periodontal disease (Divaris, Monda et al. 2013, Rhodin, Divaris et al. 2014, Schaefer, Jochens et al. 2014). However, no single marker association in these reports met strict genome-wide significance criteria, except four loci (*NIN/ABHD12B*, *WHAMM/AP3B2*, *KCNK1* and *DAB2IP*) met gene-centric statistical significance criteria (Divaris, Monda et al. 2013), which underscore the need to continue exploring for the genetic basis of this condition by using larger samples with more distinct clinic parameters and high-quality phenotypes.

Recently, our group created distinct periodontal complex traits by using principle component analysis (PCA) to interrogate in GWAS analysis to identify novel potential candidate genetic loci related to the biological basis and pathogenesis of chronic periodontitis (Offenbacher and Beck 2014). PCA was carried out among 975 participants including eight periodontal pathogens, gingival crevicular fluid IL-1β levels (GCF IL-1β) and clinical disease classification (CDC/AAP chronic periodontitis classification), which created distinct periodontal complex traits (PCTs) to interrogate in GWA. Each PCT defines a specific microbial community structure with varying levels of IL-1β. PCT5 (named *P.g* trait) is associated with the strongest emergence of *P.gingivalis* across all PCTs with a correlation coefficient of 0.882 followed by *A. actinomycetemcomitans* (0.134) and the correlation with *P.g* can be seen in (Table 2.1). Strikingly, *MORN2* locus contains a missense SNP (rs3099950) (Figure 2.1A), a novel loci significantly associated with disease within PCT5 as assessed by Meta-Analysis Gene-set Enrichment of variaNT Associations (MEGENTA) analysis. To our knowledge, the expression and function of MORN2 has not been reported in any disease. Only one study through RNAi screening of planarians identified MORN2 as being involved with phagocytosis and promoting the recruitment of LC3. LC3 (Microtubule-associated protein light chain 3) is a soluble protein distributed ubiquitously in mammalian tissues and cultured cells that is involved in autophagy, localizes to the phagosomal membrane to facilitate maturation of degradative phagolysosomes-mediated macrophage restriction of intracellular pathogens including *Mycobacterium tuberculo*sis, *L. pneumophila* and *S. aureus* (Abnave, Mottola et al. 2014).

In this report we demonstrate the first functional relevance of the identified gene *MORN2* associated with *Pg* dysbiosis and chronic periodontitis. We examine the gene-environment interaction of the *MORN2* risk locus with levels of periodontal microorganisms and disease severity. We perform structural analysis of the predicted conformational change induced by rs3099950. We further provide analysis of MORN2 protein expression in gingival samples obtained from healthy individuals and individuals with periodontitis and evaluate the role of MORN2 in macrophage clearance of periodontal micro-organisms and the innate immune response in gingiva epithelial cells and macrophages. Our findings suggest that *MORN2* might play a role in the LC3-associated phagocytosis mediated killing of *Pg* and cytokine/chemokine response by modulating Ca^{2+} flux and NF-kB activation. The function of MORN2 in mediated phagocytosis, *Pg* killing and innate inflammatory responses are demonstrated as a potential functional link between the *MORN2* genetic variant, *Pg* dysbiosis and periodontal disease.

2.2. Materials and methods

2.2.1. Reagents and antibodies

Puromycin was from Invitrogen. 3-methyladenine (3-MA) and Polybrene were from Sigma-Aldrich. Primary human gingival epithelia cells (pooled) were from CELLnTEC. RNA stabilization buffer was from Qiagen. Anti-rabbit HRP-DAB (CTS005, R&D SYSTEMS). ProLong® Diamond Antifade Mountant was from Life Technologies. Lysogeny Broth was from Thermo Fisher Scientific. Wilkins-Chalgren Anaerobe Broth was from Oxoid. Brucella Blood Agar was from Anaerobe Systems. Antibodies used in this study included the following: rabbit anti-MORN2 (ab188524, Abcam), rabbit anti-LC3 (#2775, Cell Signaling Technology), rabbit anti-phospho-IKKα/β (#2697, Cell Signaling Technology), rabbit anti-phospho-IκBα (#2859, Cell Signaling Technology), rabbit anti-phospho-NF-κB p65 (#3033, Cell Signaling Technology), rabbit anti-NF-κB p65 (#8242, Cell Signaling Technology), goat anti-beta Actin (Ab8229, Abcam), rabbit IgG polyclonal isotype control (ab27478, Abcam),

2.2.2. Bacterial strain

The bacteria strain *Porphyromonas gingivalis* 33277 was obtained from ATCC. Strains were characterized and tested for purity by colony forming on Brucella Agar plate (AS-141, Anaerobic System) and Gram staining kit according to the manufacture's instruction (212539, BD). All stocks were frozen in 10% skim milk at -80 ℃. Bacteria were grown in Wilkins-Chalgren Anaerobe Broth (CM0643, Oxoid) or Brucella Agar plate in an anaerobic chamber (Thermo Scientific) in an atmosphere of 85% N_2, 5% H_2, and 10% CO_2. An aliquot of the initial stock solution was used for each experiment without sub-culturing.

2.2.3. Cell culture

THP-1 human monocytic cell line were cultivated in suspension in RPMI-1640 medium (11875093, Gibco), supplemented with 10% fetal bovine serum (Corning) at 37 ℃, 5% CO_2. Culture medium was changed every 2nd to 3rd. THP-1 cells were differentiated into macrophages by treatment with 100 nM phorbol 12-myristate 13-acetate (PMA). Primary human gingival epithelia cells (pooled) were thawed by gentle swirling in water bath at 37 ℃ and adding necessary amount of CnT-Prime epithelial culture medium to the cell culture dish. CnT medium was changed 12 h after seeding to remove the

residual DMSO from the freezing medium. During routine cultivation, medium was changed every 2nd to 3rd day.

2.2.4. Bioinformatic approaches

The primary sequences of the wild type and a SNP variant of MORN2 were obtained from UniProtKB/Swiss-Prot accession number Q502X0 (http://www.uniprot.org/). The sequences were used to create a wild type and a SNP variant homology models using Phyre2 (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index) (Kelley, Mezulis et al. 2015). Functional significance of the SNP variant compared to the wild type including residue change, physico-chemical properties and variant position assessed using PyMol (1.7.4). The influence of SNP on the functional side chains on the protein surface was analyzed using the surface electrostatic potentials.

2.2.5. GWAS characterization of the *P.g* trait

Locus zoom plot for the MORN2 regions was created using the GWAS as described elsewhere (Offenbacher, Divaris et al. 2016) and http://locuszoom.org. Previously-reported GWAS data was used to examine the association of the lead *MORN2* locus polymorphism with levels of eight periodontal pathogens, *P.g* and *F.n*, extent of probing depth ≥ 4mm (EPD4) and interproximal attachment loss ≥5mm (IAL5).

2.2.6. *MORN2* expression analysis in gingival tissue by RT-qPCR

A total of twenty-six healthy subjects and nineteen chronic periodontitis subjects were recruited from the Department of Periodontology, University of North Carolina at Chapel Hill. Besides obtaining demographic information and periodontal clinical parameters, informed consent was obtained from each subject prior to enrollment. All procedures were approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Medical and dental histories of subjects were obtained followed by a periodontal examination. The gingival biopsies were obtained that represented interproximal samples that included epithelium and connective tissue, as well as inflammatory infiltrate, if present. The gingival biopsies were immediately placed in the RNA stabilization buffer (RNA later, Qiagen, Germany) for gene expression or fixed in 10% formalin for immunohistochemistry and immunofluorescence assays. Total RNA was extracted from the gingival tissue samples by using RNeasy Mini Kit (Qiagen, Germany) and cDNA was synthesized using the SuperScript VILO cDNA synthesis Kit (Thermo Fisher, USA)

according to the manufacturer's instructions. The total RNA concentration and purity of each sample were assessed by spectrophotometry using a NanoDrop1000. The *MORN2* (Taqman gene expression, ThermoFisher, USA) mRNA relative expression level was performed by quantitative PCR on StepOnePlus Real-Time PCR system (Applied Biosystems). The gene expression levels were calculated using ΔΔCT method and the *GAPDH* (Hs02786624_g1, ThermoFisher, USA) gene was used to normalize for cell count. Means of 2 technical replicates were generated for each of 2 biological replicates and these values were used for statistical analysis.

2.2.7. Immunohistochemistry and immunofluorescence of human gingival biopsies

Human gingival tissues were fixed in 10% neutral buffered formalin overnight at room temperature, dehydrated in graded alcohol and then embedded in paraffin. The paraffin-embedded tissue blocks were sectioned into 5-um thickness in the sagittal direction including the epithelial layer and connective tissues. The slides were subjected to immunohistochemistry and immunofluorescence to analyze the expression characteristics of MORN2. The slides were stained with anti-MORN2 (rabbit anti-human, ab188524, Abcam) and anti-rabbit HRP-DAB according to instructions provided by the manufacturer (CTS005, R&D SYSTEMS) and then counterstained with hematoxylin. Rabbit IgG, polyclonal isotype control was used as a negative control (ab27478, Abcam). Photo images for DAB staining were captured using an Olympus microscope. Sequential immunofluorescence stain with CD68 and MORN2 was carried in the Leica Bond-Rx fully automated staining system. Epitope retrieval for CD68 was done for 20 min in Bond-epitope retrieval solution 2 pH 9.0 (AR9640) and for MORN2 for 30 min in solution 1 pH 6.0 (AR9661). The epitope retrieval was followed with 5 min endogenous peroxidase blocking. After pretreatment CD68 was applied for 15 min, then Bond post primary and polymer for 8 min (Bond Refine Detection kit #DS9800); the TSA-Cy5 (#SAT705A001EA, Perkin Elmer) was used to visualize CD68. After completion of CD68 stain the epitope retrieval for the MORN2 was performed as described above, then MORN2 (1:200) was applied for 1h followed with the Bond polymer (#DS9800) and TSA-Cy3 (#SAT704A001EA, Perkin Elmer). Stained slides were counterstained with Hoechst 33258 (#H3569, Life Technologies Carlsbad, CA) and mounted with ProLong® Diamond Antifade Mountant (P36961, Life Technologies Carlsbad, CA). High resolution acquisition of IF slides in the DAPI, Cy3 and Cy5 channels was performed in the Aperio ScanScope FL (Leica) using 20x objective. Nuclei were visualized in DAPI channel (blue), MORN2 in Cy3 (green) and CD68 in Cy5 (red).

2.2.8. Lentivirus production and transduction

For MORN2 targeted shRNA bacterial glycerol stocks (Sigma Aldrich) were grown in Lysogeny broth for 16h at 37 ℃ and plasmids DNA were isolated using NucleoBond Xtra Midi Kit (Clonetech) according to manufacturer's instruction. Plasmids DNA were sent to the lenti-shRNA core facility at UNC-CH to assemble lentiviral particles. Lentivirus containing medium were aliquoted and stored in -80 ℃.*MORN2* is targeted by 2 different shRNAs driven by the U6 promoter on pLKO.1 vector backbone. Knockdown of human *MORN2* was achieved by transduction of specific shRNAs or unspecific negative control shRNA (sh-scramble) lentivirus in pooled human gingival epithelial cells (HGEs) and THP-1 cells according to the manufacturer's instruction. Transduction was facilitated by the addition of 8 μg/ml polybrene. HGEs and THP-1 cells were subjected to selection with puromycin. The quality of gene down-regulation was evaluated via RT-qPCR. MORN2 lentiviral vector (LV226383, Abm) was packaged into lentiviral particles for MORN2 overexpression in THP-1. Empty version of the vector was used as a control during lentiviral experiments.

2.2.9. Phagocytosis and survival assay of *P.g*

The *MORN2* knockdown and scrambled THP-1 cells were plated into 24-well plates and allowed to differentiate to macrophage in the presence of PMA. Before infection, culture medium were replaced by new medium and cells were exposed to *P.g* at a multiplicity of infection (MOI) of 100 for 1h, then incubated with metronidazole (200 μg/ml) and gentamicin (300 μg/ml) for 1h to kill extracelluar bacteria. Cells were lysed with 1 ml of sterile distilled water and 100 μl of 10x diluent was placed on Brucella Blood Agar, and cultured under Forma Anaerobic System (Thermo Scientific) at 37 ℃. For bacteria survival, cells were further incubated 24 hours after infected by *P.g* 1 h and extracelluar bacteria were killed by antibiotics. Cells were lysed with 1 ml of sterile distilled water and 100 μl of 10x diluent was placed on Brucella Blood Agar, and cultured under Forma Anaerobic System (Thermo Scientific) at 37 ℃.

2.2.10. LC3 puncta formation assays

Differentiated THP-1 cells seeded in 8-well Chamber Slide (Thermo Fisher Scientific) and pre-incubated with 3-Methyladenine. Cells were challenged by *P.g* 33277 at MOI 100 for 1 hour, washed twice with PBS, fixed in 4% paraformaldehyde in PBS (pH 7.4), permeabilized by 0.2% Triton X-100, blocked by 10% serum. LC3 puncta were stained by rabbit anti-LC3B antibody kit (L10382, Thermo

Fisher Scientific) followed by Alexa Fluor 647 chicken anti-rabbit dye staining (Thermo Fisher Scientific). Slides were mounted in anti-fade media after DAPI staining. Cells treated with 60 μM Chloroquine diphosphate for 14 hours were used as positive control. The average number of $LC3^+$ puncta per 50 macrophages was obtained from Zeiss LSM 700 confocal microscope.

2.2.11. Transmission electronic microscopy (TEM)

Cell monolayers grown on plastic tissue culture plates challenged by *P.g* at MOI of 100 were fixed in 3% paraformaldehyde/0.15M sodium phosphate buffer for one hour at room temperature. Following three rinses with 0.15M sodium phosphate buffer (pH 7.4), the cells were post-fixed with 1% osmium tetroxide/1.25% potassium ferrocyanide/0.15M sodium phosphate buffer for 1 hour at room temperature. After washes in deionized water, the cells were dehydrated using increasing concentrations of ethanol and embedded in Polybed 812 epoxy resin (Polysciences, Inc. Warrington, PA). The cells were sectioned en face to the substrate at 70nm using a diamond knife and Leica Ultracut UCT ultramicrotome (Leica Microsystems, Inc., Buffalo Grove, IL). Ultrathin sections were collected on 200 mesh copper grids and stained with 4% aqueous uranyl acetate for 15 minutes, followed by Reynolds' lead citrate for 7 minutes. Samples were viewed with a LEO EM910 transmission electron microscope (Carl Zeiss Microscopy, LLC, Peabody, MA) with an acceleration voltage of 80 kV. Digital images were taken using a Gatan Orius SC 1000 CCD Camera and DigitalMicrograph 3.11.0 software (Gatan, Inc., Pleasanton, CA).

2.2.12. Real-time PCR

RNA was extracted from HGEs and THP-1 cells infected by *P.g* at indicated time with the RNeasy Mini kit (Qiagen, Germany) and cDNA was synthesized using the SuperScript VILO cDNA synthesis Kit (Thermo Fisher, USA) according to the manufacturer's protocol. The expression of human genes encoding IL-1β, IL-6, TNF-α, CXCL1, CXCL2, GCP-2, IL-8, CXCL3, CCL3, RANTES, GM-CSF, G-CSF, MCP-1 and GAPDH was assessed by real-time PCR with human Taqman Gene Expression Assays (Thermo Fisher Scientific). Results were normalized to expression of the gene GAPDH and were quantified by the $\Delta\Delta$ct method.

2.2.13. ELISA

Cell culture supernatants were collected and assayed for cytokines. Cytokine production was measured by enzyme-linked immunosorbent assay of human IL-1β, IL-6, TNF-α, IL-8 according to the instruction of

manufacturer (R&D Systems).

2.2.14. Intracelluar calcium flux assay

Differentiated THP-1 cells were plated in 96-well plate and Fluo-8 dye-loading solution was added according to the manufacturer's protocol (Abcam). The calcium flux assay was performed by monitoring the fluorescence intensity at Ex/Em=490/525 nm after cells were stimulated by *P.g* at MOI of 100.

2.2.15. Westernblot

THP-1 differentiated macrophages were stimulated with *P.g* 33277 for the indicated time points at a MOI 100:1. Cells were washed twice and were lysed with RIPA buffer containing phosphatase and proteinase inhibitors. Samples were separated by SDS-PAGE with 4-12% NuPAGE Bis-Tris gels (Invitrogen), transferred into a 0.2 μm polyvinylidene difluoride (PVDF) membranes, blocked with 5% skim milk and incubated overnight at 4℃ with the respective primary antibodies: rabbit anti-LC3 (#2775, Cell Signaling Technology), rabbit anti-phospho-IKKα/β (#2697, Cell Signaling Technology), rabbit anti-phospho-IκBα (#2859, Cell Signaling Technology), rabbit anti-phospho-NF-κB p65 (#3033, Cell Signaling Technology), rabbit anti-NF-κB p65 (#8242, Cell Signaling Technology), goat anti-beta Actin (Ab8229, Abcam). Secondary antibodies against the corresponding primary antibodies were used. The blots were developed using chemiluminescence (ECL, Thermo Scientific) and visualized using ImageQuant LAS4000 luminescent image reader (GE Healthcare Life Sciences, USA).

2.2.16. Statistical analysis

Results are reported as mean ± SEM. Differences between two groups was analyzed with paired t-test. Statistical analysis of experiments was carried out using one-way ANOVA followed by Dunn's post hoc test, as appropriate, for the distribution of variance among groups. P-values < 0.05 were considered statistically significant. Statistical analyses were performed using Prism 6.0 software (GraphPad Software).

2.3. Results

2.3.1. Characterization of the *Pg* dominant trait, *MORN2* SNP identification and *in silico* analysis

Our analysis of the *MORN2* locus, as identified in our previous work (Offenbacher, Divaris et al. 2016) demonstrates that it contains a missense SNP rs3099950 ($p=1.65 \times 10^{-6}$) that is predicted to be probably damaging with a score of 1.0 by Polypen-2 (Figure 2.1B). Molecular models (PyMol) predict that rs3099950 causes a missense mutation which results in an amino acid change from glutamate, which is a highly negative charged (red area) (Figure 2.1C, D), to lysine, which is a highly positively charged (blue area) (Figure 2.1E, F). This model suggests an alteration in the surface electrostatic potential of the protein, which will cause the change of the charge distribution across a PIP3 binding region of the MORN2 structure (cleft region) that may result in impaired membrane interactions and/or phagolysosome fusion (Figure 2.1G).

2.3.2. *MORN2* SNP is associated with periodontal disease severity

We show that the newly identified SNP rs3099950 has a significant association with the level of total pathogens (ANOVA p=0.007) (Figure 2.2A), the level of *Pg* (ANOVA p=0.046) (Figure 2.2B), and *F.nucleatum* (ANOVA p=0.046) (Figure 2.2C) stratified by rs3099950 genotype. For example, the *Pg* count was 1.4 times as high in 2.2 individuals (homozygous for the minor allele, G/G) as compared to 1.1 individuals (homozygous for the major allele, A/A). This finding further supports the SNP in the *MORN2* region potentially affect the biological host response of the individual, resulting in increased numbers of periodontal pathogens present in plaque samples. rs3099950 is also significantly associated with EPD4 and IAL5 in the presence of high *Pg* or *Fn* (dichotomized at 75th percentile loading of *Pg* and *Fn*) (Figure 2.2D-G). Because the presence of SNP in the *MORN2* region is associated with an increased number of total periodontal pathogens, with strong emergence of *Pg* and *Fn*. The combination of the 2.2 genotype in the presence of high pathogen load (upper quartile) suggests a strong gene-environment interaction that results in clinical disease. These findings suggest that the presence of the minor allele frequency in *MORN2* locus may increase the susceptibility of the individual to periodontal disease by perhaps promoting dysbiosis and when the pathogens emerge, there is an association of more severe disease.

2.3.3. Identification of MORN2 mRNA expression and localization in human gingival tissue

The presence of MORN2 expression has never been evaluated in periodontal tissues. Therefore, we evaluated the MORN2 expression in full-thickness gingival tissue samples from subjects free of periodontitis and from individuals with chronic periodontitis (CP). The demographic information and clinical parameters of the participants are shown in Table 2.2. Our results indicate that, in comparison to periodontitis-free samples, the mRNA level of MORN2 was significantly lower in CP groups ($P < 0.01$) (Figure 2.3A). To identify the cellular localization of MORN2 protein expression in the cells of the periodontium, immunohistochemistry and immunofluorescence were performed. The major cell types expressing MORN2 were epithelial and infiltrated immune cells. Representative low-resolution (x10) and high-resolution (x40) images of periodontitis-free and periodontitis tissues show a similar cellular pattern of expression among samples (Figure 2.3B). In addition, co-localization of MORN2 and CD68 (macrophage marker) by confocal microscope demonstrated that the infiltrated immune cells expressing MORN2 are mostly identified as macrophages (Figure 2.3C). In periodontitis the macrophage plays a critical role in clearing invasive pathogens and in innate immune response by recruiting other immune cells such as neutrophils and lymphocytes to the lesion. This descriptive localization analysis indicates that MORN2 is expressed predominantly by macrophages within the periodontal inflammatory lesion and the epithelium.

2.3.4. MORN2 promotes the LC3-asscociated phagocytosis in macrophages

Given that MORN2 is expressed in the macrophages of periodontal gingival tissues and MORN2 facilitates phagocytosis-mediated restriction of *M. tuberculosis*, *L. pneumophila*, and *S. aureus* in macrophages (Abnave, Mottola et al. 2014). We investigated the functional implication of MORN2 in macrophage-mediated uptake and killing of *P.g.* Our functional analysis revealed that the silencing of *MORN2* slightly reduced the phagocytosis of *P.g* in a log-10 form and markedly resulted in a 1.8 ± 0.6 fold increased intracellular survival of *P.g* at 24 hours infection($p<0.05$) (Figure 2.4A, B). Currently, the most significant ultrastructural difference that distinguishes LAP from canonical autophagy is that bacteria is enclosed within single-membrane phagosomes, instead of double membrane auophagosomes (Lerena and Colombo 2011, Martinez, Almendinger et al. 2011). EM analysis of macrophages infected with *P.g.* We observed that single-membrane phagosomes (white arrow in Figure 2.4C). To elucidate the mechanism of MORN2 in phagocytosis-mediated killing of *P.g*, we next investigated whether MORN2 has a role in LC3 recruitment. The cytosolic form of LC3 (LC3-I) is conjugated to phosphatidylethanolamine

(PE) and in this form (LC3-II) it is recruited to the phagosome or autophagosome membrane. Conjugation was assessed by measuring the levels of LC3-II in the presence of 3-MA. This treatment blocks the fusion of autophagosomes with lysosomes, thereby precluding the effect of autophagy on the accumulation of LC3-II. LC3-II levels showed a decrease after MONR2 knockdown in differentiated THP-1 cells challenged by *P.g* (Figure 2.4D). The formation of LC3 puncta viewed by fluorescence microscopy is widely used as a marker for LC3 recruitment. Given that *P.g* infection of macrophages resulted in decrease in the LC3-II formation, we then assessed the formation of LC3 puncta. The silencing of MORN2 in macrophages decreased the LC3 puncta formation at 1h by immunofluorescence (Figure 2.4E). Collectively, these data demonstrate that MORN2 plays a role in the LC3-associated phagocytosis for the efficient clearance of *P.gingivalis*.

2.3.5. Role of MORN2 on production of proinflammatory cytokines and chemokines by HGEs and Macrophages

Given that MORN2 expressed both in human gingival epithelial cells and macrophages, we investigated the role of MORN2 on production of inflammatory mediators on these two type of cells. To test this prediction, we first demonstrated the knockdown efficiency (knockdown mRNA expression by 45% in HGEs and >90% in THP-1) and specificity of *MORN2* silencing in HGEs and THP-1 cells with one or two corresponding shRNAs. We observed that neutrophil specific chemokines (CXCL1, CXCL2, IL-8) and G-CSF synthesis was downregulated in MORN2 knockdown HGEs at mRNA level ($p<0.05$) (Figure 2.5A-D). The proinflammatory cytokines (IL-1β, IL-6, TNF-α) and IL-8 production was reduced at both mRNA and protein level ($p<0.05$) in MORN2 knockdown macrophages (Figure 2.6A-F). In addition, silenced MORN2 in macrophages significantly impaired mRNA levels of neutrophil specific chemokines (CXCL1, CXCL2, GCP-2), monocytic and T cells recruitment chemokines (CXCL3, CCL3, RANTES) and hematopoietic growth factors (GM-CSF, G-CSF) production ($p<0.05$), except MCP-1 expression was upregulated ($p<0.05$) (Figure 2.7A-K). Based on these results, MORN2 posit that MORN2 plays an important role in regulating inflammatory response to periodontal pathogens.

2.3.6. Knockdown MORN2 attenuates calcium flux and NF-κB activation

NF-κB is an important transcriptional factor in several types of inflammations, which results in the production of a plethora of pro-inflammatory cytokines and chemokines. Considering that NF-κB can be

stimulated through TLR to activate the IKK complex, which leads to the translocation of heterodimers of the NF-κB subunits p65 and p50 to the nucleus (Kawai and Akira 2007). To determine whether MORN2 is involved in TLR-mediated NF-κB activation, we knocked down *MORN2* expression by *MORN2*-specific shRNA in THP-1 cells and stimulated those differentiated cells with *P.g* 33277. Cell lysates were used to assess the phosphorylation of IKKα/β, IκBα and p65. We found that specific knockdown of *MORN2* diminished the phosphorylation of IKKα/β, IκBα and p65, especially at 30 min and 60 min after *P.g* stimulation, compared THP-1 cells transduced with control scramble shRNA (Figure 2.8A). Calcium signals have been described in various cells of the immune system, including T and B cells, DCs, monocytes and macrophages, in which they contribute to the cell's activation, differentiation or gene expression (Feske 2007). An influx of Ca^{2+} into cytosol occurs following the engagement of immunoreceptors on the cell surface. To further determine the molecular mechanisms by which *MORN2* knockdown affects NF-κB signaling, we performed intracellular calcium flux assay. A transient increase in Ca^{2+} was evoked at 60s after the start of *P.g* treatment and totally diminished at 300s (Figure 2.8B). Lower intracellular calcium flux was observed in MORN2 knockdown group compared to control at 60s and 90s. Therefore, the results indicate that the initial transient intracellular increase in Ca^{2+} may play a role in NF- κB activation. Taken together, these results suggest that activation of NF-κB depends on the transient increase in Ca^{2+} in *P.g* stimulated differentiated macrophages from THP-1 cells and specific knockdown of MORN2 decreases intracellular calcium flux mediated NF-κB activation in response to *P.g* stimulation.

2.4. Discussion

Studying the role of human genes in infection has been termed infectogenomics and has the potential to serve as a powerful tool to identify and understand gene function relevant to host response to pathogens and chronic infection associated diseases. Our group's previous GWAS has highlighted a potential role for MORN2 in periodontal disease (Offenbacher, Divaris et al. 2016), which was emerged from GWAS gene-centric data relative to *Pg* dominated dysbiosis and is corroborated by the role of *Pg* as keystone pathobiont in periodontitis (Hajishengallis, Darveau et al. 2012). In the present study we have identified a potentially functionally important *MORN2* SNP. Subjects with the 2.2 *MORN2* genotype (homozygous for the minor allele) have more severe disease as reflected by greater extent of probing depth ≥ 4mm and interproximal attachment loss ≥ 5mm, only in the presence of high bacterial loading, as compared to subjects with 1.1 (homozygous for the major allele) or 1.2 (heterozygous). This suggests that compromised function of MORN2 induced by the minor allele of rs3099950 in *MORN* exon 5 may render patients more susceptible to periodontal disease. Prediction analysis using UniProtKB suggested that the function of MORN2 is altered by the presence of rs3099950 due to an amino acid change from a negatively charged glutamate to a positively charged lysine at position 48. We identified that MORN2 is expressed in epithelial cells and macrophages of gingival tissues, which suggests a potential role of MORN2 in epithelial barrier function and monocytic response to periodontal pathogens. The mRNA expression of MORN2 is suppressed in chronic periodontitis, as compared to health.

The knowledge base regarding *MORN2* is scarce and its function in any disease, including periodontal disease, is unclear. To date, only one study that explored whole-transcriptome analysis of the planarian response to bacterial challenge through a functional RNAi-based screening approach has identify identified MORN2 as a critical gene for bacterial resistance in planarians with human orthologs. Using human macrophages they demonstrated that MORN2 promotes the recruitment of LC3 to microbe-containing phagosomes and phagosome-lysosome fusion (Abnave, Mottola et al. 2014). This suggests that MORN2 function may be important for bacterial clearance and phagocytic cell function. The function of MORN2 in LC3-associated phagocytosis (LAP) of the periodontal keystone pathogen P.g was studied by creating stable MORN2 knockdown human monocytic cell line. LC3 can be recruited into both phagosomes and autophagosomes, which are both involved in microbial clearance. LAP distinct from the conventional autophagy is that LC3 is recruited into the double-membrane autophagosome and LC3 is

recruited directly to the single-membrane phagosome (Sanjuan, Dillon et al. 2007, Martinez, Almendinger et al. 2011). Our study pinpoints a role for MORN2 in LAP of *P.g* (strain 33277) in THP-1 differentiated macrophage. Analysis by EM indicated most of the *Pg* are located in single-membrane phagosomes. We found that *in vitro* MORN2 in human macrophages promotes the phagocytosis and killing of *Pg*. Similarly, we provide evidence that during phagocytosis MORN2 expression promotes the cytosolic form of LC3-I complexation to membrane associated phosphatidylethanolamine (PE) to form LC3-II and increases the number of LC3-positive puncta formation independently of autophagy. This finding is consistent with a role for MORN2 in LC3 recruitment to *M. tuberculosis* containing phagosomes as previously report (Abnave, Mottola et al. 2014). Collectively, our findings support a function for MORN2 in the LAP of *Pg* in macrophages. It is not known whether this loss of MORN2 function induces *Pg* dysbiosis, as further research is indicated.

Invading pathogens are recognized by mammalian cells through receptors either in the cytoplasm (NLRs) or at the cell surface (TLRs) (Xu, Jagannath et al. 2007, Anand, Malireddi et al. 2012). It has been shown that *P.g* can interact with host-expressed TLR2 (Burns, Eliyahu et al. 2010, Papadopoulos, Weinberg et al. 2013, Maekawa, Krauss et al. 2014) and LPS of *P.g* has also been reported to engage TLR4 (Ogawa, Asai et al. 2002, Darveau, Pham et al. 2004). The TLR2 and TLR4 binding of *P.g* is strain specific due to the presence of an LPS structure that contains multiple lipid A (Darveau, Pham et al. 2004, Herath, Darveau et al. 2013). Proteomics analysis of purified phagosome membranes in cells that have engulfed different cargoes by phagocytosis revealed that the absence of TLR signaling results in failure of LC3 recruitment to phagosome membranes (Sanjuan, Dillon et al. 2007, Shui, Sheu et al. 2008). The triggering of LAP by TLRs is associated with enhanced phagosomal fusion with lysosomal compartment, results in a more efficient degradation of pathogens to reduced intracellular survival. Also, LAP might serve as another host defense strategy to protect from microorganisms by facilitating peptide presentation on MHC class II molecules to initiate adaptive immunity or delivering phagosomes cargo to other intracellular pathogen recognition receptors.

The activation of Toll-like receptors that occurs upon *P.gingivalis* exposure results in activation of the NF-κB intracellular signaling pathway (Lerena and Colombo 2011), which will triggers increased expression of many pro-inflammatory cytokines [IL-1β, IL-6, TNF-α] (Geivelis, Turner et al. 1993, Yavuzyilmaz, Yamalik et al. 1995, Salvi, Brown et al. 1998) and chemokines [IL-8, MCP-1, RANTES, etc]

(Gurkan, Eren et al. 2016, Diomede, Zingariello et al. 2017). Considering the broad implication for MORN2 in antibacterial immunity and completely unknown the role of MORN2 in modulating inflammatory mediators, it will be of interest to assess whether MORN2 is involved in TLR-induced NF-κB signaling pathway to prevent pathological consequences. To define the biological function of MORN2 in vitro, we generated MORN2 knockdown HGEs and THP-1 cells with shRNA targeting MORN2 expression. Our study using MORN2-knockdown cells provide evidence that MORN2 is a critical regulator of TLR-induced NF-κB signaling pathway. MORN2 knockdown HGEs stimulated by *P.g* produce lower levels of chemokines that target neutrophil recruitment and activation. MORN2 knockdown causes macrophages to produce less proinflammatory cytokines and chemokines in response to TLR stimulation, with the exception of MCP-1, accompanied by diminished phosphorylation of IKKα/β, IκBα and p65.

Intracelluar calcium flux is an important second messenger that plays a role in diverse array of cellular processes, including activation of NF-κB (Pahl and Baeuerle 1996, Gewirtz, Rao et al. 2000, Han and Logsdon 2000). Phosphatidylinositol 4,5-bisphosphate (PIP_2) is a minor phospholipid component of plasma membrane and serves as a precursor of important second messengers. PIP_2 can be phosphorylated by phosphatidylinositol-3 kinase (PI3K) to generate phosphatidylinositol 3,4,5-trisphosphate (PIP3) (Carnero and Paramio 2014), which is a very important molecule to orchestrate actin polymerization (Insall and Weiner 2001) and acts to recruit and regulate the function of several effectors important in phagocytosis and phagosome maturation (Vieira, Botelho et al. 2002, Bohdanowicz and Grinstein 2013, Schink, Raiborg et al. 2013). At the same time, PIP2 hydrolysis can by initiated by phospholipase C (PLC) to produce inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 bind to an IP_3-specific receptor on ER, which in turn leads to a release of intracellular Ca^{2+} to activate NF-κB. Our structural analysis found that rs3099950 in MORN2 locus is predicted to impair the PIP3 binding domain, which might influence phagocytosis and phagosome-lysosome fusion. We reported that the knockdown of MORN2 decreases intracellular calcium flux consistent with this potential pathway. Interestingly, MORN2 is a protein contains 2 MORN motifs. Although the localization and function of MORN2 have not been reported in any disease, MORN motifs were first described in junctophilin that is a component of junctional complex between the plasma membrane and ER of excitable cells and have been proposed bind to membrane phospholipids (Takeshima, Komazaki et al. 2000). It has been shown that GFP-MORN was detected primarily in plasma membrane enriched fraction isolated by aqueous two phase partitioning

and expressing the MORN domain increased the plasma membrane PIP kinase activity (Im, Davis et al. 2007).

Our findings suggest that MORN2 is likely a plasma membrane-binding protein that is associated with the cytosolic monolayer of the lipid bilayer involved both in NF-κB activation and PIP kinase activity. Our results suggest that MORN2 may play a predominant role in TLR-induced NF-κB signaling and functions as a checkpoint for the production of inflammatory cytokines in macrophages. Although a potential biologically relevant function associated with pathogenesis of periodontitis was identified, the present results have limitations. Due to the lack of a suitable antibody for immunoblot, a MORN2 antibody will be needed to investigate protein-protein interaction and confirm the knockdown efficiency at protein level. The validity of protein function with rs3099950 will need to be further examined in a new study, as well as the effects of the specific SNP variant on *Pg* and *Fn* mediated dysbiosis.

In summary, our genetic and functional study here pinpoints the broad critical function of MORN2 in phagolysosomal-mediated bacterial clearance and innate immune response. Our results suggest that it may play a role in the pathogenesis of periodontitis, and specifically support the hypothesis that *MORN2* polymorphism may compromise the innate immune response leading to a *P.g* dominated dysbiosis in periodontal disease.

2.5. Conclusions

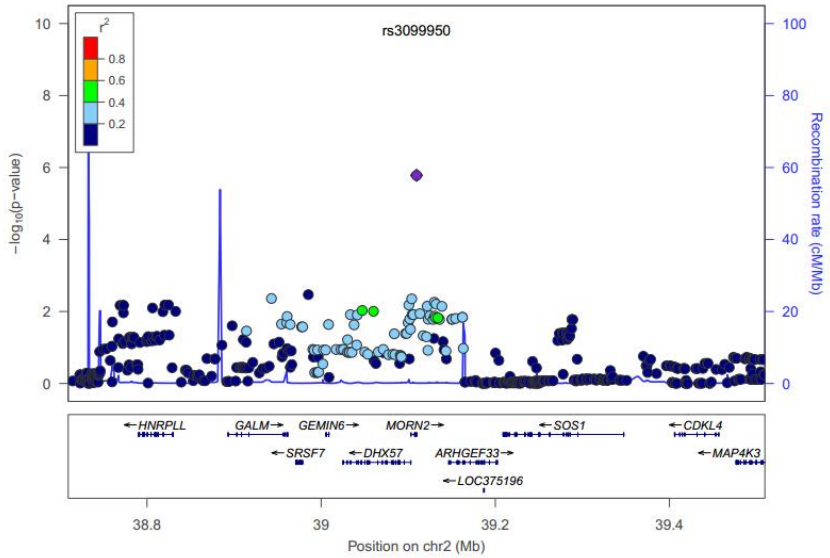
Our studies support a role of MORN2 in the pathogenesis of periodontal disease. The present results suggest that MORN2 plays a role in the LC3-associated phagocytosis mediated killing of *Pg* and cytokine/chemokine response through calcium mediated NF-kB activation. It is possible that the examined MORN2 polymorphism may compromise killing and the innate immune response leading to a *Pg* dominated dysbiosis in periodontal disease.

2.6. Figures and tables

	PCT1	PCT3	PCT5
P.g	0.179392	-0.197802	0.881999
P.i	0.387405	-0.150185	-0.232949
P.n	0.345661	-0.137211	-0.278858
T.f	0.377298	-0.13046	0.089544
T.d	0.3681	-0.158729	-0.055044
F.n	0.412358	0.072963	-0.047672
C.r	0.39499	0.176501	-0.010886
A.a	0.294662	0.340761	0.133942
GCF IL1-b	0.061093	0.840057	0.037194
CDC/AAP Entry	-0.01044	0.135934	0.239261
CDC/AAP Severe	0.06066	-0.022578	-0.006506

Table 2.1. Principle Component Trait (PCT) profiles for Socransky complex (PCT1), *Aa* trait (PCT3) and *Pg* trait (PCT5). PCA model was developed using the parameters shown. GCF IL-1b = gingival crevicular fluid interleukin 1 beta levels. The eigenvectors for each parameter is shown in the table.

A



B

PolyPhen-2 report for Q502X0 E48K (rs3099950)

Query

Protein Acc	Position	AA_1	AA_2	Description
Q502X0	48	E	K	Canonical; RecName: Full=MORN repeat-containing protein 2; AltName: Full=MORN motif protein in testis; Length: 79

Results

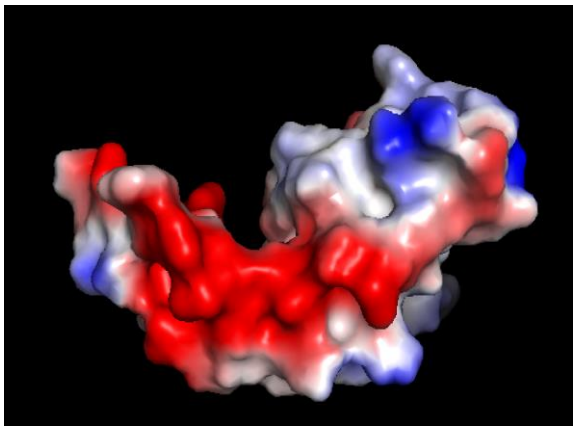
Prediction/Confidence *PolyPhen-2 v2.2.2r398*

HumDiv

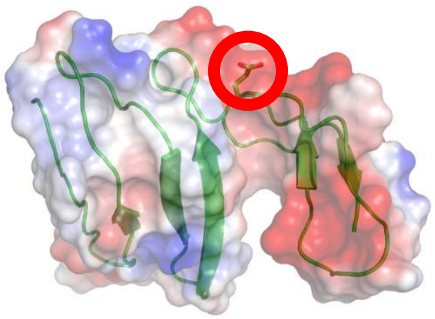
This mutation is predicted to be **PROBABLY DAMAGING** with a score of **1.000** (sensitivity: **0.00**; specificity: **1.00**)

0.00 0.20 0.40 0.60 0.80 1.00

C

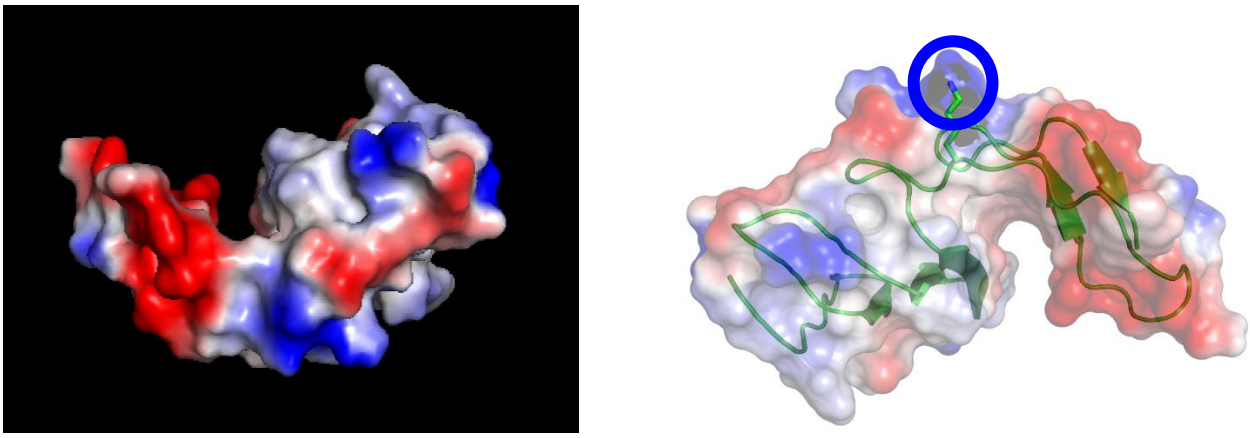


D



E

F



G

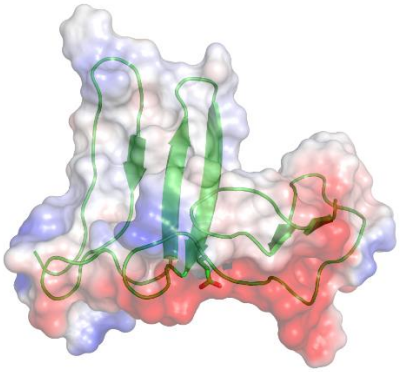
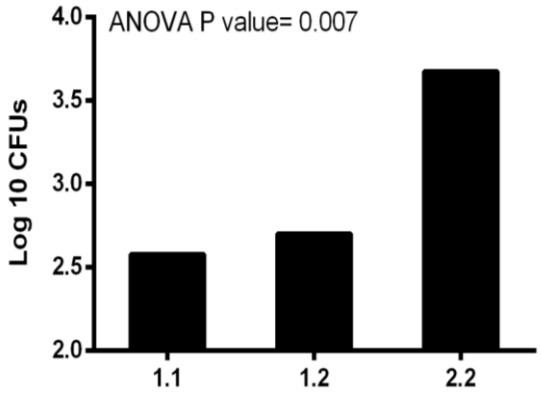
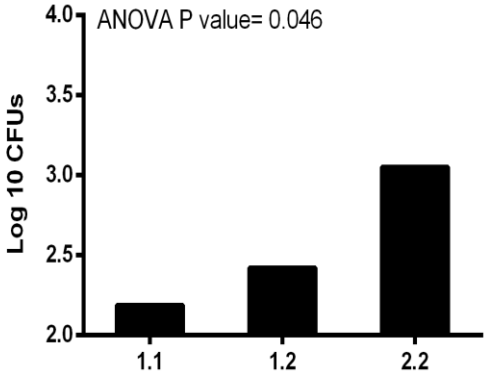


Figure 2.1. rs3099950 codes for a missense mutation in MORN2. (A) *MORN2* coding missense SNP-rs3099950. (B) Possible impact of the amino acid substitution on the structure and function of MORN2 was predicted by PolyPhen-2. (C, D) Wild type and (E, F) a SNP variant homology models were created by using Phyre2. (G) The influence of SNP on the functional side chains on the protein surface was analyzed using the surface electrostatic potentials.

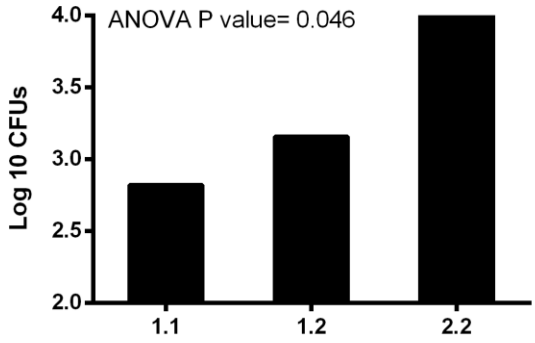
A


ANOVA P value= 0.007
Log 10 CFUs
4.0
3.5
3.0
2.5
2.0
1.1
1.2
2.2

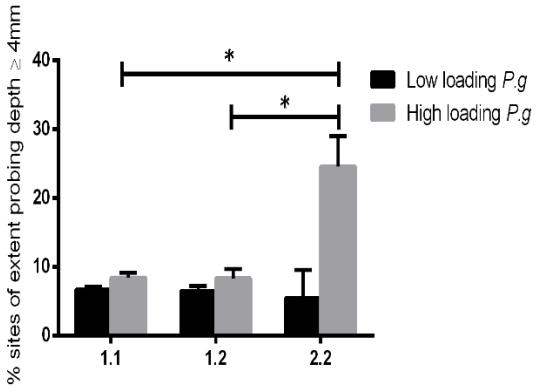
B


ANOVA P value= 0.046
Log 10 CFUs
4.0
3.5
3.0
2.5
2.0
1.1
1.2
2.2

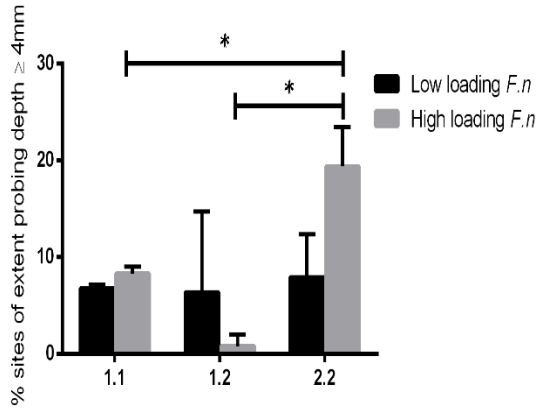
C


ANOVA P value= 0.046
Log 10 CFUs
4.0
3.5
3.0
2.5
2.0
1.1
1.2
2.2

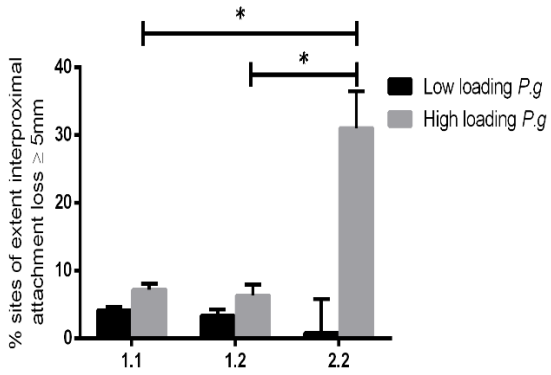
D


% sites of extent probing depth ≥ 4mm
40
30
20
10
0
*
*
Low loading P.g
High loading P.g
1.1
1.2
2.2

E


% sites of extent probing depth ≥ 4mm
30
20
10
0
*
*
Low loading F.n
High loading F.n
1.1
1.2
2.2

F


% sites of extent interproximal attachment loss ≥ 5mm
40
30
20
10
0
*
*
Low loading P.g
High loading P.g
1.1
1.2
2.2

G

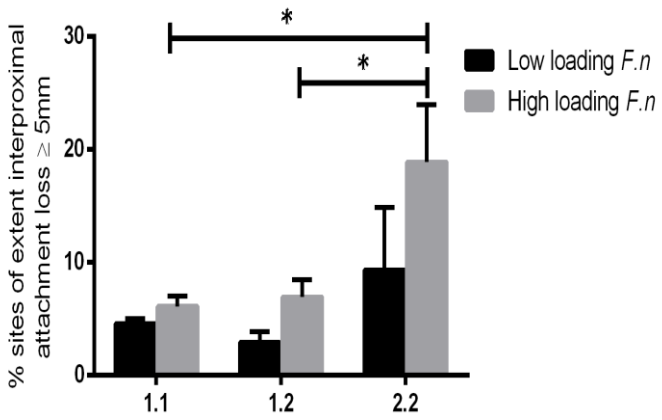


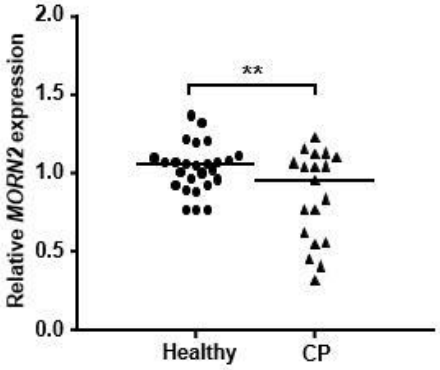
Figure 2.2. *MORN2* SNP (rs3099950) is associated with microbial burden and periodontal disease severity. (A-C) Effect of *MORN2* SNP (rs3099959) on log-transformed count of total periodontal pathogens (*P.gingivalis*, *F.nucleatum*, *P.intermedia*, *P.nigrescens*, *T.forsythia*, *T.denticola*, *C.rectus*, *A.actinomycetemcomitans*), *P.g* and *F.n* in GWAS (n=945) (1.1 is A/A, 1.2 is A/G, 2.2 is G/G). (D, E) Effect of *MORN2* SNP (rs3099950) interacted with dichotomized *P.g or F.n* loading on percentage sites of extent probing depth ≥ 4mm. (F, G) *MORN2* SNP (rs3099950) interacted with dichotomized *P.g* or *F.n* loading on percentage sites of extent interproximal attachment loss ≥ 5mm. Low loading *P.g* or *F.n* indicated 75th percentile. *p<0.05 (Student's t test).

Table 2.2. Subjects' Demographics and Clinical Parameters

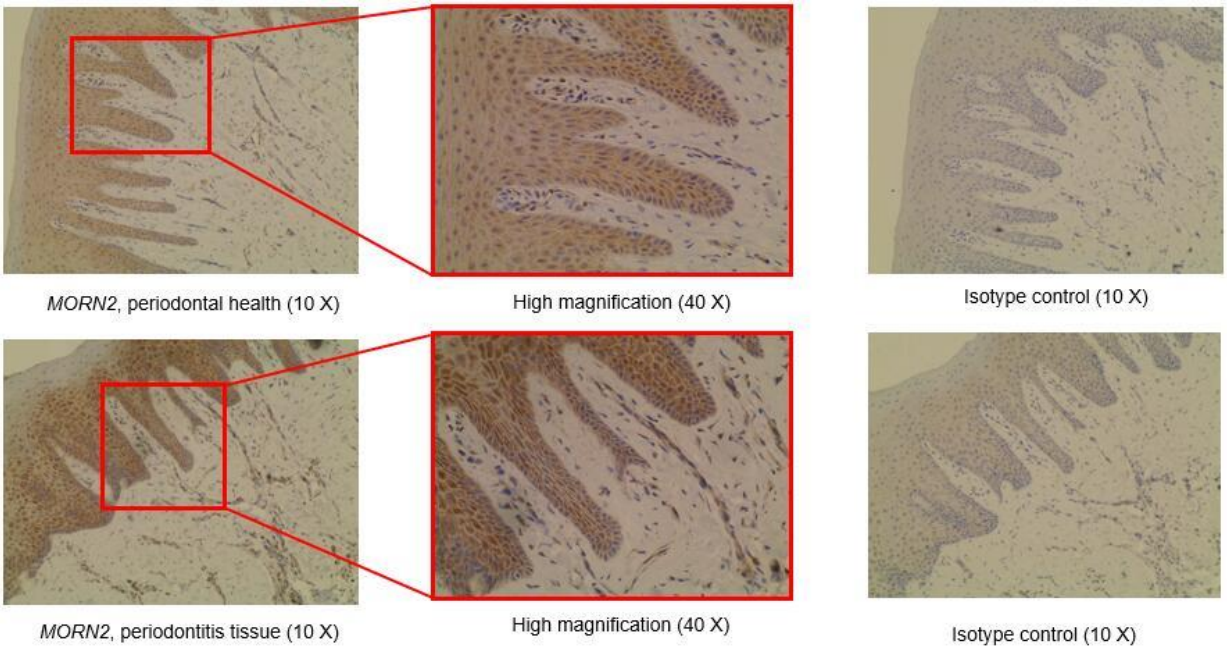
	Healthy (n=26)	Periodontitis (n=19)	P
Age	40.1 ± 12.9	48.53 ± 11.5	< 0.05
Female	18	9	0.22
Caucasian	18	10	0.35
Probing depth	2.2 ± 0.6	5.8 ± 1.6	< 0.05
Attachment loss	0.8 ± 0.6	4.8 ± 1.6	< 0.05

Values are presented in mean ± standard error or number

A


Relative MORN2 expression
2.0
1.5
1.0
0.5
0.0
**
Healthy
CP

B


MORN2, periodontal health (10 X)
High magnification (40 X)
Isotype control (10 X)
MORN2, periodontitis tissue (10 X)
High magnification (40 X)
Isotype control (10 X)

C

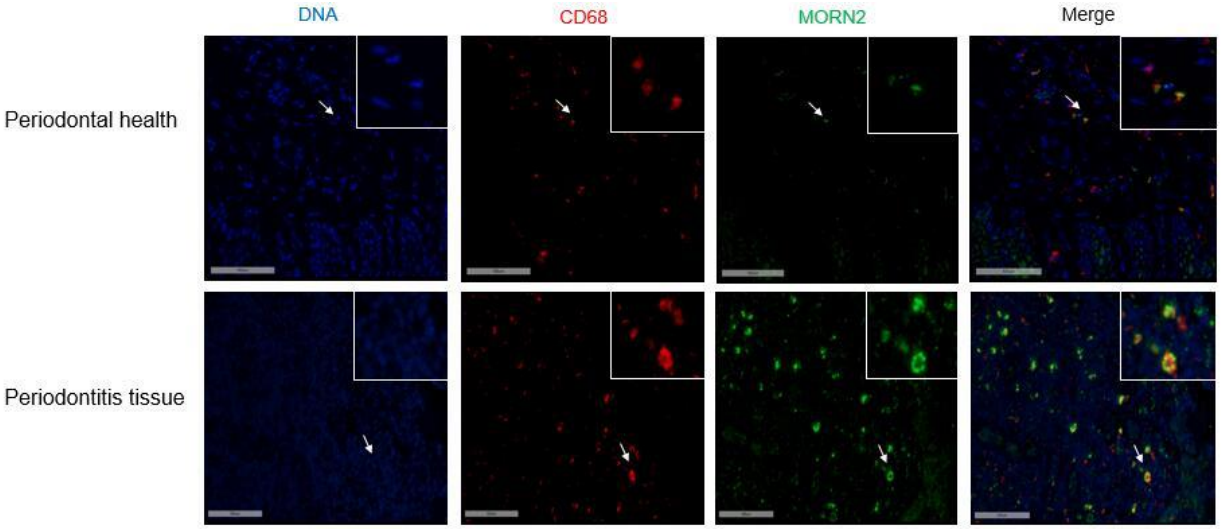
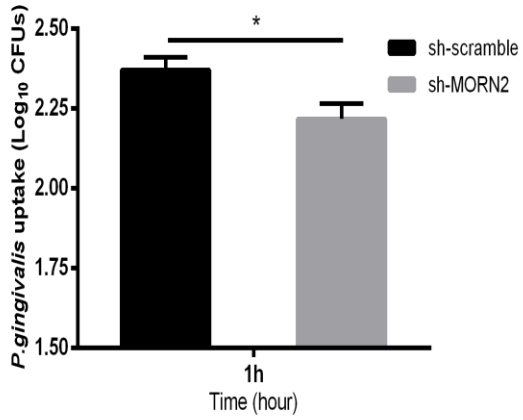
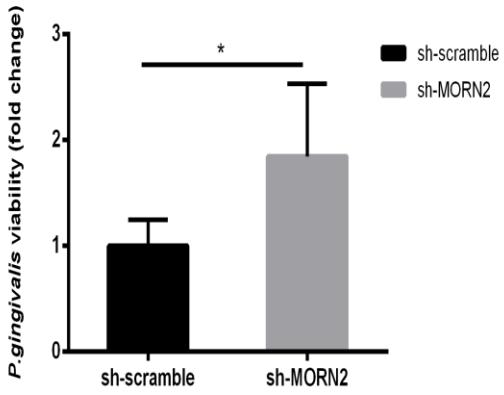


Figure 2.3. Identification of MORN2 in human gingival tissues. (A) The mRNA relative level of MORN2 was determined in gingival tissues from healthy (n=26) and chronic periodontitis (n=19) subjects by real-time PCR. **p < 0.01. (B) Representative images of tissue sections from a healthy individual and an individual with periodontal disease stained with MORN2 antibody. (C) Colocalization of MORN2 and CD68 were performed by immunofluorescence staining. Pictures were taken using confocal microscopy. Scale bar, 100 μm. All data are representative of two independent experiments.

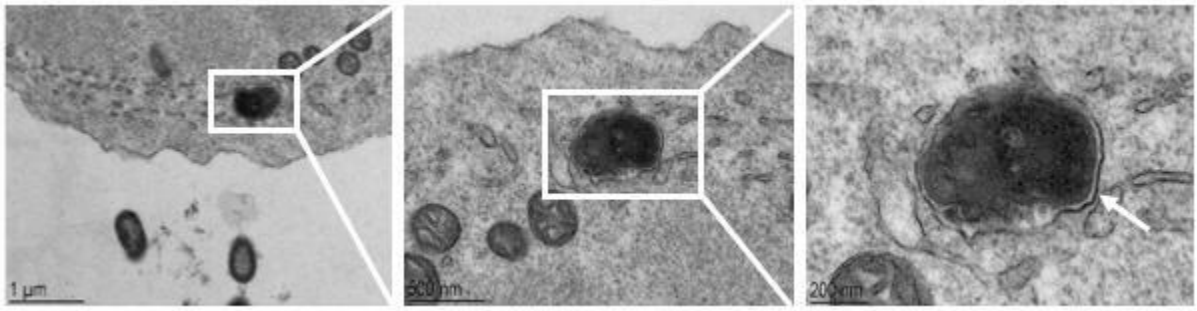
A


*
sh-scramble
sh-MORN2
P.gingivalis uptake (Log10 CFUs)
2.50
2.25
2.00
1.75
1.50
1h
Time (hour)

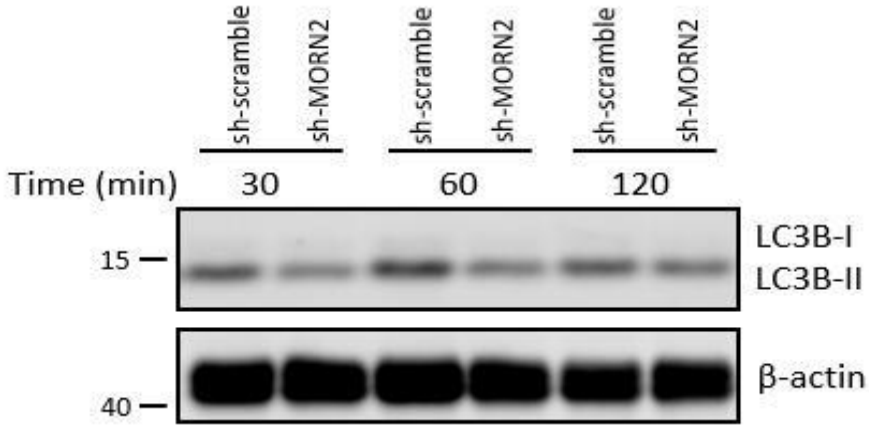
B


*
sh-scramble
sh-MORN2
P.gingivalis viability (fold change)
3
2
1
0
sh-scramble
sh-MORN2

C


1 μm
500 nm
200 nm

D


sh-scramble
sh-MORN2
sh-scramble
sh-MORN2
sh-scramble
sh-MORN2
Time (min)
30
60
120
15
LC3B-I
LC3B-II
40
β-actin

E

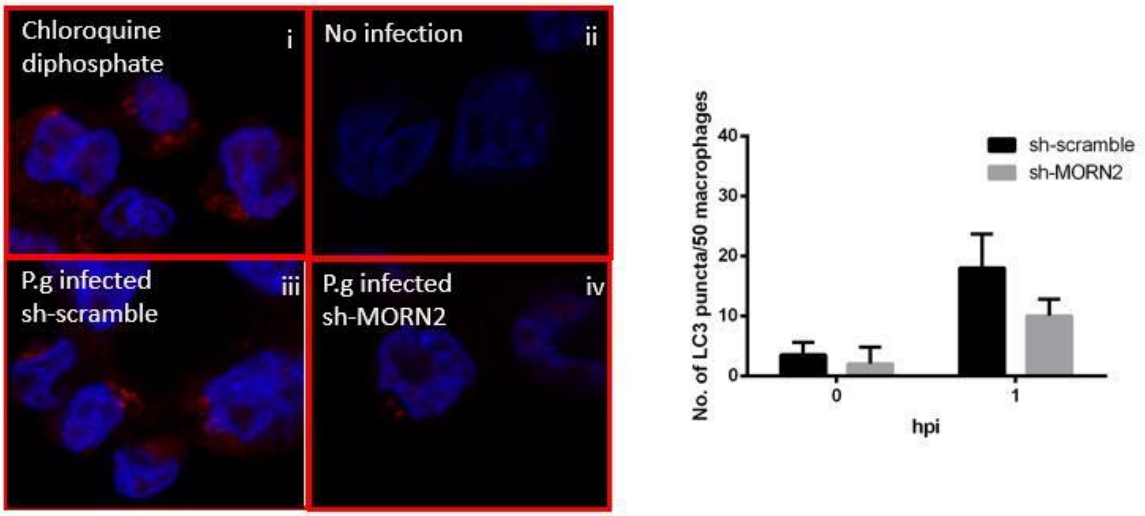


Figure 2.4. MORN2 is required for LAP. (A, B) THP-1 cells derived macrophages with shMORN2 or scrambled control shRNA were infected with *P.g* (MOI=100) and then bacterial uptake (A) and bacterial viability (B were evaluated by CFU counting. The results are expressed as mean ± SD (n=3, *p<0.05). Data are representative of three independent experiments. (C) Electron micrograph of *P.g*-infected macrophages. EM revealed the presence of single membranes (indicated by a white arrow) surrounding the phagosomes containing *P.g*. Scale bars were indicated in the figures. (D) LC3 conversion was measured in control or MORN2 knockdown cells challenged with *P.g*. LC3-II levels were measured by immunoblotting. (E) Differentiated macrophages were left untreated, treated with chloroquine diphosphate or *P.g.* LC3 puncta were assessed at indicated time. The results are expressed as mean ± SD (n=2).

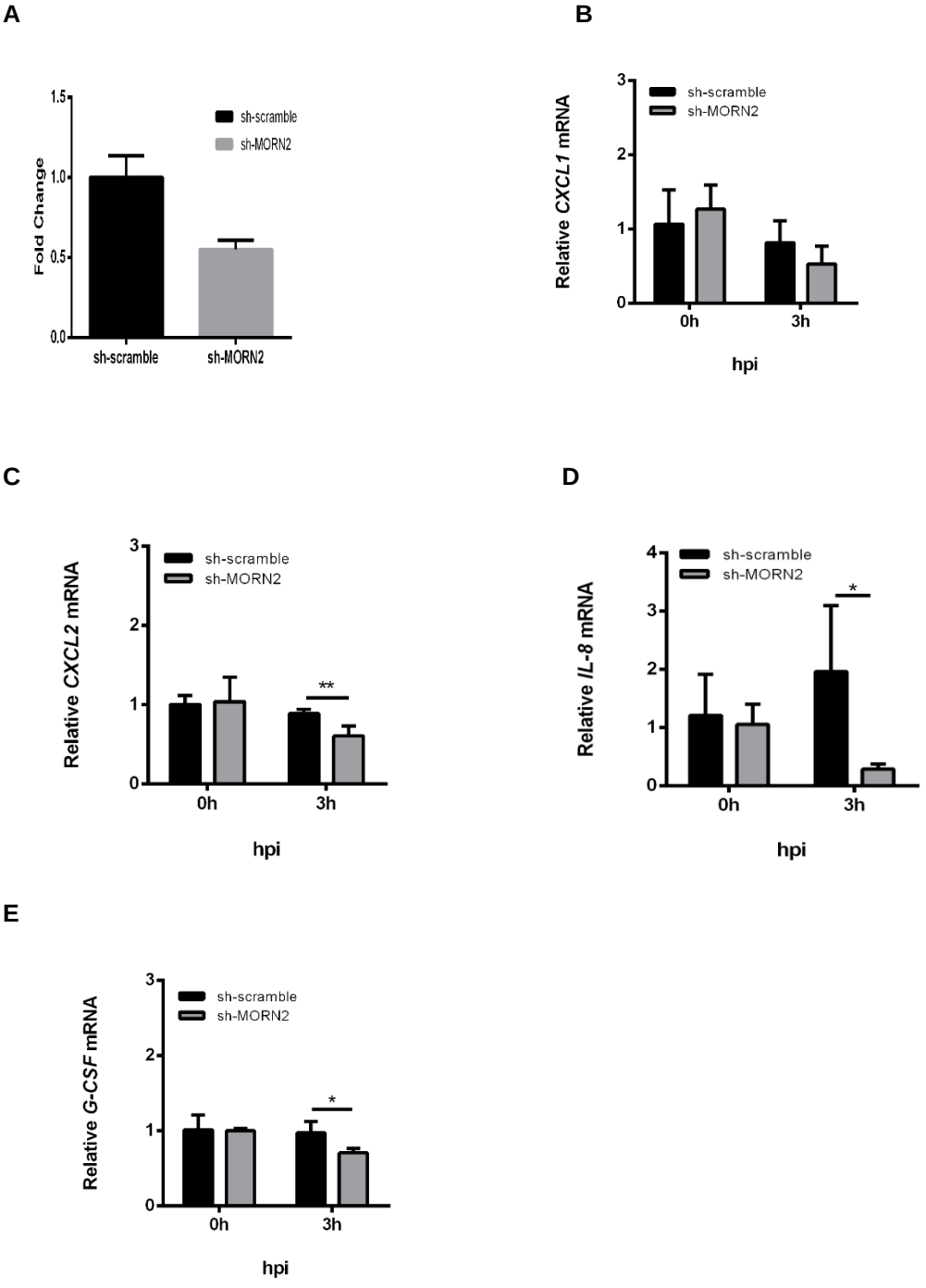


Figure 2.5. MORN2 is required for neutrophil specific chemokines and G-CSF synthesis in HGEs. (A) Fold change of MORN2 mRNA expression in HGEs. (B-E) HGEs transduced with shMORN2 or scrambled control shRNA were infected with *P.g* (MOI=100). CXCL1, CXCL2, IL-8 and G-CSF mRNA levels were determined by qPCR 3hr post-infection. Data are presented as the mean ± SD. *$p < 0.05$ (Student's t test).

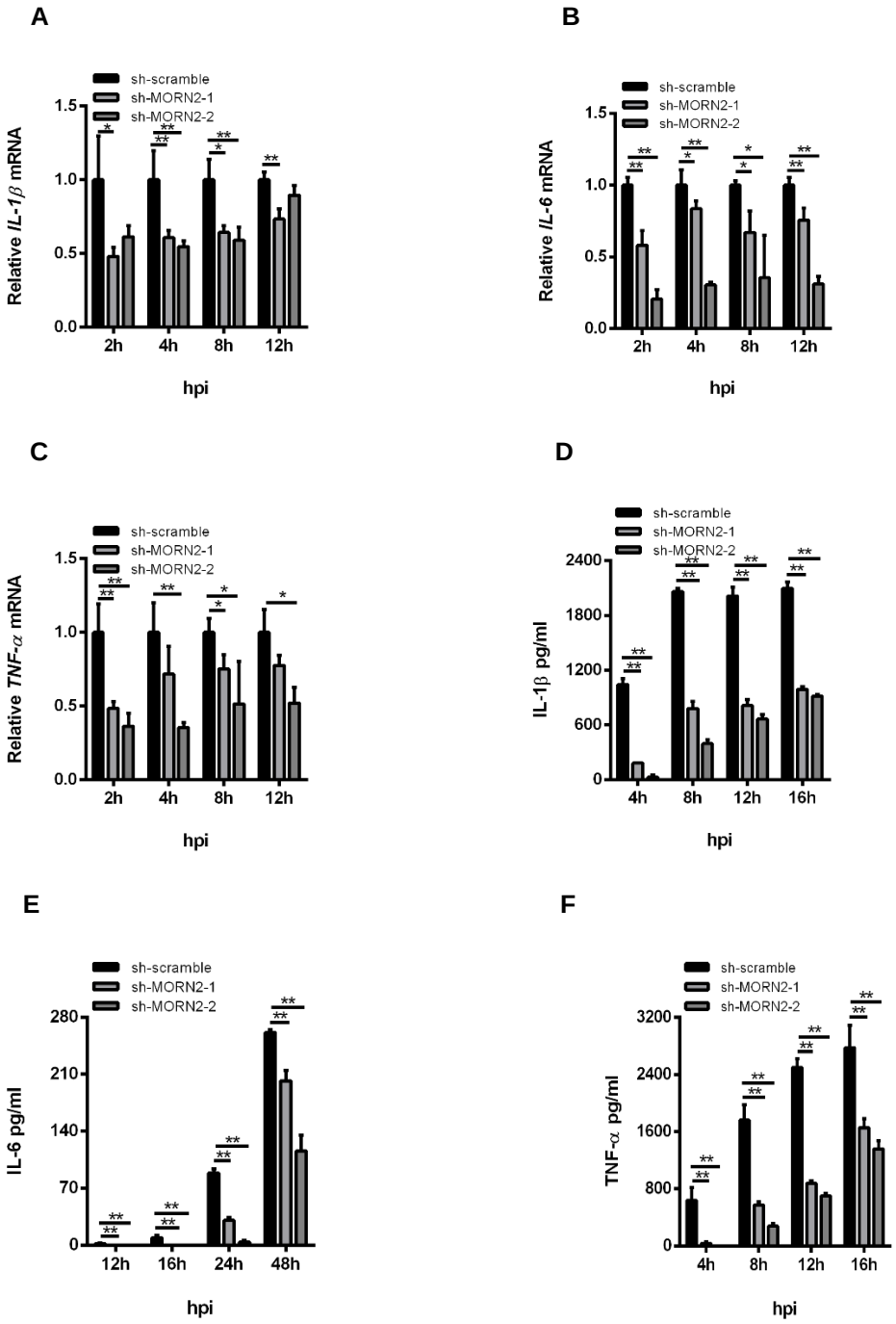
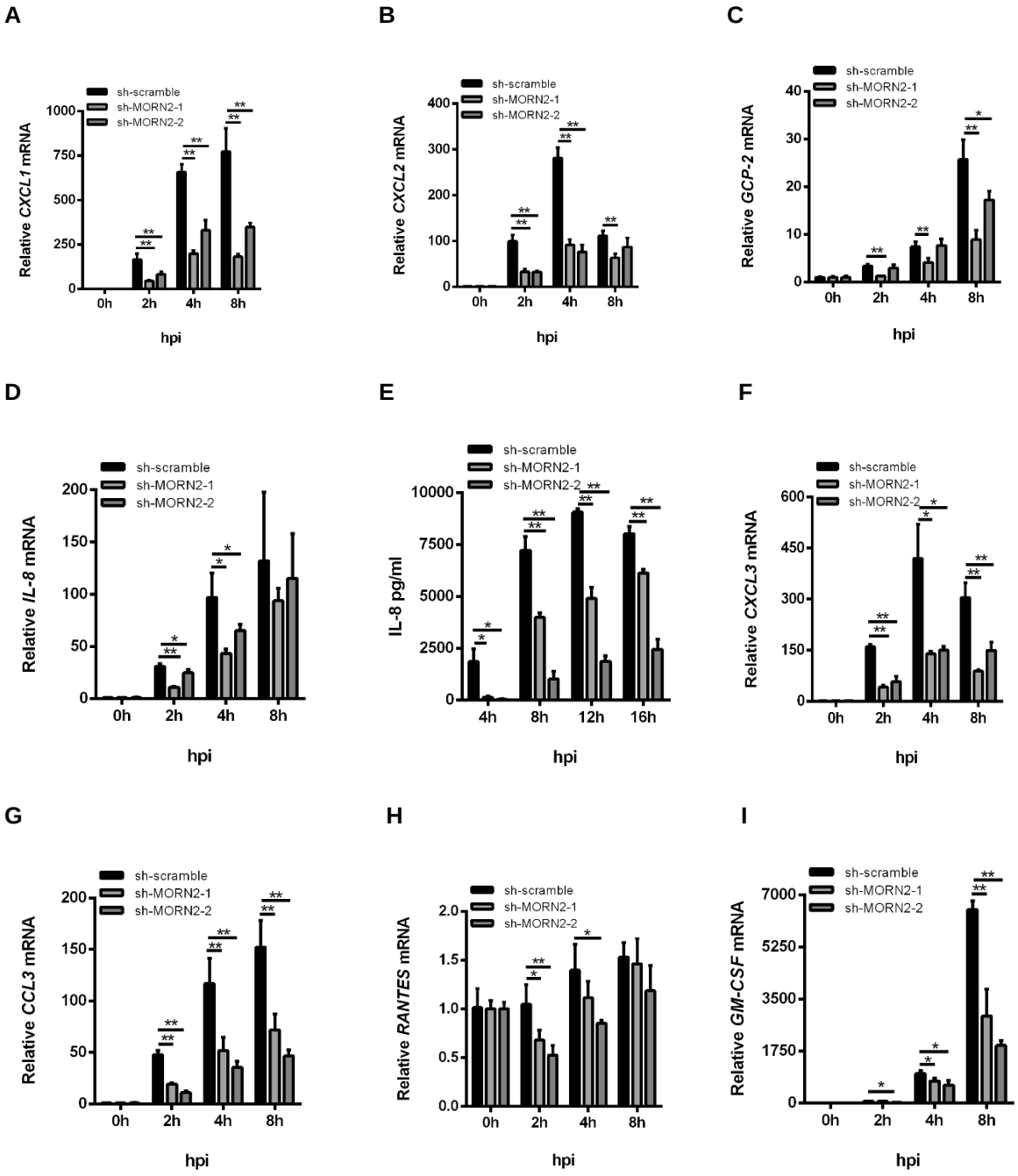


Figure 2.6. Knockdown of MORN2 decreases proinflammatory cytokine production upon *P.g* infection. (A-C) THP-1 cells containing scrambled shRNA or shRNA for MORN2 were differentiated into macrophages and infected by *P.g.* mRNA levels of IL-1β, IL-6 and TNF-α were determined by qPCR at indicated hours post infection. (D-F) protein levels of IL-1β, IL-6 and TNF-α were determined by ELISA at indicated hours post infection. All data were performed duplicate measured by duplicate and presented as the mean ± SD. *p < 0.05 (Student's t test).


A
B
C
D
E
F
G
H
I
sh-scramble
sh-MORN2-1
sh-MORN2-2
Relative CXCL1 mRNA
Relative CXCL2 mRNA
Relative GCP-2 mRNA
Relative IL-8 mRNA
IL-8 pg/ml
Relative CXCL3 mRNA
Relative CCL3 mRNA
Relative RANTES mRNA
Relative GM-CSF mRNA
0h
2h
4h
8h
12h
16h
hpi

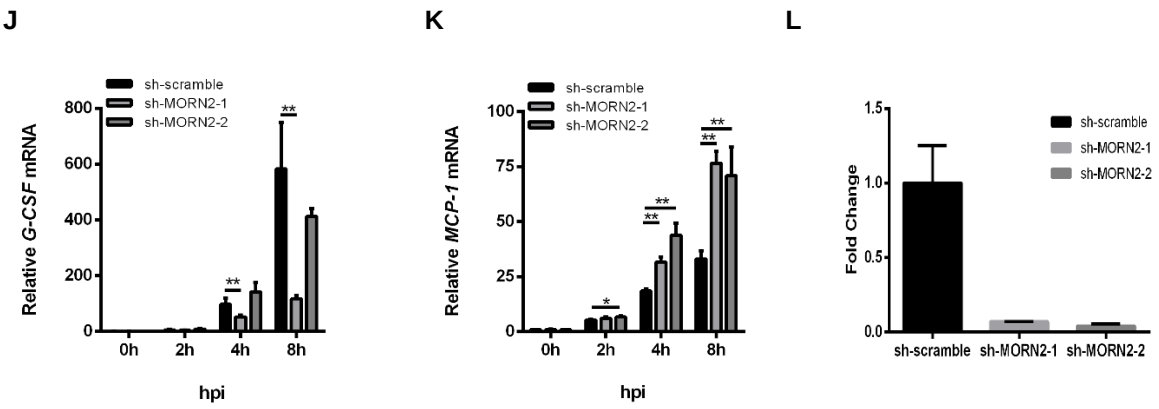
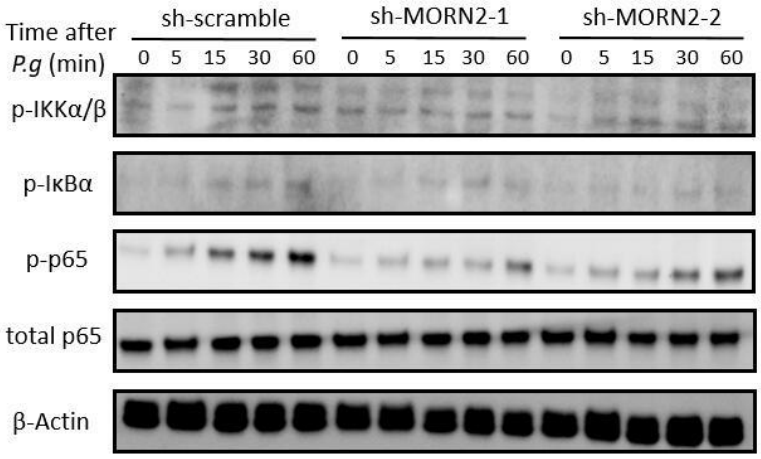


Figure 2.7. MORN2 is required for chemokines and hematopoietic growth factors production upon *P.g* infection. THP-1 cells with shMORN2 or scrambled control shRNA seeded in plates were differentiated into macrophages and infected with *P.g* (MOI=100) at different time points. (A-D) mRNA levels of CXCL1, CXCL2, GCP-2, IL-8 were measured by qPCR. (E) Protein level of IL-8 was measured by ELISA. (F-K) mRNA levels of CXCL3, CCL3, RANTES, GM-CSF, G-CSF, MCP-1 were measured by qPCR. All data were performed duplicate measured by duplicate. Data are presented as the mean ± SD. *p < 0.05 (Student's t test). (L) MORN2 knockdown efficiency in THP-1 cells.

A



B

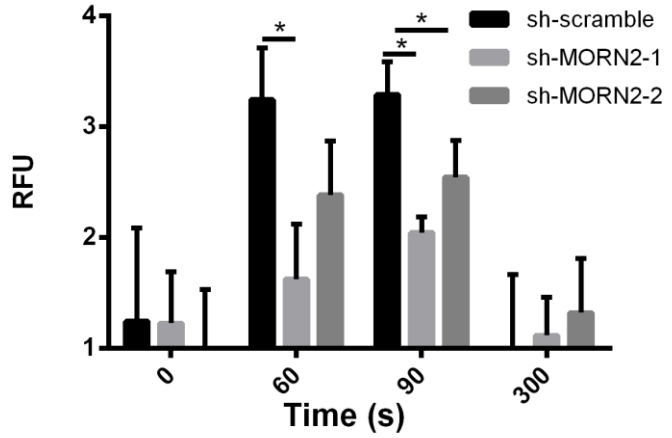


Figure 2.8. MORN2 knockdown macrophages attenuates calcium flux and activation of NF-κB. (A) THP-1 cells with shMORN2 or scrambled control shRNA were differentiated into macrophages and infected with *P.g* (MOI=100) at indicated time points. Phosphorylation of IKKα/β, IκBα and p65 were evaluated by immunoblot. β-Actin was used as loading control. (B) Calcium flux was measured by Fluo-8 at indicated time point. Data are presented as the mean ± SD. *p < 0.05 (Student's t test).

CHAPTER3: THE ROLE of TRAF3IP2 GENETIC VARIANTS IN MODULATING INNATE IMMUNE RESPONSE TO PERIODONTAL PATHOGENS

3.1. Introduction

Periodontitis is well-characterized by local tissue destruction that involves loss of alveolar bone and supporting ligament around the teeth. Although pathogenic bacteria are assumed to be essential to the destruction in host tissues, they are not a strong predictor of periodontal disease severity, suggesting that microbial challenge alone is not sufficient to cause disease. Recent evidence indicated that periodontitis is associated with compromised host responses resulting in a shift in the normal human commensal oral microbiome and destructive periodontal tissue (Genco 1992, Hajishengallis and Lamont 2012, Teles, Teles et al. 2013, Nibali 2015). Conceptually, genetic variants that will cause a functional change in the encoded protein may alter host barrier function and host innate or adaptive immunity that modifies inflammatory responses to microorganism to determine susceptibility and severity.

Recent genome-wide association studies (GWAS) of chronic periodontitis by our and other groups have highlighted loci potentially associated with clinically derived disease definitions (Divaris, Monda et al. 2013, Rhodin, Divaris et al. 2014, Shaffer, Polk et al. 2014, Offenbacher, Divaris et al. 2016) among populations of European descent. Our group created distinct periodontal complex traits by using principle component analysis (PCA) to interrogate in GWAS analysis to identify novel potential candidate genetic loci related to the biological basis and pathogenesis of chronic periodontitis (Offenbacher, Divaris et al. 2016). PCA was carried out among 975 participants including eight periodontal pathogens, gingival crevicular fluid IL-1β levels (GCF IL-1β) and clinical disease classification (CDC/AAP chronic periodontitis classification), which created distinct periodontal complex traits (PCTs) to interrogate in GWA. Each PCT defines a specific microbial community structure with varying levels of IL-1β. PCT3 (named *A.a* trait) is associated with the strongest emergence of *A. actinomycetemcomitans* across all PCTs with a correlation coefficient of 0.341 and high gingival crevicular fluid IL-1β with an eigenvalue 0.84. Strikingly, *TRAF3IP2* locus contains a non-synonymous SNP within PCT3, a novel loci significantly associated with disease

within PCT3 as assessed by Meta-Analysis Gene-set Enrichment of variaNT Associations (MEGENTA) analysis.

Th17 cells accumulate at mucosal surfaces of the skin and gut where they regulate protective immunity against a variant of bacteria. Researchers have implicated Th17 cells and Th17-asscociated cytokines, such as IL-17, in a variety of human disease based on experimental animal studies related to infectious diseases, autoimmune conditions and allergy, which makes IL-17 has recently gained prominence because of its involvement in autoimmune diseases in both human and mouse (Pene, Chevalier et al. 2008, Zhu and Qian 2012, Tabarkiewicz, Pogoda et al. 2015). IL-17 exerts its function as a protective mediator by multiple mechanisms, such as maintaining barrier integrity, producing antimicrobial peptides and regulating the recruitment and generation of neutrophils (Abusleme and Moutsopoulos 2017). Besides, IL-17A could also induce various MAPK activation and ERK is the most rapidly and strongly phosphorylated, which leads to control the stability of mRNA transcripts through the inhibition of destabilizing proteins (Hartupee, Liu et al. 2007, Herjan, Yao et al. 2013). It has been shown that homozygous deletion of IL-17RA abrogates the increase in splenic neutrophil progenitors resulting from the overexpression of IL-17A (Ye, Rodriguez et al. 2001). IL-17RA$^{-/-}$ mouse fibroblasts fail to produce CXCL1 production in response to IL-17A and in human epithelial cells, a monoclonal antibody against IL-17RA effectively blocks both IL-17A and IL-17F induced expression of G-CSF and CXCL1 (McAllister, Henry et al. 2005). IL-17RA deficient mice are susceptible to many pathogens such as *Bacteroides fragilis*, *Candida albicans*, *Klebsiella pneumoniae* and *Toxoplasmosis gondii* (Chung, Kasper et al. 2003, Huang, Na et al. 2004, Kelly, Kolls et al. 2005, Ouyang, Kolls et al. 2008)

Signaling of IL-17 family cytokines is mediated by a Type I transmembrane protein IL-17 receptor and adaptor protein TRAF3IP2, the obligate adaptor for IL-17 receptor signaling. IL-17 pathway has been shown is critical in animal model for serving as a dominant protective response to oral candidiasis through neutrophil recruitment and induction of antimicrobial factors (Conti, Shen et al. 2009). Association between over-activated IL-17 related response and various inflammatory and autoimmune diseases in humans is also supported by the findings from GWAS revealing that IL-23R variants being linked with psoriasis, psoriatic arthritis (Liu, Helms et al. 2008), Crohn's disease (Duerr, Taylor et al. 2006) and multiple sclerosis. Similarly, patients harboring mutations in molecules required for IL-17 signaling, such as IL-17RA, IL-17RC and the adaptor protein TRAF3IP2, also exhibit an increased susceptibility to

mucocutaneous, including oral candidiasis (Puel, Cypowyj et al. 2011, Boisson, Wang et al. 2013, Ling, Cypowyj et al. 2015). Meanwhile, exaggerated IL-17 response have been linked to periodontitis in the oral cavity. Johnson RB et al showed that gingival concentrations of IL-17 were highest at 4 to 5 mm sites compared to other sites and significantly lower in gingiva adjacent to a ≥ 6 mm pocket, which suggests that IL-17 concentration change as a consequence of progression of gingivitis to periodontitis (Johnson, Wood et al. 2004). *Porphyromonas gingivalis*-driven bone destruction is increased in IL-17RA knockout mice (Yu, Ruddy et al. 2007). Taken together, it is possible that the IL-17 signal pathway is a critical component of periodontal inflammation, which facilitates a shift toward a pathogenic microbial dysbiosis to further driven pathogenesis of periodontal disease.

Animal models have distinct advantages because they can mimic cellular complexities that occur in humans in vivo. Animal models in periodontal disease are well-established for understanding periodontal disease and for examining complicated host-bacteria interactions that cannot be carried out in vitro (Graves, Kang et al. 2012). Although gain or loss of function studies are often difficult to achieve in human clinical studies, it is well established the use of genetically modified animals to investigate cause and effect relationship. Rodents and humans share characteristics of periodontal disease such as anatomy, bacterial colonization, and susceptibility to experimentally induced periodontal disease.

TRAF3IP2 is the obligate adaptor protein for IL-17 signal pathway. Thus, TRAF3IP2 function is critical to IL-17 pathway to modulate immune response in oral cavity. To date, little is known for the mechanism by which periodontal bacteria regulate IL-17 pathway and the role of TRAF3IP2 in modulating innate immune response to periodontal pathogens. Thus, the purpose of this study is to unravel the genetic basis of TRAF3IP2 in the pathogenesis of periodontal disease *in vivo*.

3.2. Materials and methods

3.2.1. Bioinformatic approaches

Locus zoom plot for the TRAF3IP2 regions was created using the GWAS as described elsewhere (Offenbacher, Divaris et al. 2016) and http://locuszoom.org. The possible impact of an amino acid substitution on the structure and function of TRAF3IP2 is predicted by PolyPhen-2 score that represents probability damaging of a substitution (http://genetics.bwh.harvard.edu/pph2/).

3.2.2. GWAS characterization of the *A.a* trait

Previously-reported GWAS data was used to examine the effect of the lead *TRAF3IP2* locus polymorphism on the association of levels of periodontal pathogens and extent of probing depth ≥ 4mm (EPD4), interproximal attachment loss ≥6mm (IAL6).

3.2.3. Bacterial strain

The clinical strain *Porphyromonas gingivalis* A7436 was obtained from Oral UNC Microbiology Lab, Chapel Hill, North Carolina. Strains were characterized and tested for purity by colony forming on Brucella Agar plate (AS-141, Anaerobic System) and Gram staining kit according to the manufacture's instruction (212539, BD). All stocks were frozen in 10% skim milk at -80 ℃. Bacteria were grown in Wilkins-Chalgren Anaerobe Broth (CM0643, Oxoid) or Brucella Agar plate in an anaerobic chamber (Thermo Scientific) in an atmosphere of 85% N_2, 5% H_2, and 10% CO_2. An aliquot of the initial stock solution was used for each experiment without sub-culturing.

3.2.4. Mice

All animal procedures described in this study were approved by the institutional animal care and use committees at University of North Carolina at Chapel Hill (UNC-CH), in compliance with established federal and state polices. Specific pathogen free (SPF) wild type and TRAF3IP2$^{-/-}$ mice on a C57BL/6J background. All mice were housed under specific pathogen-free conditions in the Berryhill Hall (Laboratory Animal Medicine) at the UNC-CH. Mice were maintained in ventilated cages and given regular mouse chow diet and water ad libitum with a cycle of 12 h of light and 12 h of darkness. All surgical procedures will be conducted under the standard aseptic conditions certified by Division of Laboratory Animal Medicine personnel. All experiments were performed with the approval of UNC-CH Animal Care and Use Committee and in accordance with all relevant institutional guidelines.

3.2.5. Murine experimental periodontitis model

Periodontal bone loss was induced in mice by oral inoculation with *P. gingivalis*, as originally described by Baker (Baker, Dixon et al. 2000) with slightly modifications. Briefly, the mice were given sulfamethoxazole (0.87 mg/mL) and trimethoprim (0.17mg/mL) in their drinking water for 10 consecutive days followed by a 3 day antibiotic free period to suppress the mice normal oral flora and facilitate the subsequent bacterial inoculation enhancing colonization. After this pretreatment, the mice were randomly assigned to the different experimental groups and orally inoculated one time per day for fourteen consecutive days infected with 10^9 CFU *P. gingivalis* A7436 suspended in 2% carboxymethyl-cellulose (CMC) vehicle by oral inoculation. Sham controls from each group (wild type and TRAF3IP2$^{-/-}$) received vehicle alone. The mice were euthanized 48 hours or 6 weeks after the last oral inoculation.

3.2.6. Samples and DNA isolation

The swabbed plaque samples from four time points (before *P.g* inoculation, 48 hours, 10 days and 42 days after last oral inoculation) were used for 16s rRNA sequencing experiment. Total genomic DNA was extracted using DNeasy Blood & Tissue kit (69504, Qiagen) according to manufacturer's instructions with minor modifications. Briefly, plaque samples were added to a tube containing 180 µl PBS and 200 mg of autoclaved glass beads (G4649, Sigma). 20 µl of 200 mg/ml lysozyme solution was added into plaque samples and incubate at 37 ℃ water bath for 1 h. The samples were incubated at 56 ℃ overnight after ATL buffer and Proteinase K were added according to the manufacturer's instruction. Samples were homogenized using a TissueLyser (Qiagen) for 5 min at 25 Hz. 200 µl of Buffer AL was added to mix and was incubated at 70 ℃ for 10 min followed by adding 300 µl of 100% ethanol. DNA was isolated by Zymo-Spin Column. Quality of the isolated DNA was assessed by agarose gel electrophoresis and purtity verified using 260/230 and 260/280 ratios measured by NanoDrop 1000 instrument (Thermo Fisher Scientific, MA). DNA concentration was quantified using Quant-iT PicoGreen dsDNA Reagent (Molecular Probes, Thermo Fisher Scientific)

3.2.7. Alveolar bone measurement

Assessment of periodontal bone loss in defleshed and formalin-fixed maxillae obtained from 42 days after the last *P.g* oral inoculation were subjected to micro-computed tomography (micro-CT) image analysis. The specimens were scanned in all three spatial planes with a 100 micro-CT system as

described elsewhere (Park, Abramson et al. 2007). To assess the bone loss, the distance between cementoenamel junction (CEJ) to the alveolar bone crest (ABC) was measured on 12 predetermined points (the mesial and distal buccal root region from the first to the third maxillary molars) on the buccal surfaces of the maxillary molars by MicroView software (Parallax innovations) according to the manufacturer's instruction. The mean of the 12-site total CEJ-ABC distance of sham-infected mouse was subtracted from the mean CEJ-ABC distance for each mouse (Baker, Dixon et al. 2000). The measurements were repeated two times per site by two trained researchers and the results were expressed in mm.

3.2.8. Immunohistochemistry

Maxillae of mice sacrificed at 48 hours after the last oral inoculation with intact surrounding tissue were fixed in 4% paraformaldehyde, decalcified in 10% EDTA solution for 7 days, and embedded in paraffin. Serial mesio-distal sections (5μm thick) parallel to the long axis of the teeth (sagittal) were stained with rat monoclonal (IgG2b) to mouse Ly6g/Gr1 antibody (LS-C112469, LifeSpan BioSciences), a specific neutrophil marker (Daley, Thomay et al. 2008). The specificity of staining was confirmed by using isotype control (rat IgG2b). Anti-rat HRP-DAB according to instructions provided by the manufacturer (CTS017, R&D SYSTEMS) and then counterstained with hematoxylin (51275, Sigma). Photo images for DAB staining were captured using an Olympus microscope.

3.2.9. Oral microbial burden

To assess the oral microbial *P.g* burden at 48 hours after the last oral inoculation, the murine oral cavity was sampled for 15 s using sterile swabs. Samples were stored in PBS at -80 ℃ until bacteria DNA extraction. Total DNA extraction was performed by using MasterPure DNA Purification Kit (MC85200, Epicentre) according to the manufacturer's instructions. To establish quantitative assay, qPCR will be performed by using Maxima SYBR Green qPCR Master Mix (2X) (#K0221, ThermoFisher) through using 16S rRNA gene primers for specific *P.g* and standard curve was generated for the enumeration of *P.g* in test samples. Ten folds serial dilutions of genomic DNA (10^8, 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 cells) isolated from a pure culture of *P.g* A7436 was used in generating a standard curve. Each PCR was performed in a total volume of 25 μl containing Maxima SYBR qPCR Master Mix (2X) 12.5 μl, forward primer 0.75 μl (10 μM), reverse primer 0.75 μl (10 μM) and 1 μl of template DNA. Real-time PCR was carried out with an

initial incubation of 10 min at 95 ℃ followed by 40 cycles consisting of denaturing at 95 ℃ for 15 sec, annealing at 60 ℃ for 30 sec followed by amplification at 72 ℃ for 60 sec. After amplification, a melting curve analysis was performed to determine the specificity of the PCR products. Quantification of the *P.g* from experiment samples were calculated using the standard curve.

3.2.10. Gene expression analysis by NanoString

Gingival tissues were dissected from age-matched SPF control animals and total RNA was hybridized with reporter and capture probes for a murine customized panel (NanoString Technologies) as per manufacturer's instructions. Data were normalized to housekeeping genes and spiked positive controls. Transcript counts less than the mean of the negative control transcripts plus 1 SD for each sample were considered as background.

3.2.11. Microbiome evaluation via Illumina MiSeq 16S rRNA amplicon sequencing

DNA was amplified using primers targeting the V3-V4 region of the bacterial 16S rRNA gene and overhang adapter sequences appended to the primer pair for compatibility with IIlumina index and sequencing adapters. The complete sequences of the primers are listed in Table 3.1. Master Mixes contained 12.5 ng of total DNA and 2x KAPA HiFi HotStart ReadyMix (KAPA Biosystems). Negative controls, not containing template, were amplified for all barcode-primer sets. Each 16S amplicon was purified using AMPure XP reagent. Each sample was amplified using a limited cycle PCR program in the next step, adding IIlumina sequencing adapters and dual- index barcodes to the amplicon target. The final libraries were again purified using AMPure XP reagent (Beckman Coulter), quantified and normalized prior to pooling. The DNA library pool was then denatured with NaOH, diluted with hybridization buffer and treat denatured before loading on the MiSeq reagent cartridge (Illumina) and on the MiSeq instrument (Illumina). Automated cluster generation and paired-end sequencing with dual reads were performed according to the manufacturer's instructions.

3.3. Results

3.3.1. *TRAF3IP2* SNP is Associated with Periodontal Disease Severity

PolyPhen-2 showed that the non-synonymous SNP (rs13190932, A/G) in *TRAF3IP2* locus (Figure 3.1A) is predicted to be benign with a score of 0.00 (Figure 3.1B). rs13190932 (C/T) in LocusZoom exhibits strong linkage disequilibrium with rs33980500 ($r^2 = 0.883$) that will cause the amino acid changed from Aspartic acid to Asparagine. This mutation is predicted to be probably damaging with a score of 1.00 (Figure 3.1C). To determine the role of rs13190932 in periodontal disease severity, we examined the effect of the lead *TRAF3IP2* locus polymorphism on the association of periodontal clinical parameters from previous reported GWAS. The result shows that rs13190932 had a significantly association with EPD4 (dichotomized at 75th percentile loading of *F.n*, *T.d*, *T.f*) (Figure 3.1D-F). This finding supports the SNP in the *TRAF3IP2* locus affect the biological host response of the individual, results in increased percentage sites of extent probing depth ≥ 4mm in the 1.2 individual (heterozygous) and 2.2 (homozygous for the minor allele). rs13190932 is also significant associated with IAL6 (dichotomized at 75th percentile loading of all eight periodontal pathogens) (Figure 3.1G-N). Because the presence of SNP in the *TRAF3IP2* region lead to the severity of periodontal disease, it suggests that functional defect of TRAF3IP2 increased the susceptibility of the individual developing the periodontal disease. Taken together, the data suggest that the presence of the SNP lead to an increased predisposition of disease development accompanied by increased probing depth and clinical attachment loss.

3.3.2. *Traf3ip2*$^{-/-}$ mice are susceptible to *P.g*-induced periodontal bone loss

Next, we determined the effect of TRAF3IP2 deficiency in periodontal disease progression. The disease phenotype was evaluated among 4 groups of mice including sham- or *P.gingivalis*-infected wild-type and TRAF3IP2 knockout mice. We calculated bone loss in mice by measuring the distance between the cementoenamel junction and the alveolar bone crest of a total 12 predetermined buccal sites per mouse using micro-CT. Analyses of the mean CEJ-ABC measurements and the change in maxillary alveolar bone levels among each group revealed significantly increased bone loss in the *P.g*-induced *Traf3ip2*$^{-/-}$ mice compared to the results for the *P.g*-infected WT mice ($P < 0.05$) (Figure 3.2A, B). Thus, there was an inverse relationship between TRAF3IP2 expression and bone loss. Collectively, these data suggested TRAF3IP2 deficiency may contribute to dysregulated neutrophil recruitment and bone loss.

3.3.3. *TRAF3IP2* plays a protective role in a *P.g* colonization partially through neutrophil recruitment

We investigated the role of TRAF3IP2 in periodontal disease pathogenesis using *P.gingivalis* - induced murine periodontitis model. *P.gingivalis* colonization was assessed to confirm that the mice were successfully infected by *P.g*. *P.g* was detected in WT and *Traf3ip2*$^{-/-}$ mice at comparable levels 2 days post oral inoculation. Moreover, there was a significant increase in *P.gingivalis* loading levels in *Traf3ip2*$^{-/-}$ mice inoculated with this organism compared to the levels in WT-infected mice ($P<0.001$) (Figure 3.3A). Therefore, TRAF3IP2 deficiency significantly affect *P.g* colonization, which is important for the validity of the leukocyte recruitment since any potential differences in leukocytes recruitment between WT and *Traf3ip2*$^{-/-}$ would be attributed to differences in *P.gingivalis* colonization. Neutrophils are considered to be the first line of defense during inflammation and the most abundant leukocytes in blood. Given that neutrophil recruitment through chemotaxis is a major IL-17 function (Korn, Bettelli et al. 2009, Abusleme and Moutsopoulos 2017) and TRAF3IP2 adaptor protein is an immediate and essential signaling component of IL-17 receptor (Chang, Park et al. 2006, Qian, Liu et al. 2007), we first determined to check whether elevated *P.g* colonization was associated with changes in neutrophils recruitment. Immunohistochemical analysis of Ly6G (Lymphocyte antigen 6 complex locus G6D), a marker expressed predominantly on neutrophils, showed that the lower number of cells expressed Ly6G in the *P.g* challenged *Traf3ip2*$^{-/-}$ mice than that in WT mice (Figure 3.3B, C).

3.3.4. Altered cytokines and neutrophil related chemokines expression in the infected *Traf3ip2*$^{-/-}$ mice

To investigate molecular differences between *Traf3ip2*$^{-/-}$ mice and WT mice, we conducted transcriptome profiling of several inflammatory genes. Because TRAF3IP2-deficient mice exhibited less neutrophils level after *P.g*-infection, we hypothesized that neutrophil recruitment would be compromised in these animals. *P.g*-infected *Traf3ip2*$^{-/-}$ mice showed altered expression of some genes relative to *P.g*-infected WT mice. Although the transcription profiles of neutrophil related cytokines (*Cxcl1*, *Cxcl2*, *G-csf*) and *Ccl2* of KO and WT mice were generally similar, there are lower *Cxcl5* expression at 48 hours post-infection in *Traf3ip2*$^{-/-}$ mice compared to WT mice (Figure 3.4 A-E). Interestingly, there are no differences of *Ccl6* expression between two genotype groups, but it showed a decreasing trend upon infection in

Traf3ip2$^{-/-}$ mice whereas no significant changes was observed in WT mice (Figure 3.4F). *Il36g* functions as an agonist of NF-κB activation through the orphan IL-1-receptor-related protein 2/IL1RL2. It has been demonstrated that IL-36G exerts inflammatory effects on human oral epithelial cells and stimulates the strong expression of IL-8, CXCL1 (Huynh and Scholz 2016), which suggests that epithelium derived IL-36G may also be capable of functioning in amplifying inflammatory responses. The expression of *Il36g* was downregulated in the *Traf3ip2*$^{-/-}$ mice at baseline and early infection time point (Figure 3.4G). T cell functional differentiation is mediated by lineage-specific transcription factors and RORγ was shown to regulate Th17 differentiation (Yang, Pappu et al. 2008, Castro, Liu et al. 2017). There is higher *Rorc* expression in *Traf3ip2*$^{-/-}$ mice compared to WT mice at baseline (Figure 3.4H), which demonstrates that there is a positive-feedback of Th17 cell differentiation in *Traf3ip2*$^{-/-}$ mice at baseline. Taken together, these date complement the effects of deficiency of TRAF3IP2 associated with less neutrophils recruitment and provide one possible mechanism by which IL-17-TRAF3IP2 signaling directly on neutrophils recruitment and Th17 lineage differentiation.

3.3.5. Absence of TRAF3IP2 adversely influences epithelial barrier function

Besides neutrophils recruitment, additional mechanisms of immune subversion may contribute to the ability of *P.g* in the pathogenesis of periodontal disease. To elucidate the mechanisms by which TRAF3IP2-mediated signaling contributes to periodontal disease in this model, alternations in epithelial cell function were further assessed by measuring expressing changes of genes associated with epithelial barrier function, tight junction integrity, differentiation and antimicrobial peptides production. In line with their susceptibility to bone loss, *P.gingivalis*-infected *Traf3ip2*$^{-/-}$ mice displayed significantly lower level of expression of *Ocln* (Figure 3.5A), a critical component in maintaining the barrier properties of tight junctions (Feldman, Mullin et al. 2005), compared to the WT mice group in all time point ($p < 0.05$), particularly at the baseline time point. Next we detected lower mRNA expression levels for *Krt1*, *Krt10*, *Klf4*, *Dsc1*, *Dsg1*, *Filaggrin, Znf750* (Figure 3.5B-H), genes associated with maintenance of epithelial integrity and differentiation, in *Traf3ip2*$^{-/-}$ mice group. Antimicrobial peptides such as *S100a8*, *S100a9*, *Defb1*, *Defb4*, and *Lcn2* expressed lower levels at baseline and late time points ($p < 0.05$) (Figure 3.5I-M), consistent with the known role of IL-17 signaling in induction of anti-microbial peptides.

3.3.6. TRAF3IP2-mediated signaling promotes osteoblasts formation

To examine the role of TRAF3IP2 on osteoblasts formation, gene expression of osteocalcin and Runx2 were detected in *Traf3ip2*$^{-/-}$ mice and wild type mice infected by *P.g*. Osteocalcin is produced by osteoblasts and often be used as a marker for the bone formation process. Decreased osteocalcin expression at 42 days compared to 48h after last dose *P.g* infection was observed in *Traf3ip2*$^{-/-}$ mice whereas there is no obvious osteocalcin expression changes in WT mice (Figure 3.6A), which illustrated that deficiency of TRAF3IP2 has a negative effect on bone formation process. Runx2 protein is the first transcription factor required for the determination of the osteoblast lineage and it induces the differentiation of multipotent mesenchymal cells into immature osteoblasts, directing the formation of immature bone (Komori 2006, Komori 2010). We observed that there is significant less expression of Runx2 at mRNA level in gingiva of *Traf3ip2*$^{-/-}$ mice at 42days after last dose of *P.g* infection compared to wild type mice (Figure 3.6B), which consistent with previous observed the bone loss phenotype.

3.3.7. Microbiome shifts correlates with the absence of TRAF3IP2

Commensal communities shape tissue immunity and specific oral microbes are implicated in the development of periodontitis (Griffen, Beall et al. 2012, Abusleme, Dupuy et al. 2013). To determine the contributions of TRAF3IP2 in IL-17 signaling to the microbiota composition before and after *P.g* infection, we analyzed the microbiota isolated from the plaque before and at various times after inoculation by 16s rRNA sequencing. Samples were successfully amplified at V3-V4 region and sequenced. We found bacterial composition in mice between male and female are quite similar, but there is difference in bacterial composition between two genotypes (Figure 3.7A, B). These data allowed us to undertake a detailed examination of the mouse oral microbiome between genotypes. The α-diversity of samples was measured by Shannon entropy at different time point (Figure 3.7C-G) and suggested that there was more bacterial richness and evenness in WT mice compared to *Traf3ip2*$^{-/-}$ mice. We showed that lack of TRAF3IP2 produces profound effects on the oral microbiota with an expansion of *Firmicutes* and *Proteobacteria*, and decreased abundance of *Actinobacteria* (Figure 3.7 H). At phylum level, we did find a statistically higher abundance of *Firmicutes* and reduction of *Actinobacteria* in *Traf3ip2*$^{-/-}$ mice compared to WT mice, there is no significant difference in *Proteobacteria* but increased amount of *Fusobacteria* from baseline to 10 days after *P.g* infection in *Traf3ip2*$^{-/-}$ mice (Figure 3.7 I-L).

3.4. Discussion

Many findings implicate IL-17 is a multifaceted cytokine with diverse roles in both immune protection and also immunopathology. Some evidence showed that IL-17 plays a role for recruitment of neutrophils in limiting pathogens (Liao, Xia et al. 2012, Flannigan, Ngo et al. 2017, Burstein, Guasconi et al. 2018). Besides, IL-17 enhances antimicrobial peptide in human keratinocytes (Liang, Tan et al. 2006, Peric, Koglin et al. 2008) and the role of IL-17A in maintaining barrier function of epithelial tissues (Kinugasa, Sakaguchi et al. 2000). Meanwhile, understanding IL-17 immunopathology may require us to consider the IL-17 response in the context of other host immune mechanisms that may be inappropriate or inadequate and thereby dysregulate or amplify immune response. It has been showed that increased IL-17 level in GCF of aggressive periodontitis compared with chronic periodontitis and controls (Shaker and Ghallab 2012). Critically, overabundance of neutrophils in patients with periodontitis also linked to excessive IL-17 response. IL-17 has potential to amplify inflammation through excessive neutrophils recruitment by enhancing pro-inflammatory cytokine production and by activating osteoclasts, which would result in immunopathology and bone resorption (Moutsopoulos, Chalmers et al. 2015). It is also documented that exaggerated IL-17 responses are linked to microbial overgrown or dysbiosis (Eskan, Jotwani et al. 2012). TRAF3IP2 is the obligate adaptor for signaling by IL-17 and the specific role of TRAF3IP2 in periodontitis has not been explored previously.

Our group created biologically informed periodontal phenotypes by using PCA (Offenbacher, Divaris et al. 2016) and identified genome-wide significant loci associated with periodontal disease by using the gene-wide strategy of MAGENTA analysis. We found *TRAF3IP2* is significantly associated with periodontal disease phenotypes and identified the leading non-synonymous SNP (rs13190932) exhibits strong linkage disequilibrium (r^2=0.883) with a deleterious rs33980500 that is predicted to be probably damaging with a score of 1.0. Our GWAS revealed that subjects with 1.2 or 2.2 *TRAF3IP2* genotype (heterozygous or homozygous for minor allele) have greater extent probing depth ≥ 4mm (dichotomized at 75th percentile loading of *F.n*, *T.d*, *T.f*) and interproximal attachment loss ≥ 5mm (dichotomized at 75th percentile loading of all eight periodontal pathogens) compared to subjects with 1.1 (homozygous for major allele), which suggests that defects in protein function of TRAF3IP2 may cause individuals more susceptible to periodontal disease. Given the well-characterized role of IL-17 serves as a regulator of mucosal immunity and TRAF3IP2 is a necessary adaptor protein for IL-17 signal pathway, it becomes

important to understand the role of TRAF3IP2 in periodontal disease. Our group have identified that TRAF3IP2 is mainly expressed in epithelial cells of human gingival tissues, which suggests a potential role of TRAF3IP2 in epithelial cells response to periodontal pathogens. CXCL1, IL-8, IL-6 at both mRNA and protein level was significant decrease in *Traf3ip2* knockdown in HGEs upon IL-17 stimulation, suggesting that TRAF3IP2 is a determinant for IL-17-induced gingival epithelial cytokines and several neutrophil recruiting chemokines production. In addition, SNP variant rs33980500 impairs CXCL1 and IL-8 production at messenger level.

Nonetheless, cellular studies that take place on plastic surfaces with limited numbers of cell types present cannot mimic cellular complexities that occur in humans and are often less accurate than *in vivo* studies. Animal models in periodontal disease are particularly important at this point in understanding cause and effect relationships through using genetically modified animals. The inoculation of human strains of bacteria into the oral cavity and subsequent impact on the periodontium has been studied in various rodent models (Chang, Ramamurthy et al. 1988, Baker, Evans et al. 1994, Lalla, Lamster et al. 1998, Baker, Dixon et al. 2000). To identify the direct role of TRAF3IP2 in local inflammatory pathology, we investigated whether the periodontal disease phenotypes can be mirrored in TRAF3IP2-deficient mice by establishing experimental murine periodontitis through oral inoculating *P.g* 14 consecutive days. At weeks of 12 weeks of age, global loss of TRAF3IP2 had significantly greater periodontal bone loss than did their respective age-matched wild type mice, consistent with the hypothesis that TRAF3IP2 contributes to protective immunity in periodontal disease development. However, to exclude to possibility that the observed greater bone loss in 12 week old *Traf3ip2*$^{-/-}$ mice relative to that age-matched wild was not due to innately different periodontal bone heights, *Traf3ip2*$^{-/-}$ mice and wild type mice at various age should be examined in future.

Here we investigated underlying mechanisms of TRAF3IP2 plays a protective role in *P.g*-induced periodontal bone loss. In fact, IL-17 signaling plays its function as a protective role in innate immunity by multiple mechanisms. The first line of defense strategically positioned at barrier site is regulating the recruitment and generation of neutrophils. IL-17 promotes granulopoiesis and induces the chemotactic recruitment, activation and survival of neutrophils (Kolls and Linden 2004, Stark, Huo et al. 2005, Gaffen 2009). Notably, the increased inflammatory bone loss in *Traf3ip2*$^{-/-}$ mice was associated with diminished infiltration of Ly6G^{+} neutrophils in the gingiva, consistent with lack of TRAF3IP2-mediated regulation of

neutrophil trafficking and compromised *P.g* clearance. Quantitative analysis of the expression of mRNA encoding inflammatory mediators in the gingiva of *Traf3ip2*$^{-/-}$ mice and their wild type control at the age of 12 weeks old showed similar neutrophil-recruiting CXC chemokines, CCL2 and neutrophil differentiation and maturation cytokine G-CSF. However, we observed significant but less pronounced downregulation in the transcript abundance of IL-36G between *Traf3ip2*$^{-/-}$ mice and their wild type control at 48 h post infection. It has been documented that IL-36 is a dominant cytokine in psoriasis and prominent expressed by keratinocytes proximal to neutrophilic pustules as well as both neutrophils and neutrophil proteases activate IL-36 (Johnston, Xing et al. 2017). IL-36G has does dependently effect on the production of proinflammatory cytokines, chemokines and can induce the production of IL-17 by $CD4^+$ T cells (Vigne, Palmer et al. 2011). Collectively, these data suggesting IL-36-chemokine-neutrophil inflammatory axis is a potent driver of neutrophil chemokine expression and infiltration. The RORγ is selectively expressed in *in vitro*-differentiated Th17 cells and required for IL-17 production, as impaired Th17 differentiation was observed in mice reconstituted with the bone marrow of RORγ-deficient mice (Ivanov, McKenzie et al. 2006). Furthermore, RORγ deficiency greatly reduced the differentiation of Th17 cells even in the presence of TGF-β and IL-6, establishing RORγ as a master transcription regulator for the differentiation of Th17. RORγ expression decreased in a time-dependent manner upon infection in *Traf3ip2*$^{-/-}$ mice suggesting that the deficiency of TRAF3IP2 leads to compromised homeostasis of IL-17-producing Th17 cell differentiation compared to wild type mice. Produced IL-17 cytokines thus cannot feed forward to induce the expression of chemokines and cytokines in epithelial cells. These results indicated that TRAF3IP2-mediated signaling promotes neutrophil recruitment and Th17 cell differentiation. Thus, a positive feedback loop amplifies IL-17 production and IL-17-TRAF3IP2 signal transduction.

Another mechanism by which TRAF3IP2 contributes to mucosal immune surveillance is promoting epithelial integrity by regulating tight junction protein or inducing keratinocyte differentiation. To date, it has been shown that IL-17 regulates the cellular localization of the tight junction protein occluding and neutralizing IL-17 results in increased gut permeability and IL-17$^{-/-}$ mice suffer worse epithelial injury and enhanced gut permeability (Lee, Tato et al. 2015). IL-17 inhibition weakens intestinal epithelial barrier function and exacerbates Crohn's disease and *Abcb1a*$^{-/-}$ mouse colitis (Maxwell, Zhang et al. 2015). Moreover, IL-17C directly regulated the expression of the tight junction molecule occludin by colonic epithelial cells (Reynolds, Martinez et al. 2012). Intriguingly, we observed a prominent quantitative

difference of epithelial barrier function markers in the gingiva of *Traf3ip2*$^{-/-}$ mice compared to WT mice. Keratins are typically organized into intricate networks integrated at sites of cell-cell and cell-matrix adhesion (Hobbs, Lessard et al. 2012). Keratin1 and Keratin10 are the most abundant proteins in the upper epidermis where they form intermediate filaments (IFs) by polymerization. Keratin IFs integrating neighbor keratinocytes and ultimately the entire tissue by spanning the cytoplasm and interact with desmosomal cell-cell contacts at the plasma membrane. It has been reported that deletion of K1/K10 IFs affects desmosomal structure (Wallace, Roberts-Thompson et al. 2012) and Krt1-null mice caused perinatal lethality and barrier defect (Roth, Kumar et al. 2012). Klf4 was reported as a transcription factor required for establishing the barrier function of the skin and *Klf4*$^{-/-}$ mice die shortly after birth due the loss of skin barrier function, as measured by penetration of external dyes and rapid loss of body fluids (Segre, Bauer et al. 1999). Both desmocollin-1 (DSC1) and desmoglein-1 (DSG1) are primarily found in epithelial cells where they constitute the adhesive proteins of desmosome cell-cell junction and are required for cell adhesion and desmosome formation that is essential for epithelial barrier creates a surface seal essential for protecting animals against microbial infection. Reduced barrier integrity coincided with alternations in epithelial-cell-associated other gene expression. Loss of TRAF3IP2 leads to decreased endogenous filaggrin and ZNF750 expression was also observed in our study. It was shown that loss of filaggrin leads to poorly formed stratum corneum and recent human genetic studies strongly suggest that perturbation of skin barrier function as a result of reduction or complete loss of filaggrin expression (Sandilands, Sutherland et al. 2009). Therefore, filaggrin protects the host from the entry of foreign antigens as the frontline defense. Keratinocyte differentiation is an essential key process in formation and maintenance of the skin barrier. Various common skin disease such as psoriasis and ichthyosis involve the disintegration of epidermal skin barrier due to altered keratinocytes differentiation (Candi, Schmidt et al. 2005, Hoffjan and Stemmler 2007). ZNF750 is a regulator of keratinocyte terminal differentiation and controls epithelial homeostasis by inducing differentiation genes and mutations of ZNF750 has been shown increased psoriasis risk (Birnbaum, Hayashi et al. 2011, Debniak, Soczawa et al. 2014). ZNF750 knockdown cells presented with markedly reduced expression of epidermal late differentiation markers including gene subsets of skin barrier formation (Cohen, Birnbaum et al. 2012). Thus, it is conceivable that TRAF3IP2 mediates or promotes epithelial integrity by regulating tight junction proteins and inducing terminal differentiation of epithelial cells to maintain the epithelial barrier and keeping out periodontal pathogens.

It must be acknowledged that TRAF3IP2 may, at least theoretically, contribute to the periodontal disease phenotype in ways other than neutrophil recruitment and epithelial integrity maintenance. Given the role of IL-17 in induction of antimicrobial mediators in epithelial cells (Liang, Tan et al. 2006, Peric, Koglin et al. 2008, Mengesha and Conti 2017), we are attempting to speculate whether TRAF3IP2 plays a role in the induction of antimicrobial mediators in the experimental periodontitis mice model. Our data showed that *P.g* induces less production of S100A8/A9, β-defensin1/4 and lipocalin-2 in gingiva of *Traf3ip2*$^{-/-}$ mice compared to wild type mice, which suggests that TRAF3IP2 is emerging as a critical regulators of antimicrobial peptide production in the development of periodontal disease. This protection might be mediated by IL-17-driven upregulation of antimicrobial peptides to effectively eliminate the pathogens. However, data are accumulating to support a role for Th17 cells derived cytokines as key regulators of the antimicrobial peptide response, which may be an important mechanisms by which they confer further protection from infection. In wild type mice, the basal expression of antimicrobial proteins (β-defensin1/4 and lipocalin-2) by epithelial cells also controls the overgrowth of commensal bacteria, thereby preventing bacterial invasion in the absence of a breach in the epithelial cell barrier. Recent studies showed that the antimicrobial protein can synergize with IL-1β to increase the production of cytokines, such as IL-8 and chemokines such as CCL2 (Yu, Mookherjee et al. 2007), as well as to increase the synthesis and release of defensins (Zheng, Niyonsaba et al. 2007). Our results find that there is no proper IL-1β response in *Traf3ip2*$^{-/-}$ mice compared to WT mice, which is probably another potential mechanism that cytokine signaling cannot further increases the antimicrobial protein response. IL-1β has been shown that induces the expression of lipcalin-2, an iron-sequestering antimicrobial peptide, in the lungs (Cowland, Sorensen et al. 2003) and upregulates the transcription of genes that encode β-defensin-2, S100A7, calprotectin (heterodimer of S100A8 and S100A9) and lipocalin-2 in the skin (Bando, Hiroshima et al. 2007, Yano, Banno et al. 2008). Such findings support the notion that IL-17/TRAF3IP2-mediated regulation of antimicrobial peptides in epithelial cells.

Host immunity in health and disease can shape commensal communities and specific oral microbes can be implicated in the development of periodontitis (Griffen, Beall et al. 2012, Abusleme, Dupuy et al. 2013). It becomes important to understand what the role of TRAF3IP2 in shaping microbiome colonization in the periodontium. To date, little is known of the role of TRAF3IP2 in microbiome composition in periodontium. What is more, a possible role for the changed microbiome as a

contributor in the susceptibility to the pathogenesis of periodontal disease cannot be disregard. Therefore, evaluating microbiome-host interactions in the setting of defective TRAF3IP2 becomes greater interest. Our study shows that lack of IL-17 signaling alters the oral microbiome composition, leading to increased proportion of *Firmicutes* and *Proteobacteria*, with reductions in *Actinobacteria*, with a reduction in overall microbiome diversity and evenness. This suggests that IL-17/TRAF3IP2 signaling targets such as neutrophils and antimicrobial peptides have a significant role in maintaining the normal microbiome composition, in particular increasing colonization proportion with potentially pathogenic *Firmicutes* and reducing *Actinobacteria*, which consistent with the association of *Firmicutes* with periodontitis while the proportion of *Actinobacteria* was higher in health in previous human studies (Abusleme, Dupuy et al. 2013, Perez-Chaparro, Goncalves et al. 2014). Meanwhile, numerous studies do suggest that reduced microbiome diversity may be associated with gut diseases (Manichanh, Rigottier-Gois et al. 2006, Noor, Ridgway et al. 2010, Zhernakova, Kurilshikov et al. 2016). These reflect the lack of TRAF3IP2 mediated inflammation may cause shifts in oral microbial communities correlated with pathogenic.

In sum, although a fully understanding of host-commensal interactions in TRAF3IP2-mediated immunity remains to be determined, here we demonstrate TRAF3IP2 plays a protective role at the mucosal surface in clearance of pathogens by multiple potential mechanisms and shifts the commensal microbiota, highlighting genetic variant may increase the susceptibility of individual to periodontal disease. These data may potentially serve as evidence for a gene therapeutic intervention.

Collectively, these data indicate that IL17-RA is critical component of the receptor complex that mediates IL-17 chemokine production. TRAF3IP2 provides a protective immunity against bacterial infection and this protection is mediated by upregulation of granulopoietic cytokines, maintaining epithelial barrier integrity and inducing antimicrobial peptide production.

3.5. Conclusions

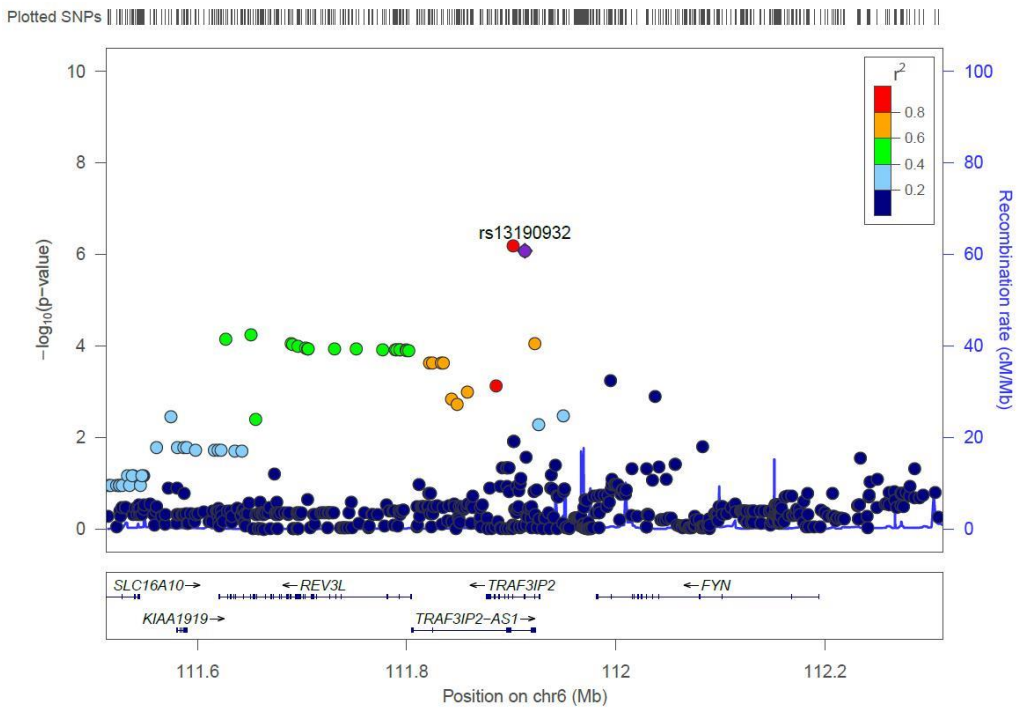
TRAF3IP2 plays a protective role in a *P.g* colonization through neutrophil recruitment, maintenance epithelial barrier and induction of antimicrobial peptides. Defective TRAF3IP2 disrupts host-microbial homeostasis and causes bone loss. It is possible that TRAF3IP2 loss-of-function polymorphisms may compromise microbial clearance and the innate immune response leading to the emergence of *A.a* dominated dysbiosis in periodontal disease.

3.6. Figures and tables

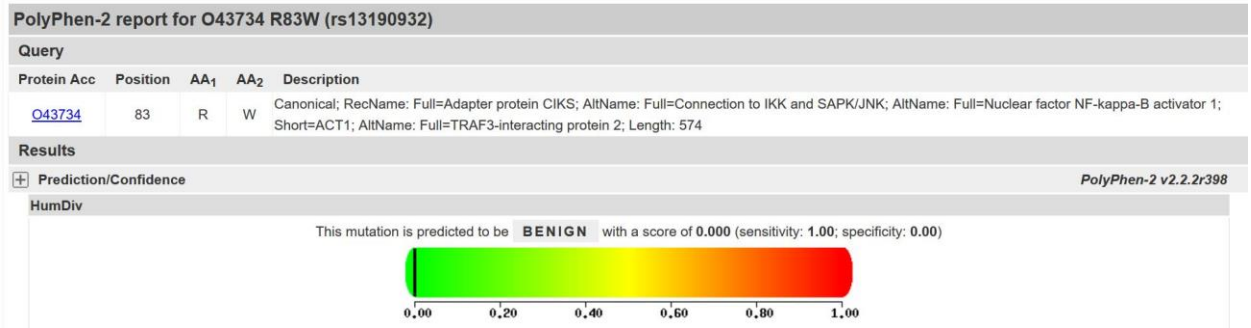
Table 3.1 Primer sequence information for 16S rRNA sequencing

Platform	Primer Name	Sequence (5' – 3')	Targeting Region
Illumina MiSeq	Forward Primer	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGCCAGCMGCCGCGGTAA	V3-V4
	Reverse Primer	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACHVGGGTWTCTAAT	V3-V4

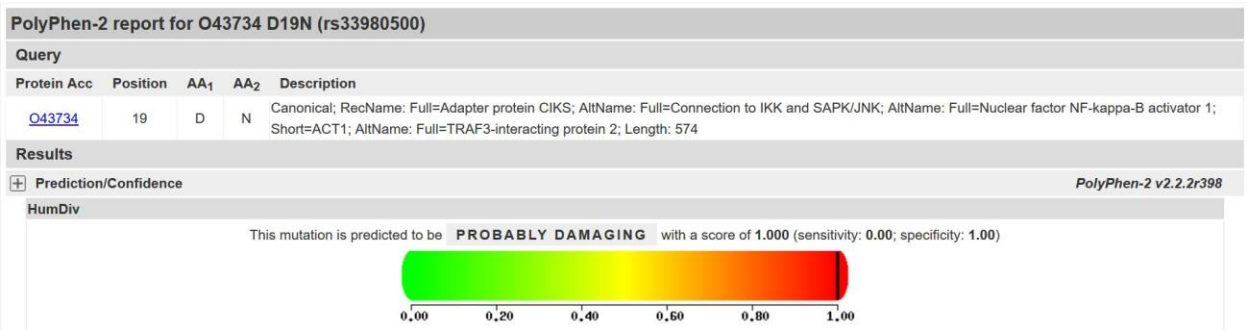
A


Plotted SNPs
rs13190932
r²
0.8
0.6
0.4
0.2
−log10(p−value)
Recombination rate (cM/Mb)
SLC16A10→
←REV3L
←TRAF3IP2
←FYN
KIAA1919→
TRAF3IP2−AS1→
111.6
111.8
112
112.2
Position on chr6 (Mb)

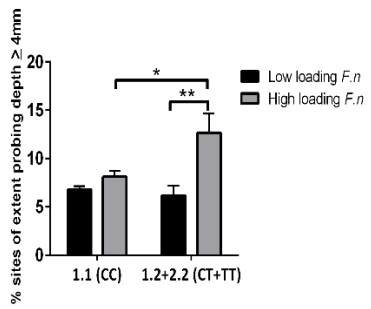
B


PolyPhen-2 report for O43734 R83W (rs13190932)
Query
Protein Acc
Position
AA1
AA2
Description
O43734
83
R
W
Canonical; RecName: Full=Adapter protein CIKS; AltName: Full=Connection to IKK and SAPK/JNK; AltName: Full=Nuclear factor NF-kappa-B activator 1; Short=ACT1; AltName: Full=TRAF3-interacting protein 2; Length: 574
Results
Prediction/Confidence
PolyPhen-2 v2.2.2r398
HumDiv
This mutation is predicted to be BENIGN with a score of 0.000 (sensitivity: 1.00; specificity: 0.00)
0.00
0.20
0.40
0.60
0.80
1.00

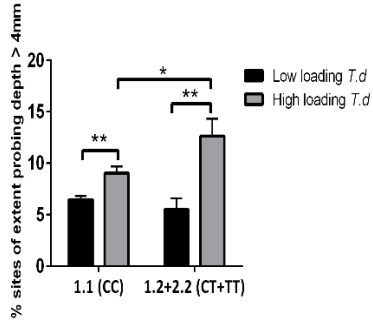
C


PolyPhen-2 report for O43734 D19N (rs33980500)
Query
Protein Acc
Position
AA1
AA2
Description
O43734
19
D
N
Canonical; RecName: Full=Adapter protein CIKS; AltName: Full=Connection to IKK and SAPK/JNK; AltName: Full=Nuclear factor NF-kappa-B activator 1; Short=ACT1; AltName: Full=TRAF3-interacting protein 2; Length: 574
Results
Prediction/Confidence
PolyPhen-2 v2.2.2r398
HumDiv
This mutation is predicted to be PROBABLY DAMAGING with a score of 1.000 (sensitivity: 0.00; specificity: 1.00)
0.00
0.20
0.40
0.60
0.80
1.00

D


% sites of extent probing depth ≥ 4mm
Low loading F.n
High loading F.n
1.1 (CC)
1.2+2.2 (CT+TT)

E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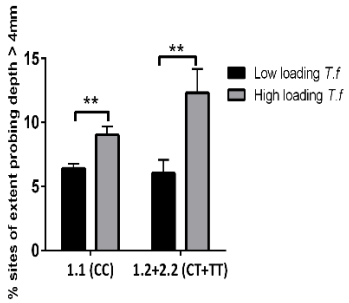
Low loading T.d
High loading T.d
1.1 (CC)
1.2+2.2 (CT+TT)

F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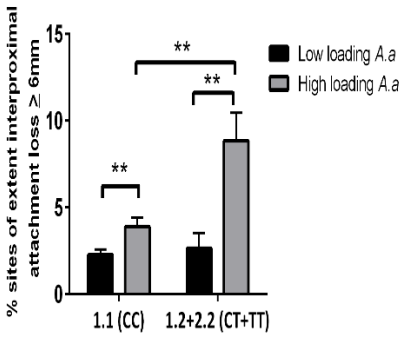
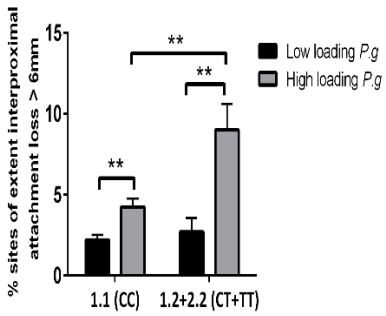
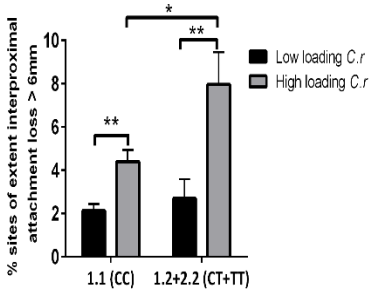
G


% sites of extent interproximal attachment loss ≥ 6mm
Low loading A.a
High loading A.a
1.1 (CC)
1.2+2.2 (CT+TT)

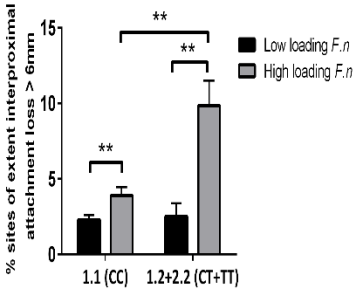
H


% sites of extent interproximal attachment loss > 6mm
Low loading P.g
High loading P.g
1.1 (CC)
1.2+2.2 (CT+TT)

I


% sites of extent interproximal attachment loss > 6mm
Low loading C.r
High loading C.r
1.1 (CC)
1.2+2.2 (CT+TT)

J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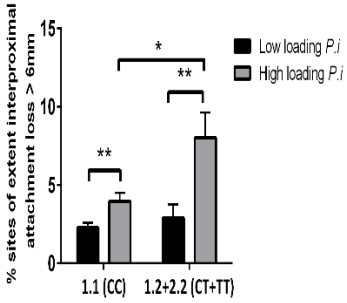
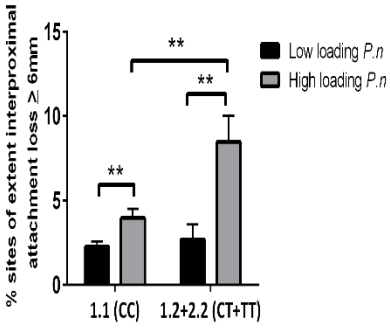
Low loading F.n
High loading F.n
1.1 (CC)
1.2+2.2 (CT+TT)

K


% sites of extent interproximal attachment loss > 6mm
Low loading P.i
High loading P.i
1.1 (CC)
1.2+2.2 (CT+TT)

L


% sites of extent interproximal attachment loss ≥ 6mm
Low loading P.n
High loading P.n
1.1 (CC)
1.2+2.2 (CT+TT)

M **N**

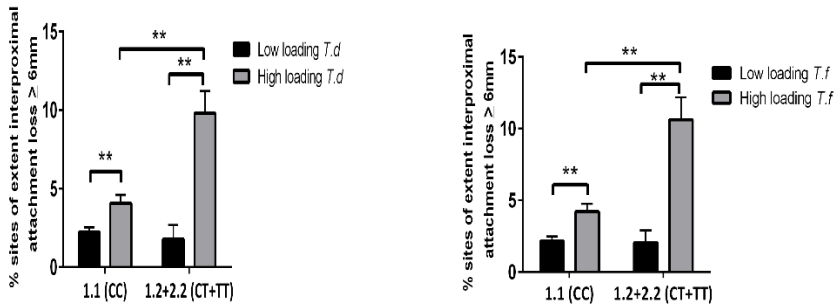


Figure 3.1. *TRAF3IP2* SNP is associated with periodontal disease severity. (A) LocusZoom of *TRAF3IP2* containing a non-synonymous SNP (rs13190932). (B, C) Possible impact of the amino acid substitution (rs13190932 and rs33980500) on the structure and function of TRAF3IP2 was predicted by PolyPhen-2. (D-F) *TRAF3IP2* SNP (rs13190932) with *F.n, T.d or T.f* loading effects on percentage sites of extent probing depth ≥ 4mm. (G-J) *TRAF3IP2* SNP (rs13190932) with eight periodontal pathogens' loading effects on percentage sites of extent interproximal attachment loss ≥ 6mm. Low loading periodontal pathogens indicated 75th percentile. *p<0.05 (Student's t test).

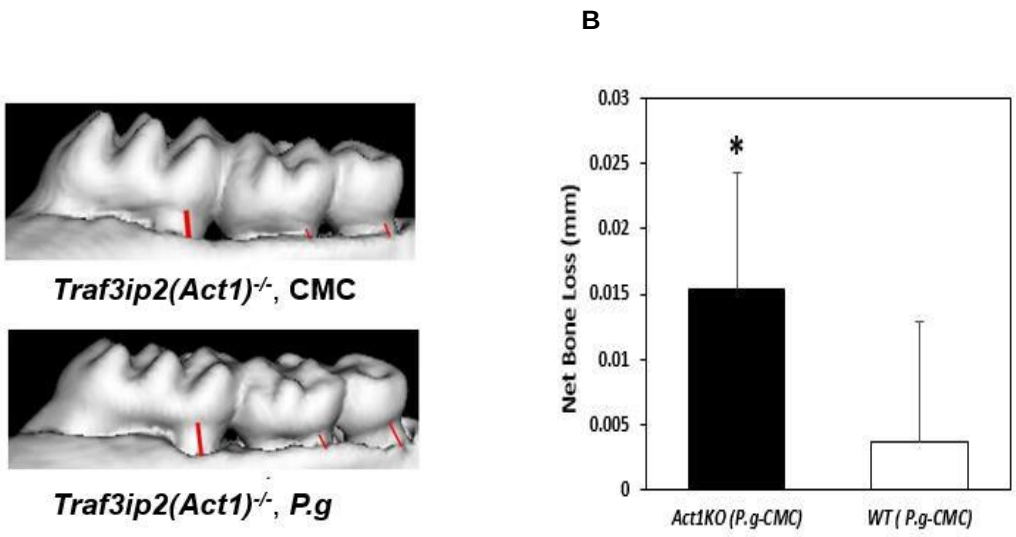


Figure 3.2 *Traf3ip2*$^{-/-}$ mice are susceptible to *P.g*-instigated periodontal bone loss. (A) Representative micro-CT images of maxillae from *P.g*-infected two groups of mice. (B) Amount of bone change in *Traf3ip2*$^{-/-}$ mice and WT mice (12 weeks of age). Values were calculated by distance between the cementoenamel junction and alveolar bone crest (CEJ-ABC distance) in *P.g*-infection groups minus sham-infection groups. * $P < 0.01$. Data are pooled from two-independent experiments with four to five mice per group in each, for a total of nine to ten mice per group.

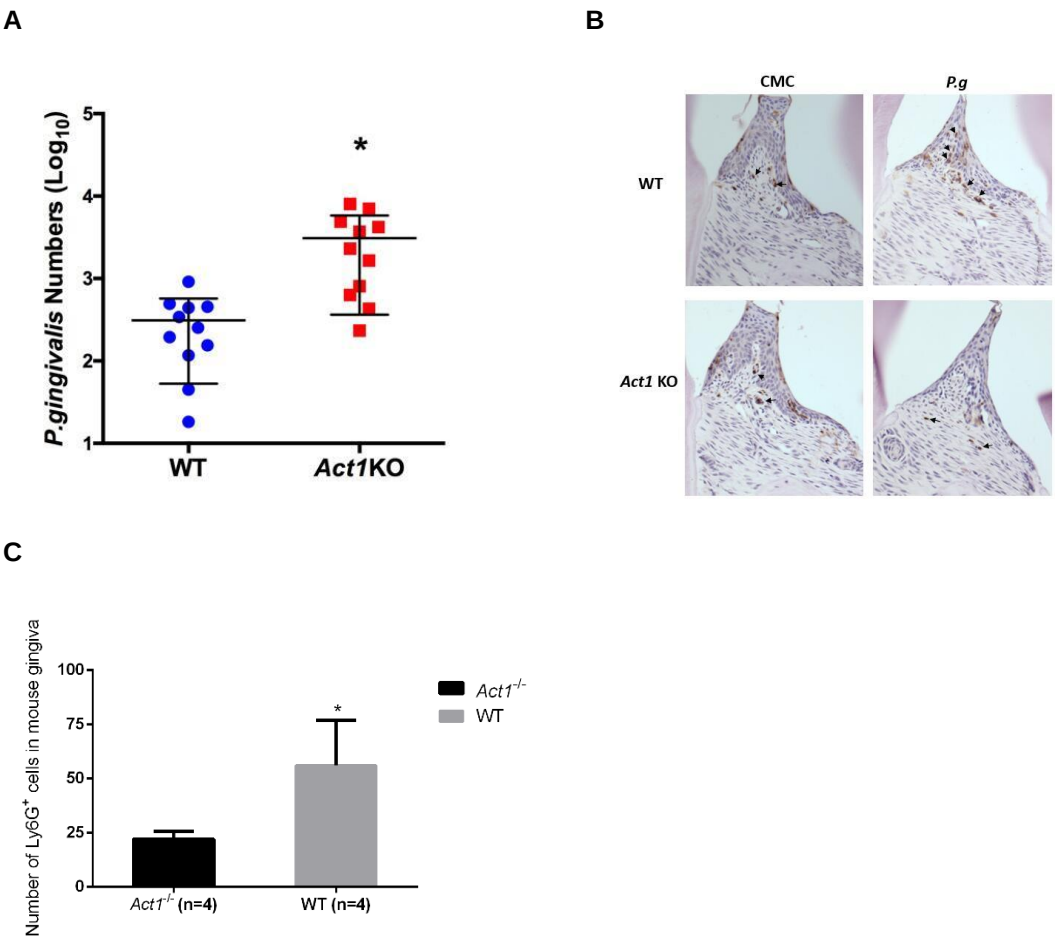
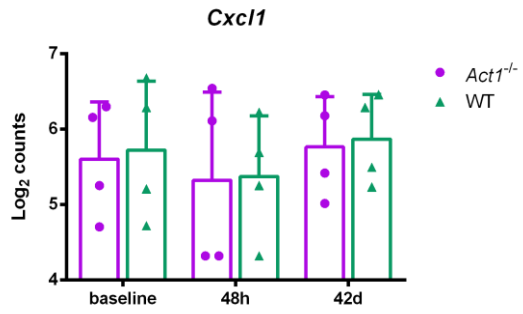
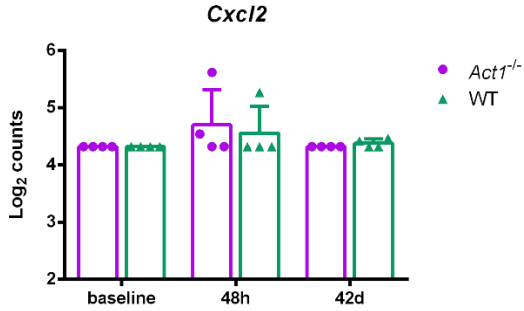


Figure 3.3. Higher colonization of *P.g* in *Traf3ip2*$^{-/-}$ mice is correlated with defective neutrophils recruitment. (A) Graph shows comparison of *P.g* in the oral cavity of 48 hours post-infection, determined by a real-time PCR assay (n = 11). (B) Representative immunohistochemical images of sagittal sections of interdental gingiva stained for Ly6G. (C) Quantification of the number of neutrophils recruitment of the images in B and additional images. * $P < 0.01$. Data are pooled from two independent experiments with four mice per group in each, for a total of eleven mice per group (Mean ± S.D. in C).

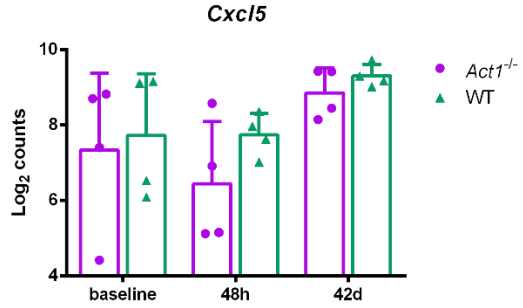
A


Cxcl1
Log2 counts
7
6
5
4
baseline
48h
42d
Act1-/-
WT

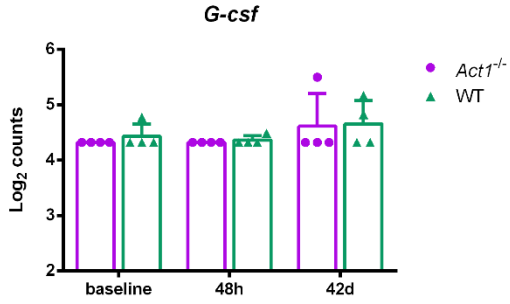
B


Cxcl2
Log2 counts
6
5
4
3
2
baseline
48h
42d
Act1-/-
WT

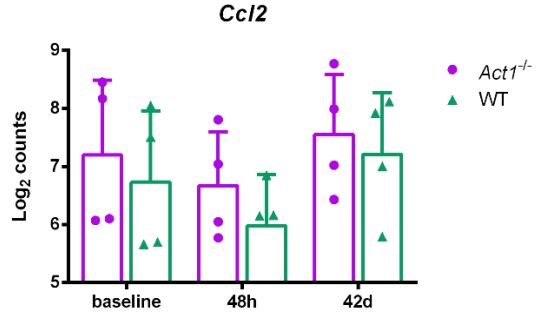
C


Cxcl5
Log2 counts
10
8
6
4
baseline
48h
42d
Act1-/-
WT

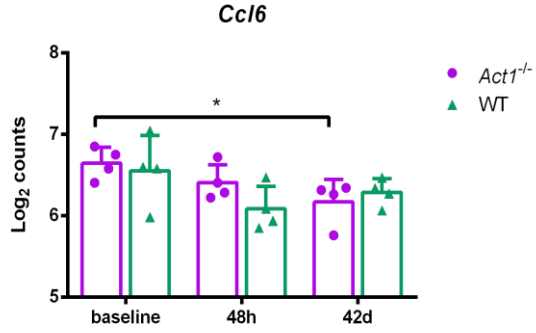
D


G-csf
Log2 counts
6
5
4
3
2
baseline
48h
42d
Act1-/-
WT

E


Ccl2
Log2 counts
9
8
7
6
5
baseline
48h
42d
Act1-/-
WT

F


Ccl6
Log2 counts
8
7
6
5
*
baseline
48h
42d
Act1-/-
WT

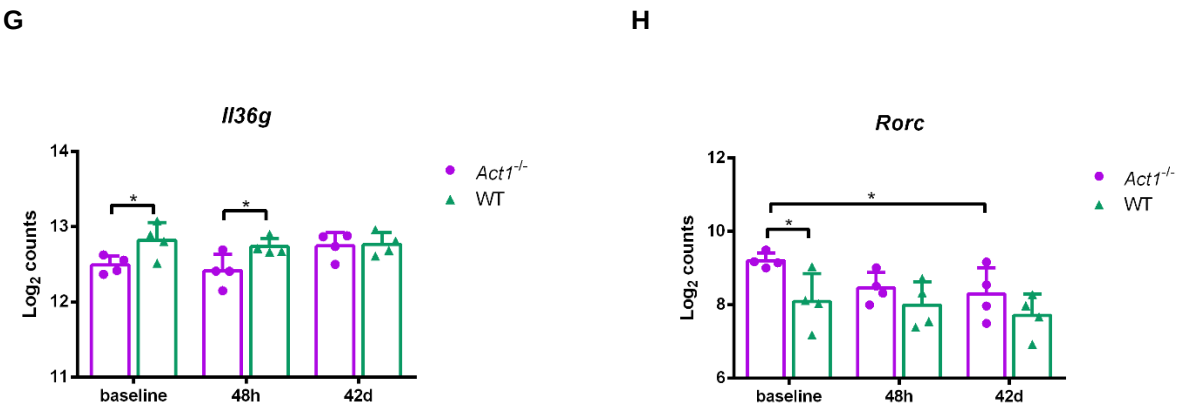
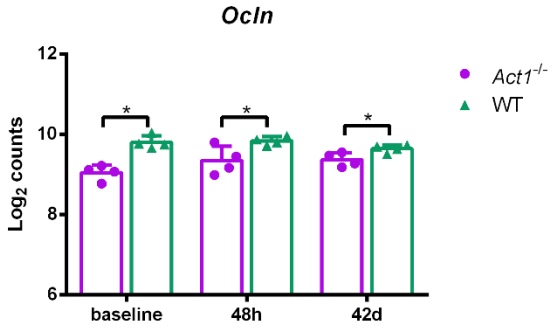
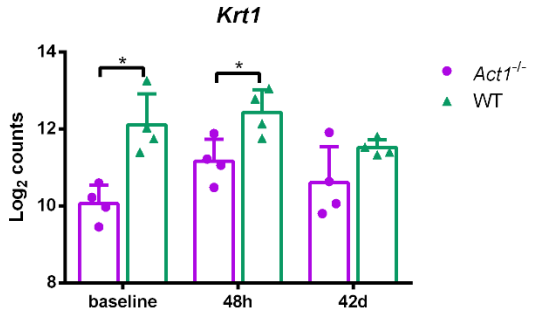


Figure 3.4. Cytokines expression in the *Traf3ip2*$^{-/-}$ mice infected with *P.g* is altered. *P.g*-infected *Traf3ip2*$^{-/-}$ mice and WT mice were euthanized at the baseline (before infection), 48 hours and 42 days (after infection). RNA was prepared for NanoString analysis from gingiva tissue taken from the same mice as above. Expression of (A) *Cxcl1*, (B) *Cxcl2*, (C) *Cxcl5*, (D) *G-csf*, (E) *Ccl2*, (F) *Ccl6*, (G) *Il36g*, (H) *Rorc* are shown. Bar graphs represent average values ± SEM. Asterisks represent statistical significance between groups. Data are representative with 4 mice per group.

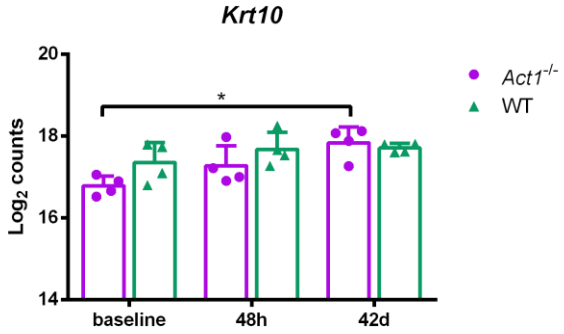
A


Ocln
Log2 counts
12
10
8
6
baseline
48h
42d
Act1-/-
WT

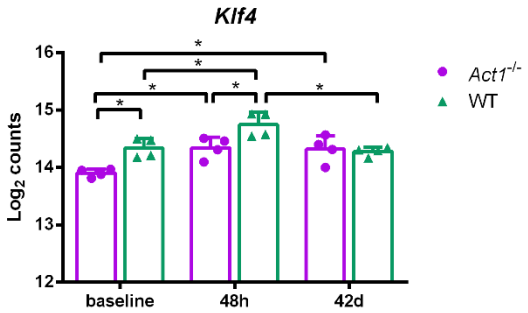
B


Krt1
Log2 counts
14
12
10
8
baseline
48h
42d
Act1-/-
WT

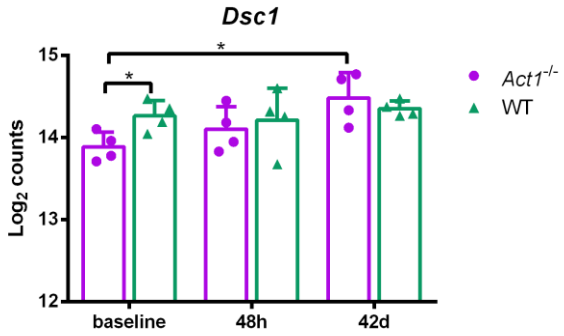
C


Krt10
Log2 counts
20
18
16
14
baseline
48h
42d
Act1-/-
WT

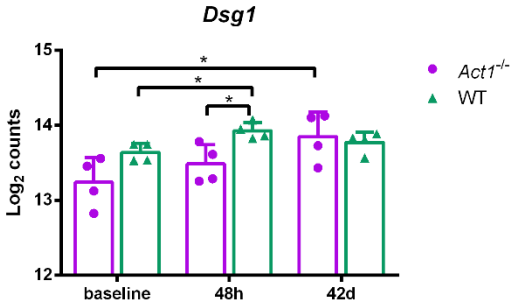
D


Klf4
Log2 counts
16
15
14
13
12
baseline
48h
42d
Act1-/-
WT

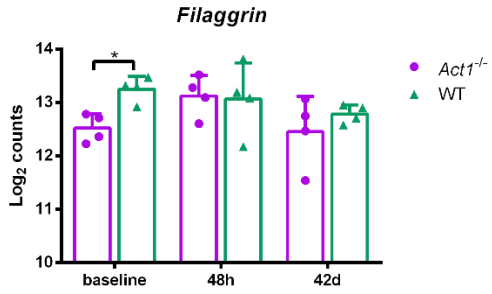
E


Dsc1
Log2 counts
15
14
13
12
baseline
48h
42d
Act1-/-
WT

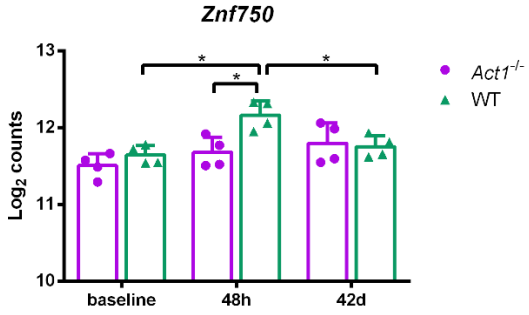
F


Dsg1
Log2 counts
15
14
13
12
baseline
48h
42d
Act1-/-
WT

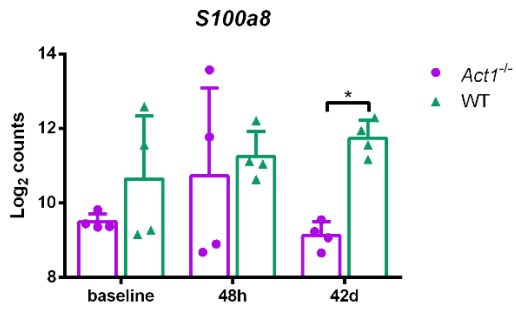
G


Filaggrin
Log2 counts
14
13
12
11
10
baseline
48h
42d
*
Act1-/-
WT

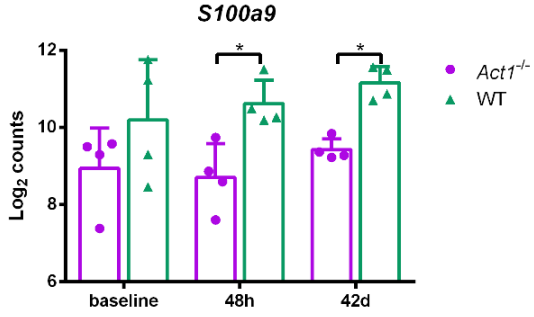
H


Znf750
Log2 counts
13
12
11
10
baseline
48h
42d
*
*
*
Act1-/-
WT

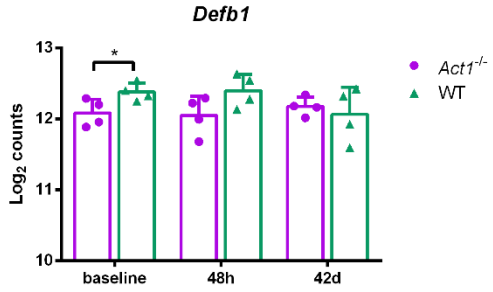
I


S100a8
Log2 counts
14
12
10
8
baseline
48h
42d
*
Act1-/-
WT

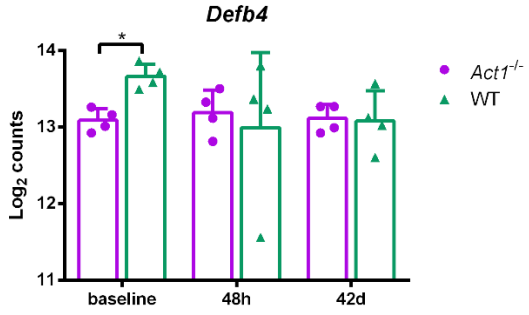
J


S100a9
Log2 counts
12
10
8
6
baseline
48h
42d
*
*
Act1-/-
WT

K


Defb1
Log2 counts
13
12
11
10
baseline
48h
42d
*
Act1-/-
WT

L


Defb4
Log2 counts
14
13
12
11
baseline
48h
42d
*
Act1-/-
WT

M

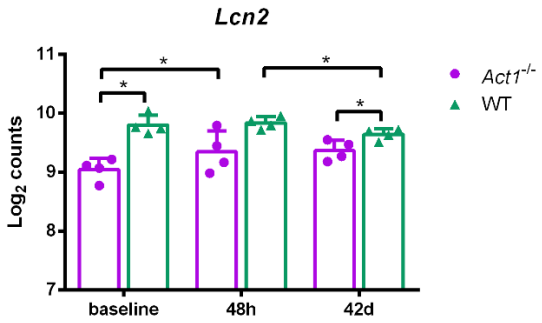


Figure 3.5. Absence of TRAF3IP2 exacerbates gingival epithelial barrier dysfunction. *P.g*-infected *Traf3ip2*$^{-/-}$ mice and WT mice were euthanized at the baseline (before infection), 48 hours and 42 days (after infection). RNA was prepared for NanoString analysis from gingiva tissue taken from the same mice as above. Expression of (A) *Ocln*, (B) *Krt1*, (C) *Krt10*, (D) *Klf4*, (E) *Dsc1*, (F) *Dsg1*, (G) *Filaggrin*, (H) *Znf750*, (I) S100a8, (J) *S100a9*, (K) *Defb1*, (L) *Defb4*, (M) *Lcn2* are shown. Bar graphs represent average values ± SEM. Asterisks represent statistical significance between groups. Data are representative with 4 mice per group.

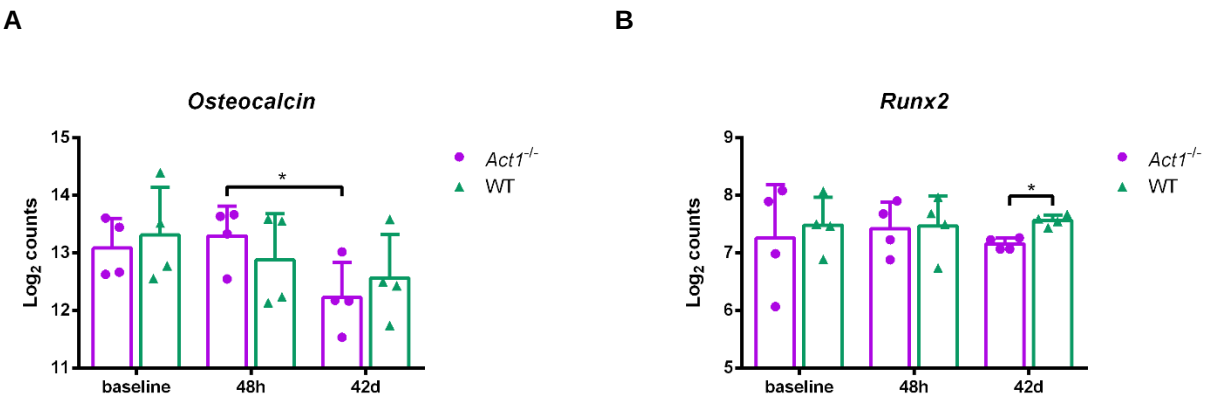
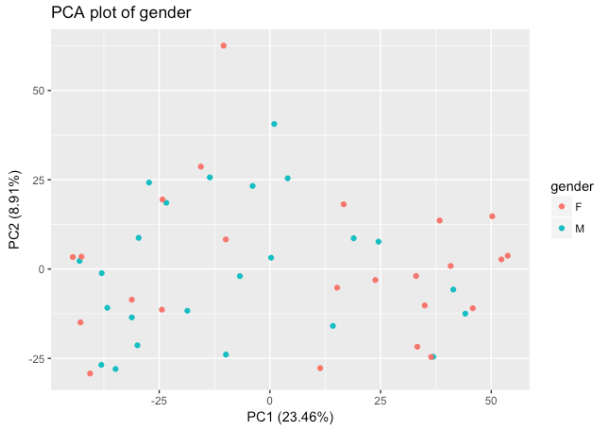
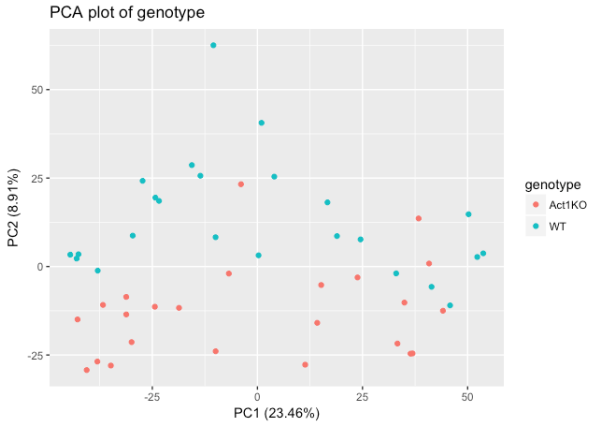


Figure 3.6. TRAF3IP2-mediated signaling promotes osteoblasts formation. *P.g*-infected *Traf3ip2*$^{-/-}$ mice and WT mice were euthanized at the baseline (before infection), 48 hours and 42 days (after infection). RNA was prepared for NanoString analysis from gingiva tissue taken from the same mice as above. Expression of (A) *Osteocalcin*, (B) *Runx2* are shown. Bar graphs represent average values ± SEM. Asterisks represent statistical significance between groups. Data are representative with 4 mice per group.

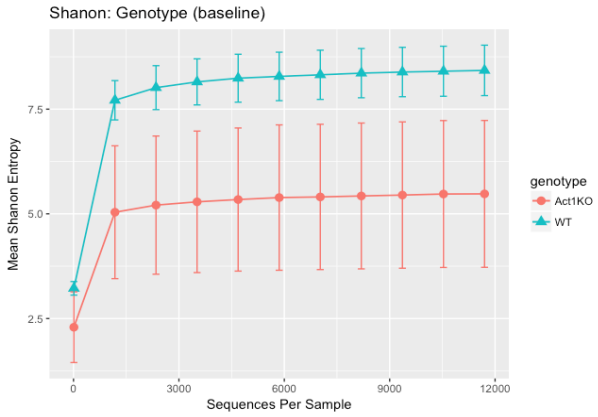
A


PCA plot of gender
PC2 (8.91%)
PC1 (23.46%)
gender
F
M

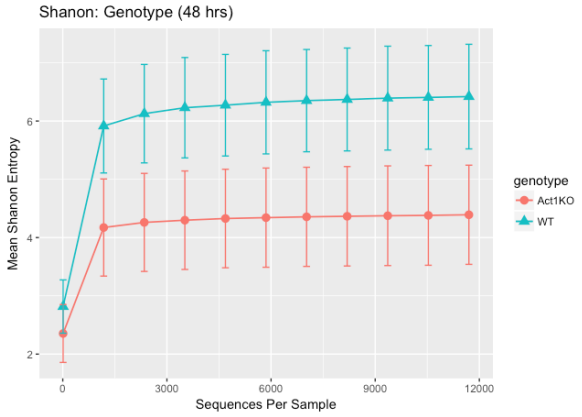
B


PCA plot of genotype
PC2 (8.91%)
PC1 (23.46%)
genotype
Act1KO
WT

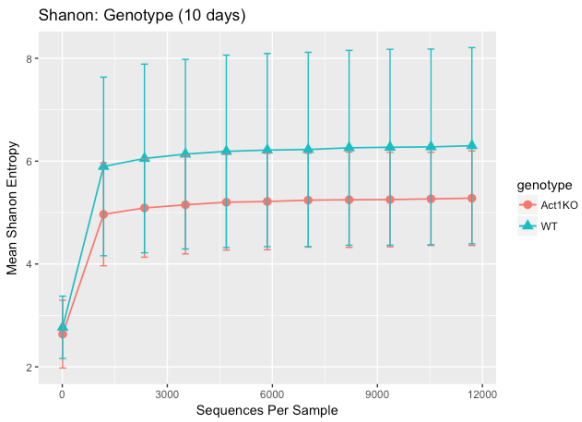
C


Shanon: Genotype (baseline)
Mean Shanon Entropy
Sequences Per Sample
genotype
Act1KO
WT

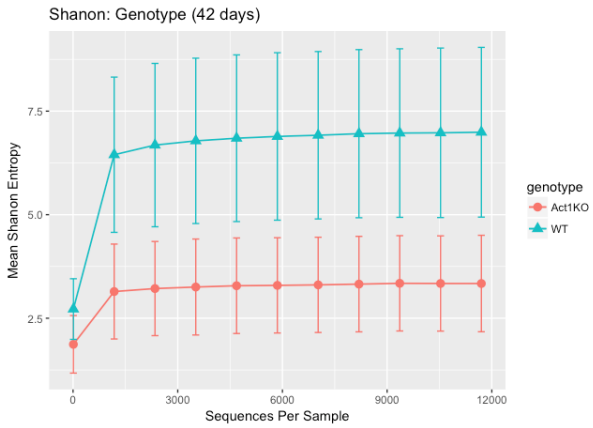
D


Shanon: Genotype (48 hrs)
Mean Shanon Entropy
Sequences Per Sample
genotype
Act1KO
WT

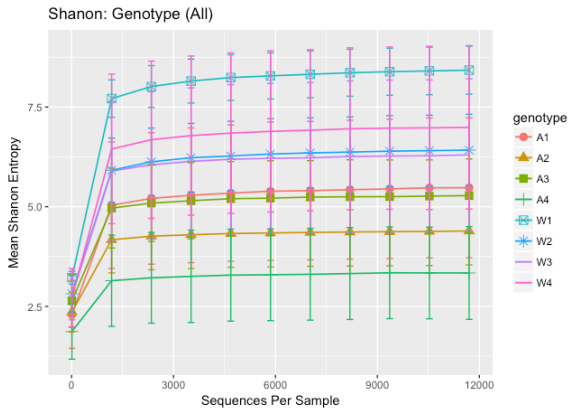
E


Shanon: Genotype (10 days)
Mean Shanon Entropy
Sequences Per Sample
genotype
Act1KO
WT

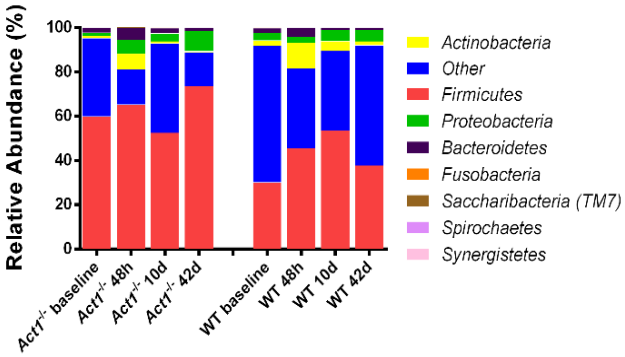
F


Shanon: Genotype (42 days)
Mean Shanon Entropy
Sequences Per Sample
genotype
Act1KO
WT

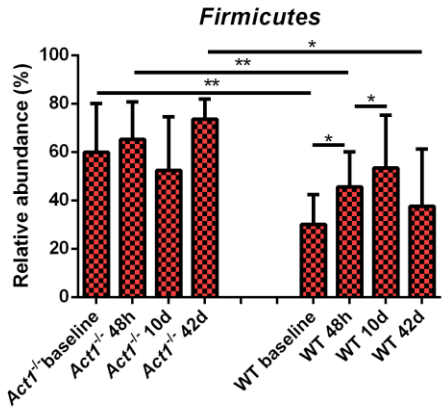
G


Shanon: Genotype (All)
Mean Shanon Entropy
Sequences Per Sample
genotype
A1
A2
A3
A4
W1
W2
W3
W4

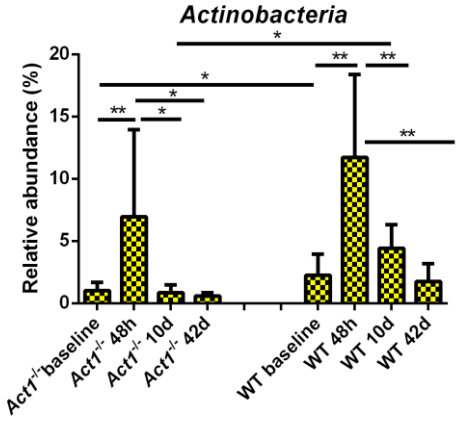
H


Relative Abundance (%)
Actinobacteria
Other
Firmicutes
Proteobacteria
Bacteroidetes
Fusobacteria
Saccharibacteria (TM7)
Spirochaetes
Synergistetes
Act1-/- baseline
Act1-/- 48h
Act1-/- 10d
Act1-/- 42d
WT baseline
WT 48h
WT 10d
WT 42d

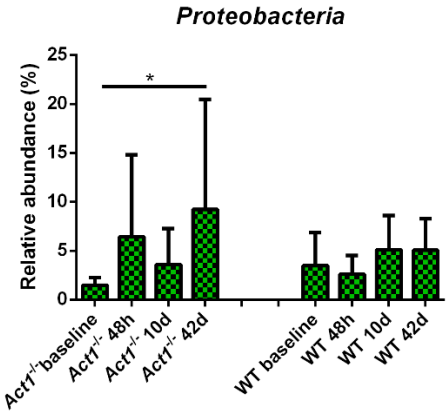
I


Firmicutes
Relative abundance (%)

J


Actinobacteria
Relative abundance (%)

K


Proteobacteria
Relative abundance (%)

L

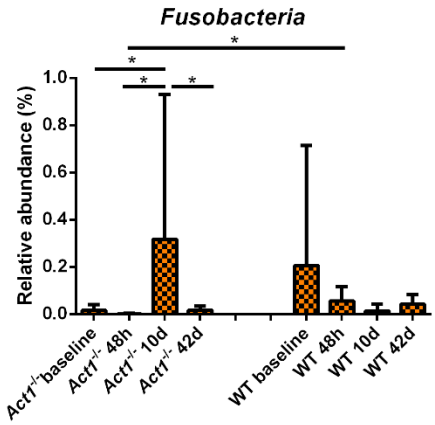

Fusobacteria
Relative abundance (%)

Figure 3.7. Differences of oral microbiota composition between *Traf3ip2*$^{-/-}$ mice and WT mice. (A, B) PCA plots based on gender and genotype showing difference in global community structure of oral microbiota profiles assessed by 16S amplicon sequencing of DNA. (C-G) Shannon Entropy of oral microbiota profiles assessed from *Traf3ip2*$^{-/-}$ mice and WT mice plaque DNA. (H) Average phylum-level composition of microbiota isolated from *Traf3ip2*$^{-/-}$ mice and WT mice in HOMD reference database. (I-L) Means ± SD of the relative abundance of *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Fusobacteria* phyla. * $P < 0.05$ by unpaired t test.

Chapter 4: DISCUSSION AND FUTURE DIRECTIONS

Periodontal disease is a polygenic condition that is associated with an exaggerated inflammatory response to the microorganism resides periodontium. Although pathogenic bacteria are assumed to be essential to initiate the periodontitis, multi-factors have been considered that play a role in the pathogenesis of periodontitis. Genetic variants that will cause a functional change in the encoded protein that may alter host barrier function and host innate or adaptive immunity that modifies inflammatory responses to microorganism to determine susceptibility and severity. A pattern of familial aggregation of severe disease demonstrated by earliest population studies (Benjamin and Baer 1967, Boughman, Halloran et al. 1986, Beaty, Boughman et al. 1987, Boughman, Astemborski et al. 1992) suggesting a genetic component, but Michalowicz in monozygotic and dizygotic pairs study (Michalowicz, Diehl et al. 2000) found that monozygotic twins were more similar than dizygotic twins for all clinical measurements, which provided the first demonstration of heritability and the variance in disease expression was attributable to genetics. The candidate gene approach to conducting genetic association studies is based on a priori knowledge of the gene's biological functional impact on the phenotypes states, which is in contrast to genome-wide association studies that can scan common genetic variation in the whole genome. GWAS of periodontitis performed recently (Divaris, Monda et al. 2012, Teumer, Holtfreter et al. 2013, Hong, Shin et al. 2015) highlighted loci that are associated with the phenotype of this disease. However, these studies need to explore the gene centric of this condition by integrating more distinct clinical parameters.

Recently, our group used PCA enriched with clinical parameters of periodontal phenotypes to identify genome-wide significant loci included genes associated with epithelial barrier function or immune response (Offenbacher, Divaris et al. 2016). We have strong evidence from a well-characterized population of 945 subjects demonstrating the central significance of MORN2 and TRAF3IP2 in controlling the periodontal inflammatory response and the severity of periodontitis. In Chapter 3, we found that *MORN2* plays a role in the LC3-associated phagocytosis - mediated killing of *P.g* and cytokine/chemokine

response through Ca^{2+} flux and NF-kB activation. It is possible that *MORN2* loss-of-function polymorphisms may compromise microbial killing and the innate immune response leading to a *P.g* dominated dysbiosis in periodontal disease. In Chapter 4, we found that *TRAF3IP2* plays a protective role in a *P.g* colonization through neutrophil recruitment, maintenance epithelial barrier and induction of antimicrobial peptides. Defective TRAF3IP2 disrupts host-microbial homeostasis and causes bone loss.

For the future directions, we will observe the effect of variant of MONR2 in THP-1 cells and human samples. Our rationale for testing the variant is that rs3099950 had significantly association with the counts of periodontal pathogens and severity of periodontal disease. We will create construct by site directed mutagenesis to generate MONR2 variant (rs3099950) lentivirus and use THP-1 cells transduced with variant MORN2 to elucidate the effects of the SNP on the inflammatory response and activation of NF-κB signaling pathway to *P.g*. The mRNA and protein levels of pro-inflammatory cytokines (IL-1β, IL-6, TNF-α) and chemokines (CXCL1, CXCL2, GCP-2, IL-8, CXCL3, CCL3, RANTES). NF-κB activation will be evaluated by phosphorylation of IKKα/β, IκBα and p65 and nuclear translocation of p65. For human study, individuals will be enrolled will be genotyped. Briefly, saliva samples will be collected from potential participants and DNA will be extracted (Qragene DNA collection kit) following manufacturer's instructions. Custom primers will be designed to amplify the region of interest from the genomic DNA and host DNA will be amplified by PCR. Genotyping will be performed using PCR based pyrosequencing methods that target the SNP variant of MORN2. The genotyped subjects will be further performed blood draws via venipuncture. Ficoll-Paque will be used to isolate peripheral blood monocytes that will be further differentiated into macrophages by adding M-CSF. The cells from wild type and variant MORN2 of participants will be stimulated by *P.g* to assess the inflammatory mediators at mRNA and protein levels.

We will also propose to use murine RAW 264.7 cells transduced with lentivirus mediated shRNA targeting Mm-MORN2 to elucidate the effects of Mm-MORN2 on the inflammatory response and NF-κB activation to P.g and LC3-asscociated phagocytosis. Our rational for testing murine MORN2 is that the murine MORN2 shares 96% sequence homology with human MORN2 including conservation of the SNP and region of interest. We will also collaborate with UNC animal model core facility to generate the *MORN2* $^{-/-}$ mice by using CRISRP/CAS9. In brief, *MORN2* specific sgRNA and Cas9 mRNA are microinjected into blastocysts that will be oviduct transferred using psuedopregnant females. We will perform immunoblot and RT-PCR to confirm *MORN2* is knocked out in new progeny and gross anatomic

abnormalities, gender distribution and weight will be assessed. Defleshed and formalin-fixed maxillae obtained from *P.g* oral inoculation periodontitis model at 42 days after the last *P.g* oral inoculation will be subjected to micro-computed tomography (micro-CT) scanning for assessing periodontal bone loss. TRAP staining will be performed for evaluation of osteoclasts expression. Maxillae of mice sacrificed at 48 hours after the last oral inoculation with intact surrounding tissue will be paraffin-embedded, sectioned and subjected to stain with mouse Ly6g/Gr1 antibody for neutrophils recruitment and mouse CD68 for macrophages recruitment. Bone marrow-derived macrophages from wild type and knockout mice can also be used for investigating inflammatory responses to *P.g* and NF-κB activation. Gene expression in gingival tissues can be analyzed by NanoString. Microbiome composition and shift can be evaluated via Illumina MiSeq 16S rRNA Amplicon Sequencing after DNA extraction from swabbed plaque samples. NETosis, an antimicrobial form of neutrophil cell death, is considered as a primary source of citrullinated autoantigens in systemic lupus erythematosus and rheumatoid arthritis. Due to the limited knowledge of MORN2, it would be interesting to investigate the role of MORN2 in neutrophil extracelluar traps by measuring histone citrullination through westernblot and confocal microscope.

For TRAF3IP2 project, we could observe the effect of variant of TRAF3IP2 in Caucasian human samples. Briefly, saliva samples from enrolled subjects will be collected for DNA extraction and genotyping will be performed using PCR based pyrosequencing methods that target the major SNP variant of TRAF3IP2. Primary human gingival epithelia cells isolated from participants of WT homozygote group and variant heterozygote/homozygote groups will be cultured (Kedjarune, Pongprerachok et al. 2001). We will use a real-time PCR based approach to evaluate the mRNA level of neutrophil specific chemokines (CXCL1, CXCL2, IL-8) and pro-inflammatory cytokines production in human gingival epithelial cells from different genotyped participants upon stimulation with IL-17. GAPDH will be used as internal control. Phosphorylation of ERK, JNK, p38 and p65 for the MAPK and NF-κB activation will also be examined by immunoblot.

We will also propose to isolate and process murine gingival tissue from wild type and *Traf3ip2*$^{-/-}$ mice maxilla for profiling sub-populations of T lymphocytes as well as their capability to express extracelluar and intracellular molecules by flow cytometry. Extracelluar markers, differentiation cytokines or transcription factors will be used to differentiate Th1, Th2, Th17 and Treg. Indirect measurements of epithelial barrier breach and systemic microbial exposure can be assessed by measuring serum

concentration of soluble CD14 (sCD14) and LPS binding protein (LBP) (Pastor Rojo, Lopez San Roman et al. 2007, Lakatos, Kiss et al. 2011). Although transcripts of *Ocln* were lower in *Traf3ip2*$^{-/-}$ mice compared to WT mice, altered intra-cellular localization could be examined for the loss of barrier function. What is more, *P.g* invasion can be examined by FISH to investigate the role of TRAF3IP2 on the epithelial barrier function. Also, isolated murine oral epithelial cells can be stimulated upon IL-17 to assess the chemokines and cytokines production. TRAF3IP2 variant transgenic mice can be established for oral infection model to investigate the role of the SNP in TRAF3IP2 locus in the pathogenesis of periodontal disease in mice. In order to test the specific contribution of TRAF3IP2 on epithelial cells, a mouse with conditional deletion of *Act1* driven by the expression of an epithelial-specific protein K18 can be utilized.

The merit of this study is based on the central importance of understanding the genetic basis of the innate immune response as a determinant of periodontal disease. Uncovering the genetic basis of pathogenesis will lead to improved diagnoses and prognoses to identify individuals at risk for disease to optimize prevention strategies. Subjects identified early in life to be at risk with the presence of SNP of *MORN2* and *TRAF3IP2* allele should receive more frequent preventive maintenance treatments in order to reduce the chance of the onset of periodontal disease. Furthermore, other microbiome conditions, like Crohn's disease and ulcerative colitis, which is associated with gut microbiota may also be modulated by this risk allele. In the future, periodontal gene therapy may provide a novel direction in sustainable inflammation control of periodontal disease.

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