

Modulation of oral homeostasis in health

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Abstract

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Over a half a century of research has identified that periodontitis is a result of dysbiosis of both the commensal microbial community as well as the host innate immune response. However, our lack of understanding oral health and how health is maintained has yet to produce a cure or sufficient treatment for this disease. The results from this investigation will bring novel insights into key innate host protective mechanisms which contribute to the maintenance of health. This work is a continuation of previous findings by Zenobia *et al.* which identified commensal bacteria directly contribute to neutrophil migration through select neutrophil chemotactic receptor, CXCR2, ligand expression and its expression is dependent on the MyD88 inflammatory pathway (Zenobia *et al.*, 2013). Furthermore, Greer *et al.* identified that neutrophils along with the expression of its chemotactic molecule was not homogeneous within oral tissues, but rather displayed site-specific neutrophil migration and chemokine expression patterns (Greer *et al.*, 2016). Chapter 2 identifies bacterial recognition receptors, toll-like receptors 2 (TLR2) and TLR4, two upstream activators of the MyD88 activation pathway, which may be utilized by oral commensal bacteria in the selective expression of CXCR2 ligands and determines the role of these receptors in neutrophil recruitment, bacterial homeostasis, and alveolar bone loss. Chapter 3 addresses the role of a neutrophil activating receptor, CXCR1, in its role in oral homeostasis determined by its effect on neutrophil recruitment, microbial homeostasis and alveolar bone loss. Lastly, Chapter 4 identifies the

immunomodulatory effects of a natural extract, Sudantha (Sud), against host and bacterially mediated production of a potent neutrophil chemoattractant, interleukin-8 (IL-8). These three chapters complement each other in that both oral commensal bacteria as well as cell intrinsic genetic regulatory mechanisms of inflammation exist to modulate health. This proposal will provide insights into the mechanisms of microbial- and host-mediated modulatory maintenance of healthy oral homeostasis, and hence a better understanding for the prevention of dysbiosis.

DEDICATION

To my parents, Susan Chang and In Hoe Chang

&

my little brother, Andrew Won Jae Chang

~

For your unconditional love, faith and support.

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Chapter 1

Health in relation to the oral microbiota

Introduction to the Human Oral Microbiota

We live on a microbial planet and are composed of predominantly microorganisms. To be exact, about 40 trillion bacteria have been identified to populate the human body, contrasted to an estimated 4 trillion human cells, producing a ratio of 10 bacterial cells to 1 human cell (Sender *et al.*, 2016). This large contrast in number not only emphasizes the importance of the host immune defense system and its role in the maintenance of health, but also conversely of the potentially indispensable relationship of the resident microbiota to its host to establish health. This microbial community together with the human host is termed “holobiont” (Rosenberg, 2013).

It is estimated that bacteria first arose about 3.8 billion years ago, while the eukaryotic lineage, which includes humans, arose 2.2 to 2.4 billion years ago (Mojzsis *et al.*, 1996; Kappler *et al.*, 2005). Moreover, it is conservatively estimated that resident microbiota have been performing metabolic functions within animal hosts for at least 500 million years (Cho & Blaser, 2012). Thus, host-microbial interactions have coexisted and coevolved over millions, if not billions, of years to produce a holobiont with mutual adaptation and functional integration; further emphasizing the importance of the microbiota and its fundamental integration to host function and health.

The oral cavity offers a heterogeneous environment for colonization. It is composed of distinct shedding mucosal surfaces: the lips, cheek, tongue, palate, and, uniquely, the only non-shedding surfaces in the body, the teeth. Moreover, despite being continuously bathed in saliva, each surface provides location-specific biological properties and environmental conditions that enriches and selects for unique microbial communities that are characteristic of each habitat

(Gibbons, 1989; Lamont & Jenkinson, 2000). These organisms are not randomly distributed but selectively attach in an orderly succession of early, intermediate, and late bacterial species colonizing surface and displaying tissue tropisms (Kolenbrander *et al.*, 2010).

Acquisition of the oral microbiota occurs by both vertical, from mother to child, and horizontal transmission through saliva, food, and the environment (Asikainen & Chen, 1999; Berkowitz & Jones, 1985; Davey & Rogers, 1984). Despite housing the second most diverse microbial community in the body, early colonization of the mouth is highly selective allowing only a few of the commonly identified species from the adult oral microbial consortium (and even to a lesser degree, species from the environment) to colonize within the mouth of newborns (Kilian *et al.*, 2016; Marsh, 2000). In newborns the predominating pioneer species are from the streptococcus genera, most notably *S. salivarius*, *S. mitis*, and *S. oralis* (Smith *et al.*, 1993; Pearce *et al.*, 1995). As tooth eruption occurs providing additional surfaces for attachment, the diversity of the oral microbial community increases to commonly include members from the genera *Neisseria*, *Veillonella*, *Actinomyces*, *Lactobacillus*, and *Rothia* (McCarthy *et al.*, 1965). At maturity, on average, a single human mouth houses 296 species, of which many have yet to be cultivated (Kilian *et al.*, 2016).

Over time, biological changes including aging and disease can affect the balance of bacterial species. For example, the aging process is characterized by a chronic, low-grade, systemic inflammatory phenomenon called “inflammaging” that affects host defences and salivary composition (Percival *et al.*, 1991; Marsh *et al.*, 1992; Feres *et al.*, 2016; Vissink *et al.*, 1997). All of these components combined can disturb the microbial balance; evidenced by an increased prevalence of *Actinomyces viscosus* and lactobacilli in healthy adults over the age of 60 (Percival *et al.*, 1991; Feres *et al.*, 2016). Similarly, during diseases such as periodontitis, uncontrolled increases of plaque biofilm can trigger a dysbiotic inflammatory response, damaging soft tissue and bone supporting structures and ultimately leading to premature tooth loss. This chronic inflammatory state favors the growth of pathogens such as *Porphyromonas gingivalis*, *Treponema*

denticola, and *Tannerella forsythia* and reduces the overall diversity of the oral microbiota (Dongmei *et al.*, 2016; Socransky *et al.*, 1998).

Advancing techniques in DNA sequencing has been able to reveal the vast diversity of the human oral microbiome, with reports of 770 microbial species to have ever populated the human oral cavity (Escapa *et al.*, 2018). These examples show how the oral microbial consortium is ever evolving in response to external factors from birth until death.

Formation of the dental plaque biofilm

Dental plaque is an example of a biofilm. It is a highly structurally and functionally organized and interactive community of diverse microorganisms embedded in an extracellular matrix composed of both host- and microbial-derived polymers (Marsh, 2004). The composition of dental plaque varies greatly within an individual by distinct surfaces (i.e. gingival crevice, tooth fissures and smooth surfaces, implants, and even dentures) and also between individuals (Costello *et al.*, 2009). Plaque formation is a natural part of life, found present in both healthy and diseased sites, although the predominating species and characteristics of the biofilm differs between the two sites (Marsh & Zaura, 2017; To *et al.*, 2015).

The formation of the dental plaque biofilm preferentially begins at stagnant sites in distinct phases (acquired or salivary pellicle formation, reversible adhesion leading to irreversible attachment, co-adhesion, and multiplication) and in ecological succession (Marsh, 2004; Kolenbrander *et al.*, 2010). Initial formation begins with immediate adsorption of both host and bacterial molecules (i.e., lipids, glycoprotein and antimicrobial and antifungal peptides) onto newly erupted or freshly cleaned tooth surfaces to create a conditioning layer known as the acquired or salivary pellicle (Al-Hashimi & Levine, 1989; Vukosavljevic *et al.*, 2014). It has been shown that individual components that make up the salivary pellicle can directly influence the initiation and accumulation of dental plaque. For example, active bacterially-derived glucosyltransferases (enzymes which polymerize sucrose to glucans) can become incorporated into the salivary pellicle

to synthesize and modify glucans, providing selective binding sites for oral bacteria (Kopec *et al.*, 2001). Formation of the salivary pellicle is succeeded by weak and long-range reversible adhesion between passively transported bacteria and the pellicle-coated tooth surface, which can lead to stronger adhesin and receptor-mediated irreversible attachment (Busscher & van der Mei, 1997; Marsh, 2004). This selective adhesin-receptor attachment is believed to explain the observed tissue tropisms. Subsequent co-adhesion of secondary colonizers such as *Fusobacterium nucleatum*, an indiscriminate bridging organism, to primary colonizers facilitates structural and functional organization (Kolenbrander *et al.*, 2000). Once established, exopolysaccharide production and multiplication of this diverse community results in a highly organized and complex three-dimensional plaque biofilm structure that can remain stable and active over time (Marsh, 2004).

Characterization of the oral microbiome

Historically, the oral microbiome has been investigated using a variety of conventional microbiological methods including bacterial culture, microscopy, PCR and enzymatic and immuno-assays, all of which are still widely used today. More recently, advancement of culture-independent high-throughput “-omics” technologies, such as genomics (the study of genes and their functions), proteomics (the study of proteins), metabolomics (the study of metabolites), and transcriptomics (the study of mRNA) combined with the reduction in assay costs have revolutionized the field of microbiology. Meta-omics tools thus enhances traditional microbiological approaches of single species investigations by bridging together multi-level approaches of genes, proteins, metabolites, mRNA, and bacteria to better understand a community and its activities as a whole.

The most common genomics approach to profiling the oral microbiome is based on the 16S ribosomal RNA (16S rRNA) gene. The 16S rRNA gene is comprised of approximately 50 functional domains and encodes for the 30S small ribosomal subunit that is evolutionarily

conserved in all prokaryotes, making it a universal target for bacterial identification. In addition, due to its highly conserved nature, variabilities in its sequence would more likely reflect random mutations as opposed to selected ones that could potentially alter its function and hence doubles as a “molecular chronometer” allowing the changes to mark evolutionary distances and relatedness between organisms time (Clarridge, 2004). This gene can be extracted from heterogeneous samples, sequenced with massively parallel sequencing technology known as next-generation sequencing (NGS), and matched to reference genomes in publicly accessible databases such as the Greengenes 16S rRNA Database (DeSantis *et al.*, 2006) and the Human Oral Microbiome Database (HOMD) (Escapa *et al.*, 2018).

Consequently, up to a third of the diversity identified in the oral cavity by NGS remains unculturable with traditional techniques, and little is known about these species except for their presence in the mouth and their 16S rRNA gene sequence (Vartoukian *et al.*, 2016). This uncultivated population including individual members to entire bacterial phyla are referenced as the “great uncultivated majority” and “microbial dark matter” (McLean, 2014). The 16S rRNA gene sequence alone is unable to provide proper insight to functional properties and characteristics that still requires cultivation, experimentation, and validation. To combat this, there are growing efforts to advance culturing methods with the new approach of “helper strains” and addition of small compounds, such as siderophores, which are high-affinity iron-chelating compounds secreted by microorganisms to aid in the cultivation of novel strains, implying their dependency of signaling and/or nutritional interactions with other bacterial members (Vartoukian *et al.*, 2016). This further emphasizes the need to understand the multi-species oral microbial consortium as a whole, identifying the functions and interactions within the biofilm community, in contrast to investigating independent monocultures or structural components of this biofilm.

A large portion of the sequences from microbial studies fall into a category known as “orphan reads,” sequences with no assigned reference genome (McLean, 2014). Reference genomes are imperative to the assignment, identification, and characterization of reads from

metagenomic and metatranscriptomic analyses. The most comprehensive database for the human oral cavity is the expanded Human Oral Microbiome Database (eHOMD). This version is currently comprised of 770 microbial species and over 1500 oral reference genomes (Escapa *et al.*, 2018). Similarly, the Human Microbiome Project (HMP) provides a more comprehensive dataset of “baseline” sequences from a cohort of 300 disease-free healthy adults across 9 sites within the oral cavity (subgingival dental plaque, supragingival dental plaque, gums, palate, cheek, tonsils, throat, saliva, and tongue) and includes over two terabytes of oral microbiome data (Human Microbiome Project Consortium, 2012). Nevertheless, current databases lag behind the rapid collection of new metadata and impedes efforts to assign reads to genes and hence taxonomic identification (McLean, 2014). This limitation can be readily observed in the inability to identify multiple species in the mouse oral cavity in Chapter 2.

The interdisciplinary combination of microbiology, molecular biology, and bioinformatics is fueling further advancements and innovations with the goal to better tackle the complex interactions and ecology of the oral microbiome within the community context to ultimately reach a better understanding of health.

The junctional epithelium

Eruption of teeth through the gingival mucosa present a unique challenge of the epithelial barrier to provide continuous protection against constant bacterial challenge from dental plaque and mastication. At this interface, the structural continuity is secured by the junctional epithelium (JE) which is firmly attached to the cervical surface of the tooth by 15 to 30 cells coronally and 1 to 3 cells at its most apical location (Bosshardt & Lang, 2005). This loosely organized, nonkeratinized specialized tissue exhibits unique characteristics (expression of defensive factors, direct attachment of cells to the tooth surface, rapid cell turnover, and permeability) that differ from other oral epithelial tissues and plays an important role in host defense and periodontal health.

At the cell level, JE cells secrete a multitude of defense factors that protect against microbial challenge and maintain tissue integrity, such as antimicrobial peptides β -defensins (Dinuto *et al.*, 2003) and S100A8 (Nishii *et al.*, 2013), and a protease inhibitor, secretory leukocyte protease inhibitor (SLPI) (Hayashi *et al.*, 2010). Moreover, they constitutively express regulatory chemokines and cytokines including keratinocyte-derived chemokine (KC/CXCL1/Gro- α), macrophage inflammatory protein-2 (MIP-2/CXCL2/Gro- β) and IL-1 β that direct immune surveillance (Tsukamoto *et al.*, 2012; Zenobia *et al.*, 2013).

Structurally, the JE is stratified and protected by two biochemically distinct basal laminas, the internal and the external basal laminas (Pöllänen *et al.*, 2003). The internal basal lamina mediates attachment of the JE to the tooth surface by cells that are “directly attached to the tooth” (DAT cells). It has been suggested that DAT cells, like the external basal lamina cells along the connective tissue, have the ability to proliferate at a suprabasal location providing the JE with two proliferating populations (Pöllänen *et al.*, 2003). Possibly due to this feature, the JE is characterized with rapid cell turnover that is about twice the rate of the oral epithelium or shown to be about 5 days in nonhuman primates (Skougaard, 1970). This rapid shedding of epithelial cells armed with 2 distinct physical barriers provide an effective method of removing and defending against adherent bacteria (Pöllänen *et al.*, 2003).

Another important protective feature of the JE is its permeability. Between the tight seal of the double basement membrane, the JE is characterized with wide intracellular spaces allowing continuous flow of gingival crevicular fluid (GCF) and inflammatory cells, such as neutrophils, into the gingival crevice (Schroeder & Listgarten, 1997). Neutrophils, also known as polymorphonuclear leukocytes, are an indispensable part of the host innate immune defense system and contribute to the antimicrobial properties of the JE. In the absence of disease, approximately 30,000 neutrophils transit through the human JE per minute (Schiött & Löe, 1970). They are found near the basal cell layer, near the tooth surface and in the central region of the JE (Bosshardt & Lang, 2005; Schroeder & Listgarten, 1997; Schroeder, 1973, 1977).

Significance of the neutrophilic barrier to oral health

In this process of health, neutrophils play a pivotal role in the maintenance of host-bacterial homeostasis, making up about 90% of leukocytes present in GCF. They provide a rapid broad spectrum response against bacteria by phagocytosis and release of superoxides and peroxides, and loss or disruption of this neutrophilic barrier results in periodontal disease (Johnson & Ward, 1981; Rashmi *et al.*, 2006; Deas *et al.*, 2003). It has been shown that inability to regulate proper neutrophil numbers (neutropenias), recruitment (Chediak-Higashi syndrome and Papillon-Lefèvre syndrome), or adhesion (LAD-I, LAD-II, and LAD-III), as seen in patients with congenital neutrophil deficiencies and induced neutropenia, or conversely with the failure to downregulate neutrophil transit resulting in uncontrolled neutrophil recruitment or prolonged exposure, have all been shown to increase alveolar bone loss (Cortes-Vieyra *et al.*, 2016; Hart & Atkinson, 2007; Deas *et al.*, 2003; Carrassi *et al.*, 1989; Attstrom & Schroeder, 1979; Sima *et al.*, 2018; Waldrop *et al.*, 1987). Furthermore, abnormalities in neutrophil lifespan, activation, and function, such as with decreased phagocytosis or increased superoxide production, was also found to be associated with this disease (Lucas *et al.* 2010; Aboodi *et al.* 2011; Ryder 2010). Therefore, in the healthy periodontium, neutrophil homeostasis is tightly regulated in the presence of commensal bacteria to control bacterial plaque overgrowth without eliciting tissue damage. However, the decades of investigations focused on disease in an attempt to understand health has provided inadequate insight into the maintenance of health, but more specifically into the modulation of oral homeostasis in the healthy periodontium.

Reiterating the importance of this neutrophilic barrier, the periodontium contains a highly orchestrated expression of select innate host defense mediators, which regulate the extravasation of neutrophils from the vasculature through gingival tissue and ultimately to the gingival crevice where they form a protective barrier between the JE and the dental plaque biofilm (Kornman *et al.*, 1997; Ye *et al.*, 2000, Heymann *et al.*, 2001; Tonetti *et al.*, 1998; Tonetti *et al.*, 1994; Moughal

et al., 1992; Tonetti, 1997; Greer *et al.*, 2016; Tsukamoto *et al.*, 2012). Interleukin-8 (IL-8), also known as chemokine CXCL8, and its analogs, CXCL1 (Gro- α), CXCL2 (Gro- β), CXCL3 (Gro- γ), CXCL5 (ENA-78), CXCL7 (NAP-2) are potent neutrophil chemoattractants which mediate their movements through distinct receptors, CXCR1 (IL-8R1 or IL-8RA) and CXCR2 (IL-8R2 or IL-8RB) (Baggiolini *et al.*, 1994).

In humans, gingival keratinocytes have been reported to show differential spatial expression patterning of IL-8 and its receptors (Sfakianakis *et al.*, 2002). The authors observed that IL-8 and its more specific receptor, CXCR1, were more abundantly expressed in the gingival epithelial layers closest to external stimuli in the oral cavity (keratin and granular layers), and its expression decreased deeper in the corono-apical direction, away from external stimuli (spinous layer) (Chuntharapai & Kim, 1995). These findings confirm previous reports of a similar patterned gradient expression of IL-8 (Tonetti *et al.*, 1998). In contrast, the expression of CXCR2, a less specific IL-8 receptor which is able to bind to IL-8 and its analogs, was found to be more evenly distributed within all layers of the epithelium. Neutrophil migration across mucosal barriers requires complex coordination between the epithelial cells of the mucosal barrier and neutrophils. In a urinary tract infection model, it was shown that IL-8 bound CXCR1, but not CXCR2 on epithelial cells lining the urinary tract, guiding neutrophil transmigration across the mucosal barrier to the site of infection (Godaly *et al.*, 2000).

On human neutrophils, it has been shown that CXCR2 has a higher binding affinity for IL-8 compared to that of CXCR1, being internalized at lower agonist inputs and with faster kinetics than CXCR1 (Rose *et al.*, 2004). Therefore, it is postulated that in the course of inflammation, the higher affinity CXCR2 is responsible for the initial recruitment of neutrophils from distant sites. As neutrophils are recruited closer to the source of IL-8, bound CXCR2 receptors become internalized allowing for the more specific CXCR1 receptor to dominate IL-8 responses (Chuntharapai & Kim, 1995). However, there is widespread disagreement as to the chemotactic ability of CXCR1, as contradictory reports have been described in different tissues (Loetscher *et*

et al., 1994; Aul *et al.*, 2012; Godaly *et al.*, 2000). Interestingly, neutrophil elastase release in response to IL-8 is exclusively triggered by CXCR1 (Jones *et al.*, 1996). Furthermore, cleavage of CXCR1 (Hartl *et al.*, 2007) or a mutation affecting the function of CXCR1 (Swamydas *et al.*, 2016) in human neutrophils results in impaired bacterial and fungal killing.

Altogether, these examples demonstrating the utilization of differential spatial patterning of receptors and chemokines reveal that a complex network of host factors are required for the regulation of periodontal homeostasis and neutrophil homing and does not represent redundancy as once believed (Sadik *et al.*, 2011). In fact, it is becoming widely recognized that although many host defense mediators share overlapping core functions, each mediator possesses unique features that allowed for its preservation and selection through evolution, and patterns of distinct temporal and spatial expression are strategically employed to effectively direct and mount an immune response (Mantovani, 2018).

Relationship of the oral microbiota and health

The host is armed with defense mechanisms that are able to differentiate self from nonself. Despite this ability, years of coadaptation and coevolution have selected for mechanisms, which are still not fully understood, that establish a delicately balanced relationship between resident microbes and the host to exist under normal conditions without causing disease. This balanced relationship is termed “homeostasis” and disruption of this balance results in disease which has the potential to disseminate outside of the local environment of the oral cavity to other parts of the body and has been associated with cardiovascular disease (Chukkapalli *et al.*, 2014; Velsko *et al.*, 2014; Herzberg *et al.*, 2001), Alzheimer's (Poole *et al.*, 2015; Hammond *et al.*, 2010; Kamer *et al.*, 2009), oral and colorectal carcinomas (Binder *et al.*, 2015; Inaba *et al.*, 2014), diabetes (Sugiyama *et al.*, 2012; Nishimura *et al.*, 2003), adverse pregnancy outcomes (Kaur *et al.*, 2014; Han *et al.*, 2010; Katz *et al.*, 2009) and bacterial pneumonia (Heo *et al.*, 2011; Hajishengallis *et*

al., 2008). Therefore, there is a need to establish a clear understanding of the homeostatic mechanisms employed in the oral cavity to maintain health.

Bacterial colonization is not essential for life (Wallace *et al.*, 2011). However, germ-free (GF) studies show an inextricable link between the microbiota and the healthy development and existence of the host. For example, GF mice, which are born and raised completely devoid of bacteria, have a poorly developed gut with an enlarged cecum (Wostmann & Bruckner-Kardoss, 1959), reduced intestinal surface area, and number of villi (Gordon & Bruckner-Kardoss, 1961). Benefits of bacterial colonization is evident by the fact that despite consuming 30% more calories than conventionally raised specific-pathogen free mice (SPF) to maintain similar body weight, they still show deficits in digestion, nutrient absorption, and metabolism (Hooper & Gordon, 2001; Wostmann *et al.*, 1983). GF mice have been reported to have comparable frequencies of immune cells to SPF mice (Dutzan *et al.*, 2017). However, these mice are presented with blunted immune responses, expressing decreased or absent levels of certain bacterial recognition receptors such as Toll-like receptors (TLRs), reduced antibody secretion, and smaller immune surveillance centers called Peyer's patches within the gut (Wostmann *et al.*, 1970; Abrams *et al.*, 1963). Bacterial colonization has been shown to reverse many of these anatomical and physiological deficiencies. Therefore, in the gut these studies demonstrate that bacterial colonization facilitates normal tissue development and immune protection.

In contrast to the gut microbiota, little is known about the contributions of the oral microbiota to the development, structure, and function of clinically healthy periodontal tissue as well as its role in conditioning local immunity. However, there is growing evidence showing the presence of both microbiome dependent and independent regulation of oral homeostasis. Histological examinations between GF and SPF mice, rats, and dogs show no difference in the morphology of the JE, indicating that bacterial colonization does not influence the wide intercellular spaces nor the presence of neutrophils within this tissue, but rather suggests that it is a microbiome independent and genetically predetermined feature (Heymann *et al.*, 2001;

Yamasaki *et al.*, 1979; Listgarten & Heneghan, 1971). While GF and SPF mice both contain neutrophils within the JE, SPF mice have been reported to contain a higher number of neutrophils compared to GF mice indicating that both microbiome dependent and independent mechanisms regulate neutrophil recruitment into the JE (Tsukamoto *et al.*, 2012; Zenobia *et al.*, 2013). Moreover, the authors noted that bacterial colonization increased the area of the JE as well as the number of PCNA-positive epithelial cells, a marker of cell activation for proliferation (Tsukamoto *et al.*, 2012). Interestingly, controlled subclinical levels of inflammation, believed to be induced by oral commensal bacteria, lead to marginal alveolar bone loss and are a part of the natural process of normal healthy oral homeostasis (Dixon *et al.*, 2004; Hajishengallis *et al.*, 2011; Nishii *et al.*, 2013; Tsukamoto *et al.*, 2012).

Select innate defense mediators have also been shown to depend on commensal colonization. For example, chemokines CXCL1 and CXCL2 are constitutively expressed in the JE, however it was found that CXCL2 but not CXCL1 is selectively upregulated in the presence of commensal colonization in a spatially regulated manner (Zenobia *et al.*, 2013; Greer *et al.*, 2016) indicating that microbiome dependent mechanisms may be responsible for up-regulating innate defense functions of the JE. Similarly, bacterial colonization has been shown to differentially upregulate the expression of a key ligand involved in the resolution of inflammation (Nasser *et al.*, 2017; Rothlin *et al.*, 2015). TYRO3, AXL, MERTK, collectively known as the TAM receptors and their ligands, growth arrest-specific 6 (GAS6) and protein S (PROS1) have the ability to down-regulate innate inflammatory responses, clear necrotic or apoptotic cells (efferocytosis), and restore vascular integrity (Lemke & Burstyn-Cohen, 2010). Nasser *et al.* reported that GF mice had significantly lower expression of GAS6 in the JE and in adjacent oral epithelial tissues compared to SPF controls. Furthermore, its expression was able to be restored with the introduction of bacteria. In contrast, despite close structural homology, PROS1 expression did not show significant differences with or without the presence of bacteria. Altogether, these examples demonstrate that the host defense is comprised of a complex network

of discrete modulatory molecules with distinct roles that are spatially controlled by both microbiome dependent and independent mechanisms to maintain homeostasis.

Colonization of commensal bacterial has been shown to promote health. It is believed that colonization limits available binding sites and nutrients in the oral cavity to potential pathogens, termed “colonization resistance” (Vollaard & Clasener, 1994). This phenomenon becomes evident during antibiotic treatment when the natural oral microbial community becomes disrupted and infections by opportunistic pathogens occur (Sullivan *et al.*, 2001). Additionally, studies show health associated bacteria can promote protection against invading pathogens. For example, *Streptococcus salivarius* strain K12 produces antimicrobial peptides to inhibit the growth of Gram-negative species associated with disease and oral malodour (Wescombe *et al.*, 2009; Burton *et al.*, 2006). Moreover, subgingival plaque samples obtained from clinically healthy sites of adult patients showed the unique ability to antagonize bacterial recognition receptor TLR4 specific activation by *Fusobacterium nucleatum* lipopolysaccharide (LPS) suggesting that the local microbial community may work in a coordinated manner to modulate the immune responses (To *et al.*, 2016).

Oral bacteria have also been implicated in the maintenance of cardiovascular health in relation to nitrate metabolism. Nitric oxide is a key determinant of vascular health, exerting vasorelaxation, antiplatelet, antithrombotic, and anti-inflammatory effects within the vasculature (Jin & Loscalzo, 2010). About 40% of ingested nitrate is secreted through saliva, where oral bacteria reduce nitrate to nitrite (Spiegelhalder *et al.*, 1976; Wade, 2013). Nitrite is then absorbed into the bloodstream and converted into nitric oxide (Spiegelhalder *et al.*). Disruption of the oral microbiome with the use of an antimicrobial mouthrinse has potential side effects that reduce nitrite levels in plasma and affects the gastroprotective and blood pressure lowering effects following nitrate ingestion (Govoni *et al.*, 2008; Petersson *et al.*, 2009). These studies show that commensal oral bacteria have positive implications in promoting cardiovascular health as well as protection against infections.

Flow cytometric analysis of oral tissues revealed that gingival tissues contained the highest number of inflammatory cells compared to any other site during health (Dutzan *et al.*, 2016). This idea is further evident in that the JE constitutively expresses several types of inflammatory mediators such as the CXC chemokines, IL-1 β and SLPI, which are necessary for neutrophil recruitment and cross-talk and is augmented by bacterial colonization (Dixon *et al.*, 2004; Hayashi *et al.*, 2010). Increased tissue surveillance is necessary to maintain a homeostatic barrier against continuous triggers by the plaque biofilm as well as against mechanical damage from chewing and dental hygiene. Clinical studies investigating inflammatory changes over time in healthy patients abstaining from oral hygiene reported increases in gingival inflammation and microbial diversity and load that can occur within days (Theilade *et al.*, 1966; Loe *et al.*, 1965). Reduction of microbial load by administration of oral hygiene or antibiotics have been proven to reverse clinical signs of disease (Feres *et al.*, 2015) demonstrating that the JE has the ability to regenerate (Nanci, 2013) and restore this delicate balance of homeostasis (Moutsopoulos & Konkel, 2018). However, the mechanisms that are in play to regulate this fine balance is still not well understood and a subject of this dissertation.

Mice as a model to investigate periodontal health

Rodents are the most extensively utilized animal model for the investigation of oral health and disease due to the similarity in periodontal anatomy and structure to that observed in humans (Yamasaki *et al.*, 1979). Moreover, ease of handling and the ability to genetically manipulate strains and modify microbial factors, such as with germ-free models, provide useful tools to break down complex questions to individualized components in order to ascribe their role in the overall process of the investigation.

Structurally, the rodent JE is attached to the root surface with a shallow gingival sulcus, similar to that of humans, and it provides a pathway for bacterial products and host inflammatory mediators that models what occurs in humans. In contrast, rodents continuously grow a partially

enamel-encased incisor that lacks a root structure. Moreover, unlike in humans, the sulcular epithelium is keratinized, and the most superficial cells of the gingival epithelium are characterized as having desmosomal contact to nonkeratinized cells within the JE (Listgarten, 1975). Under natural conditions rodents are extremely resistant to periodontal diseases, but it can be induced by tying ligatures, introducing bacteria, affecting diet or with chemicals (Page & Schroeder, 1982; Oz & Puleo, 2011).

Additional differences arise within immune modulatory molecules. Chemokines are a superfamily of structurally related cytokines with diverse roles in neutrophil activation and recruitment. Members of the CXC family have invariant cysteines that are separated by one non-cysteine amino acid residue demarcated as an X. Within this family, a subclass of CXC chemokines are characterized by a reactive glutamic acid-leucine-arginine (ELR) extracellular loop motif (Rovai *et al.*, 1998) and are potent neutrophil chemoattractants. The best studied ELR CXC chemokine is human IL-8 (also known as CXCL8); however six additional ELR CXC chemokines have been identified: CXCL1 (Gro- α), CXCL2 (Gro- β), CXCL3 (Gro- γ) (Richmond *et al.*, 1988; Tekamp-Olson, 1990), CXCL5 (ENA-78) (Walz *et al.*, 1991), CXCL6 (GCP-2) (Proost *et al.*, 1993; Ravai *et al.*, 1997) and CXCL7 (NAP-2) (Walz & Baggiolini, 1990). CXCR1 and CXCR2 are two human receptors which bind to ELR chemokines. CXCR1 is highly selective for IL-8 and CXCL6 (Devalaraja & Richmond, 1999), while CXCR2 binds to all 7 ELR chemokines (Baggiolini *et al.*, 1994). In mice, the gene encoding IL-8 is absent, and its function is speculated to have become adopted by other genes most notably the keratinocyte-derived protein chemokine (KC), macrophage inflammatory protein-2 (MIP-2), and LPS induced CXC chemokine (LIX) (Modi & Yoshimura, 1999; Watanabe *et al.*, 1993; Smith & Herschman, 1997). All three, KC, MIP-2 and LIX contain the ELR motif, and their expression has been shown to be induced by LPS (Watanabe *et al.*, 1993; Rovai *et al.*, 1998; Modi & Yoshimura, 1999; Smith & Herschman, 1995). Interestingly, murine neutrophils express a homologous receptor to human CXCR2 that is able to mediate chemotaxis to human IL-8 and to murine KC and MIP-2 (Bozie *et al.*, 1994; Lee *et al.*,

1995; Wuyts *et al.*, 1996; Rovai *et al.*, 1998). Similarly, mice express a human homologue of CXCR1, which is engaged by human CXCL6 (GCP-2) and CXCL8 (IL-8), but not responsive to human CXCR2 ligands CXCL1 (Gro- α), CXCL2 (Gro- β), CXCL3 (Gro- γ), CXCL5 (ENA-78), and CXCL7 (Nap-2) like the human CXCR1 (Fan *et al.*, 2007). It has been determined that KC is related to human CXCL1, while MIP-2 to human CXCL2, and lastly, LIX to human CXCL5 (Watanabe *et al.*, 1994; Smith *et al.*, 1997). Henceforth, all murine chemokines will be referred to by their human equivalent chemokine in this dissertation. Despite these physiological differences and the limitations they may pose, mice models have proved to be a valuable tool for investigating host-microbial interactions (Zenobia *et al.*, 2013), tissue homeostasis (Watanabe *et al.*, 2004), and tissue regeneration (Masaoka *et al.*, 2009).

Immunomodulatory effects of natural products

Balance between the oral microbial community and the innate host inflammatory response is key for oral health (Darveau, 2010; Marsh, 2012). Similarly, the most effective strategy to maintain this balance is prevention, normally with proper tooth brushing and adequate dental provider care visits. Nonetheless, over the past 25 years oral health has not improved, with periodontal disease affecting an estimated 47.2% in adults aged 30 and over to 70.1% in adults 65 and older in the United States alone and on a global scale about 56% of adults aged 35 to 44 years of age (Kassebaum *et al.*, 2017; Eke *et al.*, 2012; Ide *et al.*, 2007). The most common manifestations of disease in the periodontium are gingivitis and periodontitis. Both diseases are associated with dysbiosis: 1) of the microbial community, with alterations in load and composition as well as 2) of the host, with a highly dysregulated immune response (Hajishengallis, 2015). As more evidence linking the health status of the oral cavity to overall health emerges, there is mounting pressure to identify alternative strategies to prevent, restore, and maintain oral homeostasis.

Currently, the most efficacious treatment modalities of infectious diseases are targeted to the microbial community either by physical removal or by narrow spectrum antimicrobials (Axelsson *et al.*, 2004; Chapple *et al.*, 2015). Efforts to target the host inflammatory response have been unsuccessful (Cekici *et al.*, 2013) and may be due to the presence of multiple inflammatory pathways which prevent single target anti-inflammatory approaches from being successful (Cottrell & O'Connor, 2010; Lee *et al.*, 2004; Souza *et al.*, 2012). Moreover, the natural state of healthy homeostasis in the oral cavity by nature results in a constant state of controlled subclinical inflammation that proves to be difficult to differentiate from disease associated inflammation (Dixon *et al.*, 2004; Hajishengallis *et al.*, 2011; Nishii *et al.*, 2012; Tsukamoto *et al.*, 2012; Heasman & Seymour, 1990; Souza *et al.*, 2012). This is further complicated by the fact that the same inflammatory mediators utilized for protection during health are also associated with disease (Barros *et al.*, 2016).

Recently, various forms of alternative or traditional medicinal treatments, such as Ayurveda, have gained popularity due to their natural origin, cost effectiveness, availability, cultural familiarity, and minimal side effects (Pal & Shukla, 2003; Jayashankar *et al.*, 2011). According to fossil records, humans have used natural products, particularly medicinal plants, to alleviate and treat diseases since prehistoric times (Shi *et al.*, 2010; Fabricant & Farnsworth, 2001). Ayurveda is a time tested system of natural medicine reportedly dating more than 3,000 years from the Indian subcontinent (Meulenbeld, 1999). The word *Ayurveda* is Sanskrit for *ayur* (life) and *veda* (science of knowledge). Together they come to mean “knowledge of life and longevity” (Fields, 2001). Ayurveda emphasizes the importance of oral hygiene in the promotion of health (Tokinobu *et al.*, 2018; Jayashankar *et al.*, 2011; Howshigan *et al.*, 2015). A core concept of this ancient medicine focuses on the maintenance of balance amongst the bodily elements, whereas imbalance results in disease.

Much of the substances used in Ayurveda and are plant based, comprised of roots, leaves, fruits, bark, or seeds. Specifically, *Acacia chundra* Willd., *Adhatoda vasica* Nees., *Mimusops*

elengi L., Piper nigrum L., Pongamia pinnata L. Poir., Quercus infectoria Olivier., Syzygium aromaticum L., Terminalia chebula Retz. and Zingiber officinale Roscoe., have been shown to have antibacterial, antioxidant, antifungal, anticarcinogenic (Doshi *et al.*, 1983; Modder & Amarakoon, 2002), antiparasitic and anti-inflammatory (Khanna *et al.*, 2007; Zhang *et al.*, 2016) effects (Jayashankar *et al.*, 2011; Howshigan *et al.*, 2015; Stoyell *et al.*, 2016). For example, eugenol from clove extracts has been in widespread use in the dental field, being incorporated into some sealants, cements, and temporary restorations because of its antibacterial, antifungal and antiseptic properties (Saha *et al.*, 2019). Plant derived polyphenols are a class of natural anti-inflammatory compounds that can inhibit key mediators of the inflammatory response, such as MAP kinases and nuclear transcription factors (Santangelo *et al.*, 2007) and have been proposed for the prevention and treatment of oral diseases (Palaska *et al.*, 2013). Previous clinical trials have indicated successful applications of herbal based approaches for the maintenance of oral health and the potential to modulate host inflammatory responses (Jayashankar *et al.*, 2011; Howshigan *et al.*, 2015).

Summary

Over the last 60 years, billions of dollars have been invested into dental research. Nevertheless, translation from basic science to clinical practice has only been moderate in comparison to other medical fields. This problem lies in our lack of understanding the fundamental components that are necessary for the maintenance of health in the oral cavity. To date, much of oral research has been focused on disease in an effort to better understand health. However, this has led the field to a deeper understanding behind the mechanisms which cause disease, but not of the mechanisms which maintain health. In contrast to previous strategies, the subsequent chapters of this dissertation attempts to address the determinants of health by studying health and the components involved in its regulation and maintenance.

Currently, the modulation of homeostasis in oral health is not known. Understanding the mechanisms behind this carefully balanced status of health is essential not only for health in the oral cavity, also for the body as a whole. The overall approach of this dissertation is that commensal bacteria, as well as natural products, utilize the host immune axis to regulate homeostasis through selective expression and modulation of inflammatory mediators in the oral cavity. Aim one will elucidate the roles in which the TLR host bacterial recognition receptors contribute to the maintenance of bacterial homeostasis and regulation of neutrophils in the JE (Chapter 1). Aim two will examine how a neutrophil activation receptor, CXCR1 contributes to the maintenance of homeostasis (Chapter 2), and aim three will examine the modulatory effects of plant extracts in regulating host immune surveillance (Chapter 3). Observations from these studies will elucidate the mechanisms employed to modulate and maintain oral homeostasis. Moreover, this work will provide reference data for future work into both health and disease. Ultimately to inspire the innovation of novel therapeutic approaches to go beyond the treatment of disease and towards reestablishment of homeostasis and health.

Chapter 2

Toll-like Receptor 2 and Toll-like Receptor 4 responses regulate neutrophil infiltration into the junctional epithelium and significantly contribute to the composition of the oral microbiota

Introduction

The oral cavity is unique in that controlled subclinical levels of inflammation, induced by oral commensal bacteria, lead to marginal alveolar bone loss and is part of the active process of maintaining normal healthy oral homeostasis (Dixon *et al.*, 2004; Hajishengallis *et al.*, 2011; Nishii *et al.* 2013; Tsukamoto *et al.* 2012). Neutrophils are a key component of health, where individuals with deficiencies in neutrophil numbers or defects in leukocyte adhesion (LAD1 and LAD2) develop periodontitis (Waldrop *et al.*, 1987; Hajishengallis & Hajishengallis, 2014; Hart *et al.*, 1994). Similarly, failure to downregulate neutrophil transit resulting in increased neutrophil numbers or prolonged neutrophil retention has been shown to increase alveolar bone loss (Eskan *et al.*, 2012; Nussbaum & Shapira, 2011). Furthermore, abnormalities in neutrophil activation and function, such as with decreased phagocytosis (Carneiro *et al.*, 2012; Van Dyke *et al.*, 1986) or increased superoxide production (Tapashetti *et al.*, 2013), was also found to be associated with this disease. Therefore, in health, neutrophil homeostasis is finely tuned in the oral cavity to prevent commensal dental plaque bacteria overgrowth without an over response that would elicit tissue damage.

The relationship between oral bacterial colonization and the establishment of a healthy homeostatic innate defense inflammatory state is not well characterized. However, it is becoming clear that periodontal health is an active and collaborative process between commensal bacteria and host defenses, requiring regulated gene expression, controlled inflammatory responses, active neutrophil monitoring, neutralization and repair, and others yet still to be identified, all

working in homeostasis (Tsukamoto *et al.*, 2012; Darveau, 2009; Darveau, 2010). One mechanism by which host-commensal bacterial interactions contribute to oral health is exemplified by the selective usage of chemotactic ligands targeting chemokine receptor CXCR2 to recruit neutrophils to the periodontal tissues (Zenobia *et al.*, 2013).

Human gingival tissues express a wide range of toll-like receptors (TLR1-9) (Manhanonda & Pichyangkul, 2007; Beklen *et al.*, 2008), which are part of the innate immune system and are key mediators for inducing innate immunity, inflammation, proliferation, and cell survival. TLRs specifically, TLR2 and TLR4, are capable of recognizing an extensive variety of bacteria through highly conserved pathogen-associated-molecular-patterns, such as lipoteichoic acid on gram-positive bacteria and lipopolysaccharide on gram-negative bacteria, respectively (Kawai & Akira, 2005). It was previously reported that neutrophil migration into the JE was dependent on commensal colonization and the myeloid-differentiation-primary-response-88 (MyD88) activation pathway (Tsukamoto *et al.*, 2012; Zenobia *et al.*, 2013), a downstream signaling co-adaptor molecule utilized by the interleukin-1 and -18 receptors (IL-1R and IL-18R) and all TLRs except TLR3 (Kawai & Akira, 2005). However, it is not known if oral commensal bacteria utilize these TLRs to either elicit or maintain neutrophil homeostasis. If bacteria utilize these TLRs for modulation of neutrophil homeostasis, without them, we hypothesized to see a decrease in the number of neutrophils in the junctional epithelium. Therefore, to further elucidate the MyD88-dependent mechanism(s) by which oral commensal bacteria contribute to neutrophil trafficking into the JE, we investigated the role of TLR2 and TLR4 or both (TLR2/4) in their potential involvement in neutrophil recruitment in a mouse model.

In this study, specific-pathogen-free (SPF) mice deficient in TLR2, TLR4 or both (TLR2/4) were used to investigate the contribution of these TLRs to healthy homeostasis by measuring neutrophil recruitment in the junctional epithelium (JE), oral microbial composition, and alveolar bone height. Since TLR2 and TLR4 are upstream components of the MyD88 signaling cascade, it was hypothesized that their deficiency would impair neutrophil recruitment into the JE.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of the University of Washington in compliance with established federal and state policies.

Animal resource

Mice with targeted deletions of TLR2 and TLR4 were obtained from Dr. S. Akira (Osaka, Japan) (Hoshino *et al.*, 1999; Takeuchi *et al.*, 1999) and backcrossed to C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) to reduce genetic variations for at least six generations. TLR2 and 4 double deleted mice were created by crossing TLR2 homozygous deleted and TLR4 homozygous deleted mice (Skerrett *et al.*, 2007). Genotypes were confirmed with tail-snip DNA.

Thirty specific-pathogen-free (SPF) female and male TLR2, TLR4, TLR2/4 KO and wild-type (WT) C57BL/6 mice (12 to 14 weeks-old and 5 to 6 mice/group) were raised and maintained under SPF conditions at the University of Washington under the direction of the Department of Comparative Medicine.

Histology

Similar to previously published methods (Greer *et al.*, 2016), mice were euthanized by cervical dislocation and tissues were harvested for histological processing. Maxillae and mandibles were immediately fixed overnight on a shaker at 4 °C and rinsed with 70% ethanol daily for 1 week, gradually decreasing the rinses to once every other day until dissection. After dissection, tissues were demineralized in EDTA-cacodylate decalcification solution and processed following standard histological procedures and embedded in paraffin. Using a

microtome, five mandibular halves per group were examined and sectioned (5 μ m) from mesial to distal in the coronal plane. Two serial sections were collected per charged glass slides, resulting in approximately 100 sections per tooth (50 slides per tooth). Slides were numbered and imaged in consecutive order.

Immunohistochemistry

Detection of neutrophils on paraffin-embedded samples by immunohistochemistry (IHC) was performed on every 5th slide. Each slide was picked serially from the root-associated first molar to the root-associated second molar as previously described (Greer *et al.*, 2016), resulting in approximately 30 sections (60 JE; 15 slides) per half mandible. Tissue sections were deparaffinized and rehydrated using a gradation of diluted ethanol. Sections were blocked in 1.5% hydrogen peroxide in methanol solution and targeted with a primary neutrophil elastase monoclonal antibody at 1 μ g/mL (Neutrophil Marker Sc-71674, Santa Cruz Biotechnology, Dallas, TX, USA). Primary antibodies were conjugated to an avidin-biotinylated secondary antibody, developed with a peroxidase substrate, and counterstained with hematoxylin. The WT samples processed alongside experimental samples with and without the addition of primary antibody served as positive and negative controls, respectively. All quantitative analyses were manually counted under 200X magnification by a single blinded examiner, A.M.C., and repeated.

Histomorphometry

Linear distances from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) were measured using publicly available software (ImageJ Version 1.50b National Institutes of Health, Bethesda, MD, USA) on all IHC stained sections (150 sections per group) on both buccal and lingual surfaces of the tooth. Images (200X) were acquired in a blinded manner (SPOT CCD camera and software, Diagnostic Instruments, Sterling Heights, MI, USA). Area measurements

were determined similarly on the blinded images (200X). All measurements were calibrated using a 0.01 mm stage micrometer and repeated.

Oral Swabbing and Microbial Genomic DNA Extraction

Bacterial oral swabs of 5 mice per group were obtained from teeth, gingival surfaces, and tongue immediately after sacrifice and stored at -80 °C until extraction. Bacterial genomic DNA was extracted using a DNA extraction kit (QIAamp DNA Microbiome Kit, Qiagen, Germantown, MD, USA) and collected in low bind tubes (DNA LoBind eppendorf Tubes, Eppendorf, Hauppauge, NY, USA). Samples were further purified, concentrated (DNA Clean & Concentrator Kit, Zymo Research, Irvine, CA, USA) and stored at -20 °C until sequencing. Similarly, bacterial oral swabs of 6 mice per group were obtained, and genomic DNA was extracted for sequencing. Separate samples were used for the quantification of total bacterial load.

V3-V4 Amplification and Sequencing Strategy

The V3-V4 hypervariable region of the 16S rRNA was targeted using region specific primers containing an adapter sequence (Illumina flow cell adapter sequence, Illumina, San Diego, CA, USA) and purified using magnetic beads (Agencourt AMPure XP beads, Beckman Coulter, Inc., Pasadena, CA, USA). PCR products were verified by gel for visualization of the correct size after each PCR step. Amplicons were then indexed (Nextera XT Index Kit V2 SetA, Illumina, San Diego, CA, USA), purified with magnetic beads, and analyzed by gel electrophoresis (Agilent 4200 TapeStation System, Santa Clara, CA, USA) to assess library quality. Concentrations were determined individually with a fluorometer (Qubit3, Invitrogen, Carlsbad, CA, USA) and normalized to a concentration of 3-6 nM (SequalPrep Normalization Plate Kit, Invitrogen). Ultimately, the library was pooled, denatured with sodium hydroxide and sequenced (300 paired end MiSeq, Illumina). The PhiX control (PhiX Control v3 library, Illumina) was spiked

at 20% to serve as an internal control and to balance for possible low diversity and base bias present in the 16S amplicon samples as recommended by provider (Illumina).

Analysis and further downstream applications of sequencing data

300 base pair (bp) paired-end 16S rRNA gene sequences were de-multiplexed, and the adaptor sequences were removed and filtered for quality. The approximately 130 bp overlapping sequences were merged (Pandaseq; Masella *et al.*, 2012) within the V3-V4 region and processed with publicly available tools (Qiime version 1.9.1; Caporaso *et al.*, 2010). Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity, aligned, and assigned a taxonomy in reference with Greengenes 16S rRNA database version 13_8 (DeSantis *et al.*, 2006). OTU tables were rarefied to a sequence depth of 15,200 reads/sample to calculate alpha diversity. For beta diversity, weighted and unweighted Unifrac (Lozupone & Knight, 2005) distances were calculated and further analyzed by principal coordinates analysis (PCoA) (Gower, 1966). Significant differences in overall beta diversity was conducted (R Studio 3.0.2, Vienna, Austria) and determined with PERMANOVA(adonis) statistical testing (R Core Team, 2013). Linear discriminant analysis effect size (LEfSe) was used to determine differentially abundant features at the species level (Segata *et al.*, 2011). Publically available packages (“phyloseq”, McMurdie & Holmes, 2013; “ggPlot2”, Wickham, 2009) were used to generate Shannon’s and Fisher’s alpha diversity index charts and then the pairwise Wilcoxon Rank Sum test was applied.

Total bacterial load quantification by quantitative real-time PCR

Absolute quantitation of total bacterial load of 6 mice per group was measured by quantitative real-time PCR (qPCR) targeting the 16S rRNA gene. Genomic DNA from mouse oral swabs, nuclease-free water for negative controls, or standard (2 µl) was added to 10 µl of mastermix (SsoAdvanced Universal Probes Supermix, Bio-rad, Hercules, California, USA), 600 nM concentrations each of forward primer (5'-CGCTAGTAATCGTGGATCAGAATG-3') and

reverse primers (5'-TGTGACGGGCGGTGTGTA-3'), and 250 nM of probe (5'-FAM-CACGGTGAATACGTTCCCGGGC-TAMRA-3') (Yoshida *et al.*, 2003). Nuclease-free water was added to bring the total volume of the reaction to 20 μ l. The qPCR conditions were run at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The number of bacteria was determined using a serially diluted internal standard curve of *F. nucleatum* ATCC 10953 genomic DNA and analyzed using the second derivative maximum method (Rasmussen, 2001). Internal standards were of high quality, with efficiencies of 2.0 and approximate errors of 0.20.

Additional statistical analysis

Mann-Whitney U test was performed (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, California, USA) to determine significance for average neutrophils, total bacterial load, and bone loss. An α value below 0.05 was considered significant.

Results

TLR2 and TLR4 contribute to the regulation of neutrophil recruitment into the JE

The ability of TLR2, TLR4, and TLR2/4 KO mice to recruit neutrophils into the JE was examined by immunohistochemistry. All TLR KO mice showed a significant increase in average neutrophils/mm² of JE ($p = 0.0079$) when compared to that of WT mice (Fig. 1A, C). This demonstrates that TLR2 and TLR4, two upstream components in the MyD88-dependent inflammatory cascade, are not necessary for neutrophil migration into the JE. Instead, since the total JE area did not vary between mice strains (Fig. 1B), it is possible that TLR2 and TLR4 may play roles in limiting neutrophil infiltration into the JE. Alternatively, the lack of TLR2 and TLR4 may indirectly affect neutrophil migration and regulation through the alteration of the oral microbiome into a dysbiotic oral community.

TLR4 responses prevent overgrowth of the mouse oral microbiota

To address the potential role of an alteration in the oral microbiome for the observed dysregulation of neutrophil recruitment into the JE of the TLR KO mice, genomic DNA from oral swabs were extracted and their total bacterial load was determined. Bacterial load was significantly greatest ($p < 0.01$) in TLR4 KO mice with 1.2×10^5 mean copy numbers (Fig. 2). Similarly, TLR2/4 KO mice also showed an increase in bacterial load with 5.6×10^4 mean copy numbers ($p < 0.05$) compared to WT samples which had 4.6×10^4 mean copy numbers (Fig. 2). In contrast, TLR2 KO mouse samples showed no difference in total bacteria present compared to WT samples. This data reveals that the host's ability to control the growth of the microbial community, as measured by total bacterial load, may be specifically disrupted in TLR4 KO mice, demonstrating a novel role for TLR4 in regulating oral microbial homeostasis.

TLR2, TLR4, and TLR2/4 KO and WT mice all display distinct and unique oral microbial compositions

In order to more closely analyze the disruption of bacterial homeostasis observed from the TLR KO mice, the 16S rRNA gene was sequenced from oral swab samples to assess microbial composition and the diversity was analyzed. There are two approaches to analyze diversity, alpha and beta diversity. Alpha diversity measures the diversity within a sample (Who is there? How many are there?) (Whittaker, 1972). In contrast to beta diversity, which measures the diversity between samples (How similar are the pairs of samples?) (Whittaker, 1972). There are multiple ways to measure alpha diversity. For example, by the measurement of richness or alternatively, evenness. Richness measures the number of different types of organisms present in a sample and can be measured by the total count of OTUs present or estimated by combining both present/absence of organisms as well as its abundance with the Chao1 diversity index (Kim *et al.* 2017). Evenness, in contrast, measures how equally abundant those organisms are in a sample and is often done with the Fisher's alpha or Shannon diversity indices which takes into

account both abundance and evenness (Kim *et al.* 2017). Beta diversity can be represented by a distance metric called the unweighted UniFrac, which calculates the branch lengths of a phylogenetic tree that is shared or unshared by the OTUs between samples (Lozupone & Knight, 2005). Next, an ordination method such as the principal coordinate analysis (PCoA) can take these distance matrices and plot them in a n-dimensional space to visualize for similarity or dissimilarity by clustering (Kruskal, 1964).

Analyses of alpha diversity measuring total observed operational taxonomic units (OTUs) shows close similarity in the mean number of OTUs in all mice groups, with a trend, but no statistical significance towards a higher OTU and Chao1 diversity in TLR4 KO mice (Fig. 3A). Although no significant difference was found in the total number of observed OTUs between mice, deeper investigation at the OTU sequence level shows individual KO-specific OTU representation (Fig. 3B). A total of 156 OTUs were shared among all of the mouse groups, with TLR2/4 KO mice having 2, TLR4 KO mice having 16, TLR2 KO mice having 6 and WT mice having 22 unique OTUs. Moreover, Chao1 diversity analysis shows that TLR2 KO mice ranked the lowest on the diversity index when compared to WT in contrast to the rest of the KO mice groups, but these differences were not statistically significant (Fig 3C). Similar results were obtained when alpha diversity was also measured by the Shannon and Fisher Indexes (Supplemental Figure 1).

Additional analyses of beta diversity by UniFrac PCoA shows clear spatial segregation and clustering between each TLR KO and WT mice, which is more pronounced in the unweighted (phylogenetic distance alone) than the weighted (combined abundance and phylogenetic distance) PCoA analysis (Fig. 3D). Weighted PCoA analysis shows moderate clustering within each group and minor separation between KO groups (Fig. 3E). The tight clustering of individual mouse samples within each KO strain group and the distinct separation of these groupings from each other in the unweighted PCoA demonstrates that each KO strain is uniquely different from the other. Moreover, the uniqueness of each strain is further reflected by nonparametric multivariate statistical analyses utilizing distances from the unweighted PCoA analysis (employed

in Adonis package), which shows a minimum $p \leq 0.05$ for all groups, with the most similarity ($p < 0.05$) between TLR2 and TLR2/4 double KO mice and the most dissimilarity ($p < 0.01$) between TLR2 and TLR4 KO mice (Supplemental Figure 2). Altogether, alpha and beta diversity analyses reveal there are clear differences in microbial composition, but not in total number of OTUs present.

Major differences in predominating bacterial genera among mouse groups

Supporting clear differences represented by the PCoA analyses and the Adonis statistical testing, alterations in bacterial composition were observed at the genus level (Fig. 4A). TLR KO mice exhibited a bacterial composition strikingly different from WT, with all TLR KO groups showing a predominance of Pasteurellaceae/unidentified#2. This TLR KO associated Pasteurellaceae/unidentified#2 is a unique and distinct member from the Pasteurellaceae identified from WT mice, labeled as Pasteurellaceae/unidentified#1. Both identified Pasteurellaceae groups in TLR KO and WT mice are uncharacterized and unnamed distinct genera in the Family Pasteurellaceae.

Focusing on statistically significant differences in bacterial composition, LEfSe analysis confirmed the dominance of the Pasteurellaceae/unidentified#1 in WT mice compared to that of Pasteurellaceae/unidentified#2 in the TLR KO mice (Fig. 4B). Moreover, WT mice show a high prevalence of both gram-negative and gram-positive members such as *Aggregatibacter*, *Microbacterium*, *Enterobacteriaceae/unidentified*, and *Fusobacterium* in contrast to TLR KO mice which show a dominance of gram-negative species such as Pasteurellaceae/unidentified#2 and *Actinobacillus*. Specific differences amongst KO groups can further be observed when analyzed at the broader family level (Fig 4C); where, the presence of 5 unique families (Weeksellaceae, Rikenellaceae, Micrococcaceae, Deferribacteraceae, and Campylobacteraceae) are only found in TLR4 KO mice and absent in the rest of the mouse groups. Furthermore, TLR2 KO mice exclusively harbor Pseudonocardiaceae, Brucellaceae, and Aerococcaceae, while TLR2/4 KO

mice show an abundance of Caulobacteraceae. Collectively, analysis of oral bacterial composition between normal, non-infected TLR KO mice show that each TLR KO mouse group establishes a unique oral environment that in each case supports a distinct microbial community.

The predominant members from the Pasteurellaceae family and their abundance are different between WT and TLR KO mice

In order to characterize and distinguish between the Pasteurellaceae/unidentified#1 from WT and the Pasteurellaceae/unidentified#2 from TLR KO mice, their respective OTU sequences were aligned and compared in a phylogenetic tree alongside reference sequences and, concomitantly, with the total observed OTU counts (Fig. 5). Among the reference sequences the 16S rRNA gene sequence of a potentially novel Pasteurellaceae strain, NI1060, was also included into the analysis in an effort to identify and characterize these Pasteurellaceae. The NI1060 is a recently identified murine oral bacterium shown to induce periodontitis independently without the cooperation of the oral microbiota in a murine ligature-induced disease model and found to be closely related to *Aggregatibacter actinomycetemcomitans* (Darzi *et al.*, 2016; Jiao *et al.*, 2013), a species with strong correlation to the development of aggressive periodontitis in humans (Newman & Socransky, 1977; Slots *et al.*, 1980). Lastly, additional Pasteurellaceae OTUs identified in the samples were also included into this analysis to check for distinctions between the Pasteurellaceae in TLR KO and WT groups.

Sequence analysis shows OTU82728, identified as Pasteurellaceae/unidentified#2 in TLR KO mice samples, and OTU226, identified as Pasteurellaceae/unidentified#1 in WT mice, share a common ancestor, but come from distinctly different lineages that are both closely related to the reference sequence of *Actinobacillus muris* S004536332. Moreover, sequence count data correlates with previous analyses, where TLR KO OTU82728 is predominant in all TLR KO mice samples, approximately 12,000 to 24,000 sequence counts, but in low abundance, approximately 2,000 sequence counts in WT samples. In contrast, WT OTU226 is found in large sequence

abundance in all WT samples, but in low abundance in TLR KO samples. Sequence comparison of the Pasteurellaceae OTUs identified in WT and TLR KO mice to the novel NI1060 strain reveal that both TLR KO OTU82728 and WT OTU226 have 96% sequence similarity, where 97% sequence similarity represents species equivalence. More interestingly, OTU99051 shows 99% sequence similarity and is present in low numbers in all WT and select TLR2/4 double KO samples, but not in TLR2 or TLR4 KO samples. These analyses reveal that the presence of NI1060, a bacteria previously shown to induce periodontal disease (Alves-Filho *et al.*, 2009), is also present in the oral cavity of these normal healthy mice. Overall, TLR KO mice have a distinctly different member of Pasteurellaceae from that of WT.

Dysregulation of neutrophil recruitment and an alteration of the microbial composition does not affect alveolar bone levels

To understand the clinical implications of dysregulated neutrophil recruitment and an alteration of the microbial burden and composition, alveolar bone levels were measured histomorphometrically with linear distances from the alveolar bone crest (ABC) to the cemento-enamel junction (CEJ). Histomorphometric analysis of alveolar bone loss at the buccal and lingual sides revealed comparable levels with no statistically significant difference in bone loss between TLR KO and WT mice (Fig. 6). Altogether, our data points out that the dysregulation of neutrophil recruitment and alteration of the oral microbial burden and composition by loss of TLR2 or TLR4 or both TLR2 and 4 does not disrupt alveolar bone levels.

Discussion

Previous studies demonstrated a significant reduction in neutrophil transit in the JE of MyD88 KO mice (Tsukamoto *et al.*, 2012; Zenobia *et al.*, 2013). Therefore, it was anticipated that TLR2 and/or TLR4 deficiency would show a reduction in the number of neutrophils that transit into the JE as both signaling pathways engage the TLR adaptor protein MyD88. However, in

contrast to our hypothesis, all KO mice showed a significant increase in average neutrophils/mm² of JE when compared to WT mice demonstrating that neither TLR2 nor TLR4 are required for neutrophil transit. This increase was likely not a result of an increase in total circulating blood neutrophils, as TLR-deficient mice were reported to show similar numbers of total neutrophils to WT mice (Hoy *et al.*, 2015; Sabroe *et al.*, 2005). Consistent with the notion that TLRs are not required for neutrophil migration into the JE, but may rather play a role in the modulation of neutrophil trafficking, similar increases in bacterial load and neutrophil numbers in the peritoneal cavity of TLR4 deficient mice were observed in a sepsis model (Sabroe *et al.*, 2003). Possible explanations for the increase in neutrophil numbers in the JE include the increase in bacterial load observed in the TLR4 and TLR2/4 KO mice and/or the significant change in the oral microbial composition observed in all TLR KO mice.

Oral microbiome analysis revealed that TLR KO mice have characteristically different compositions, with increases in mostly gram-negative species for TLR2 and TLR2/4 KO mice, while TLR4 KO mice show an increase in gram-positive species. In addition, TLR KO mice showed a strong selection for a Pasteurellaceae species more related to *Actinobacillus muris*, while the WT group has a Pasteurellaceae species more closely related to *Haemophilus influenza-murium*. At the same time, each group harbored distinctly unique species within each TLR KO group. Cage-to-cage variation may potentially explain the unique compositional differences (Kennedy & DeLeo, 2009) amongst TLR KO and WT mice and remains a limitation of this study. However, if cage to cage variation were to contribute to the observed results, it may explain only a fraction of the differences in composition since samples from each experimental group were collected across different litters and cages over multiple generations and yet still maintained close compositional similarities within the group that were distinct from the others. Therefore, although cage to cage variation remains a limitation of this study, a preponderance of evidence supports the contention that TLR2 and TLR4 significantly shape the oral microbial composition.

Another possible explanation for the increased as opposed to decreased neutrophil infiltration in the JE is that additional MyD88 dependent pathways such as TLRs 1, 5 and 6, and the IL-1 receptor regulate neutrophil transit independent of TLR2 or 4. Alternatively, the absence of TLR2 or TLR4 may indicate a critical role for these TLRs in limiting excessive neutrophil infiltration to sites of infection via dampening chemokine receptor expression or functions. Lending support to this possibility, ex vivo experiments in human neutrophils (Chukkapalli *et al.*, 2017; Zenobia *et al.*, 2017) and in vivo experiments in mouse models (Andonegui *et al.*, 2003) demonstrated that TLR2 and TLR4 activation downregulates the cell surface expression of CXCR2 on neutrophils, impairing chemotaxis. Furthermore, it was observed that deficiency of TLR2 prevented CXCR2 downregulation and relieved impairment of neutrophil numbers in lung tissue in a mouse model of sepsis (Andonegui *et al.*, 2003). Moreover, these mutations may have additional effects on neutrophil lifespan by the inhibition of apoptotic factors and mechanisms and/or inducing mediators which prolong neutrophil survival (Kennedy & DeLeo, 2009). Collectively, our data indicate that TLR2 and TLR4 are not required for neutrophil recruitment, but instead play a key role in regulating neutrophil homeostasis in the oral cavity—possibly directly, by limiting chemotactic responses, or indirectly, by promoting the development of a dysbiotic oral commensal community.

Interestingly, the dysregulated recruitment of neutrophils into the JE and alteration in microbial composition of these TLR KO mice did not affect alveolar bone levels. This result is consistent with previous observations in a mouse model, which found no significant differences in alveolar bone loss between uninfected control TLR2 and TLR4 KO mice (Chukkapalli *et al.*, 2017) and also in a rabbit model of disease investigating the contribution of periopathogens to initiate dysbiosis in oral communities, which concluded that not all dysbiotic communities induced bone loss (Zenobia *et al.*, 2017). This suggests that periodontitis is a complex disease process that requires more than a dysbiotic microbial community and may require engagement of additional components yet to be elucidated.

This study also revealed the potential hazard in the interpretation of data obtained from the current mouse models of periodontitis. It has been shown that one major effect of the addition of *Porphyromonas gingivalis* is a change in the microbial composition. Clearly, TLR2 and TLR4 KO mice display significantly different oral microbial compositions, therefore differences in the initial microbial composition in the different TLR KO mice preclude definitive conclusions concerning the specific contribution of either TLR2 or TLR4 to *P. gingivalis* induced disease as has been previously reported (Lima *et al.*, 2010; Burns *et al.*, 2006; Gibson *et al.*, 2008; Lin *et al.*, 2017). Furthermore, although the presence of N1060, a bacterium shown to initiate periodontitis in mice without the cooperation of the other oral microbiota represents a valid study with appropriate conclusions (Jiao *et al.*, 2013), the observation that it was not found in all the groups of mice underscores the need for a complete oral microbial analysis of the strains being employed in all periodontitis mouse model of disease.

Conclusion

In conclusion, our findings demonstrate that in health, TLR2 and TLR4 are not required for promoting neutrophil trafficking into the JE, but rather may significantly contribute to shaping the composition of the microbiota, which may in turn modulate neutrophil homeostasis. This study demonstrates that at least in mice, direct recognition of bacterial components by either TLR2 or TLR4 is not required for neutrophil migration into the gingival sulcus. Rather, the key activation pathway that elicits neutrophil migration in healthy gingiva remains unknown, but appears to be dependent upon both MyD88 and bacteria (Tsukamoto *et al.*, 2012; Zenobia *et al.*, 2013; Greer *et al.*, 2016).

Fig. 1

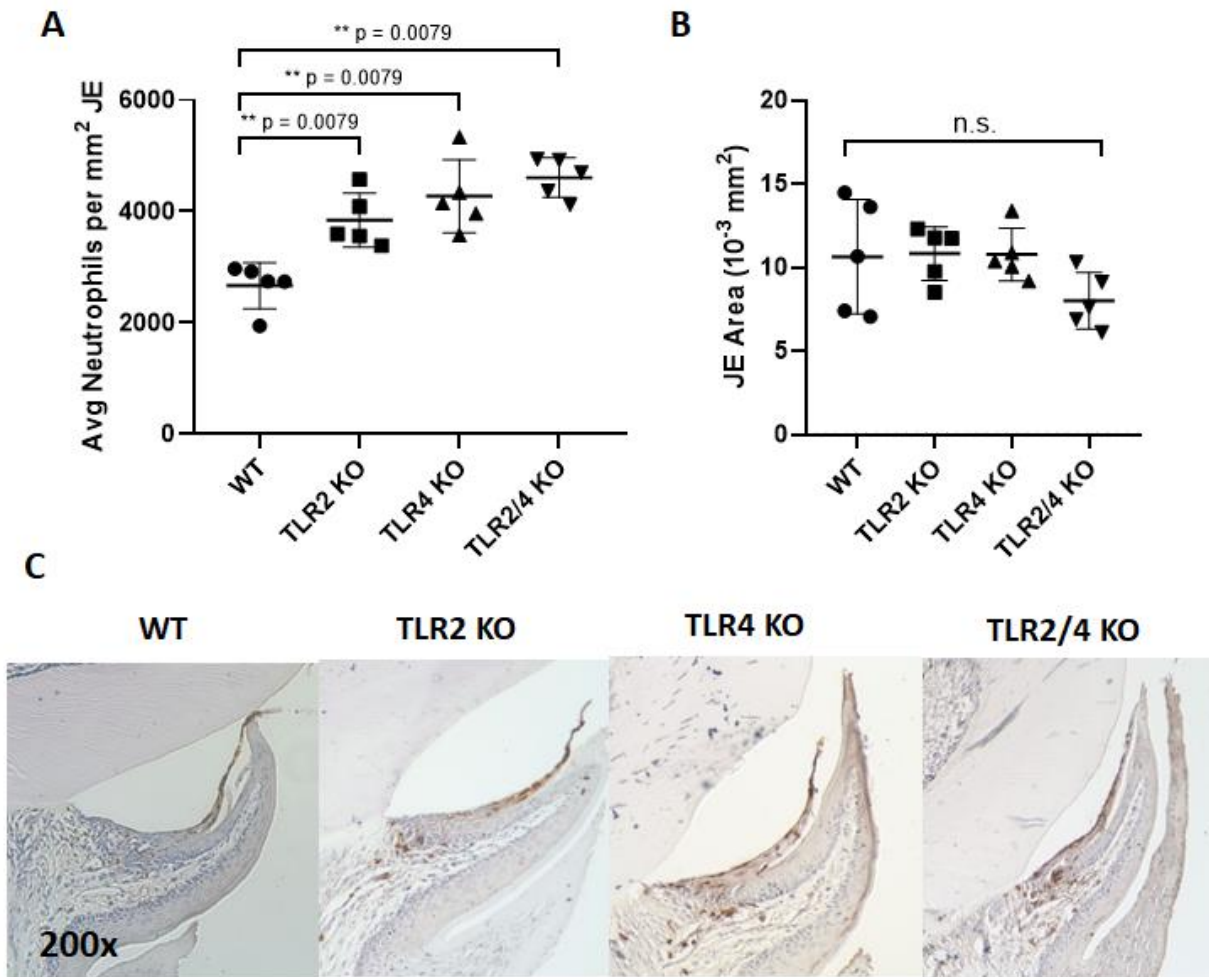


Fig 1. TLR2 and TLR4 are not required for neutrophil recruitment into the junctional epithelium (JE). (A) Scatter dot plot shows average neutrophil numbers standardized to their respective JE areas. (B) Scatter dot plot shows average JE area of each mouse. Each point is represented as an average calculated from 30 JE sections / mouse, with 5 mice per group with error bars representing standard deviation. (C) Representative images of histology sections at 200x magnification. Significant statistical differences were calculated using the Mann-Whitney test. (***) $p < 0.001$ and (n.s.) not significant.

Fig. 2

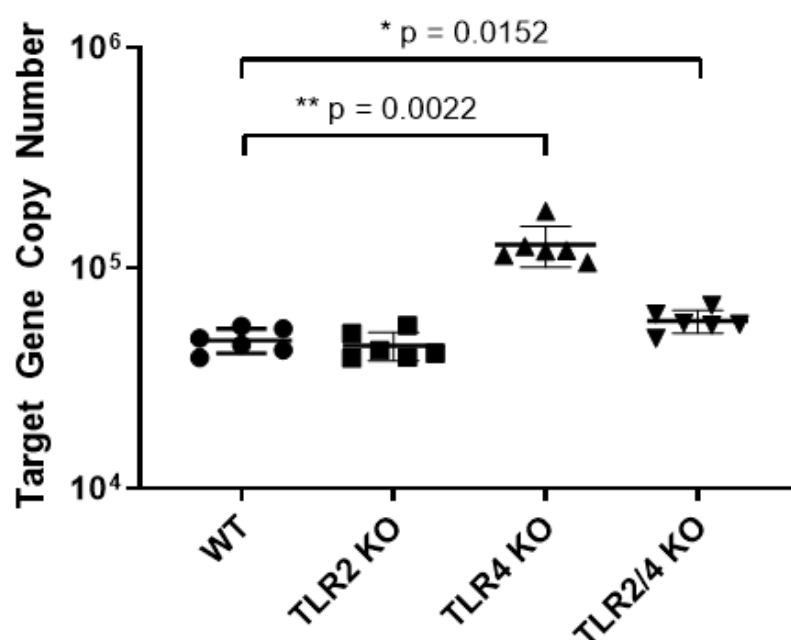


Fig 2. Bacterial load reveals disruption of bacterial homeostasis in some but not all TLR KO mice. Total bacterial load is represented by the copy numbers of 16S rRNA gene for each oral swab sample ($n = 6$ / group) by real-time PCR assays in a scatter plot. Error bars represent standard deviation. Significance was determined using the Mann-Whitney U test. (*) $p < 0.05$ and (**) $p < 0.01$

Fig. 3

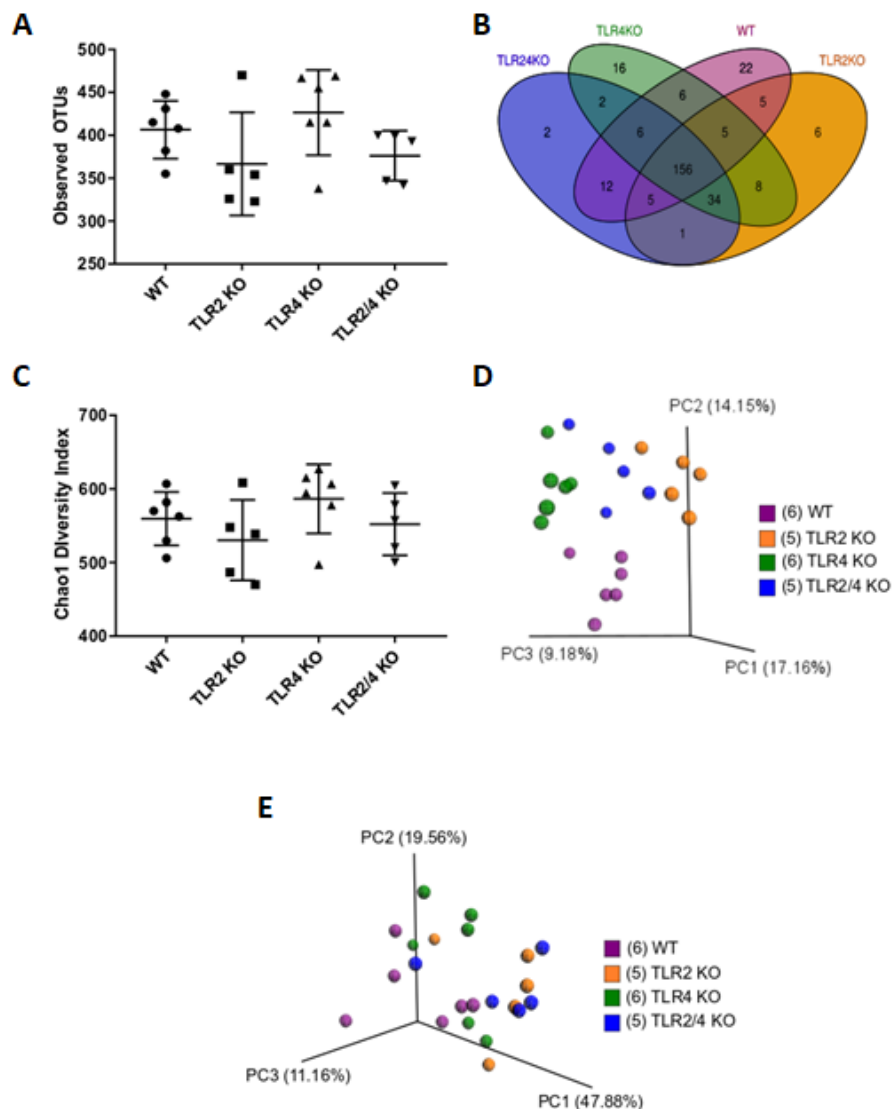


Fig. 3. Beta diversity, but not alpha diversity measurements show clear differences in microbial compositions between TLR and WT mice (n=5-6 mice / group).

A. Scatter dot plot comparing total observed operational taxonomic units (OTUs). Alpha diversity measure by total OTUs showed no statistically significant difference between groups.

B. Venn diagram comparing OTU specific sequences among groups reveal the number of both unique and shared OTU representations in and amongst each mouse group.

C. Scatter dot plot comparing the Chao1 diversity index did not show differences in species richness amongst mouse groups.

D. Beta diversity patterns using Principal Coordinate Analysis (PCoA) of unweighted UniFrac distances show clustering of mice within the same group and spatial segregation between different groups.

E. PCoA of weighted UniFrac distances reveal moderate clustering within each group and moderate spatial segregation between different groups.

Each point represents a single mouse and each color represents a mouse group. Error bars represent standard deviation.

Fig. 4

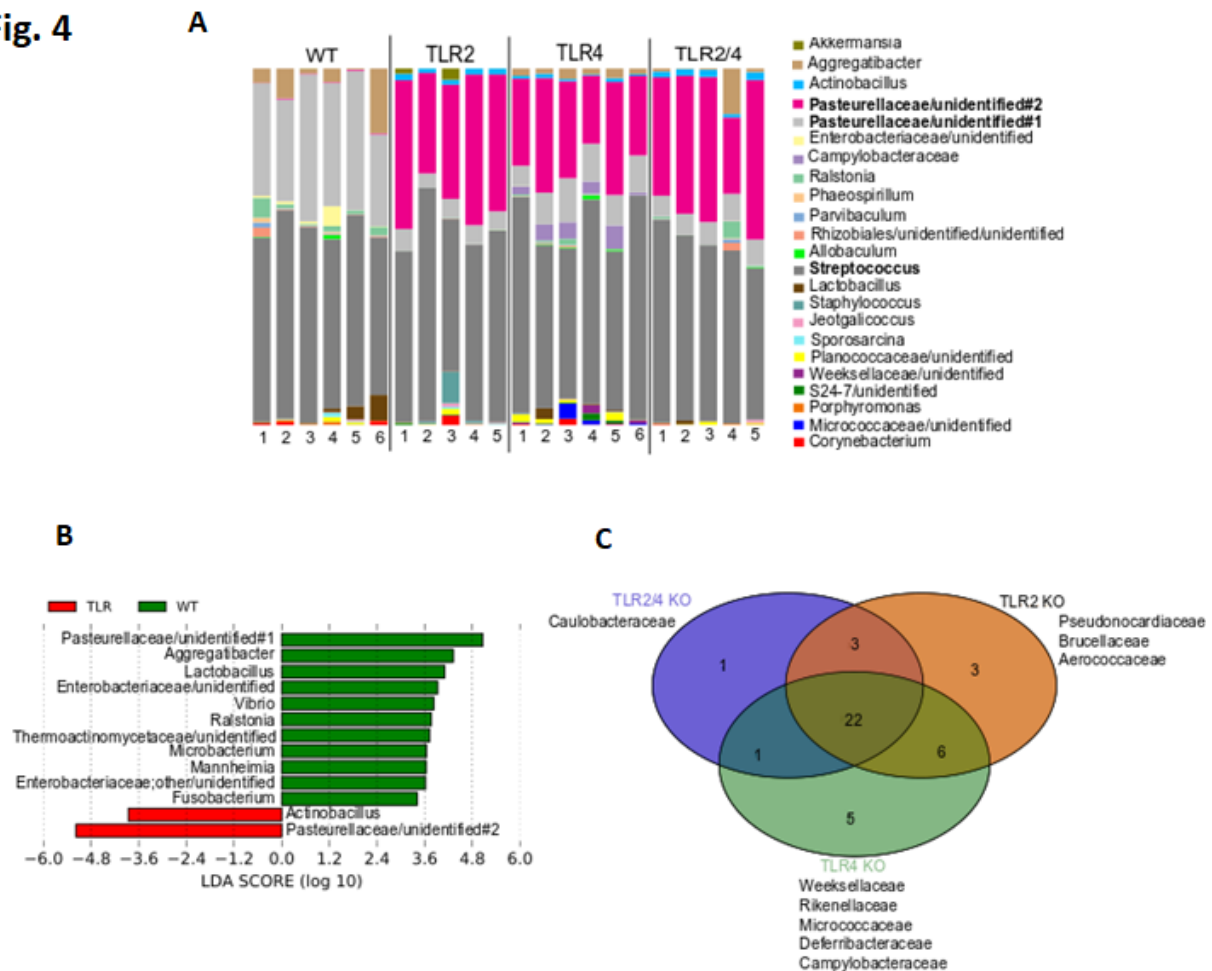


Fig 4. TLR KO mice show distinct complexity and representation by unique taxa that differ from each other and WT (n=5-6 mice / group).

A. Bacterial composition between TLR2, TLR4, TLR2/4 double KO and WT mice at the genus level. Each color is represented by a genus in the figure legend.

B. Linear discriminant analysis effect size (LEfSe) analysis showing those OTUs that were significantly differentially abundant between WT (green) and TLR KO (red) mice, ranked by effect size (All linear discriminant analysis (LDA) scores > 3.4)

C. Venn diagram showing specific family representation amongst the TLR KO mice.

Although the Greengenes 16S rRNA database version 13_8 identified unique genera, limited characterization of some sequences could not be labeled by its respective genus and is referred by its family level identity followed by “unidentified” for its genus level identification.

Fig. 5

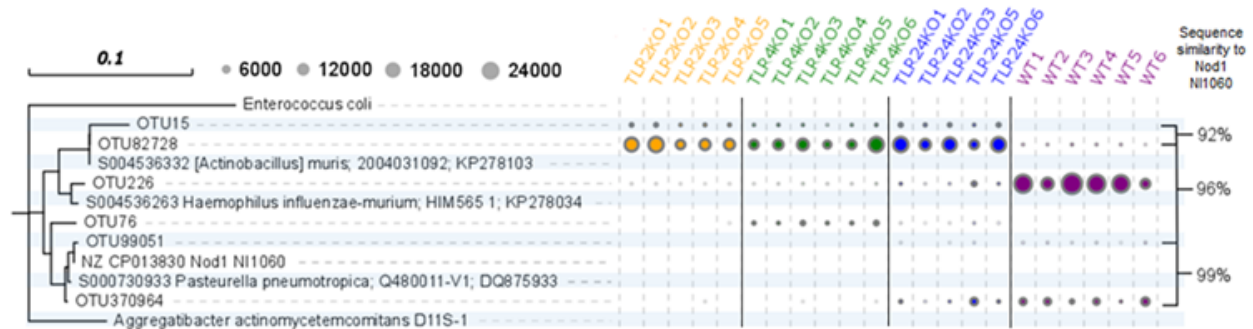


Fig 5. TLR KO mice harbor different species of Pasteurellaceae from WT and WT contains a Pasteurellaceae strain, NI1060.

A phylogenetic tree comparing specific WT and TLR KO identified Pasteurellaceae OTU sequences at the species level and their total sequence counts in relation to reference strains. Sequences from WT from this study were 99% similar to a novel strain of Pasteurellaceae that is closely related to *Aggregatibacter actinomycetemcomitans*, a species with strong correlation to the development of aggressive periodontitis in humans. Circles represent OTU sequence counts and a sequence similarity of ~97% is accepted as species equivalence.

Fig. 6

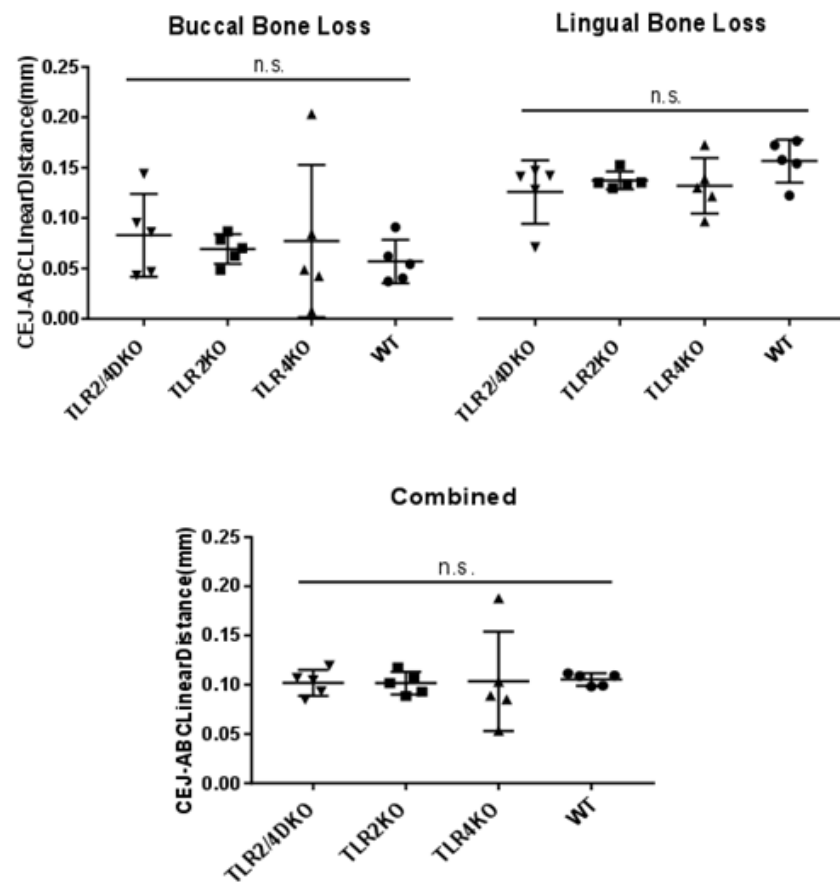
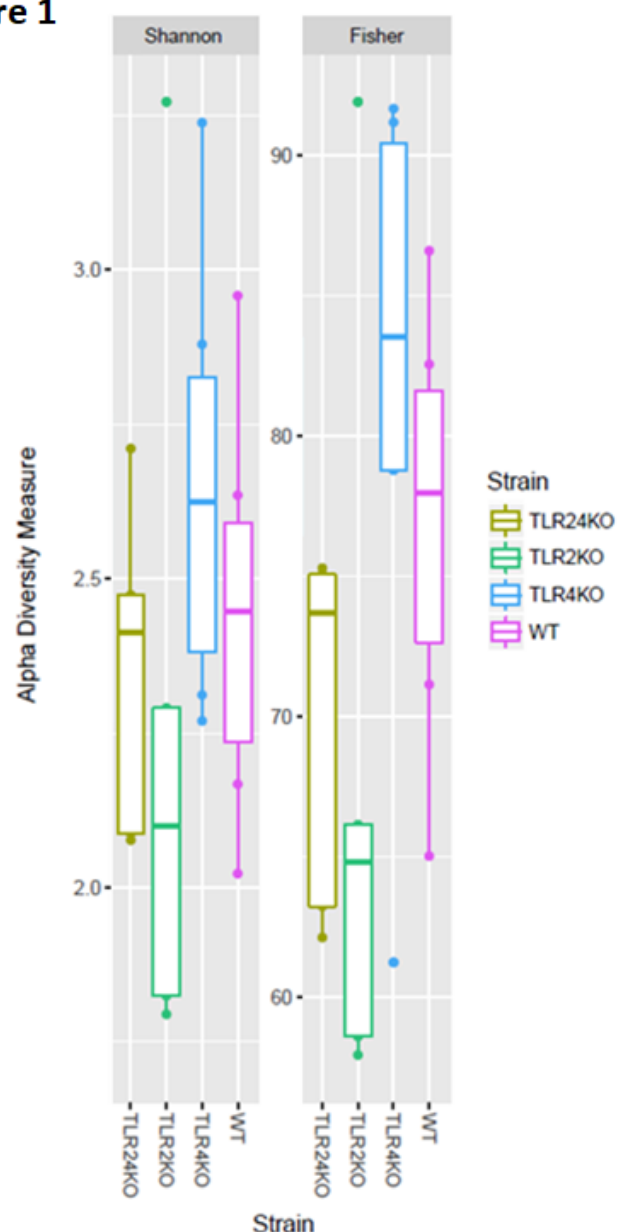


Fig 6. No difference in bone loss was observed between TLR KO and WT mice. Scatter dot plot shows no significant difference in bone loss when determined by histomorphometric analysis of linear distances from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) on buccal and lingual surfaces of the tooth (300 JE per group). Measurements of buccal and lingual sides were then combined for analysis of combined measurements. Images were acquired at 200x and measured blinded two independent times by the same observer. Error bars represent standard deviations. (n.s.) not statistically significant.

Supplementary Figure 1



Supplementary Figure 1. Alpha diversity measurements do not show differences between mouse groups. 16S rDNA V3-V4 sequence data were filtered and assigned a taxonomy using the Greengenes 16S rRNA database version 13_8 then rarefied to a sequence depth of 15,200 reads/sample to calculate alpha diversity measures. OTUs were defined as groups of sequences that differ by $\leq 3\%$ at the DNA level. Charts were generated through R 3.0.2 ($n = 5-6/\text{group}$). Box and whisker plot comparing Shannon's (left) and Fisher's (right) alpha diversity indices. ($n = 5-6$).

Supplementary Figure 2

	WT	TLR2	TLR4	TLR2/4
WT		0.006	0.005	0.012
TLR2	0.006		0.003	0.022
TLR4	0.005	0.003		0.009
TLR2/4	0.012	0.022	0.009	

* p<0.05

** p<0.01

Supplementary Figure 2. Nonparametric multivariate statistical analysis using distances from the unweighted PCoA results reveal significant compositional differences among groups. Statistical significance testing was performed in R 3.0.2 and determined with PERMANOVA(adonis) statistical testing followed by a pair-wise Wilcoxon Rank Sum test. Each mouse group corresponds with an n = 5-6. (*) p < 0.05 and (**) p < 0.01

Chapter 3:

Chemokine receptor CXCR1 responses regulate neutrophil infiltration into the junctional epithelium and significantly contribute to the composition of the oral microbiota

Introduction

Neutrophils are a key component of periodontal health, providing a neutrophilic “wall”-like barrier between the plaque biofilm on the tooth surface and the host tissues (Darveau, 2010). They follow a chemokine gradient of IL-8 (CXCL8) from low to high, to the site of infection, where highly regulated activity of neutrophils inhibits overgrowth of the bacterial biofilm without over responding to elicit tissue damage (Darveau, 2010). IL-8 is a chemotactic cytokine expressed by a variety of cells including keratinocytes, leukocytes, endothelial cells, and vascular smooth muscle cells (Sfakianakis *et al.*, 2002) and contributes to angiogenesis, inflammation, and neutrophil recruitment (Devalaraja & Richmond, 1999). It binds to CXCR1 and CXCR2, and the expression of IL-8 and its receptors are reported in both clinically healthy and chronically inflamed gingival tissues (Sfakianakis *et al.*, 2002). In the literature there is disagreement as to which CXCR receptor has a higher affinity for IL-8, but it is routinely reported that CXCR1 is a highly specific IL-8 receptor while CXCR2 is more promiscuous binding to various C-X-C ligands including IL-8 (Chuntharapai & Kim, 1995). It has been shown that CXCR2 has a higher binding affinity for IL-8 compared to that of CXCR1 on human neutrophils and is postulated that in the course of inflammation, the higher affinity CXCR2 is responsible for the initial recruitment of neutrophils from distant sites. As neutrophils are recruited closer to the source of IL-8, bound CXCR2 receptors become internalized allowing for the more specific CXCR1 receptor to dominate IL-8 responses (Chuntharapai & Kim, 1995). However, the chemotactic capability of CXCR1 for neutrophil recruitment in the junctional epithelium is still unclear.

CXCR1 has been implicated in later stages of neutrophil recruitment, but more recently, also in neutrophil activation (Swamydas et al., 2016; Chuntharapai & Kim, 1995). In humans, allelic mutations in the CXCR1, CXCR1-T276, showed impaired neutrophil degranulation and increased risk for bacterial infections associated with pyelonephritis and bronchiectasis, indicating a more active role in neutrophil activation rather than neutrophil migration (Javor *et al.*, 2012; Swamydas *et al.*, 2016; Boyton *et al.*, 2006). Similarly, characterization of neutrophils from CXCR1 deficient (CXCR1 KO) mice revealed that neutrophils were not impaired in their ability to traffic into *Candida* infected kidneys, but displayed cell intrinsic defects in degranulation and non-oxidative fungal killing (Swamydas *et al.*, 2016). The authors also reported that CXCR1 deficient neutrophils were not impaired in their survival, maturation, or phagocytic ability (Swamydas *et al.*, 2016). Moreover, immunohistological staining of gingival tissues showed that CXCR1 expression was localized primarily to gingival keratinocytes closest to the external stimuli, showing differential spatial distribution within the periodontal tissue layers (Sfakianakis et al., 2002). Together, these data suggest an important role of the neutrophil activating receptor, CXCR1, in mediating host responses after CXCR2 dependent recruitment into inflamed tissues.

In this study, WT and CXCR1 deficient (CXCR1 KO) mice were used to investigate the contribution of CXCR1 to healthy homeostasis by measuring neutrophil recruitment in the junctional epithelium (JE), oral microbial composition, and alveolar bone height. Since CXCR1 is implicated in neutrophil activation and killing, it was hypothesized that CXCR1 may play a role in shaping the commensal microbial composition and hence influence innate defense and healthy oral homeostasis.

Materials and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. All protocols were

approved by the Institutional Animal Care and Use Committee of the University of Washington in compliance with established federal and state policies.

Animal resource

CXCR1 KO mice were obtained from Dr. Michail Lionakis from the NIH National Institute of Allergy and Infectious Diseases. A total of 5-6 mice per group of mixed gender specific pathogen free (SPF) CXCR1 and wild-type (WT) C57BL/6 mice were raised and maintained in a SPF barrier facility at the University of Washington under the direction of the Department of Comparative Medicine.

Histology

Similar to previously published methods (Greer *et al.*, 2016), 12 to 14 week-old mice were euthanized by cervical dislocation and tissues were harvested for histological processing. Maxillae and mandibles were immediately fixed overnight on a shaker at 4 °C and rinsed with 70% ethanol daily for 1 week, gradually decreasing the rinses to once every other day until dissection. After dissection, tissues were demineralized in EDTA-cacodylate decalcification solution and processed following standard histological procedures and embedded in paraffin. Using a microtome, five mandibular halves per group were examined and sectioned (5 µm) from mesial to distal in the coronal plane. Two serial sections were collected per charged glass slides, resulting in approximately 100 sections per tooth (50 slides per tooth). Slides were numbered and imaged in consecutive order.

Immunohistochemistry

Detection of neutrophils on paraffin-embedded samples by immunohistochemistry (IHC) was performed on every 5th slide. Each slide was picked serially from the root-associated first molar to the root-associated second molar as previously described (Greer *et al.*, 2016), resulting

in approximately 30 sections (60 JE; 15 slides) per half mandible. Tissue sections were deparaffinized and rehydrated using a gradation of diluted ethanol. Sections were blocked in 1.5% hydrogen peroxide in methanol solution and targeted with a primary neutrophil elastase monoclonal antibody at 1 µg/mL (Neutrophil Marker Sc-71674, Santa Cruz Biotechnology, Dallas, TX, USA). Primary antibodies were conjugated to an avidin-biotinylated secondary antibody, developed with a peroxidase substrate, and counterstained with hematoxylin. The WT samples processed alongside experimental samples with and without the addition of primary antibody served as positive and negative controls, respectively. All quantitative analyses were manually counted under 200X magnification by a single blinded examiner, A.M.C., and repeated.

Histomorphometry

Linear distances from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) were measured using publicly available software (ImageJ Version 1.50b National Institutes of Health, Bethesda, MD, USA) on all IHC stained sections (150 sections per group) on both buccal and lingual surfaces of the tooth. Images (200X) were acquired in a blinded manner (SPOT CCD camera and software, Diagnostic Instruments, Sterling Heights, MI, USA). Area measurements were determined similarly on the blinded images (200X). All measurements were calibrated using a 0.01 mm stage micrometer and repeated.

Oral Swabbing and Microbial Genomic DNA Extraction

Bacterial oral swabs of 5 mice per group were obtained from teeth, gingival surfaces, and tongue immediately after sacrifice and stored at -80°C until extraction. Bacterial genomic DNA was extracted using a DNA extraction kit (QIAamp DNA Microbiome Kit, Qiagen, Germantown, MD, USA) and collected in low bind tubes (DNA LoBind eppendorf Tubes, Eppendorf, Hauppauge, NY, USA). Samples were further purified, concentrated (DNA Clean & Concentrator Kit, Zymo Research, Irvine, CA, USA) and stored at -20°C until sequencing. Similarly, bacterial

oral swabs of 6 mice per group were obtained, and genomic DNA was extracted for sequencing. Separate samples were used for the quantification of total bacterial load.

V3-V4 Amplification and Sequencing Strategy

The V3-V4 hypervariable region of the 16S rRNA was targeted using region specific primers containing an adapter sequence (Illumina flow cell adapter sequence, Illumina, San Diego, CA, USA) and purified using magnetic beads (Agencourt AMPure XP beads, Beckman Coulter, Inc., Pasadena, CA, USA). PCR products were verified by gel for visualization of the correct size after each PCR step. Amplicons were then indexed (Nextera XT Index Kit V2 SetA, Illumina, San Diego, CA, USA), purified with magnetic beads, and analyzed by gel electrophoresis (Agilent 4200 TapeStation System, Santa Clara, CA, USA) to assess library quality. Concentrations were determined individually with a fluorometer (Qubit3, Invitrogen, Carlsbad, CA, USA) and normalized to a concentration of 3-6 nM (SequalPrep Normalization Plate Kit, Invitrogen). Ultimately, the library was pooled, denatured with sodium hydroxide and sequenced (300 paired end MiSeq, Illumina). The PhiX control (PhiX Control v3 library, Illumina) was spiked at 20% to serve as an internal control and to balance for possible low diversity and base bias present in the 16S amplicon samples as recommended by provider (Illumina).

Analysis and further downstream applications of sequencing data

300 base pair (bp) paired-end 16S rRNA gene sequences were de-multiplexed, and the adaptor sequences were removed and filtered for quality. The approximately 130 bp overlapping sequences were merged (Pandaseq; Masella *et al.*, 2012) within the V3-V4 region and processed with publicly available tools (Qiime version 1.9.1; Caporaso *et al.*, 2010). Sequences were clustered into operational taxonomic units (OTUs) at 97% similarity, aligned, and assigned a taxonomy in reference with Greengenes 16S rRNA database version 13_8 (DeSantis *et al.*, 2006). OTU tables were rarefied to a sequence depth of 15,200 reads/sample to calculate alpha

diversity. Taxa bar plots were generated with publicly available tools (Qiime2 docker version 2019.4; Boylen *et al.* 2018) with filtered taxa which contain a minimum of 600 OTU counts to reduce the number of low abundance taxa.

Total bacterial load quantification by quantitative real-time PCR

Absolute quantitation of total bacterial load of 6 mice per group was measured by quantitative real-time PCR (qPCR) targeting the 16S rRNA gene. Genomic DNA from mouse oral swabs, nuclease-free water for negative controls, or standard (2 µl) was added to 10 µl of mastermix (SsoAdvanced Universal Probes Supermix, Bio-rad, Hercules, California, USA), 600 nM concentrations each of forward primer (5'-CGCTAGTAATCGTGGATCAGAATG-3') and reverse primers (5'-TGTGACGGGCGGTGTGTA-3'), and 250 nM of probe (5'-FAM-CACGGTGAATACGTTCCCGGGC-TAMRA-3') (Yoshida *et al.*, 2003). Nuclease-free water was added to bring the total volume of the reaction to 20 µl. The qPCR conditions were run at 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 58 °C for 1 min. The number of bacteria was determined using a serially diluted internal standard curve of *F. nucleatum* ATCC 10953 genomic DNA and analyzed using the second derivative maximum method (Rasmussen, 2001). Internal standards were of high quality, with efficiencies of 2.0 and approximate errors of 0.20.

Additional statistical analysis

Mann-Whitney U test was performed (GraphPad Prism version 7.00 for Windows, GraphPad Software, La Jolla, California, USA) to determine significance for average neutrophils, total bacterial load, and bone loss. An α value below 0.05 was considered significant.

Results

CXCR1 is not required for neutrophil migration into the junctional epithelium during the healthy state

The ability of CXCR1 KO mice to recruit neutrophils into the JE was examined by immunohistochemistry. Compared to WT mice, CXCR1 KO mice showed a trend towards having fewer average neutrophils/mm² of JE (Fig. 7a, c), but was not found to be statistically significant ($p = 0.1$). This demonstrates that CXCR1, a receptor involved in neutrophil recruitment and activation, is not necessary for neutrophil migration into the JE. Similar to average neutrophils, deficiency of CXCR1 resulted in a reduction of JE area ($p = 0.05$) showing a mean of 5.98×10^{-3} mm² ($n=6$) compared to WT mice which had a mean of 10.65×10^{-3} mm² ($n = 5$) (Fig. 7b).

CXCR1 responses prevent overgrowth of the mouse oral microbiota

Since CXCR1 plays a role in neutrophil activation and killing, total bacterial load from oral swabs from CXCR1 KO mice were analyzed. Deficiency of CXCR1 resulted in a significant increase of bacterial load ($p < 0.01$) represented by 5.2×10^6 mean 16S rDNA copy numbers compared to WT mice with 4.7×10^4 mean 16S rDNA copy numbers (Fig. 8). This data reveals that the ability of the host to control growth of the microbial community, as measured by 16S rDNA, is specifically disrupted in CXCR1 KO mice, confirming the role of CXCR1 in neutrophil activation and killing and showing the importance of this receptor for regulating oral microbial homeostasis.

CXCR1 display differences in predominating bacterial genera from WT

Oral swabs were more closely examined for disruption of microbial communities by high throughput sequencing of the 16S rRNA gene. Taxonomic analysis at the genus revealed clear distinctions of bacterial composition between WT and CXCR1 KO mice (Fig. 9).

WT mice predominantly carried members from an unidentified genus of the Pasteurellaceae family (29%) labeled here as Pasteurellaceae/unidentified#1 along with members from the Streptococcus genus (49%). In contrast, CXCR1 KO mice did not harbor members of the Pasteurellaceae/unidentified#1 genus (0%), but instead shared only the predominance of members from the Streptococcus genus (77%). Moreover, there is a complete loss of the Aggregatibacter genus in the CXCR1 KO mice compared to WT mice (5.6%). Overall, taxonomic analysis of mice deficient of CXCR1 reveal disruption of bacterial composition that deviates from WT mice.

Deficiency of CXCR1 and an alteration of the microbial composition does not affect alveolar bone levels

To better understand the clinical implications of CXCR1 and its role in oral health, alveolar bone levels were measured histomorphometrically with linear distances from the alveolar bone crest (ABC) to the cemento-enamel junction (CEJ). Histomorphometric analysis of alveolar bone loss revealed comparable levels with no statistically significant difference in bone loss between CXCR1 KO and WT mice (Fig. 10). This suggests that in healthy mice aged 12 to 14 weeks, the deficiency of CXCR1 leads to disruption of the oral microbial burden and its composition, but does not significantly alter alveolar bone levels.

Discussion

The role of CXCR1 in neutrophil recruitment has not been well established and remains inconclusive. For example, in the urinary tract, CXCR1 was required for neutrophil migration across bacterially infected epithelial cell layers (Godaly *et al.*, 2000) while in contrast, CXCR1 was not required for neutrophil migration into fungally infected kidneys (Swamydas *et al.*, 2016). These divergent results for the function of CXCR1 in neutrophil chemotaxis may be attributed to tissue specific programming or by modulation by infection type and has yet to be clearly

established. Moreover, the function of CXCR1 for neutrophil recruitment into the junctional epithelium is not yet known.

In health, it was found that CXCR1 was not required for neutrophil migration into the JE. Immunohistochemical analysis of neutrophils within the JE showed a trend towards a reduction in numbers, but after standardization to JE area, was not found to be statistically significant. Furthermore, despite showing a significant increase in the bacterial burden, CXCR1 KO mice revealed a trend towards a reduction in JE area ($p=0.05$). Interestingly, GF mice have also been reported to have a reduced JE area compared to conventional WT mice (Tsukamoto *et al.*, 2012). Those authors identified commensal bacteria as an important factor for the up-regulation of gingival inflammation and epithelial cell proliferation, which have been associated with JE thickness (Tsukamoto *et al.*, 2012; Celenligil-Nazliel *et al.*, 2000). In our investigations, deficiency of CXCR1 resulted in the disturbance of the microbial burden and composition, where CXCR1 KO mice displayed profound deviations in microbial composition and a significant increase in microbial burden ($p = 0.01$) compared to WT mice. Since commensal bacteria have been shown to selectively modulate inflammatory host responses (Tsukamoto *et al.*, 2012; Zenobia *et al.*, 2013; Greer *et al.*, 2016), the significant alteration in the proportions and presence of individual microbial members in CXCR1 KO mice may affect epithelial cell proliferation and influence junctional epithelial thickness. Alternatively, the reduction in size may not be due to an increase in epithelial cell proliferation, but by an increase in intercellular spaces and would be an area for further investigation.

Oral microbiome analysis revealed that CXCR1 KO mice have characteristically different compositions which deviate from WT mice. WT mice showed a predominance of members from *Streptococcus*, *Pasteurellaceae/unidentified#1*, and *Aggregatibacter* genera which consist of both gram- positive and negative species. In contrast, CXCR1 KO mice predominately house members from the *Streptococcus* genus and lose genera consisting of gram negative members. Cage-to-cage variation (Kennedy & DeLeo, 2009) may potentially explain the differences in composition

between CXCR1 KO and WT mice and remains a limitation of this study. However, individual experimental samples within a group were collected over different litters and cages over multiple generations which were able to maintain similar taxonomic compositions within the group. Moreover, similar neutrophil counts were obtained within the CXCR1 KO mice that did not differ from Wt mice, suggesting that cage variation may only partially explain the difference in composition. This initial investigation shows that CXCR1 is not required for neutrophil recruitment but may play a role in oral microbial homeostasis in health. Therefore, additional studies using littermate control that will control for environmental and microbial variables will need to be performed in order to confirm these results.

Deficiency of CXCR1 resulted in a significant increase in bacterial load and an alteration of the microbial composition which did not affect alveolar bone loss. This result is consistent with previous observations in mice (Chapter 2, Chukkapalli *et al.*, 2017) and rabbit models of health and disease, respectfully, which confirms previous observations that not all dysbiotic communities induce alveolar bone loss (Zenobia *et al.*, 2014). It has been observed that wild mice, not laboratory raised mice, have naturally occurring bone loss similar to what is observed in the natural process of health in humans (unpublished observations). Therefore, it can be speculated that the reproducibility of this protective phenotype against alveolar bone loss may suggest a vast network of host modulatory mechanisms that are in constant adjustment to protect against disruptions of homeostasis. Alternatively, as it has been reported that laboratory bred mice are highly resistant to periodontal disease (Page & Schroeder, 1982; Oz & Puleo, 2011), this phenotype could also be attributed to the highly resistant nature of laboratory bred mice and remains as one of the limitations of this study which requires further investigation. Altogether, this data suggests that periodontitis is a complex disease process that cannot occur with microbial dysbiosis alone and requires engagement of multiple host components that needs further elucidation.

Fig. 7

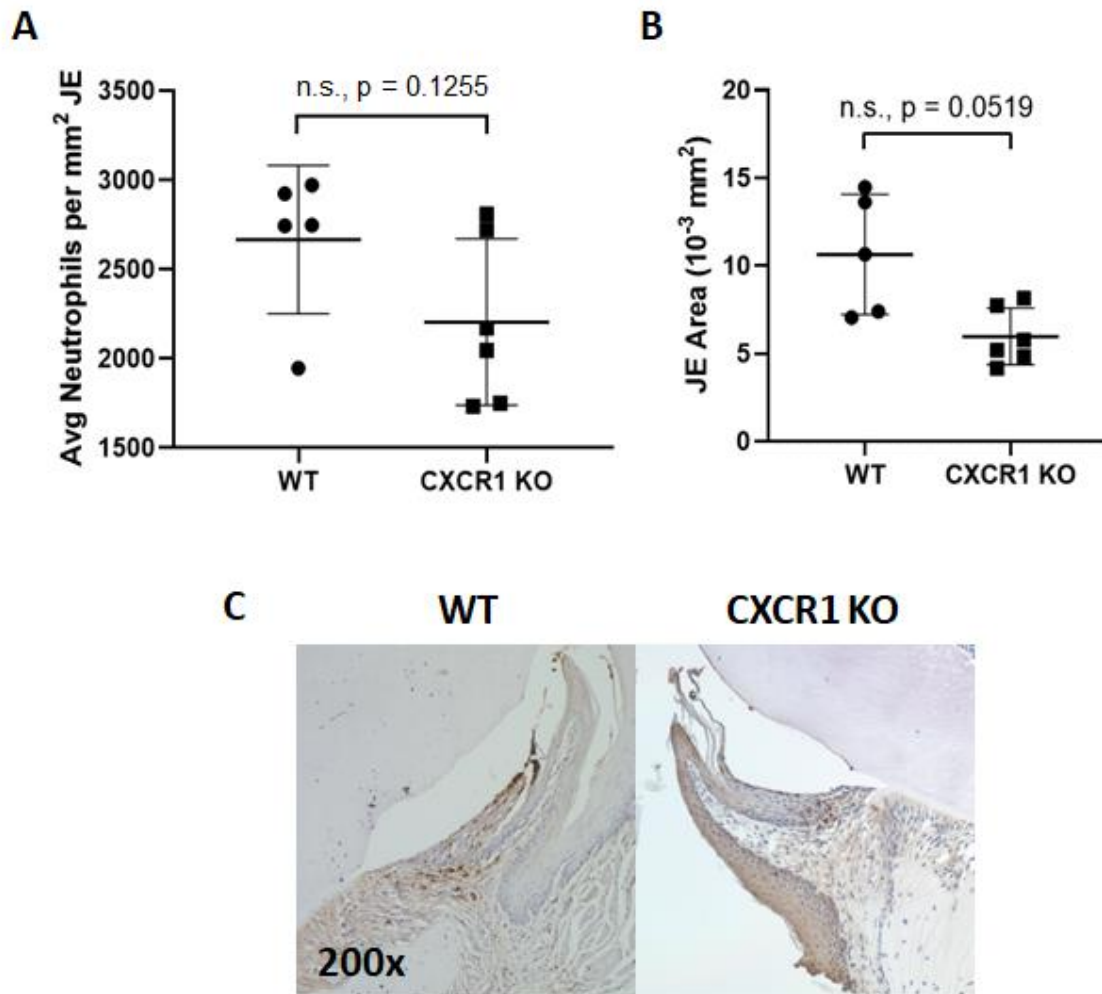


Fig 7. CXCR1 is not required for neutrophil recruitment into the junctional epithelium (JE). (A) Scatter dot plot shows average neutrophils standardized to its respective JE area. (B) Scatter dot plot shows average JE area of each mouse. (C) Representative images of histology sections at 200x magnification. Each dot is represented as an average calculated from 30 JE sections / mouse, with 5-6 mice per group. Neutrophil counts were counted two independent times by two different observers. Significant statistical differences were calculated using the Mann-Whitney test. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

Fig. 8

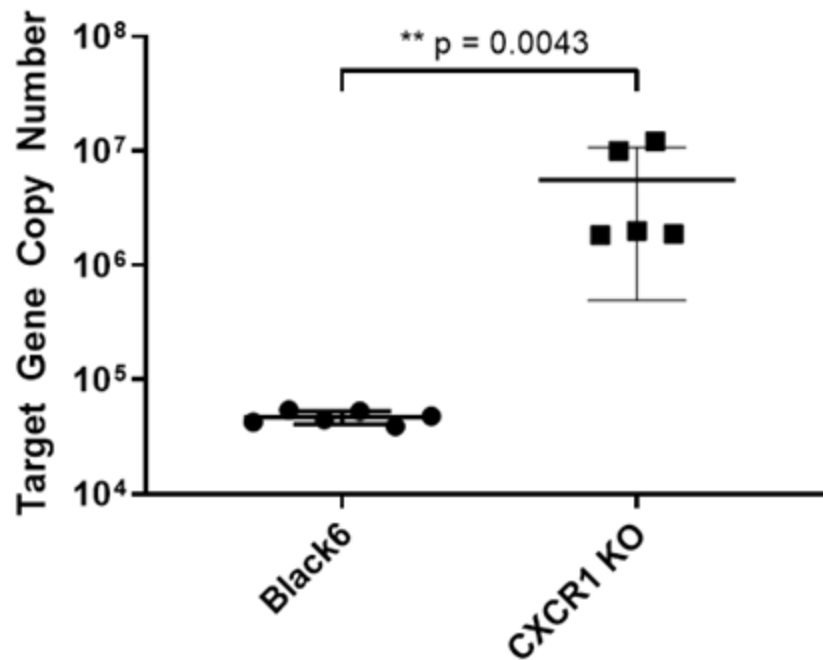


Fig 8. Bacterial load reveals disruption of bacterial homeostasis in CXCR1 KO mice. Total bacterial load is represented by the copy numbers of 16S rRNA gene for each oral swab sample (n=5-6 / group) by real-time PCR assays in a scatter dot plot. Significance was determined using the Mann-Whitney test. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$

Fig. 9

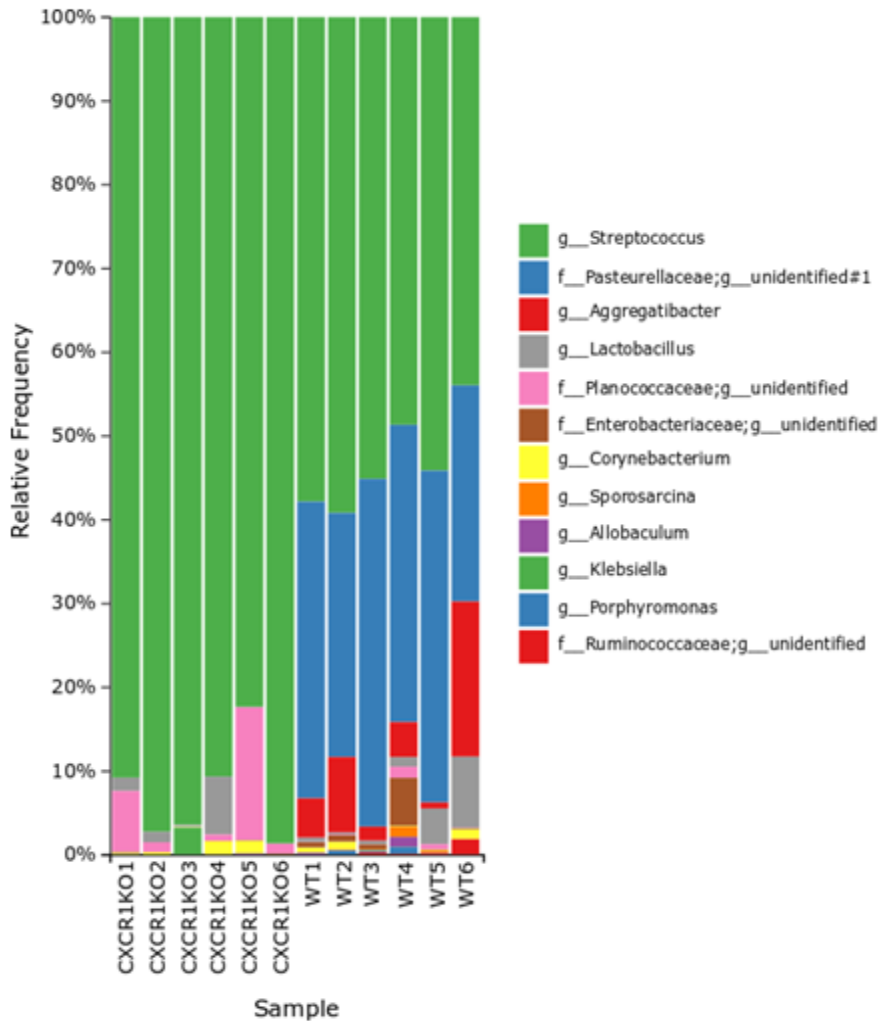


Fig 9. CXCR1 KO mice show unique taxonomic complexity and representation from WT (n=6 mice / group). Bacterial composition between CXCR1 KO and WT mice at the genus level. Each color is represented by a genus in the figure legend. Although the Greengenes 16S rRNA database version 13_8 identified unique genera, limited characterization of some sequences did not allow it to be labeled up its respective genus identity and is referred up its family level identity followed by “unidentified” for its genus level identification.

Fig. 10

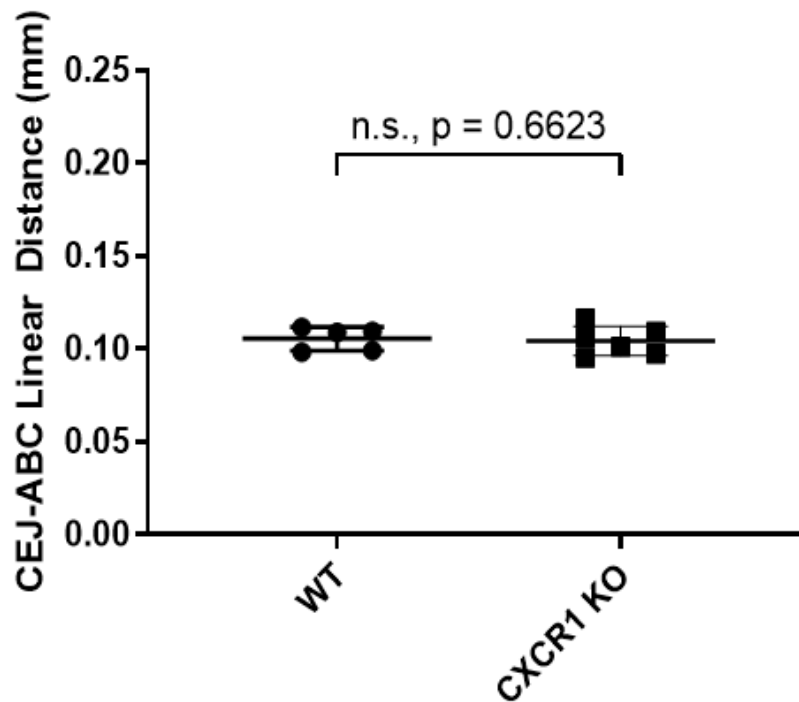


Fig 10. Deficiency of CXCR1 does not affect alveolar bone loss during health. Scatter dot plot shows no significant difference in bone loss when determined by histomorphometric analysis of linear distances from the cemento-enamel junction (CEJ) to the alveolar bone crest (ABC) (300 JE per group). Images were acquired at 200x and measured two independent times by two different observers. (*) $p < 0.05$, (**) $p < 0.01$, (***) $p < 0.001$, (n.s.) not statistically significant.

Chapter 4:

An ayurvedic herbal extract modulates oral epithelial cell inflammatory responses to *Fusobacterium nucleatum*

Introduction

Maintenance of oral health is the most effective strategy for prevention of disease. Any irremediable disturbance to this carefully balanced homeostatic state between the host inflammatory response and the microbial community can ultimately lead to disease and is characterized by dysbiosis of both the microbial community and host immune response (Darveau, 2010; Marsh, 2012). Currently, vague understanding of the complex inflammatory networks that are modulated in both health and disease limits western dental treatments to incompletely address this imbalance by partially targeting the microbial community without addressing the dysbiotic host response (Axelsson *et al.*, 2004; Chapple *et al.*, 2015). More recently, there has been more interest towards therapeutic interventions and approaches that are able to target both host and microbial responses.

Naturopathic medicine has been in practice for millennia in various forms around the globe. It encompasses a dynamic philosophy which recognizes the self-inherent healing ability within the host and emphasizes its practices around the prevention, identification, and removal of obstacles to facilitate and augment the body's natural healing processes. Corresponding with these philosophies, plant extracts have been shown to have potent antibacterial, antifungal, and anti-inflammatory qualities, which have been shown to inhibit key mediators of the inflammatory response and have been used for many centuries to maintain oral hygiene (Santangelo *et al.*, 2007).

In Sri Lanka, a time tested Ayurvedic recipe of blended plant extracts is incorporated into a toothpaste, registered as Sudantha® (Reg.No.02/01/PV/08/143), and has shown to exhibit these

types of beneficial properties (Howshigan *et al.*, 2015; Jayashankar *et al.* 2011). Among the plants used in this product are: heartwood of cutch tree (*Acacia chundra* Willd.), malabar nut leaf (*Adhatoda vasica* Nees.), Spanish cherry bark (*Mimusops elengi* L.), black pepper (*Piper nigrum* L.), pongam oil tree root (*Pongamia pinnata*(L.) Poir.), Aleppo oak galls (*Quercus infectoria* Olivier.), clove (*Syzygium aromaticum* L.), myrobalan fruit (*Terminalia chebula* Retz.), and ginger (*Zingiber officinale* Roscoe). A randomized double-blind placebo-controlled study on the effects of this herbal extract on oral hygiene and gingival health showed that this proprietary mixture of herbs significantly reduced gingival bleeding, dental plaque formation, and salivary anaerobic bacterial counts as early as 4 weeks of its use compared to the placebo group (Jayashankar *et al.*, 2011). Furthermore a follow-up clinical trial investigating therapeutic benefits for patients with gingivitis confirmed these results, which observed reduction in gingival bleeding, plaque formation, total salivary anaerobic bacterial counts, as well as with periodontal pocket depth (Howshigan *et al.*, 2015). Together, these randomized clinical studies provided robust evidence of the effective antiplaque and antigingivitis effects of this toothpaste for both the maintenance of health and treatment of disease.

To further elucidate the mechanisms by which this medicinal extract Sudantha (Sud) contributes to the promotion of gingival health and homeostasis, its immunomodulatory effects on host IL-8 production in response to bacterial products, *F. nucleatum* cell wall and live *F. nucleatum*, and host inflammatory mediators, IL-1 β and TNF- α , were examined *in vitro* on telomerase immortalized gingival keratinocytes (TIGK) (Moffatt-Jauregui *et al.*, 2013). This study found that Sud inhibited expression of the proinflammatory cytokine, IL-8, by gingival epithelial cells agonized with bacterial products (*F. nucleatum* cell wall extracts or live *F. nucleatum*) and host inflammatory mediators (IL-1 β and TNF- α) in a dose-dependent manner. Additionally, it showed the ability to modulate basal levels of IL-8, which required a 2-fold higher concentration of Sud than that required to inhibit IL-8 expression by live *F. nucleatum* and IL-1 β . Inhibition of IL-8 expression by TNF- α was the most sensitive and required the lowest dose of Sud, while there

was no significant difference in the concentration required for the inhibition of IL-8 expression mediated by *F. nucleatum* and IL-1 β .

Materials and Methods

Bacterial culture and crude cell wall

F. nucleatum ATCC 25586 was obtained from the Darveau laboratory bacterial collection and grown overnight in trypticase soy yeast broth (TYK) supplemented with 10 μ g/mL and 1 mg/mL menadione at 37°C under anaerobic conditions (80% N₂, 10% CO₂, 10% H₂). *F. nucleatum* crude cell samples were prepared as previously described (Krisanaprakornkit et al. 2000) using a french cell pressure at 15,000 lb/in².

TIGK cell culture and infection

The immortalized human gingival keratinocyte cell line, TIGK, was generously provided by Dr. Richard J. Lamont from the School of Dentistry at the University of Louisville and maintained in Ker-SFM containing 25 μ g/mL bovine pituitary extract, 0.2 ng/mL human recombinant epidermal growth factor, 0.4 mM calcium chloride, and 10% penicillin-streptomycin (Sigma-Aldrich). Antibiotics were excluded for experiments with live bacteria.

TIGK cells were plated into 96-well plates at a density of 2×10^4 cells/well and allowed to grow for 48 hours until a confluency of approximately 90%. Test wells were stimulated in triplicate for 4 hours at 37°C and 5% CO₂ with the indicated ligands: live *F. nucleatum* bacteria at an MOI of 1:500 and *F. nucleatum* cell wall components, IL-1 β , and TNF- α all at 100 ng/ml.

Measurement of secreted IL-8 by enzyme-linked immunosorbent assay (ELISA)

After termination of the 4 hour infection, culture supernatants were collected and diluted 2.5 fold in 1% bovine serum albumin (Sigma-Aldrich) for determination of secreted IL-8 by standard sandwich ELISA. IL-8 monoclonal primary capture antibody (M801) and secondary

biotin labeled detection antibody (M802B) (Thermo Fisher Scientific) were used for ELISA and detected with avidin-horseradish peroxidase enzyme (HRP) (eBioscience) and tetramethylbenzidine (TMB) substrate (Sigma-Aldrich). Optical densities were read at 450/570 nm (VMax Microplate Reader, Molecular Devices, Sunnyvale, California, USA) and concentrations were calculated from a standard curve using known concentrations of serially diluted recombinant human IL-8 (eBioscience, San Diego, CA).

Half-maximal inhibitory dose (ID₅₀)

Half-maximal inhibitory dose (ID₅₀) was estimated from an experimentally derived dose-response curve for each concentration using GraphPad Prism software version 8.

Cell Viability

After removal of supernatant for IL-8 protein determination, cell viability was assessed using a fluorometric assay (CellTiter-Glo, G9241, Promega) based on the manufacturer protocol. Briefly, 50 µl of medium was added to cells. Following, 50 µl of CellTiter-Glo reagent was added and luminescence was measured after 10 minutes at room temperature using a microplate luminometer (Centro LB 960, Berthold Technologies, Wildbad, Germany).

Results

*Sud suppresses *F. nucleatum* cell wall extract induced IL-8 expression by gingival keratinocytes*

F. nucleatum represents a common gram negative species found in gingival plaque obtained from periodontally healthy sites and have been shown to elicit a potent IL-8 response from gingival epithelial cells (Park *et al.*, 2015; Han *et al.*, 2000; Peyret-Lacombe *et al.*, 2009). TIGK cells infected with 100 ng/ml of *F. nucleatum* cell wall extracts produced a potent IL-8 response (1,152 pg/ml) that was suppressed by the addition of Sud in a dose dependent manner that was not a result of cell death within 4 hours (Fig. 11). Suppression of IL-8 by *F. nucleatum*

cell wall was able to be first observed at low concentrations of 0.24 µg/ml Sud, while Sud at a range of 62.5-250 µg/ml worked optimally to reduce IL-8 down to basal levels without affecting cell viability. Higher concentrations of 500 µg/ml Sud showed cytotoxicity and was excluded from further experimentation. Interestingly, basal levels of IL-8 expression by TIGK cells was also suppressed in response to Sud in a dose dependent manner.

Sud suppresses live F. nucleatum induced IL-8 expression by gingival keratinocytes

To further characterize the anti-inflammatory effects of Sud against *F. nucleatum* induced IL-8 production, TIGK cells were infected with live bacteria at an MOI of 1:500. Infection of TIGK cells with live *F. nucleatum* for 4 hours slightly affected cell viability, which was able to be rescued with Sud (Fig. 12B). Similar to bacterial cell wall, live *F. nucleatum* at an MOI of 1:500, produced a potent IL-8 response (Fig. 12A) greater than that observed with *F. nucleatum* cell wall alone (5,087 pg/ml) and its expression was similarly damped by Sud first observed at concentrations of 3.9µg/ml. This inhibition occurred in a dose dependent manner that at brought IL-8 expression levels down to 61 pg/ml with 250 µg/ml of Sud, almost to levels of IL-8 elicited by controls (~22 pg/ml).

Sud suppresses IL-1β and TNF-α host mediator induced IL-8 expression by gingival keratinocytes

IL-1β and TNF-α are a potent inflammatory cytokines reported to be involved in cellular proliferation, activation, and differentiation (Turner *et al.*, 2014). These host cytokines have been shown to induce IL-8 secretion from gingival epithelial cells (Schaumann *et al.*, 2013; Jo *et al.*, 2014). Consistent with previous reports of IL-1β and TNF-α induced expression of IL-8 by gingival keratinocytes, TIGK cells infected with 100 ng/ml of each cytokine for 4 hours induced expression of 793 pg/ml and 1,386 pg/ml IL-8, respectively (Fig. 13A). Inhibition of IL-8 occurred with the addition of Sud in a dose-dependent manner that was able to bring down IL-8 expression to 65

pg/ml and 83 pg/ml, respectively, almost down to control levels of 23 pg/ml IL-8 without disrupting cell viability (Fig. 13B).

Differential inhibition of agonist stimulated IL-8 by gingival keratinocytes

Sud was able to dampen both *F. nucleatum* and host cytokine induced gingival epithelial IL-8 inflammatory responses. Therefore, the 50% inhibitory dose (ID50₅₀), the concentration of the test compound required to inhibit the agonist induced cytopathogenic effect by 50% (Dymińska, 2015), was examined for preferential inhibitory effects between bacterial and host agonists. ID50₅₀ results suggest differential inhibitory effects of the extract between host cytokines. Gingival epithelial cells secrete basal levels of IL-8 (Fig. 14) which was able to be dampened by Sud to 50% expression with concentrations of 55.10 µg/ml Sud. In contrast to mechanisms related to basal expression of IL-8, agonist stimulated IL-8 was damped to 50% inhibitory levels at a much lower concentration. TNF-α stimulated IL-8 response was most sensitive to Sud with ID50₅₀ concentrations at 11.39 µg/ml Sud. While, IL-1β and live *F. nucleatum* required higher ID50₅₀ concentrations around 28.84 µg/ml and 25.77 µg/ml Sud respectfully. This suggests that Sud is able to differentially dampen agonist stimulated IL-8 responses and has the potential to be a valuable tool for the modulation of agonist induced inflammation without disrupting protective basal level inflammation.

Discussion

Health is the result of a homeostatic balance between the innate host inflammatory response and oral bacteria. Meaning that any disturbances to this balance will invariably affect both host and bacterial components. However, the lack of understanding this healthy balance and the innate host modulatory mechanisms in effect to maintain it has severely compromised the oral health field and its current approaches to treatment. To date, much of the interventions remain skewed one-sidedly towards microbial approaches, such as scaling and root planing,

without much success in targeting host components (Axelsson *et al.*, 2004; Chapple *et al.*, 2015) which may be due to the fact that the same inflammatory mediators associated with disease are also associated with health at lower concentrations (Barros *et al.*, 2016). Therefore, it is difficult to differentiate anti-inflammatory targets in the immune surveillance pathway that maintain health from the ones which lead to disease.

In general the goal of treatment is to aid and enhance the natural inherent ability of the host's defense mechanisms against disruption of homeostasis. Consistent with this approach, the use of plant derived polyphenols as a natural anti-inflammatory compounds have been intensively investigated (Santangelo *et al.*, 2007; Casati *et al.*, 2013; Correa *et al.*, 2018). They have been shown to inhibit key mediators of the inflammatory cascade, including MAP kinases and nuclear transcription factors (Santangelo *et al.*, 2007). Sud, a proprietary mixture of natural herbs, has recently shown clinical success in its ability to maintain gingival and periodontal health while providing antimicrobial activity (Howshigan *et al.*, 2015; Jayashankar *et al.*, 2011). The aim of this study was to expand on the anti-inflammatory mechanisms exhibited by Sud and determine its ability to dampen bacterial or cytokine induced gingival epithelial cell IL-8 inflammatory responses *in vitro*.

IL-8 is a proinflammatory cytokine produced by a wide variety of cells including gingival epithelial cells, endothelial cells, gingival fibroblasts, neutrophils, monocytes, and phagocytes and plays a distinct role in neutrophil migration and activation (Turner *et al.*, 2014; Tonetti *et al.*, 1994). The importance of its role in neutrophil function has been recognized in oral health and disease (Bickel, 1993; Darveau, 2009). Irregular and uncontrolled expression of IL-8 contributes to neutrophil mediated local tissue destruction of periodontal tissue (Darveau, 2009; Darveau, 2010). In the present study, *F. nucleatum* cell wall and live *F. nucleatum* induced expression of IL-8 by gingival epithelial cells was successfully suppressed by Sud at concentrations that did not affect cell viability. This confirms previous protective anti-inflammatory effects of this extract found clinically (Howshigan *et al.*, 2015; Jayashankar *et al.*, 2011).

IL-1 β and TNF- α are potent proinflammatory mediators secreted in response to bacteria and are associated with the pathogenesis and progression of periodontal disease (Agace *et al.*, 1993; Yumoto *et al.*, 1999; Graves & Cochran, 2003). They induce the upregulation of adhesion molecules on neutrophils and endothelial cells, stimulate the production of chemotactic molecules to induce neutrophil migration, and enhance inflammatory signals which potentiate inflammatory responses (Gomes *et al.*, 2016). Consistent with this, gingival epithelial cells stimulated with IL-1 β and TNF- α induced expression of IL-8. Similar to results observed with bacterial agonists, addition of Sud inhibited IL-8 expression in a dose dependent manner. Furthermore, Sud showed selective inhibition between host modulators; TNF- α induced IL-8 required 11.39 μ g/ml of Sud, while IL-1 β induced IL-8 required 2-fold this amount 28.84 μ g/ml of Sud. More interestingly, Sud was able to dampen basal levels of IL-8 expression by gingival epithelial cells which required 5-fold TNF- α induced IL-8 inhibitory concentrations of 55.10 μ g/ml. The difference in inhibitory concentrations required by these different host inflammatory modulators suggests each pathway: health-, TNF- α -, and IL-1 β -mediated IL-8 expression in gingival epithelial cells must utilize different inflammatory pathways. Moreover, similarities in the 50% inhibitory doses required by live *F. nucleatum* and IL-1 β suggest they may share a common IL-8 inflammatory pathway.

It has been reported that gingival epithelial cells in health, specifically junctional epithelial cells of mice, produce cell intrinsic constitutive expression of TNF- α and IL-1 β that was not altered with the presence of commensal bacterial colonization, but was rather suggested to be genetically programmed (Tsukamoto *et al.*, 2012). Therefore, it may be possible that high levels of exogenous TNF- α and IL-1 β may produce IL-8 by alternate inflammatory pathways that are different than ones utilized for health or, alternatively, these mediators may activate multiple inflammatory pathways simultaneously. Furthermore, since there was a distinct difference in the inhibitory concentrations of Sud required to suppress IL-8 in response to TNF- α from that of IL-1 β and live *F. nucleatum*, it can be suggested that TNF- α induces IL-8 by a different inflammatory pathway than that may be shared between IL-1 β and live *F. nucleatum*.

Altogether, *in vitro* experiments of Sud on its ability to dampen host immune response in relation to IL-8 expression supports the clinical beneficial effects of Sud for the maintenance of periodontal and gingival health. However, additional experimentation is required to expand upon the potential of this extract to selectively modulate host inflammatory pathways without disturbing the cell intrinsic host inflammatory surveillance.

Fig. 11

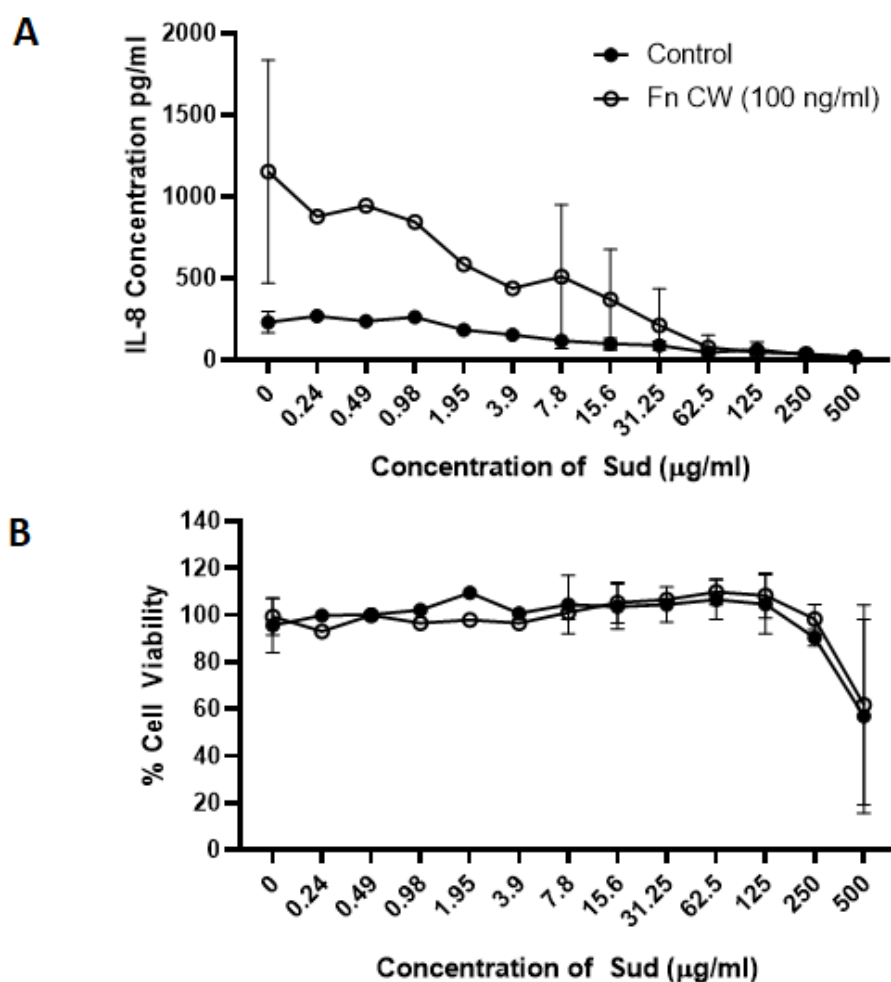


Fig 11. Sud suppresses *F. nucleatum* cell wall induced IL-8 expression by gingival keratinocytes. (A) IL-8 expression measured by ELISA of TIGK cells infected for 4 hours by *F. nucleatum* cell wall (100 ng/ml) with Sud. (B) Percent cell viability output of the cells used in (A). Error bars represent standard deviations for experiments with concentrations at 0 and above 7.8 µg/ml which were performed 3 independent times in triplicate.

Fig. 12

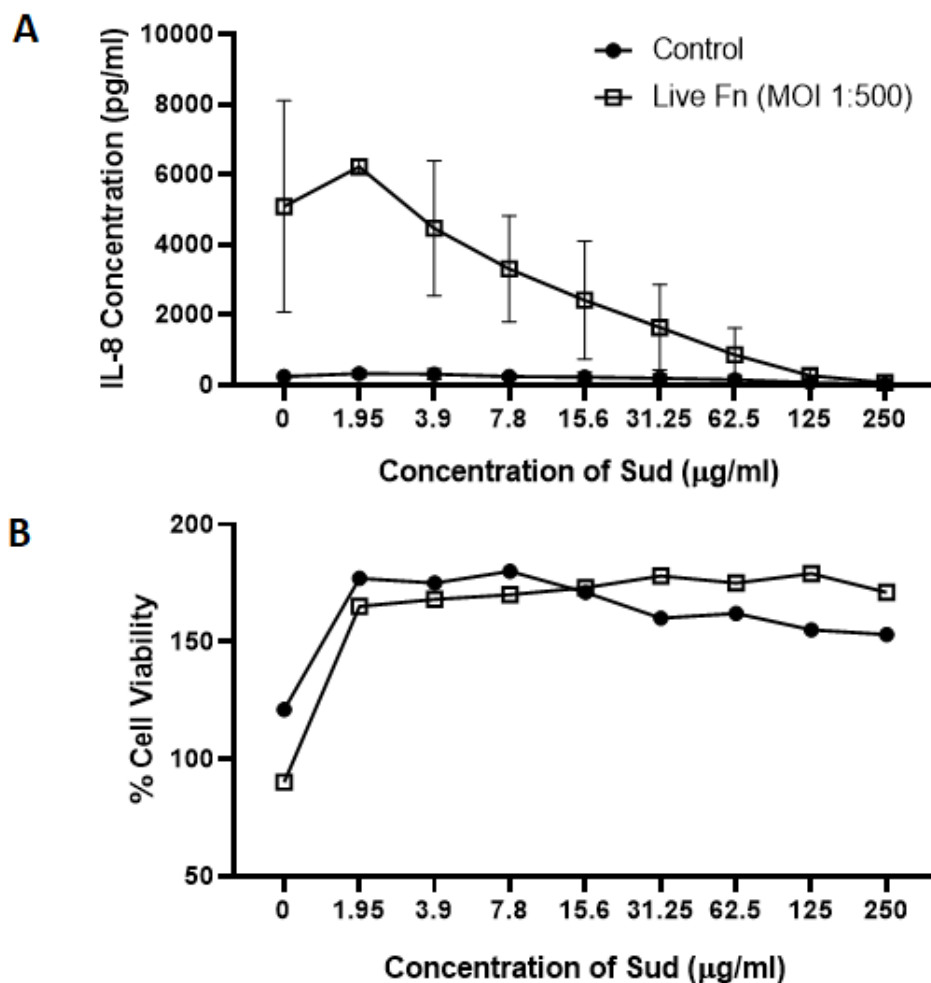


Fig 12. Sudantha (Sud) suppresses live *F. nucleatum* induced IL-8 expression by gingival keratinocytes. (A) IL-8 expression measured by ELISA of TIGK cells infected for 4 hours by live *F. nucleatum* (MOI 1:100) with Sud. (B) Percent cell viability output of the cells used in (A). Error bars represent standard deviations of IL-8 ELISA experiments performed 4-5 independent times in triplicate. Cell viability assay is a representative of a single experiment performed in triplicate.

Fig. 13

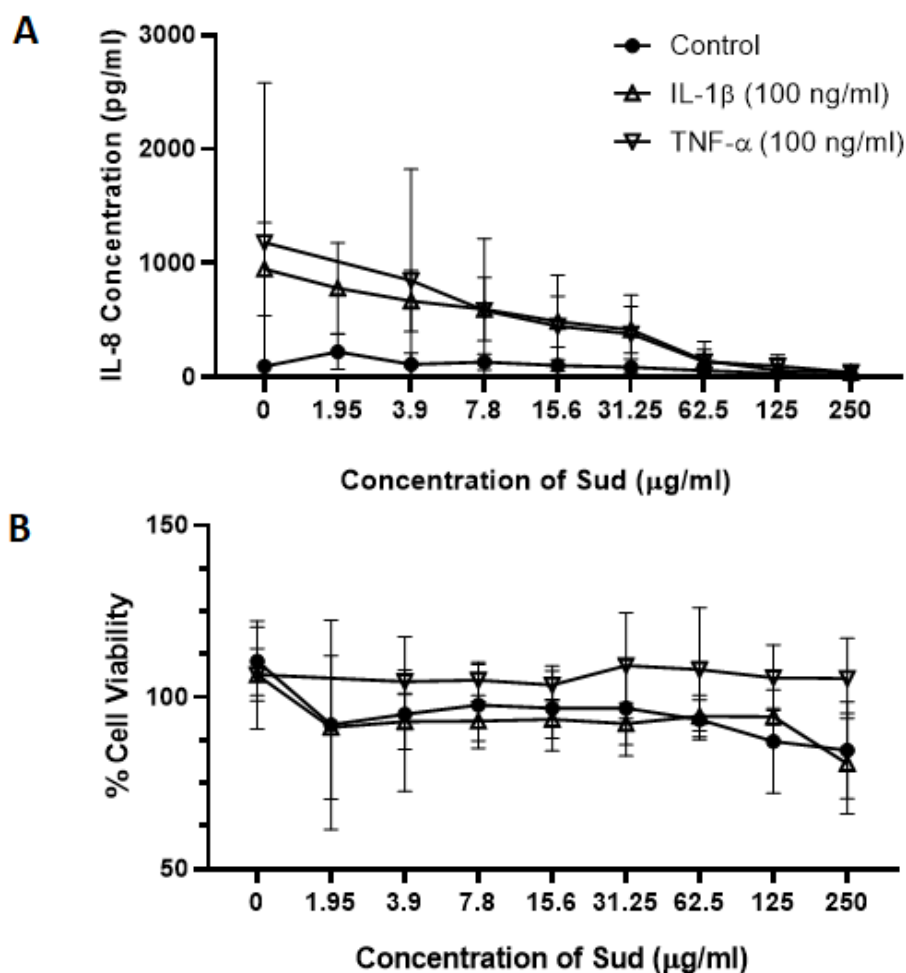


Fig 13. Sud suppresses IL-1 β and TNF- α host mediator induced IL-8 expression by gingival keratinocytes. (A) IL-8 expression measured by ELISA of TIGK cells infected for 4 hours with exogenous IL-1 β and TNF- α at 100 ng/ml. (B) Percent cell viability output of the cells used in (A). Error bars represent standard deviations of experiments performed 3 independent times in triplicate.

Fig. 14

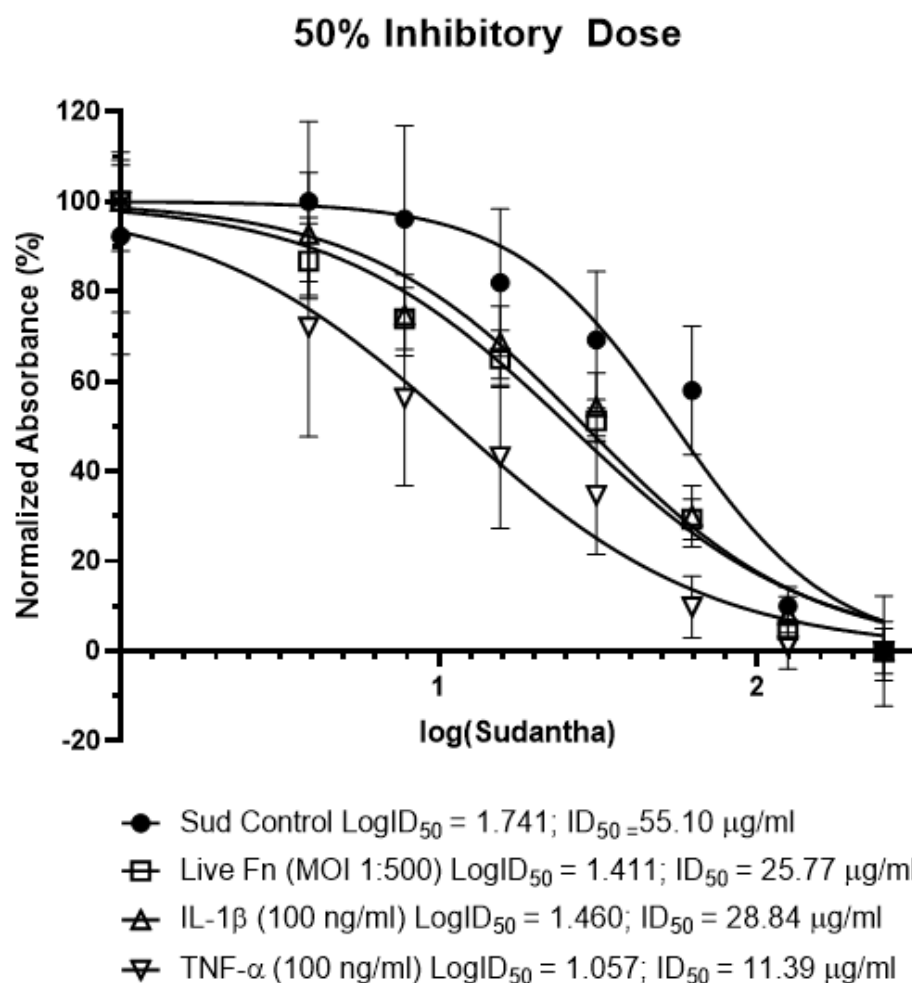


Fig 14. Differential inhibition of agonist stimulated IL-8 by gingival keratinocytes. Fifty percent inhibitory dose (ID₅₀) estimated from an experimentally derived dose-response curve for each concentration of Sudanthal (Sud) against basal levels of IL-8 (Sud Control) and IL-8 induction by live *F. nucleatum* (MOI 1:100), IL-1β, and TNF-α (both at 100 ng/ml) performed 3 independent times. Error bars represent standard error of means.

Chapter 5:

Summary and future directions for oral health

Summary

Health is a state of homeostasis. It requires continuous adaptation to maintain stability to combat change. In this process of health, commensal bacterial colonization is not essential for life, but is an indispensable component of health, playing a significant role in modulation of both health- and disease- related host responses. Concomitantly from the host side, immune cells, specifically, neutrophils work in concert to counter the microbial burden and maintain host-bacterial homeostasis. Neutrophils are a key component of oral health and disruption of this neutrophilic barrier leads to periodontal disease (Johnson & Ward, 1981; Rashmi *et al.*, 2006; Deas *et al.*, 2003). The overall aim of this dissertation is to establish a better understanding of the modulatory mechanisms that maintain healthy neutrophil oral homeostasis.

Chapter 2 is a continuation of work by Zenobia *et al.* (2013) and Greer *et al.* (2016), which identified that commensal oral bacteria play a key role in location dependent modulation of select innate host immune responses, such as neutrophil recruitment, during health and that these responses were dependent on a MyD88 pathway. MyD88 is a co-adaptor molecule utilized by IL-1R, IL-18R, and TLRs1-9 except TLR3 (Kawai & Akira, 2005). Therefore, in chapter 2, two upstream bacterial recognition receptors of the MyD88 pathway, TLR2 and TLR4 were examined for their role in health dependent immune modulation by commensal bacteria. Using mice deficient in TLR2, TLR4 and both TLR2 and TLR4, it was found that these TLRs are not required for neutrophil recruitment into the junctional epithelium, but instead may play a regulatory role for dampening recruitment. Moreover, despite having increased neutrophil recruitment into the JE, there was dysregulation of the oral bacterial burden and composition.

Chapter 3 investigated the role of CXCR1, a receptor involved in neutrophil activation and recruitment, on oral homeostasis. Current literature presents contradictory involvement of CXCR1 to neutrophil recruitment (Swamydas et al., 2016; Chuntharapai & Kim, 1995; Godaly et al., 2000) which may suggest its role may be adjustable by exposure to tissue specific signals. However, its role in the JE had yet to be established. In the JE of mice deficient in CXCR1, there were no significant differences in the number of neutrophils. However, similar with the mice deficient in TLRs, CXCR1 deficient mice had a significant increase in oral bacterial burden. Moreover, the composition of this community was significantly altered.

In chapter 4, the ability of a blend of natural medicinal herbs to modulate host immune responses was examined. A time tested ancient Ayurvedic blend of natural herbs used in a registered toothpaste, Sudantha®, was found to have the ability to modulate host inflammatory responses to self-inflammatory signals, such IL-1 β and TNF- α , as well as to live *Fusobacterium nucleatum*.

Altogether, this work provides a glimpse into the complex networks that are in constant modulation for the maintenance of oral health. This chapter explores possible future studies to further elaborate on the modulatory mechanisms that are in play for the maintenance of health in the oral cavity.

Future Directions for Oral Health

Commensal bacteria and neutrophil recruitment in health

Neutrophils are indispensable for health in the oral cavity. Zenobia et al. (2013) first demonstrated the immunomodulatory role of commensal bacteria and its active role in periodontal health. It was shown that neutrophils are present at a baseline level in the germ free state and that commensal bacterial colonization increased the number of neutrophils present within the JE. Moreover, it was found that neutrophil recruitment was dependent on the chemotactic receptor CXCR2 and mice devoid of this receptor were mostly detected in vessels and rarely found in the

JE. Investigating the influence of commensal bacterial colonization on the expression of CXCR2 ligand expression, it was found that bacterial colonization selectively increased expression of CXCL2, but not CXCL1. Furthermore, the expression of these ligands were dependent upon an MyD88 pathway, where MyD88 knockout mice showed similar neutrophil recruitment phenotypes as germ free mice.

Chapter 2 investigated two upstream components of the MyD88 pathway, TLR2 and TLR4. However, it was found that TLR2 and TLR4 are not the receptors utilized by commensal bacteria to induce neutrophil recruitment that was observed by Zenobia *et al.* (2013) suggesting the involvement of alternate MyD88-dependent pathways. The MyD88 co-adaptor molecule is converges multiple pathways including, IL-1 receptor (IL-1R), IL-18R, and all TLRs except TLR3 (Kawai & Akira, 2005). Since TLR2 and TLR4 are not required for neutrophil recruitment into the JE, investigations into additional bacterial recognition receptors, such as TLR1, 5, and 6 as well as inflammatory receptors IL-1R and IL-18R can be proposed using a similar histological approach.

Expansion of CXCR2 ligand expression

CXCR2 is required for neutrophil recruitment into the JE and binds multiple ligands in the mouse: CXCL2 (MIP2) and CXCL1 (KC), CXCL5 (LIX) and CXCL7 (Zlotnik & Yoshie, 2000). It was found that commensal bacterial colonization selectively upregulates CXCL2, but not CXCL1 (Zenobia *et al.*, 2013). This is further expanded by Greer *et al.* (2016) which demonstrates location specific induction of CXCL2 and neutrophil recruitment by single-species bacterial colonization that was not observed with CXCL1. This data corroborates with the hierarchical process of neutrophil chemotaxis and activation that is directed by the orchestration of multiple ligands and the utilization of their different binding characteristics and kinetics to CXCR2 (Rovai *et al.*, 1998; Chuntharapai & Kim, 1995; Rose *et al.*, 2004). Altogether, these data suggest that collaborative immunomodulation by commensal bacteria and the host is not homogenous or

uniform, but rather, is launched in a clearly controlled, nonredundant and spatially defined manner.

Chapter 2 revealed that TLR2 and TLR4 deficiency results in an increase of neutrophil recruitment into the JE and an alteration of the microbial composition. The resulting increase in neutrophil recruitment was not expected in that, since TLR2 and TLR4 are two mechanisms used by the host to initiate an inflammatory cascade, we hypothesized if these pathways were utilized by commensal bacteria, we expected to see a decrease in neutrophil recruitment or if these pathways were not utilized, we expected to observe no change. As CXCR2 has been shown to be required for neutrophil recruitment into the JE and it is postulated that TLR2 and TLR4 may play a role in this regulation, further insight into the influence of TLRs in the spatial expression of CXCR2 ligands, CXCL1, CXCL2, CXCL5 and CXCL7 in the JE is necessary. Moreover a carefully designed investigation controlling the differences in microbial composition must also be considered. Finally, if CXCL5, a ligand which is also involved in neutrophil activation and killing, is different from WT, it may have the potential to explain the bacterial dysbiosis observed in these mice.

Oral microbiome analysis of animal models

In chapters 2 and 3, it was found that host receptors, TLR2, TLR4 and CXCR1 are not required for neutrophil recruitment, but was involved in the maintenance of oral health by the regulation of the numbers of recruited neutrophils along with the oral microbial burden and composition. Mice within each experimental group were collected across different cages and generations to minimize confounding effects of the environment. Furthermore, despite the limitations to control for these variables, mice within each experimental group shared group specific phenotypes such as microbial composition and burden, as well as with neutrophil recruitment. However, unknown environmental effects may still influence the observed results.

Therefore, a littermate controlled study to control for unknown environmental variables should be examined to confirm the effects of TLR2, TLR4 and CXCR1 to oral health.

A littermate is a member of a group of animals born in the same litter. A littermate controlled study utilizes littermates to investigate the role of a specific gene in controlling an immunological phenomenon and eliminates environmental confounding factors that may influence the result. Typically a mouse deficient in the gene of interest, for example, CXCR1 KO mice, is backcrossed a large number of generations onto a strain of interest, in our case, C57BL/6 mice, to produce a litter that contains both mice of interest along with control or in our case again CXCR1 deficient and WT mice. In this way, both control and experimental mice groups are introduced to the same microbiome from birth (maternal effect, housing) and influenced by the same external factors (handling, bedding, food). To date, in the oral health field, much of the animal models mapping the role of a particular gene on an immunological trait has been performed without the use of littermate controls. As a result, incorrect conclusions may be drawn and hard to reproduce. Therefore, future studies using littermate controls should be performed to confirm TLR2, TLR4 and CXCR1 related influences on the modulation of oral homeostasis, for example on the effect of these genes on influencing the composition of the oral microbiome.

Further characterization of Sud on host modulation

Chapter 4 introduced the beneficial effects of a natural plant based product, Sud, on its ability to modulate host inflammatory responses to stimulation by the addition of exogenous host inflammatory mediators, as well as to bacteria. Furthermore, Sud displayed the potential to differentially inhibit levels of IL-8 by bacteria and host mediators. In order to fully exploit and characterize the potential selective inhibitory properties of this extract, further investigations with additional pro- and anti-inflammatory modulators, along with its host modulatory effects in response to both health and disease associated oral bacteria should be investigated.

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- Zlotnik, A. and Yoshie, O. (2000). Chemokines: a new classification system and their role in immunity. *Immunity* 12, 121-127.

Vitae



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Research Interests

I am interested in researching areas of host-pathogen interaction, particularly in mechanisms and modes of immune evasion and bacterial persistence.

Education

- | | |
|-------------------|--|
| Sept 2014-Present | PhD, Oral Health Sciences
University of Washington, Seattle
<i>Thesis: "Contribution of Oral Commensal Bacteria to Oral Health through regulation of Neutrophils" – in Progress</i>
Faculty Advisor: Dr. Richard P. Darveau |
| June 2012 | B.S., Microbiology
University of Washington, Seattle
<i>Thesis: "Regulation of Porphyromonas gingivalis phosphatase, PG1773"</i>
Faculty Advisor: Dr. Richard P. Darveau |
| June 2012 | B.A., Biochemistry
University of Washington, Seattle |

Honors & Awards

- | | |
|----------|--|
| Mar 2018 | Burroughs Wellcome Fund Trainee Travel Award
Translational Science 2018 Meeting in Washington, DC
Association for Clinical and Translational Science |
| 2014 | Graduate School Top Scholar Award
Dept. Oral Health Sciences, University of Washington, Seattle |

Scholarships & Fellowships

- | | |
|-------------------|---|
| Mar 2018-Mar 2019 | TL1 Fellowship Awardee |
| Jun 2017-Feb 2018 | Institute of Translational Health Sciences, University of Washington, Seattle |

Jun 2017-Aug2017	<p>East Asia and Pacific Summer Institutes (EAPSI) Showa University School of Dentistry, Tokyo, Japan National Science Foundation <i>[Retraction due to conflicting funding of a concurrently awarded fellowship]</i></p>
Teaching Experience	
Fall Quarter 2016	<p>Graduate Teaching Assistant ORALB 580: "Introduction to Molecular Biology Laboratory" Dept. of Oral Health Sciences, University of Washington, Seattle Supervisor: Dr. Richard Presland</p> <ul style="list-style-type: none"> Assisted in laboratory instruction of graduate students Supervised lab methods: DNA/RNA extraction, PCR, Western, Southern, Cloning Aided in experiment set up Answered student's questions and e-mails
Fall Quarter 2018	Graduate Teaching Assistant
Fall Quarter 2017	DENTFN513: "Oral Microbiology"
Fall Quarter 2016	<p>Dept. Oral Health Sciences, University of Washington, Seattle Supervisor: Dr. Jeffrey McLean</p> <ul style="list-style-type: none"> Assisted with syllabus and learning objectives Maintained gradebook and course website through Canvas Prepared online quizzes and final exam Graded weekly in-class activities Input questions and design quizzes in Examsoft Answered students questions
Laboratory Experience	
Sept 2014-Present	<p>Graduate Student Dept. Oral Health Sciences, University of Washington, Seattle Supervisor: Dr. Richard P. Darveau</p>
Jun 2012-Sept 2014	<p>Research Assistant Dept. of Periodontics, University of Washington, Seattle Supervisor: Dr. Sumita Jain</p>
Apr 2010-Jun 2012	<p>Student Research Assistant Dept. of Periodontics, University of Washington, Seattle Supervisor: Dr. Sumita Jain</p>

Publications

1. Kapila Y, Kamarajan P, Ateia I, Shin J, Fenno J, Le C, Zhan L, **Chang A**, Darveau R. Periodontal Pathogens Promote Cancer Aggressivity via Integrin Signaling that is Bacteriocin Responsive. *Manuscript under review*.
2. Erben P, **Chang AM**, Darveau RP, Fong H, Johnson JD, Paranjpe A. Evaluation of the bactericidal potential of 2780nm ER,CR:YSGG and 940nm Diode lasers in the root canal system. *Lasers in Dental Science*. 2019; 3:137-146.
3. **Chang AM**, Liu Q, Hajjar AM, Greer A, McLean JS, Darveau RP. Toll-Like Receptor 2 and Toll-Like Receptor 4 Responses Regulate Neutrophil Infiltration into the Junctional Epithelium and Significantly Contribute to the Composition of the Oral Microbiota. *J Periodontol*. 2019; [Epub ahead of print] doi: 10.1002/JPER.18-0719.
4. Jain S, **Chang AM**, Singh M, McLean JS, Coats SR, Kramer RW, Darveau RP. Identification of PGN_1123 as the gene encoding lipid A deacylase, an enzyme required for Toll-like receptor 4 evasion, in *Porphyromonas gingivalis*. *J Bacteriol*. 2019; 20: e00683-18. doi: 10.1128/JB.00683-18.
5. Biswas K, **Chang A**, Hoggard M, Radcliff FJ, Jiang Y, Taylor MW, Darveau R, Douglas RG. Toll-like receptor activation by sinonasal mucus in chronic rhinosinusitis. *Rhinology*. 2016; 55: 59-69. doi: 10.4193/Rhin16.201. PubMed PMID: 28025987.
6. Greer AR, Irie K, Hashim A, Leroux BG, **Chang AM**, Curtis MA, Darveau RP. Site Specific Neutrophil Migration and CXCL2 Expression in Periodontal Tissue. *J Dent Res*. 2016; 95(8): 946-52. doi: 10.1177/0022034516641036. PubMed PMID: 27013641; PubMed Central PMCID: PMC4935833.
7. Jain S, Coats SR, **Chang AM**, Darveau RP. A novel class of lipoprotein lipase-sensitive molecules mediates TLR2 activation by *Porphyromonas gingivalis*. *Infect Immun*. 2013; 81:1277-1286. doi: 10.1128/IAI.01036-12. PubMed PMID: 23381996; PubMed Central PMCID: PMC3639599.

Oral & Poster Presentations

- Jan 2019 **Chang AM**, Liu Q, Hajjar AM, Greer A, McLean JS, Darveau RP. Toll-like-receptor-2 and -4 responses regulate neutrophil infiltration into the junctional-epithelium. Poster presentation at the University of Washington School of dentistry 2019 Research Day in Seattle, WA on Jan 29, 2019. Award first place poster for SOD Research Day Graduate Division.
- May 2018 **Chang AM**, Hajjar AM, Darveau RP. The contribution of commensal oral bacteria to periodontal health through the regulated expression of neutrophil chemotactic molecules in the murine junctional epithelium. Poster presentation at the 2018 ITHS Translational Science Expo at the University of Washington in Seattle, WA on May 11, 2018.

- Apr 2018 **Chang AM**, Hajjar AM, Darveau RP. The contribution of commensal oral bacteria to periodontal health through the regulated expression of neutrophil chemotactic molecules in the murine junctional epithelium. Poster presentation at the Translational Science 2018 Meeting at the Washington Marriott Wardman Park hotel in Washington, D.C. on April 19, 2018. Awarded top 25% of abstracts.
- Jan 2018 **Chang AM**, Liu Q, Hajjar AM, Greer A, McLean JS, Darveau RP. TLR2 and TLR4 regulate neutrophil infiltration into the junctional epithelium and contributes to the oral microbial composition in a KO mouse model. Poster presentation at the University of Washington School of Dentistry 2018 Research Day in Seattle, WA on Jan 30, 2018.
- Sept 2017 **Chang AM**, Liu Q, Hajjar AM, Greer A, McLean JS, Darveau RP. TLR2 and TLR4 significantly contribute to maintain oral microbial homeostasis in a KO mouse model. Oral presentation at the 2018 Oral Health Sciences Research Symposium and Retreat, University of Washington in Seattle, WA on Sept 20, 2017.
- Jun 2016 Rajapakse S, Kulasekara H, **Chang A**, Darveau R. Antimicrobial and antibiofilm effects of an extract mixture in a herbal toothpaste. Poster presentation at the 2016 meeting of the International Association of Dental Research-Asia Pacific Region (IADR-APR), Seoul, Korea on June 22-25, 2016.

Workshops and Conferences Attended

- Jan 2019 Programming in Python
Software Carpentry
eScience Institute, University of Washington, Seattle
- Oct 2018 CMiST Microbiome Symposium
Community and Connectivity in the Microbiome
University of Washington, Seattle, WA
- May 2018 2018 ITHS Translational Science Expo
Institute of Translational Health Sciences
University of Washington, Seattle, WA
- Apr 2018 Translational Science 2018 Meeting
Association for Clinical and Translational Science (ACTS)
Washington Marriott Wardman Park Hotel, Washington, D.C.
- Feb 2018 17th Annual Mark Wilson Conference
28th Annual Meeting of the Oral Immunology/Microbiology Research Group
Cancun, Mexico
Attended as a Guest

Jan 2018	Grant Writing 101 Adeluisa 'Dely' G. Judal University of Washington, Seattle, WA
Oct 2017 – Mar 2018	Career Planning/Leadership Dinners Dr. Rick Baugh, Dr. Lee Huntsman, Dr. Matt O'Donnell Graduate School, University of Washington, Seattle
Dec 2017	CMiST Microbiome Symposium Microbiome-pathogen interactions in infectious disease University of Washington, Seattle, WA
May 2017	2017 ITHS Translational Science Expo Institute of Translational Health Sciences University of Washington, Seattle, WA
May 2016	Current Topics and Methods in Microbiome Research Workshop Center for AIDS Research, University of Washington Seattle Children's Research Institute, Seattle, WA
Mar 2016	Programming in R Software Carpentry eScience Institute, University of Washington, Seattle
Computer Skills	
Qiime2, Rstudio, Graphpad, MS office, Inkscape	
Language Skills	
Fluent in Korean (written and spoken)	
Leadership and Volunteer Experience	
Oct 2017-2018	Graduate Student Representative Dept. of Oral Health Sciences, University of Washington, Seattle
Jul 2018	RSVP Packet Stuffer Cascade Bicycle Club, Woodinville, WA
Jun 2018	Bicycle Rest Stop Volunteer 2018 Woodinville Wine Ride Cascade Bicycle Club, Woodinville, WA

Oct 2017	Dental Escort and Waiting Areas Volunteer Seattle/King County Clinic (SKC Clinic), Seattle, WA
Jul 2018	Seattle to Portland Bicycle Ride Finisher
Jul 2017	Cascade Bicycle Club, Seattle, WA
2013	Peer Mentor Pipeline Project, University of Washington, Seattle, WA
2013	Art Teacher Assistant Greenwood Elementary School Early Childhood and Education, University of Washington, Seattle
2012	High School Mentor Federal Way High School Dream Project, University of Washington, Seattle, WA
2009-2011	Study Zone Tutor Shoreline Library King County Library System, Seattle, WA

References

1. Dr. Richard R. Darveau, M.S., Ph.D.
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Professor, Department of Oral Health Sciences, University of Washington
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