Oral Polymicrobial Communities in Health and Disease

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Abstract

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The oral microbial community represents the best-characterized consortium associated with the human host. There are strong correlations between the qualitative composition of the oral microbiota and clinically healthy or diseased states. However, additional studies are needed to elucidate the mechanisms that define these microbial/host relationships. In the following chapters, the relationship between host and polymicrobial communities in the oral cavity will be explored in the context of health and disease. The oral and intestinal host tissues both carry a heavy microbial burden. It is well established that the commensal bacteria contribute to healthy intestinal tissue structure and function however; their contribution to oral health is poorly understood. A crucial component of periodontal health is the recruitment of neutrophils to periodontal tissue. Within the subsequent pages, examination of polymicrobial effects on the process of neutrophil homing will reveal a unique role for commensal bacteria in oral health. Further, it appears that the commensal-host relationship in health is a delicate balance between boosting host-immune functions and commensal induced bone loss. As we will see during an *in vivo* investigation of *Porphyromonas gingivalis* heterogeneous lipid A that immunomodulation caused by different agents can cause disruption and dysbiosis to the commensal community. The unique dysbiotic communities were each associated with a similar progression of disease. The data presented in the following pages will hopefully give insight into the complex nature of oral commensal microbiota in health as well as disease and provide a foundation for future studies.

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Chapter 1: The relationship of the oral microbiota to periodontal health and disease

History of oral microbiology

One milliliter of human saliva from a healthy adult contains approximately 100 million bacterial cells. Given a normal salivary flow rate of 750 ml per day, approximately 8×10^{10} bacteria are shed from the surfaces of the mouth every 24 hours, equivalent to 5–10g of wet weight of bacterial cells. These bacteria originate from the highly specialized and distinctive communities of organisms that reside on a variety of different environmental niches in the human mouth. Hence, the human oral microbiome can be viewed as a summation of discrete microbial communities drawn from, for example, the mucosal surfaces of the tongue, cheeks, palate and tonsils and the microbial biofilms that accumulate on the hard, non-shedding surfaces of the teeth.

The ease of accessing and sampling the mouth and the long acknowledged role of bacteria in dental caries and periodontal disease, two of the most common diseases of humans, have driven extensive investigations on the microbial communities on the tooth surface. As a result, oral bacteria are now the most well characterized microbiota of the human microbiome. These studies extend back to over 3 centuries to the very first description of bacterial cells by Antonie van Leeuwenhoek, who in 1676 used his newly manufactured microscopes to describe the “animalcules” in biofilms from human teeth. In the intervening period, our understanding of the complexity, site specificity and environmentally driven nature of these microbial communities has expanded with each technological advance in microbial identification and classification. These advances include the introduction of standardized culture techniques on

solid media, the development of anaerobic culture systems, the introduction of non-culture techniques based on molecular phylogeny through nucleic acid analyses using DNA:DNA hybridization, PCR, Sanger sequencing and the more recent developments in high throughput pyro-sequencing-based analyses and metagenomics (Wade, 2011). These culture- and non-culture-based investigations have culminated in the development of the Human Oral Microbiome Database (http://www.homd.org), which lists all bacterial species found in the human mouth (Dewhirst *et al.*, 2010) and more recently CORE (http://microbiome.osu.edu) (Griffen *et al.*, 2011), a phylogenetically curated 16S rDNA database of the core oral microbiome that is representative of the bacteria that regularly reside in the human oral cavity.

Current understanding of the oral microbiome

As in other environments, a significant proportion of the total oral microbiota remains unculturable and therefore non-culture methods are required to describe the overall species richness of the oral microbiome. Sequence analysis of 16S ribosomal RNA has been the method of choice because of its universal presence in all organisms and because, through PCR primer design, it is possible to describe either all the species present in a given sample or to target specific genera. The application of this approach has led to the description of 11 phyla in the domain *Bacteria* in the oral microbiome in addition to methanogenic species of the *Methanobrevibacter* genus from the domain *Archaea*. The phyla of the domain *Bacteria* that are reliably present include *Actinobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Tenericutes, Fusobacteria, Proteobacteria, Spirochaetes, Synergistetes* and two currently un-named phyla, SR1 and TM7. Several hundred distinct species are contained within these divisions representing the highly diverse microbial communities of the mouth. The periodontal microbiota is particularly heterogeneous and over 400 species have been described in this habitat alone using a

16S rRNA amplification, cloning and Sanger sequencing approach (Dewhirst *et al.*, 2010).

While these findings have significantly enhanced our understanding of the oral microbiome, they have also highlighted the likelihood there may be an additional large number of low abundance species that have remained undetected using this standard methodological approach, largely because of the relatively time consuming and laborious nature of the techniques. This issue is now being addressed through the application of deep sequencing methods in particular pyro-sequencing technologies, which enable a more comprehensive coverage of the 16S rRNA sequences in large numbers of samples. Although relatively few large-scale studies have been undertaken, there are indications that it may be necessary to revise our estimates of the species richness of the oral microbiome, perhaps by a factor of 10. For example, in a study of the microbiota of saliva and supragingival plaque from 71 and 98 healthy adults, respectively, amplicons from the V6 hypervariable region of the small subunit ribosomal RNA gene were generated by PCR and sequenced by 454 technology. 197,600 sequences were analyzed and suggested to represent 22 phyla comprising 3621 and 6888 species-level phylotypes in the saliva and plaque, respectively (Keijser *et al.*, 2008).However, these early data need to be viewed with some caution as it is well recognized that the errors inherent in pyrosequencing, particularly of homopolymeric tracts, may lead to an over estimation of the total number of unique sequences in a given sample. The development of increasingly sophisticated software to minimize these problems should lead to increasingly accurate estimates of the species diversity of the oral microbiome and indeed more recent investigations have tended to be more conservative (Zaura *et al.*, 2009). Nonetheless, the application of high throughput sequencing approaches, particularly to comparative analyses of health and disease, are likely to lead to increasing insights into the range of bacterial species associated with the development of

pathology.

A further issue to consider is the value of non-culture approaches to our understanding of the microbial pathogenesis of oral disease. Strategies based upon the sequence analysis of 16S rRNA limit the description of the microbiome to species level identifications. However, it is well established that there can be significant genomic variation within a species such that while a core set of genes may be common to all strains, there are others with a more restricted distribution. Along with other genetic modifications, including mutations, deletions and inversions, variability in the gene content contributes to the population diversity of that species – a heterogeneity that is not detected based on 16S rRNA analysis.

Formation of the dental plaque biofilm

The formation of the dental plaque biofilm has been well studied both in vitro and in vivo (Kolenbrander *et al.*, 2010). It is clear that there is an orderly succession of early, intermediate, and late colonizing species. Colonization of the tooth surface begins with highly specific interactions between oral bacteria, mostly *Streptococcus* species, and the tooth pellicle. The pellicle is a thin layer of both saliva and gingival crevicular fluid that coats the dentin surface of the tooth. Oral bacteria have evolved highly specific adhesions to pellicle proteins and carbohydrates, not unlike specific adhesins found in other commensal and pathogenic bacteria that display highly specific tissue tropisms. After early colonizers have established themselves on the tooth surface through host derived pellicle interactions, these bacteria themselves then serve as additional binding sites for intermediate and late colonizers. This process, which has been elegantly studied and described by Kolenbrander's group, reveals that each step in dental plaque biofilm formation is highly specific and represents co-evolution between different oral

bacterial species as well as the host (Socransky *et al.*, 2005). Recently, with the use of 16S or 18SrRNA probes the microbial spatial distribution of in vivo dental plaque biofilm was examined by fluorescent in situ hybridization (FISH) (Zijnge *et al.*, 2010). This study confirmed many early observations yet also provided microbial definition to the species composition in different layers of the dental plaque biofilm. In addition, the demonstration that *P. gingivalis* directly binds *Streptococcus gordonii*, an early colonizer, provides evidence that potential perio-pathogens can colonize the biofilm early and this may represent a reservoir for the organism (Nobbs *et al.*, 2009).

Clinically Healthy Periodontal Tissue/Bacterial Interactions

The presence of a large and diverse microbial load on the tooth surface places a polymicrobial consortium in juxtaposition to host periodontal tissue. Nevertheless, normally, the periodontium remains healthy largely due to the numerous host protection mechanisms that operate in the oral cavity (Darveau, 2010).

Similar to the intestinal approach of handling a large microbial load, the oral cavity employs the tactic of first limiting exposure to host tissues. Perhaps the single most important component that limits the numbers of bacteria that can accumulate on the tooth surface is saliva. Saliva contains numerous components that contribute to either limiting bacterial accumulation or directing killing bacteria in the oral cavity. For example, similar to the intestine, saliva contains mucin proteins; however, in contrast to their function in the intestine, the mucins in the oral cavity do not form a thick layer that bacteria may need to penetrate through in order to approach host tissue. Rather, oral mucins induce bacterial aggregation that prevents the bacteria from attaching to the tooth or oral epithelial cell surface and promotes their removal upon swallowing

(Kolenbrander *et al.*, 2010). The interaction of oral bacterial species with salivary mucins is specific, and the same host receptors that facilitate bacterial removal by aggregation and swallowing also initiate ecological succession on the tooth surface, which is coated by saliva.

Perhaps the most unique and significant host protection mechanism in the periodontium is the constant transit of neutrophils from the underlying highly vascular periodontal tissue, through the connective and epithelial cell layers and into the gingival crevice. It has been calculated that approximately 30,000 polymorphonuclear neutrophils (PMNs) transit through periodontal tissue every minute (Schiott *et al.*, 1970), which facilitates nearly constant contact between host neutrophils and the dental plaque biofilm. The high incidence of periodontitis in those individuals with low circulating neutrophils or congenital defects in neutrophil extravazation provides strong evidence that this neutrophil transit is a key component of periodontal innate defense (Darveau, 2010). Accordingly, the structure of the periodontal tissue surrounding the tooth surface is fashioned such that neutrophils can transit through to the tooth surface and inhibit biofilm growth. In particular, a specialized epithelium, termed the junctional epithelium surrounds the tooth surface and forms the “junction” between the inanimate tooth and host tissue. The junctional epithelium is highly porous with large intracellular spaces and it contains no tight junctions and a lower number of desmosomes than the adjacent oral or succular epithelium (Bosshardt *et al.*, 2005). It also expresses a higher concentration of the cell adhesion molecule CEACAM1, which through homophilic binding to itself may serve as the major cell adhesion molecule in this tissue (Heymann *et al.*, 2001). Furthermore, clinically healthy junctional epithelial tissue expresses high levels of IL-8, a potent neutrophil chemoattractant that draws neutrophils to the adjacent dental plaque biofilm. Other host defense mediators associated with neutrophil exit from the vasculature and transit through the connective tissue, such as

ICAM-1 and E-selectin, are also expressed in the appropriate tissues in clinically healthy periodontal tissue (Tonetti *et al.*, 1998).

We are just beginning to learn the relative contributions of oral commensal bacteria or host directed expression programs to the highly specialized tissue organization and orchestrated expression pattern of select inflammatory mediators in clinically healthy periodontal tissue. Early histological studies revealed that germ free (GF) and specific pathogen free (SPF) mice were very similar with respect to the unique junctional epithelium architecture and that both GF and SPF contained neutrophils in the junctional epithelium (Heymann *et al.*, 2001). This has been confirmed subsequently in a study that examined the expression of CEACAM1 in GF and SPF rats and mice (Heymann *et al.*, 2001). It was found that expression of this cell adhesion molecule, which is expressed developmentally during tooth eruption, localizes to the developing junctional epithelium where it is postulated to serve an important role in structural integrity. Therefore, commensal bacteria are not required for its expression. Likewise, another study examined the expression of secretory leukocyte protease inhibitor (Slpi) in GF and SPF mice (Hayashi *et al.*, 2010). This defense protein, which protects the host from host-mediated protease-induced tissue damage, was found to be highly expressed in the junctional epithelium, an area where a strong inflammatory process is occurring, yet the oral commensal community was not required for its expression. However, commensal colonization has been shown to influence the periodontal innate host defense status. A pilot study revealed that SPF mice contain higher levels of IL-1β (Dixon *et al.*, 2004), an inflammatory cytokine normally associated with inflammation. Perhaps more importantly, IL-1β was found in the GF mice underscoring the contribution of "inflammatory" cytokines in normal tissue homeostasis programs in the absence of a microbial stimulus.

Therefore, much more needs to be learned concerning if and how oral commensal bacteria contribute to the highly orchestrated inflammatory response seen in this tissue. Not only do we need to better understand how alterations in cytokine levels by commensal bacteria in the absence of disease may positively or negatively affect periodontal tissue homeostasis programs, but also there have been no studies that have directly examined the molecular mediators associated with neutrophil transit in germ free mice. If these mediators are solely developmentally expressed, as in the case of the epithelial cell adhesion molecule CEACAM1 and secretory leukocyte protease inhibitor (Slpi), then what are the host signals that regulate their expression in such a highly organized fashion? Likewise, if additional germ free studies reveal that commensal bacteria contribute to the neutrophil transit process, it may represent novel therapeutic avenues to "augment" innate defense with the use of probiotic approaches. The contribution of oral commensal bacteria to expression of defensins, CD14, lipopolysaccharide-binding protein, all of which have been shown to be present in clinically healthy tissue, also needs to be determined.

Periodontitis/Bacterial Interactions

Periodontitis, similar to inflammatory bowel disease, is a clinical syndrome that can have multiple etiologies. The generally accepted view is that periodontitis results from the interaction between a microbial challenge derived from the subgingival biofilms on the tooth surface and a deregulated host response in the periodontal tissues (Page *et al.*, 1997). Disruption of this interaction through debridement of the tooth surfaces, supplemented occasionally by antibiotic delivery, is the standard and in most instances broadly effective treatment strategy.

The complexity of the sub-gingival microbiota has hindered the identification of the

precise microbial etiology of periodontitis although very strong correlations between the amount and composition of the dental plaque biofilm and disease have been described (Socransky *et al.*, 1998). Furthermore, extensive microbial compositional analysis, based originally on culture techniques and subsequently extended by large scale DNA:DNA hybridization methodologies, has identified potential periopathogens, designated the red complex because of their co-localization to diseased tissue. Examination of potential virulence characteristics shared by red-complex bacteria, *Porphyromonas gingivalis*, *Tannerella forsythia* and *Treponema denticola,* has not yielded clear associations with disease. However, one shared attribute is their ability to either inhibit or evade innate host responses. This has led to the speculation that the strong association of these bacteria with diseased sites may be related to their ability to disrupt periodontal innate defense functions facilitating a deregulated host response that affects the entire dental plaque community (Darveau, 2010). Dental plaque communities obtained from either healthy or diseased sites are both potent activators of TLR2 and TLR4 (Yoshioka *et al.*, 2008) and are therefore capable of disrupting established host homeostasis programs (Bosshardt *et al.*, 2005). Nevertheless, "red" complex bacteria can be found in clinically healthy sites, albeit at lower numbers, indicating that their presence alone is not responsible for disease.

Adult type chronic periodontitis appears to be truly a microbial community-associated disease. Consistent with this, it has been reported that the stability of the dental-plaque microbial composition maybe a good predictor of periodontal health and that changes in this community are associated with changes in the clinical status of the adjacent tissue (Kumar *et al.*, 2006). However, the factors that lead to changes in the plaque microbiota that are associated with periodontitis are not known. A greater understanding of potential "triggers" that initiate these changes by either altering innate defense function or selecting for a different microbial

community may be obtained from examination of environmental and endogenous factors that are associated with an increased incidence of periodontitis. These include oral hygiene, smoking, obesity, stress and potential genetic associations (Stabholz *et al.*, 2010). Almost certainly there are multiple potential mechanisms by which normal host homeostasis may be disrupted, eliciting alterations in either the host protective status or the microbial composition or both. Understanding the effects of these risk factors on the microbial/host relationship should uncover additional mechanisms by which host homeostasis can be detrimentally disrupted.

Summary

The relationship between the highly characterized periopathogenic microbial community and chronic adult type periodontitis, the most common form of disease, remains to be determined. Additional characterization of the composition of the microbiota in periodontal health versus disease may lead to the description of additional species, which one could presumptively associate with disease causation. For example in one investigation (Kumar *et al.*, 2005), 16S rRNA gene cloning and sequencing identified several novel disease associated organisms including *Peptostreptococcus stomatis, Filifactor alocis* and species drawn from the *Desulfolobulus, Dialister* and *Synergistetes* genera. The potential contribution of clonal types and the influence of environmental triggers in altering the oral microbial composition need to be further explored to determine how a healthy microbiotia is altered into one associated with a destructive host response. Incorporation of polymicrobial approaches into *in vitro* and *in vivo* systems to examine the potential of bacterial–bacterial communication events on the host response are also required (Curtis *et al.*, 2011b).

In the subsequent chapters, two animal models are used to expand the current knowledge of the contribution of commensal polymicrobial community in health and disease. Currently, the

mechanisms by which the oral microbiota positively or negatively affect homeostasis programs in the periodontal tissue are poorly understood; to address this, the following chapter will explore the effects of commensal bacteria on the highly orchestrated inflammatory responses with regard to neutrophil migration. Although we are just beginning to appreciate functions of the polymicrobial communities in health and disease, many studies have focused on virulence factors that are specific to the bacteria termed 'red' complex. These studies have shown that a common trait to these 'periopathogens' is an ability to subvert the host immune response. It is becoming accepted that periopathogens may inhibit or evade host immune functions, which in turn could have pleiotropic effects on the bacterial community. Therefore, to examine effects of a 'periopathogen' on the oral polymicrobial community, chapter three will explore changes in the oral microbiota of plaque samples in a rabbit model of periodontal disease. Taken together, the results from these studies will expand our current understanding of commensal bacteria effects on host innate immune function, how a periopathogen can perturb the polymicrobial community and allow a greater appreciation for activities of the oral microbiome in periodontal tissues.

Chapter 2: Oral Polymicrobial Community Effects in Healthy Tissues

Select expression of MyD88-dependent CXCL2 by commensal bacteria contributes to periodontal tissue homeostasis

Introduction

A state of "controlled" inflammation that normally exists in the intestine has been attributed to both the quality and quantity of intestinal commensal microorganisms (Cebra, 1999, Chadwick *et al.*, 1992). However, little is known concerning the contribution of the oral commensal microbiome to periodontal tissue structure and function (Fig 1). As discussed in previous chapter, this is apparently due to the fact that in contrast to intestinal tissue, few structural or functional differences have been reported when GF and SPF mice have been compared (Curtis *et al.*, 2011b). This is surprising since one of the main mechanisms by which periodontal tissue regulates the numbers of oral commensal bacteria that reside on the tooth and its root surface depends on the constant transit of neutrophils through gingival tissue and into the gingival crevice (Attström *et al.*, 1979, Carrassi *et al.*, 1989, Hart *et al.*, 1994, Page *et al.*, 1987, Waldrop *et al.*, 1987). This process involves a highly orchestrated expression of select innate host defense mediators that safely guide extravasated neutrophils though connective tissue, and finally through the junctional epithelium, a loosely organized specialized epithelial tissue which connects the tooth surface to periodontal tissue and provides the first line of defense to bacterial invasion (Gemmell *et al.*, 1994, Moughal *et al.*, 1992, Nylander *et al.*, 1993, Tonetti, 1997, Tonetti *et al.*, 1994). The requirement for neutrophil transit through the periodontium for the maintenance of healthy periodontal tissue is well documented in both humans (Attström *et al.*, 1979, Carrassi *et al.*, 1989, Hart *et al.*, 1994, Page *et al.*, 1987, Waldrop *et al.*, 1987) and mice (Niederman *et al.*, 2001).

The regulation of the number of neutrophils that migrate through periodontal tissue is important in both health and disease. Defective recruitment of neutrophils to the periodontal tissue reviewed here (Nussbaum *et al.*, 2011), or excessive recruitment of neutrophils, as seen in the absence of the regulatory endothelial protein Del-1 (Eskan *et al.*, 2012) are conditions that both lead to periodontitis. Hence periodontal health is fundamentally dependent on a *critical level* of neutrophil recruitment to the gingival tissues: inappropriate under *or* over recruitment to this critical level can lead to disease. The regulation of neutrophil recruitment is therefore a key determinant of periodontal health.

In this chapter we demonstrate that mouse chemokine receptor CXCR2 is the major mediator of neutrophil recruitment to periodontal tissue. We then show that two different CXCR2 ligands, CXCL1 and CXCL2, are expressed in germ free tissues. However, CXCL2 but not CXCL1, is significantly increased in SPF mice and is controlled by the MyD88 activation pathway. These data demonstrate the select usage of CXCR2 ligands in periodontal tissue and indicate that the different usage of CXCR2 ligands is one mechanism by which the periodontal tissue regulates neutrophil migration.

GF and SPF gingival tissues display relatively minor differences in cytokine protein expression levels

In an initial pilot study, we have previously reported a small but statistically significant increase in gingival tissue IL-1β protein levels in SPF mice as compared to GF (Dixon *et al.*, 2004). Therefore, we initially sought to confirm this observation and determine if other host cytokines in gingival tissue were significantly modulated by commensal colonization. ELISAs were performed on the homogenized tissues obtained from GF and SPF mice for IL-1β, TNF-α, INF-γ and CXCL2 (Fig 2A). Although a trend for increases in IL-1β and CXCL2 and decreases

in TNFα and INF-γ were observed when SPF mice were compared to GF mice, the data was not statistically significant when assays performed from different tissue collections were compared (Student T Test). It is striking that many of the inflammatory mediators are present in similar amounts in both groups of mice, perhaps indicating that other programs outside of microbial stimulation are responsible for the low level of inflammation in the gingiva however; we suspect that the inability to accurately collect a standardized gingival tissue sample combined with the highly localized expression of select cytokines and chemokines precluded our ability to obtain statistically significant differences in the levels of these cytokines. These data demonstrate that the effects of commensal colonization on the expression levels of these cytokines are not significant enough to be detected in whole gingival tissue ELISA assays.

Commensal colonization significantly increases the number of neutrophils found in the junctional epithelium

Recently it has been reported that SPF mice contain more neutrophils in the junctional epithelium when compared to GF mice (Tsukamoto *et al.*, 2012). Employing a different strain of mice and utilizing a different quantitation method this observation has been confirmed (Fig. 2B). Neutrophils were stained with a neutrophil antibody and found to be concentrated in the junctional epithelium of both mouse groups with only occasional staining in the vasculature and no staining in the oral, sulcular epithelium or the connective tissue (Fig 2C). Four different mice were examined in each group of GF and SPF mice such that 100 sections/group were counted for neutrophils. Analysis of the results revealed that there were no statistical differences in neutrophil numbers within a group ($p > 0.01$, Random mixed model). However, in contrast, the analysis revealed that germ free mice contained significantly ($p < 0.0001$, Mann-Whitney Test) fewer neutrophils than age- and strain- matched SPF controls. These data and the previous

report (Tsukamoto *et al.*, 2012) demonstrate that oral commensal bacteria significantly contribute to increased neutrophil migration to the junctional epithelium.

CXCR2 is the major mouse periodontal neutrophil homing receptor

CXCR2 is a major neutrophil chemokine receptor in mice, located on neutrophils, facilitates neutrophil migration into host tissues (Cacalano *et al.*, 1994, Shuster *et al.*, 1995) and contributes to neutrophil homeostasis by maintaining normal neutrophil numbers in the circulation (Mei *et al.*, 2012, Yu *et al.*, 2007). Maturation of neutrophils causes up-regulation of CXCR2, which allows release from the bone marrow (Borregaard, 2010). CXCR2 KO mice have also been shown to develop spontaneous periodontitis (Hajishengallis *et al.*, 2011, Yu *et al.*, 2007); this is suspected to be due to the lack of neutrophil migration to periodontal tissue since multiple mechanisms of neutrophil chemotaxis that may occur at sites of bacterial colonization (Phillipson *et al.*, 2011). The contribution of CXCR2 to neutrophil migration to the junctional epithelium was examined in CXCR2 KO mice (Fig. 3). Of the over 50 tissue sections examined, only a rare and apparently transient neutrophil was ever identified in the junctional epithelium. In almost all sections, the junctional epithelium was completely void of neutrophils. There were however, neutrophils identified in the vessels and occasionally in the sub-epithelial vasculature. These data demonstrate that while neutrophils are present in the vessels, without proper signaling to the CXCR2 receptor the neutrophils are unable to migrate to the junctional epithelium, in contrast to the normal neutrophil recruitment in strain-matched wild-type mice (Fig. 2)

CXCL2 but not CXCL1, expression in the junctional epithelium is significantly dependent on commensal colonization

Next, the expression of two major ligands for CXCR2, CXCL1 and CXCL2, were examined since CXCR2 was essential for neutrophil migration to the junctional epithelium.

CXCL1 and CXCL2 antibody concentrations for immunohistochemical analysis were optimized for maximum sensitivity (Fig. 4). The slides were then blindly read and the expression level for each chemokine was then expressed as the relative staining intensity on a scale of 0 (weakest) to 3 (strongest). A multi level regression analysis for CXCL1 expression revealed that there was no significant difference in the relative expression levels between GF and its strain matched SPF strain (germ free versus SPF, p=0.9; N=120). More specifically, nearly identical percentages of relative intensity scores were found for both GF and SPF mice. In contrast to CXCL1, CXCL2 expression levels were significantly different (GF versus SPF, p=0.0001; N=120) in the junctional epithelium. These data demonstrate that CXCL2 expression, but not CXCL1, is modulated by oral commensal bacterial colonization.

Neutrophils and CXCL2 but not CXCL1 expression in the junctional epithelium is significantly dependant on the MyD88 signaling pathway

MyD88 is a co-adaptor protein facilitating host response after bacterial recognition through TLRs and three members of the IL-1 receptor family (Kawai *et al.*, 2005). Therefore, to determine if commensal bacteria may utilize a TLR-dependent activation pathway to augment neutrophil migration in SPF mice as shown in Fig 2, the number of neutrophils in the junctional epithelium of SPF-MyD88KO and strain matched wild type were determined as described above. A similar staining pattern of neutrophil accumulation in the junctional epithelium was observed in both MyD88 KO and wild type SPF mice (see Fig. 2 for example). Quantitative analysis performed as described above for GF and SPF comparisons revealed a significant decrease in the number of junctional epithelium-associated neutrophils in the MyD88 KO mice (p<0.0001, Mann Whitney Test, Fig. 5). This data demonstrates that a MyD88 dependent signaling pathway is required for optimal neutrophil migration in SPF mice indicating that commensal bacteria are

inducing. Furthermore, a multi level regression analysis (Fig. 5) for CXCL1 expression revealed that there was no significant difference in the relative expression levels between MyD88KO and its strain matched WT strain (WT versus myd88ko, $p=0.9$, $N=120$). In contrast, CXCL2 expression levels were significantly different (MyD88 versus wild type $p<0.0001$, $N=120$: multi level regression analysis). These data demonstrate that CXCL2 expression, but not CXCL1 is modulated by the MyD88 activation pathway.

Discussion

We found that in contrast to intestinal tissue, gingival tissue did not display readily observable differences in tissue structure when germ free and SPF mice were compared. The analysis of select cytokine expression levels also did not reveal major alterations due to oral colonization in SPF mice. These data are consistent with an early report that described neutrophils in the periodontium of germ free rats (Rovin *et al.*, 1966) and more recent analyses that have shown that GF and SPF mice express comparable levels of SLPI (Hayashi *et al.*, 2010) and CEACAM-1 (Heymann *et al.*, 2001), two highly specialized host molecules expressed by junctional epithelial cells involved in neutrophil crosstalk and specialized adhesion junctions, respectively. In addition, immunohistological staining revealed that both GF and SPF mice expressed CXCL1 and CXCL2, two ligands which contribute to neutrophil migration. These data provide evidence that bacterial colonization of the oral cavity is not needed for neutrophil migration through periodontal tissue, a key function of innate defense of periodontal tissue. Furthermore, these data demonstrate that, in contrast to intestinal tissue, bacteria-free periodontal tissue can develop the apparently appropriate structural and functional attributes normally associated with a tissue in close contact with commensal bacteria. The mechanisms which tailor the structure and function of this tissue in the absence of a bacterial signal are not known.

The contribution of oral commensal bacteria to the structure and function of periodontal tissue appears to be more subtle than that observed in intestinal tissue. It required quantitative analysis of neutrophil numbers present in the highly specialized junctional epithelial tissue in the work presented here and from a recent publication (Tsukamoto *et al.*, 2012) to demonstrate that SPF mice contain a significant increase in neutrophils numbers when compared to their germ free counterparts. Furthermore, commensal colonization altered the expression of CXCL2, a CXCR2 ligand associated with neutrophil chemotaxis. This difference was detected using a semi-quantitative immunohistochemical analysis where the expression levels CXCL2, but not CXCL1 were significantly increased in SPF mice when compared to germ free mice. Similarly, ELISA analysis of gingival tissue trended towards higher CXCL2 expression in SPF mice but was not significantly different than germ free mice (Fig. 2). However, the ELISA data relied on tissue collected from around the teeth, which additionally included connective and vascular tissues (determined by FACS analysis, data not shown) where cytokine expression levels could vary within the different tissue environments. This could explain why significant differences in the highly localized junctional epithelial tissue were not detected. A recent report employing laser capture microdissection of junctional epithelial tissue has shown that the message for both CXCL1 and CXCL2 is greater in SPF mice when compared to germ free animals (Hayashi *et al.*, 2010) which would appear to contradict our findings on the increased expression of CXCL2 but not CXCL1. However, the protein levels were not compared in that study and it is possible that similar to our previous work (Dixon *et al.*, 2004) and that of others (Hajishengallis *et al.*, 2011) mRNA levels may not always correlate to protein expression. It is particularly appropriate to consider mismatched transcriptional and translational expression patterns with respect to chemokine receptor ligands and other mediators of inflammation, where instability sequences in

the mRNA sequence regulate protein expression levels (Tebo *et al.*, 2000). Therefore, both the subtle effects of commensal colonization and the technical challenges associated with characterizing small amounts of highly specialized tissues in the periodontium, such as the junctional epithelium, have contributed to the lack of an adequate description of how oral commensal bacteria contribute to periodontal tissue structure and function.

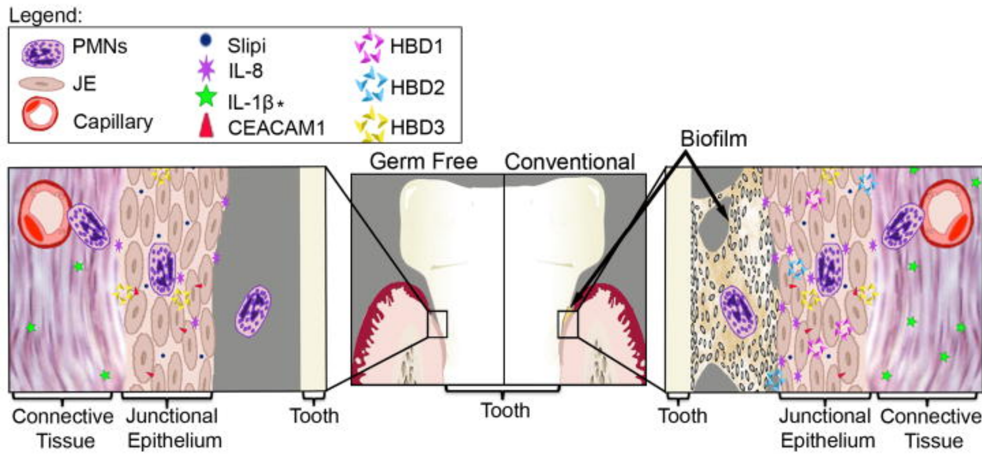
The use of MyD88 $^{-/-}$ mice identified this adapter protein as required for both the increase in neutrophil numbers and CXCL2 expression observed in SPF mice. MyD88 is a required adaptor protein for all TLR's except TLR3 and TLR4 and for three members of the IL-1 receptor family (Kawai *et al.*, 2005). Although TLR4 utilizes Myd88, it can also employ the co-adapter TRIF in response to certain bacterial components (Kawai *et al.*, 2005). This data, in MyD88 $^{-/-}$ mice, provides strong evidence that oral commensal bacteria either directly though TLR activation or indirectly though IL-1β expression facilitate the increase in both neutrophil migration and CXCL2 expression associated with SPF mice. The Myd88 activation pathway did not modulate the level of CXCL1 expression consistent with its expression being independent of commensal colonization.

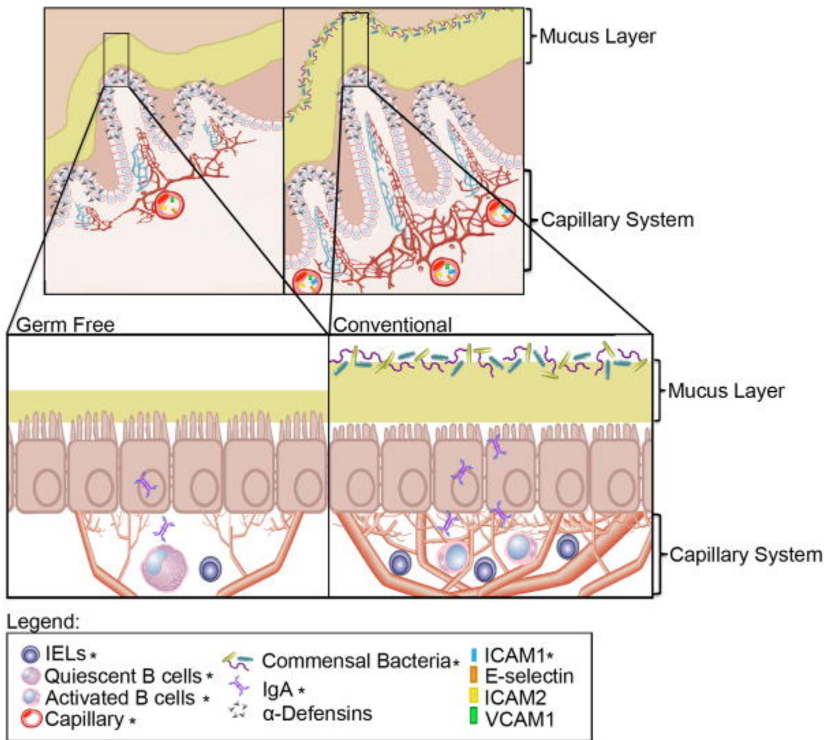
The data presented here provide the first evidence for the selective use of ligand CXCL2 in healthy periodontal tissue. CXCR2 has been shown to be a critical component maintaining periodontal homeostasis (Yu *et al.*, 2007). In this study, we demonstrate that CXCR2 $^{-/-}$ mice fail to properly recruit neutrophils to the junctional epithelium revealing this receptor as the major mechanism of neutrophil transmigration into periodontal tissue. Previous studies in different inflammatory models of disease in mice have shown that the selective use of these chemokine ligands facilitate highly specific temporal and tissue selective neutrophil homing responses (Borregaard, 2010, Lee *et al.*, 1995, McDonald *et al.*, 2010a, McDonald *et al.*, 2010b, Ritzman *et*

al., 2010, Rovai *et al.*, 1998, Sadik *et al.*, 2011, Wuyts *et al.*, 1996). Consistent with the findings that CXCL2, but not CXCL1, was regulated by commensal bacteria and the MyD88 activation pathway, GF mice displayed similar expression of CXCL1 compared to SPF, whereas SPF mice had stronger expression of CXCL2 than GF. Additional studies will be required to examine other CXCR2 ligands and their activation pathways to determine their contribution to the neutrophil homing process, which will be discussed in the subsequent chapter relating to future directions.

To conclude, the community of commensal bacteria has been shown to modulate host innate immune functions in healthy tissues. It has been postulated that a single species could alter host responses thereby disrupting the commensal community resulting in dysbiosis and disease (Darveau, 2010, Hajishengallis *et al.*, 2012). In the following chapter, polymicrobial interactions will be examined in the context of disease initiated by the bacterium, *Porphyromonas gingivalis* to determine how *P. gingivalis* host-immunomodulation affects the community of oral bacteria and contributes to disease.

Figure 1





A. Current knowledge of microbial influence on the junctional epithelium (JE) based on cumulative data from human and mouse studies. The architecture of JE tissue and presence

of PMNs are similar between germ-free and conventional mice. Several molecules appear to change dramatically with the addition of bacteria but many are unchanged (Darveau, 2010). B. Overview of current knowledge of microbial influence on the intestinal epithelium. The architecture of the intestinal tissue is changed markedly with the addition of bacteria; the crypts are deeper, the capillary network is more extensive, the mucus layer is reduced, cilia are shorter and many differences are seen with immune cells and molecules as indicated (Hooper, 2004). The figure indicates the relative location and abundance of innate immune cells/molecules. (*indicates changes due to microbial interactions confirmed in germ free studies).

Figure 2

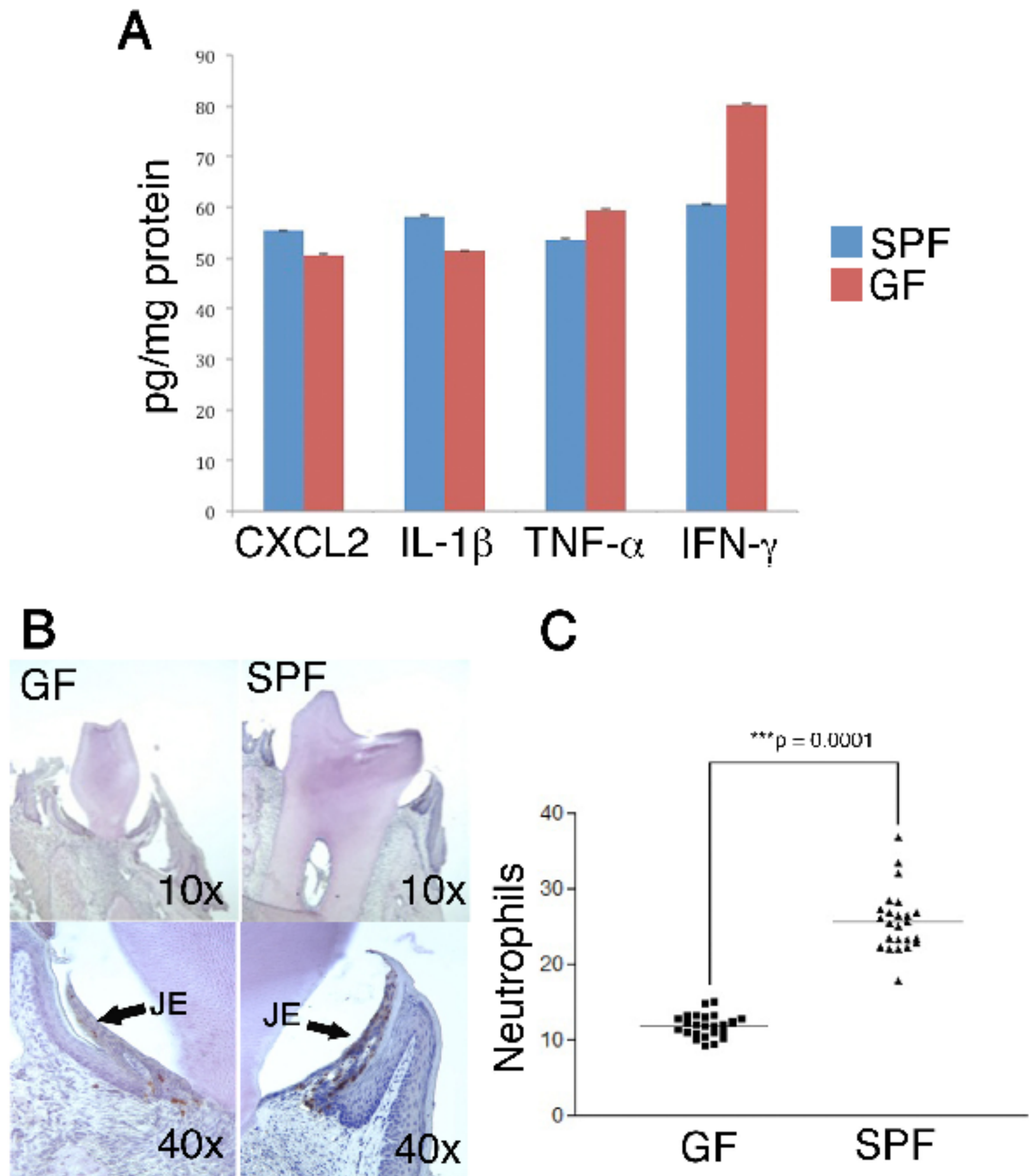


Figure 2 SPF and GF mice contain minor cytokine level but major neutrophil number differences in gingival tissue A. Cytokine analysis by ELISA from specific pathogen free (SPF) and germ free (GF) mice, 7 mice/group (pooled). B. Neutrophil staining of GF and SP mouse gingival tissue (X10 and X40 mag). A few neutrophils were also detected in both the gingival and pulp vasculature (data not shown). C. Neutrophils, stained and quantified shown here as average neutrophils per section, were mainly located in the junctional epithelial (junctional epithelium) tissue. Quantification includes 25 sections per mouse. *** denotes statistically significant difference (Mann-Whitney test).

Figure 3

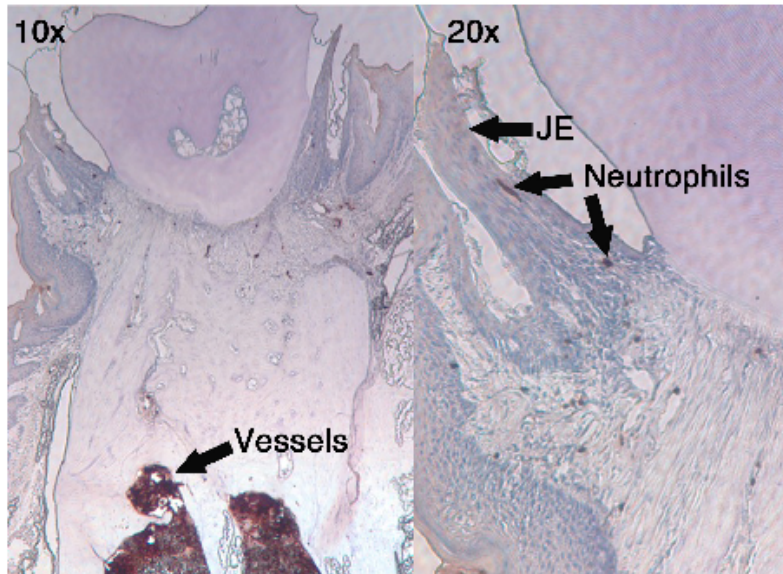


Figure 3 Lack of neutrophils in the gingival tissue of CXCR2KO mice. Gingival tissue obtained from CXCR2 KO mice were stained for neutrophils as described in the text. (10x, 20x). Most neutrophils were detected in vessels while occasional neutrophils were observed in the vasculature and only rarely in junctional epithelium.

Figure 4

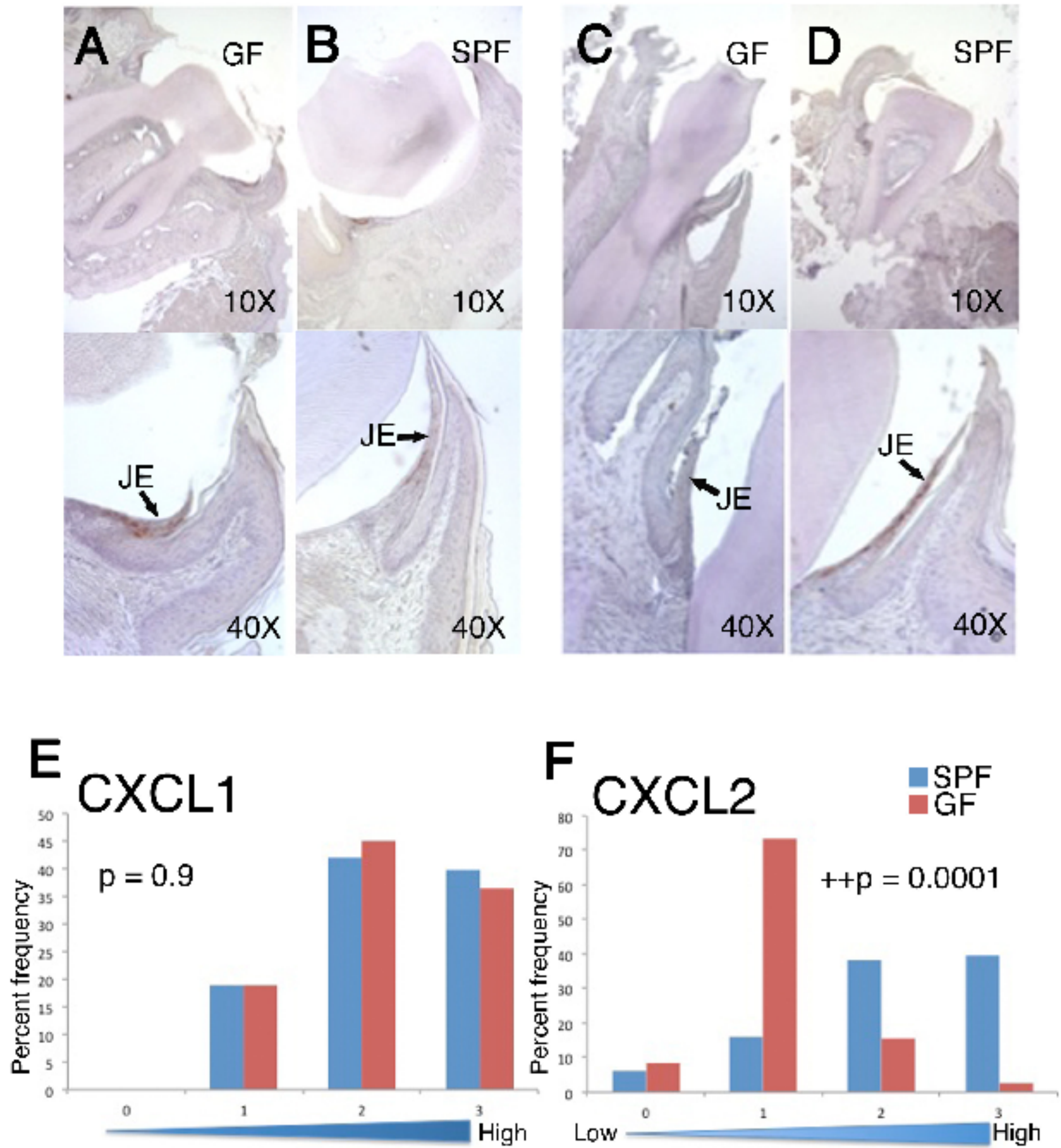


Figure 4 *SPF mice express significantly higher levels of CXCL2 in the junctional epithelium.* CXCL1 and CXCL2 immunohistochemical (IHC) staining of oral tissue. CXCL1 (A and B) and CXCL2 (C and D) expression was determined in GF (A and C) and SPF mice (B and D). Local structures are indicated as well as magnification with neutrophil staining detected in the junctional epithelium. The junctional epithelium appears detached from the tooth surface due to the fixing procedure. Lower panels represent a semi-quantitative analysis of CXCL1 (E) and CXCL2 (F) expression as described in the text: Y-axes indicate the percentage of sections examined; X axes indicate staining intensity scores. ++, denotes statistically significant difference (Multi-level logistic regression test, N=120 tissue sections).

Figure 5

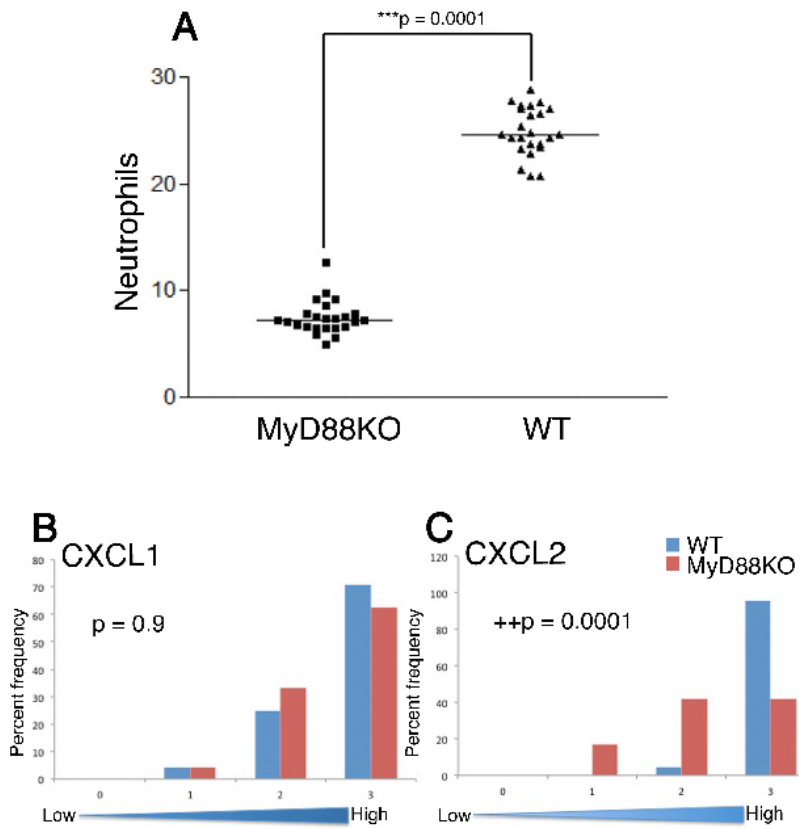


Figure 5 Myd88 mice display defects in neutrophil migration and CXCL2 expression. Neutrophil staining and CXCL1 and CXCL2 expression in MyD88KO and strain matched WT mice. The number of neutrophils in the junctional epithelium (A) were determined as described in the text. ***, denotes statistically significant difference (Mann-Whitney test). Semi-quantitative analysis of CXCL1 (B) and CXCL2 (C) expression was performed as described in the text. ++, denotes statistically significant difference (Multi-level logistic regression test, N=120 tissue sections)

Chapter 3: Oral Polymicrobial Perterbations in Disease:

Multiple dysbiotic dental plaque communities elicit periodontal disease in the rabbit ligature model of disease

Periodontitis is a polymicrobial disease consisting of inflammation, degradation of host tissue and bone that arises from a complex community of bacteria. *Porphyromonas gingivalis* is a gram-negative periopathogen that has been designated a keystone species due to its ability to induce a dysbiotic oral polymicrobial community that results in disease. *P. gingivalis* contains lipid A with structural heterogeneity that has been postulated to contribute to the formation of dysbiotic oral communities by modulating the host response. In this chapter two *P. gingivalis* lipid A phosphatase mutants which contain different "locked" lipid A structures that differ in their ability to modulate the host response were examined for the presence of dysbiotic microbial communities associated with periodontitis. In addition, the LPS containing the different lipid A structures found in these mutants was also examined. A rabbit model of ligature induced periodontal disease was employed and both the oral microbial composition and amount of bone loss were determined after addition of *P. gingivalis* wild type and mutant strains and their respective isolated LPS preparations. It was found that the wild type but not the mutant *P. gingivalis* strain was able to colonize the rabbit periodontium. Furthermore, profound and significant changes in select oral bacteria were detected in rabbits when bacterial and LPS experimental groups were compared. However, although significantly different microbial communities were observed in different experimental groups, all experimental groups displayed significant bone loss indicative of periodontitis. These data demonstrate that in the rabbit ligature model of periodontitis multiple dysbiotic dental plaque communities elicit periodontal disease.

Introduction

Porphyromonas gingivalis (*P. gingivalis*) is a gram negative, anaerobic bacterium that is associated with periodontitis. Periodontitis is a complex disease that arises due to a shift from aerobic Gram-positive bacteria to anaerobic Gram-negative bacteria whose interactions with host cells brings about degradation of host tissues, alveolar bone reduction, chronic inflammatory disease and ultimately tooth loss. Over 600 bacterial taxa have been identified in the oral cavity yet, only a relatively small percentage has been associated with disease (Dewhirst *et al.*, 2010). Although *P. gingivalis* has been shown to cause disease in several different animal models (Oz *et al.*, 2011), the mechanism by which it transforms healthy microbial/ host homeostasis to destructive periodontitis is not clear.

However, two *in vivo animal studies* have implicated commensal bacteria as having a role in *P. gingivalis* induced periodontal disease. The first study, utilizing a rabbit model of periodontal disease showed that inoculation of *P. gingivalis* via ligature leads to an outgrowth of oral bacteria as well as a shift in commensal profile and bone loss (Hasturk *et al.*, 2007). While there was no direct implication that commensal bacteria contributed to disease, it was clear that introduction of *P. gingivalis* caused the outgrowth of the oral bacterial community. In a more recent study, with a mouse model of periodontal disease, *P. gingivalis* infection by oral gavage was followed by an increase of oral bacteria that resulted in bone loss while *P. gingivalis* infected germ-free mice lacking oral bacteria were protected from disease (Hajishengallis *et al.*, 2011). This latter study clearly demonstrated that oral commensal bacteria were required for disease in the mouse gavage model and that *P. gingivalis* orchestrated the shift in the bacterial community from a healthy to a periopathogenic, dysbiotic community. Due to its significant

contribution to commensal bacteria community remodeling resulting in disease it was termed a keystone pathogen (Hajishengallis *et al.*, 2012).

P. gingivalis contains several virulence factors that may contribute to its ability to modulate the oral microbial composition (Bostanci *et al.*, 2012); one of the virulence factors, the lipopolysaccharide (LPS), has been proposed to contribute changes to the oral microbial because of the lipid A structural composition and hence subsequent innate host responses (Darveau, 2009). For example, *P. gingivalis* can alter its lipid A phosphate composition in response to different environmental conditions resulting in lipid A structures that are either agonists or antagonists for inflammatory activation at TLR4 (Al-Qutub *et al.*, 2006, Coats *et al.*, 2009, Curtis *et al.*, 2011a). Alterations in the host environment by modulation of host TLR4 activity can have global effects on the microbial community by enhancing the growth of different members of the oral community (Darveau, 2010).

In this chapter the ability of two *P. gingivalis* mutants that are unable to modulate their lipid A structural composition and display significantly different phenotypes with respect to interactions with innate host components TLR4 and antimicrobial peptides. One mutant expresses a lipid A that is a TLR4 agonist whereas the other mutant expresses a lipid A that is capable of antagonizing TLR4. It is this antagonist structure that could potentially disrupt host cell functions and cause overgrowth of the oral microbiota. Therefore, these two mutants were examined for their ability to create oral dysbiotic communities and induce periodontitis. In addition, LPS isolated from these two mutant strains was also examined. It was found that although both mutants failed to colonize the rabbit periodontium in the ligature induced disease model both the mutants as well as the isolated LPS preparations were able to create dysbiotic oral communities that resulted in periodontitis. These data demonstrate that at least in the rabbit

ligature model of periodontitis multiple different oral dysbiotic microbial communities can disrupt host homeostasis and result in disease.

Results:

Phenotypic characterization of the P. gingivalis lipid A mutants employed in this study

Two phosphatase mutants that result in *P. gingivalis* bacteria that have different lipid A profiles were created to test lipid A phenotypes in the rabbit model of periodontal disease. The initial identification and characterization of *P. gingivalis* lipid A mutants that are unable to modify their lipid A structural composition and display significantly different lipid A structural profiles was performed in strain 33277 (Coats *et al.*, 2009). However, since previous work in the rabbit ligature model was performed with *P. gingivalis* strain A7436, in order to prevent potential undefined strain variability effects in the rabbit model, the lipid A mutants were constructed in strain A7436. The mutant strains, designated PG1587 and PG1773, contain deletion mutations in the lipid A 4' (PG1587) and 1 (PG1773) phosphatase genes, respectively (Coats *et al.*, 2009). Characterization of the lipid A structural composition confirmed that similar to strain 33277, strain A7436 containing these mutations displayed an altered lipid A structural composition in that PG1587 accumulated the di-phosphate lipid A structural peak designated m/z 1770, whereas strain PG1773 accumulated peak m/z 1449 (Fig. 1). This data confirms that the genes PG1587 and PG1773 result in the same alterations in the lipid A structural profile in both *P. gingivalis* strains 33277 and A7436.

Next, the mutant strains and their respective LPS preparations were examined for their TLR4 and TLR2 responses. Similar to the results previously shown in strain 33277 (Coats *et al.*, 2009) TLR4 displayed differing responses to PG1587 and PG1773 in A7436. PG1587 bacteria

as well as their isolated LPS demonstrated strong TLR4 agonist activity (Fig. 2A). Likewise, LPS obtained from PG1773 but not PG1587 displayed TLR4 antagonism (Fig. 2B). Interestingly, and different from that observed in strain 33277 both PG1587 and PG1773 strain in A7436 displayed increased TLR2 activation compared to WT (Fig. 2B). The reasons for this are not known.

Finally, it has been previously reported that PG1587 and PG1773 in strain 33277 display significantly different susceptibilities to polymyxin B, a cationic antimicrobial peptide antibiotic (Coats *et al.*, 2009). Examination of these mutations in A7436 yielded similar results in that PG1587 was exquisitely susceptible to polymyxin B whereas the wild type strain and PG1773 were completely resistant (Fig. 2D). These data confirm that the lipid A phosphatase mutations previously described in strain 33277 display a nearly identical phenotypes when contrasted in A7436. This validates the use of this strain for use in the rabbit study.

Ligature model of periodontal disease shows P. gingivalis induces changes to oral bacteria

The model used in the present study is based on previous work that showed *P. gingivalis* induced changes to oral microbiota including the overgrowth and a shift to predominately anaerobic oral bacteria (Hasturk *et al.*, 2007). This rabbit model of periodontal disease uses a *P. gingivalis* soaked ligature to induce disease. To determine if *P. gingivalis* lipid A modification alters the microbial composition, ligatures were placed on the second premolar of both mandibular quadrants and a slurry was applied topically containing either 10^6 CFU or 35ng LPS from *P. gingivalis* WT or phosphatase mutants, four rabbits per test group as shown in Table 1. Amounts of bacteria used were matched to the previous study and the quantity of LPS used was based on the amount of LPS found in 10^6 bacteria. Samples of plaque from the ligature area were

taken at three time-points including time zero, three weeks and six weeks (see Table 1). A checkerboard DNA hybridization assay was used for a semi-quantitative analysis of a panel of known human oral bacteria. Briefly, known bacterial species are used to detect specific species in an unknown sample by DNA hybridization. This is a quick method to evaluate the presence of many different oral species of bacteria.

Although the DNA checkerboard hybridization has limitations, such as high DNA requirement and species specific probes this particular method allows for the analysis of a large panel of known bacterial species. The checkerboard assay has been compared to the 16S rDNA-based PCR method and found to be comparable in sensitivity for prevalent species although the sensitivity diminished with species at lower concentrations (Siqueira *et al.*, 2002). The panel of bacteria analyzed in this study includes the original panel of eighteen human oral bacterial probes used in the previous rabbit study and eleven additional species.

Porphyromonas gingivalis requires lipid A modulation to colonize and cause overgrowth of commensal bacteria

To examine the *P. gingivalis* colonization and changes to total bacteria, chemiluminesence units from DNA-checkerboard were plotted for plaque samples from each treatment in Figure 3. Remarkably, *P. gingivalis* wild type appears to be the only treatment group to colonize robustly (Fig 3A). PG1587 also displayed a significant increase in *P. gingivalis* colonization at 6 weeks, although much lower than wild type, (Fig. 3A). It is possible that the low chemiluminesence from the uninfected rabbits is indicative of an endogenous strain of *P. gingivalis* that may have increased over the time-course of infection with the PG1587. The test group LPS1587 shows a similar increase however, no bacteria were added and therefore this

increase although not significant, strongly implicates an endogenous strain could be effected by treatments.

Next the relative biomass of the dental plaque was examined for the different experimental groups (Fig. 3B). Biomass was determined by the total chemiluminesence activity found in the dental plaque samples and it was found that plaque samples from each experimental group (except plaque from treatment group LPS 1773ko) showed a drastic reduction in total bacteria at three weeks from uninfected (Fig3B). At six weeks, the colonization of wild type was also the only treatment to cause significant increase in commensal biomass (Fig3B). This result was not found in any other treatment group demonstrating that both lipid A phosphate mutants were unable to either significantly colonize the rabbit periodontium or significantly increase the total dental plaque biomass.

Ph*osphatase mutants exert distinct affects on oral bacterial communities compared to wild type while LPS treatments yield a simple but discrete bacterial profile*

To examine major increases and/or decreases to specific bacteria, the changes in abundance of bacterial species that experienced a significant change ($p \geq 0.01$) after six weeks of treatment were plotted in Table 2. Although wild type *P. gingivalis* was the only treatment group to generate an increase in oral bacteria at six weeks, all experimental groups had affects on the communities of oral bacteria. The increase of total oral bacteria is markedly increased and significant without the inclusion of *P. gingivalis* WT. Of the three species of *Campylobacter* that were identified in uninfected rabbits, two species (*C. concisus* and *C. curva*) were sensitive to almost all experimental groups and showed remarkable reductions. Both bacteria are microaerophillic and therefore, their disappearance/reduction is most likely due to the deep pocket depths that occur during disease progression that result in anaerobic conditions. However,

the presence of *P. gingivalis* wild type did not reduce the *C. concisus* species seen in all other treatment groups. This suggests that the environment created by the modulation of lipid A in wild type *P. gingivalis* may be required for *C. concisus* to maintain its niche; this niche may change to allow some bacteria to bind where others may lose their binding sites. The changes to oral bacteria induced by wild type *P. gingivalis* affected 16 species of the panel of 29 (59% G- and 41% G+). None of the other treatment groups affected as many bacterial species as *P. gingivalis* wild type. However, the phosphatase mutants induced unique profiles predominately in the Gram-negative communities. The changes to bacteria seen from LPS treatment groups were very few and virtually identical indicating a phenotype common to all LPS types caused the discrete changes to the bacterial communities in these treatments.

In order assess the changes to Gram-negative and Gram-positive communities, the relative abundance was plotted in a percentage plot (Fig4A). To better visualize prominent Gram-negative bacteria, *P. gingivalis, C. curva* and *C. sputigena* were separated by color (or pattern). The animals are uninfected at the first (zero) timepoint and while most animals appear to have similar profiles, there is some variability between groups that could be clarified with the use of error bars but where not shown due to the cumbersome column graph. The first major change observed was the reduction is in the Gram-negative community at three weeks in all treatment groups, which was principally due to the marked decline of *C. curva*. At the end of six weeks, we see the *P. gingivalis* wild type profile is predominately made up of Gram-negative bacteria; this can be attributed largely to the colonization of *P. gingivalis*. In addition, low abundance of *C. sputigena* was seen in uninfected rabbits, and again at three weeks however at six weeks, the LPS treatment groups show a remarkable increase in *C. sputigena* while the wild type and PG1587 show decreases. The LPS treatment groups show no significant change in

Gram-negative percentages from uninfected to 6 weeks since the dramatic loss of *C. curva* is evidently made up by the increase in *C. sputigena* masking overall changes to Gram-negative bacteria while the phosphatase mutant bacteria and wild type have reduced amounts of *C. sputigena* in addition to the reduction of *C. curva*.

To examine the effect of treatment groups on *C. sputigena*, chemiluminesence units were plotted in Fig4B. All three LPS treatment groups yield remarkable increases in *C. sputigena* while the bacterial treatment groups see a marked reduction (Fig4B). This phenomenon could be due to particular host-response mechanisms specific but different for bacteria and LPS; please see discussion for details.

Clinical parameters of disease show similar bone loss for all treatment groups

In order to evaluate amount of periodontal disease caused by *P. gingivalis* or LPS treatments, clinical disease parameters including pocket depth (distance between crest of the gingiva and soft tissue attachment on the tooth), intrabony defect depth (vertical dimension of bone loss), tooth mobility by morphometric analysis were evaluated and osteoclast activity was assessed by histological staining of tissue samples. Osteoclasts were identified by positive staining for their tartrate resistant acid phosphatases (TRAP). Strikingly, all experimental groups show similar levels of bone loss. The only difference found was in the intrabony defect assessment; treatment groups PGWT, PG1773 and LPS1773 had significant bone loss over ligature alone while other differences were not statistically significant. It is interesting that this difference was common to all treatments that had predominately the 1-phosphorylated, tetra-acylated lipid A structure capable of inhibiting host responses. However, all other parameters of disease show similar bone loss and osteoclast activity between treatment groups. Together, these

findings indicate that regardless of etiology, changes to lipid A or bacterial communities, all treatment groups cause similar periodontal disease.

Discussion

P. gingivalis has a number of virulence factors that contribute to disease such as proteases (gingipains), lipopeptides, fimbriae, hemagluttinins and lipopolysaccharide. Although some of these factors have been explored (Bostanci *et al.*, 2012), the function of *P. gingivalis* unique heterogeneous LPS in disease *in vivo* has not been examined until now. In the current study, the use of phosphatase mutants that are unaffected by environmental stimuli and fixed in their lipid A moiety made clear that *P. gingivalis* mutants that are unable to remodel their lipid A structural composition do not colonize well or induce overgrowth of the oral microbiota. However, the *P. gingivalis* mutants and their respective isolated LPS preparations induced significant changes in the oral bacterial communities and caused similar disease profiles. This observation supports the idea that multiple different microbial compositions result in dysbiosis and disease (Darveau, 2010).

Modifications to lipid A resulted in phenotypic changes. We have previously reported that PG grown in high temperature or lacking the 1587 phosphatase can result in bacteria susceptible to antimicrobial peptides (AMPs) (Coats *et al.*, 2009, Curtis *et al.*, 2011a). A potential explanation for this may be revealed by a recent study of *Helicobacter pylori* lipid A phosphatases that showed the location of the phosphate group determines susceptibility to polymyxin B (Cullen *et al.*, 2011). Perhaps the dominant lipid A structure possessing the phosphate at the 1 position in the PG1773 and *P. gingivalis* wild type confers resistance to polymyxin B whereas the lipid A with the 4'-phosphate is susceptible. Further, we recently demonstrated that P. gingivalis grown in higher temperatures had a lipid A with a 4'-phosphate

that was susceptible to human β-defensins 2 and 3 (Curtis *et al.*, 2011a). Since PG1587 was susceptible to polymyxin B, it was postulated that this mutant could be resistant to anti-microbial peptides *in vivo*. Interestingly, despite the antimicrobial peptide resistance of PG1773, it was unable to colonize. Presumably the phosphatase mutants could not colonize due to the inability to modify the lipid A moiety but for reasons not associated with antimicrobial peptide sensitivity. However, other pleiotropic effects have not thoroughly been examined. For example, we have demonstrated here that TLR2 activities are different between whole bacteria wild type and phosphatase mutants; presently, there is no explanation for this and therefore more investigation is required. Regardless of phosphatase phenotype or ability to colonize, all bacteria treatments caused changes to bacterial communities.

The changes to bacterial communities were very different between the PGWT, PG1587 and PG1773. PGWT showed the most complex changes to oral microbiota. All changes that were seen in PG1587 and PG1773 were shared with PGWT. For example, increases of *T. denticola, F. Ss Vincetii* and *V. parvula* were common to PGWT and PG1587 while rises in abundance of *E. coli, L. acidophilus* and *P. bivia* were caused by PGWT and PG1773. It is important to note that the increase of bacteria associated with PG1587 and PTWT are predominately part of the 'red complex' of bacteria described by Socransky but the bacteria common between PGWT and PG1773 are not (Socransky *et al.*, 1998). Perhaps a shared phenotype common to PGWT and PG1587 produced a suitable environment for the 'red complex' while a shared phenotype of PGWT and PG1773 caused an environment favorable for commensal bacteria. For example, the agonist lipid A from PG1587, also produced by PGWT when environmental conditions allow (Al-Qutub *et al.*, 2006, Coats *et al.*, 2009) could elicit an inflammatory host response favorable to the 'red complex'. Alternately, the antagonist lipid A common to PGWT and PG1773 could

inhibit the host response and allow an outgrowth of commensal bacteria. Interestingly, PGWT, PG1773 and LPS1773 treatment groups caused similar intrabony defects again indicating a shared phenotype such as the antagonist lipid A structure; it is possible that this particular lipid A structure may be directly involved in disease progression since there are similarities between these treatment groups disease profiles but not bacterial profiles. However, the abundance and species of oral bacteria that resulted from each treatment were distinctly different from each other yet all resulted in disease. In short, the agonist lipid A from PG1587 could result in an environment favorable for 'red complex' bacteria while the suppressive-TLR effects of PG1773 could inhibit host response that allow for outgrowth of commensal bacteria, which all contribute albeit slightly differently to disease.

LPS treatments do not appear to have any specific alterations rather; similar changes are made to bacterial profiles indicating a general environmental alteration. The striking feature common to all purified LPS was the robust TLR2 activity due to a recently discovered a lipopeptide contaminant in P. gingivalis LPS that appears in phosphatase mutants as well (S. Jain *et al.* IAI in press 2013); this alteration of host-environment could be the cause of the similar bacterial profiles observed. Further, the LPS treatments appear to alter the environment in a way that is beneficial to *C. sputigena* not seen in the whole bacteria preparations.

The present study indicates that lipid A modulation is a likely component in *P. gingivalis* colonization and commensal overgrowth however, modulation does not appear to be the cause for disease. In a recent study using a mouse model of periodontal disease by gavage, it was shown that *P. gingivalis* caused an overgrowth in commensal bacteria (Hajishengallis *et al.*, 2011) that contributed to progression of disease. This was the first study that implicated a direct role for commensal bacteria in periodontal disease. The rabbit model for this work was chosen

because of previous work that revealed infection with *P. gingivalis* could induce an increase of specific oral microbes and it was thought that changes to the lipid A moiety might create an environment that would benefit other microbial communities. However, the current study suggests that despite major differences in TLR activities and ability to colonize, all treatments caused microbial dysbiosis that resulted in very similar disease. This provides evidence for the first time that multiple different bacterial communities can result in periodontal disease.

The results presented here are very interesting for a number of reasons; it was unexpected all treatments would cause similar disease or change the profile of bacteria so profoundly. It appears that the ligature model is a very severe alteration that manifests in bone loss as well as differences to microbial composition in every treatment case. It is possible that this model of periodontal disease is particularly sensitive and therefore it will be interesting to explore similar studies in other models. In the following chapter, I have chosen to discuss future directions in more broad terms due to the apparent sensitivity with the rabbit ligature model. Fortunately, there is much work to understand the role of commensal bacteria in periodontal disease and many more models to be explored.

Figure 1

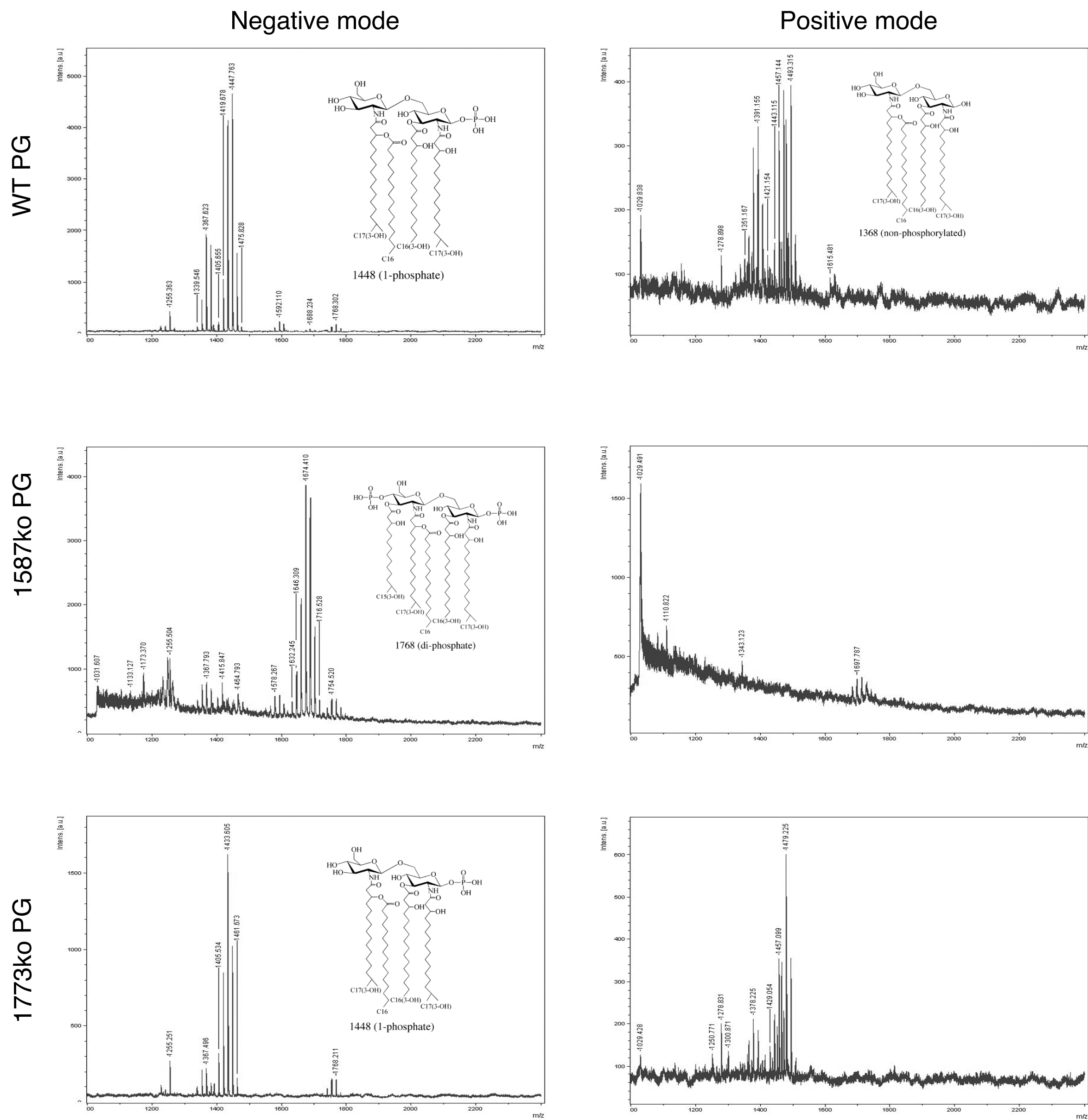


Figure 1 *P. gingivalis* expresses both lipid A 4′- and 1-phosphatase activities. Lipid A isolated from wild-type *P. gingivalis* A7436 or mutant bacteria bearing deletions in PG1587 and PG1773 gene loci were examined by MALDI-TOF MS to elucidate the position of the lipid A phosphates.

Representative structures of lipid A corresponding to peak are shown. Lipid A samples were examined in the negative ion mode or the positive ion mode for each sample.

Figure 2

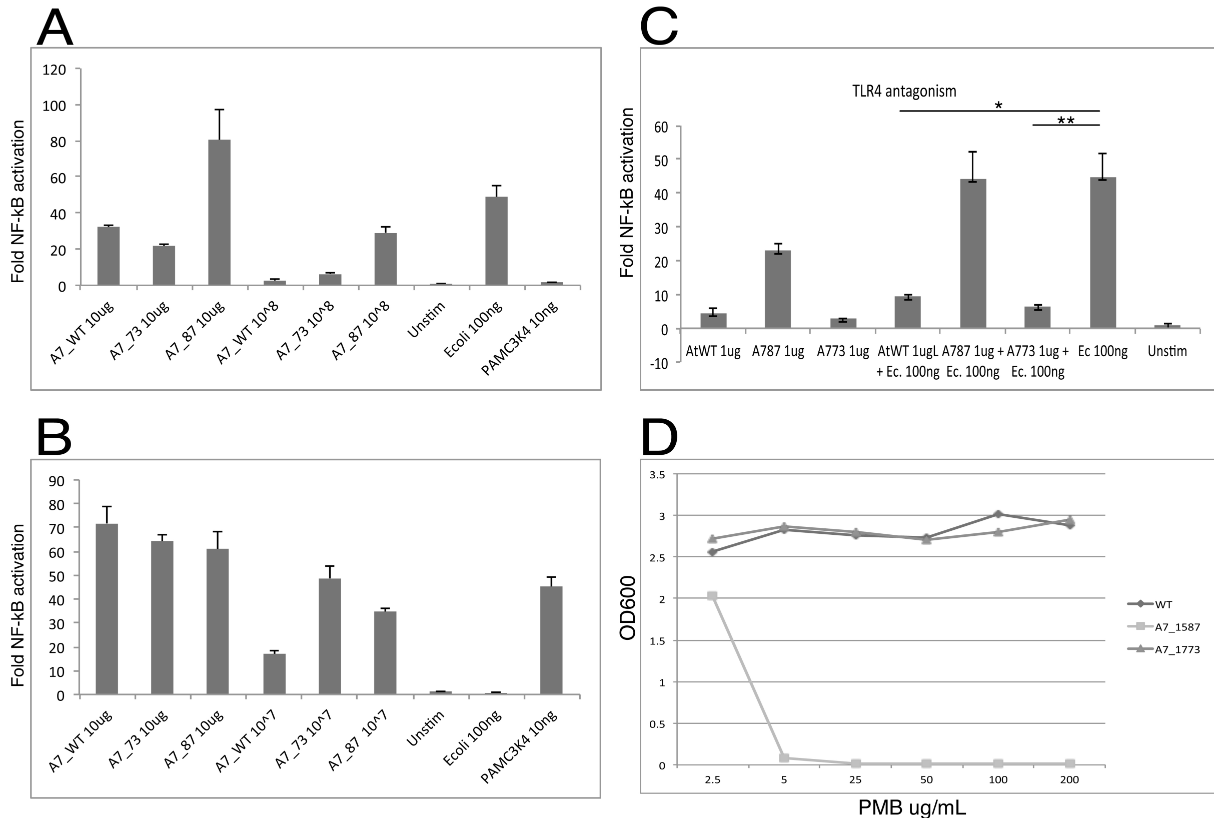


Figure 2 Lipid A phosphatases allow *P. gingivalis* to evade host innate immune differences. Phosphatase mutants have different phenotypic host innate effects through toll like receptors and antimicrobial susceptibility. HEK293 cells expressing either human TLR4 and MD-2 (A, C) or TLR2 and TLR1 (B) were exposed to the indicated doses of LPS or whole bacteria for 4 h. The fold activation of NF-kB over the media control was determined by measuring inducible Firefly luciferase activity. Positive control for TLR4 is E. coli and TLR2 is PamC3K4. The results shown are means ± SD of triplicate samples from one of three independent experiments. Asterisks indicate statistically significant differences ($P < 0.001$; two-tailed unpaired *t-tests)* in the potency of TLR4 antagonism by PGWT or PG1773. (D) Lipid A phosphatases confer *P.*

gingivalis resistance to killing by antimicrobial cationic peptides. The indicated strains of *P. gingivalis* were plated on TYHK-agar plates containing polymyxin B (PMB) (0, 5 and 200 mg ml-1) and measured by spectrophotometry OD600.

Table 1

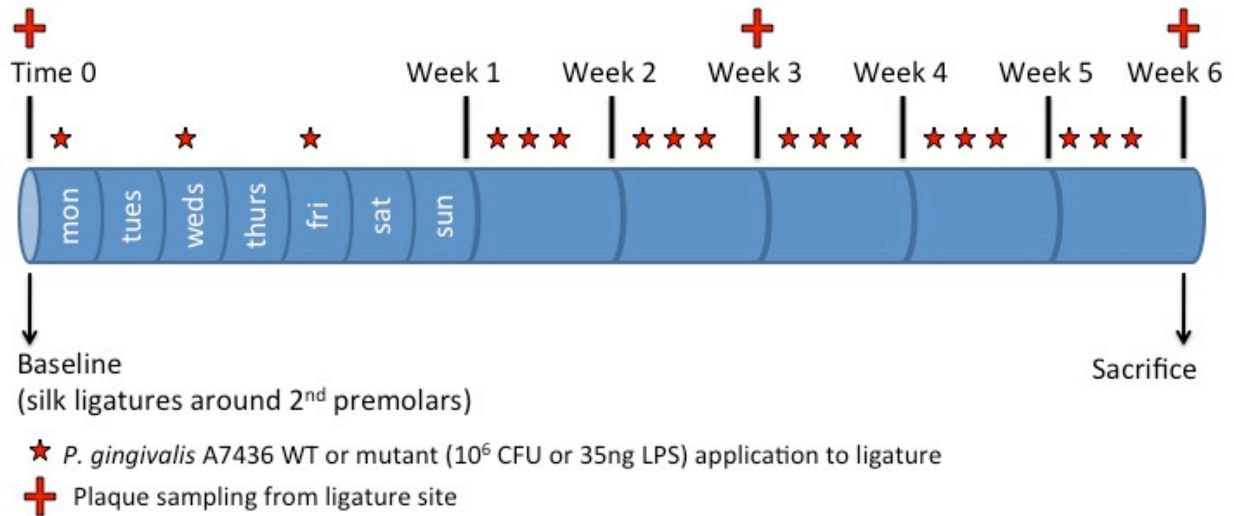


Table 1 Timeline of experimental design. 3-0 silk ligatures were tied to second premolars in mandibular quadrants at baseline in all groups, and *P. gingivalis*, phosphatase mutants or purified LPS was applied in methylcellulose slurry three times per week (M-W-F) for 6 wk. Plaque samples were taken for microbial analysis at 0, 3 and 6 weeks during treatment. At 6 wk, all were sacrificed, and the extent of disease was determined.

Figure 3

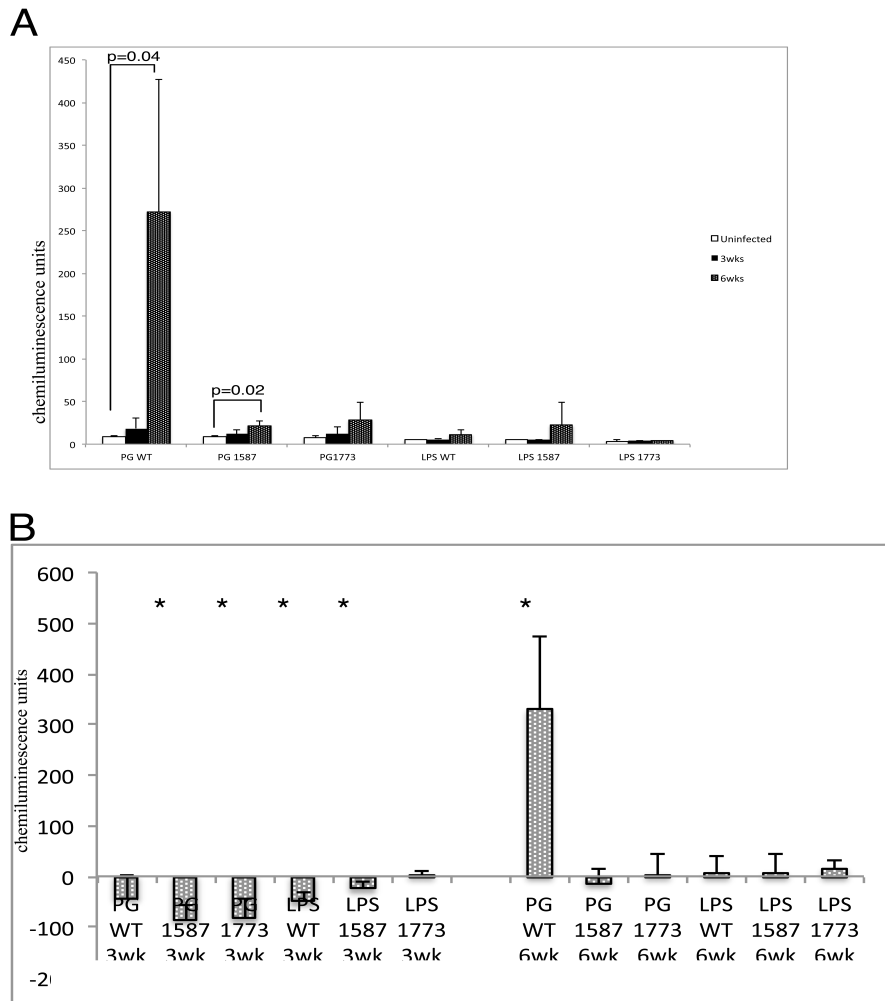


Figure 3 *Porphyromonas gingivalis* phosphate modulation is required for colonization and commensal overgrowth. Chemiluminescence units of DNA-DNA checkerboard hybridization assay specific to (A) *P. gingivalis* or (B) the summation of all bacteria analyzed (see *Materials and Methods*) ($P < 0.01$; Mann-Whitney Test)

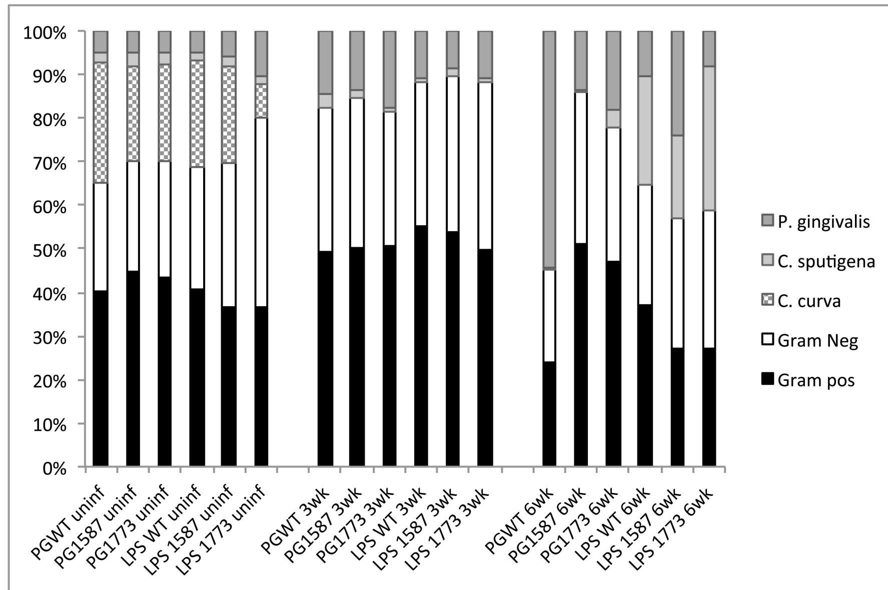
Table 2

	G- bacteria												G+ bacteria				
	P. gingivalis	***C. concisus***	***C. rectus***	***C. curva***	***T. denticola***	***C. ochracea***	***E. coli***	***C. sputigena***	***P. bivia***	***F. Ss Vincetti***	***S. noxia***	***V. parvula***	***S. sanguis***	***P. micros***	***A. viscosus***	***F. alocis***	***L. acidophilus***
WT bacteria	+		+	-	+	+	+	-	+	+	+	+	+	+	+	+	+
1587ko bacteria	+	-		-	+	+		-		+	+	+	+				
1773ko bacteria		-		-		+	+		+		+		+				+
WT LPS		-		-				+									
1587ko LPS		-		-				+									
1773ko LPS		-		-		+		+									

Table 2 Significant changes to chemiluminescence signal from specific oral bacteria at 6 weeks compared to uninfected controls ($P < 0.01$; Mann-Whitney Test)

Figure 4

A



B

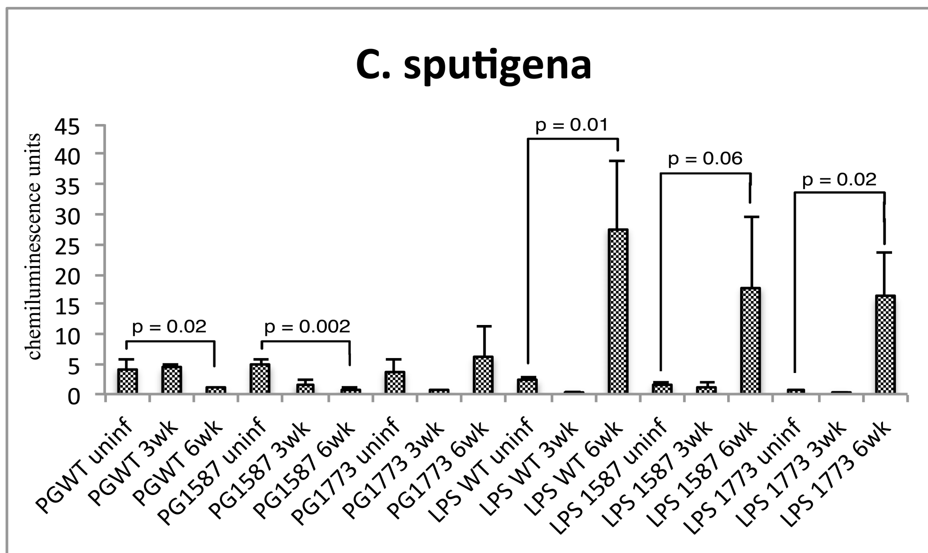


Figure 4 Distinct changes to bacterial communites result from application of *P. gingivalis*, phosphatase mutants or LPS. Relative biomass of Gram-negative bacteria including *P. gingivals, C. curva* and *C. sputegina* and Gram-positive bacteria profiles (A) and chemiluminescence units

of *C. sputegina* plotted for each treatment group over time. Statistics were scored using Mann-Whitney Test

Figure 5

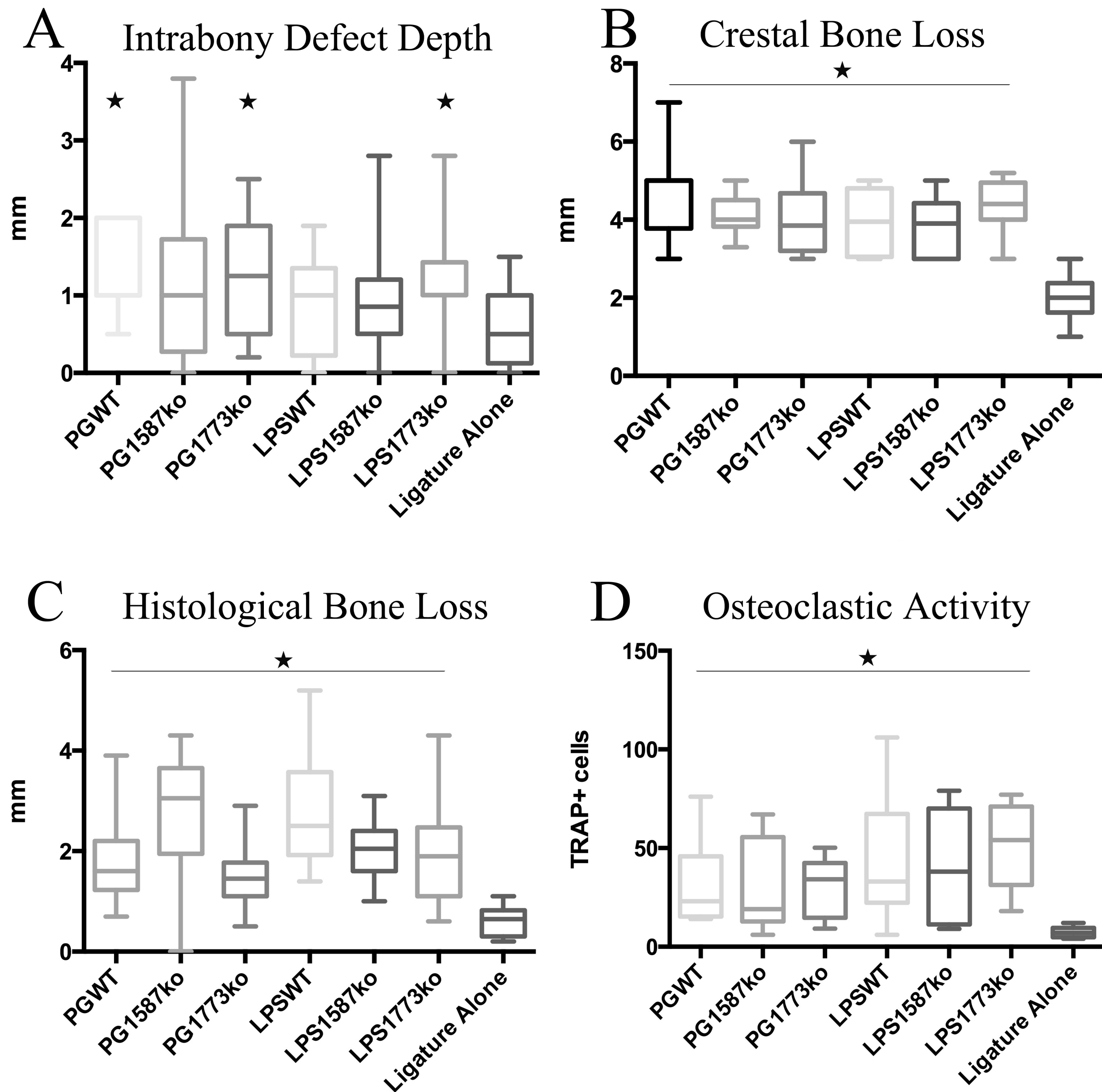


Figure 5 Analyses of periodontal disease from P. gingivalis, phosphatase or LPS-treated rabbit lesions. Alveolar bone loss for all animals was directly measured on defleshed jaws (see *Materials and Methods)* for characteristics of human periodontitis including soft (A, intrabony defect) and hard tissue (B, crestal bone loss) destruction. Rabbit mandibles were harvested and prepared for histologic analysis (see *Materials and Methods).* Histologic analysis and

quantification of histomorphometric changes (C). Osteoclastogenesis in the alveolar bone was assessed by TRAP staining (D) (see *Materials and Methods)*. Asterisks indicate statistically significant differences ($P < 0.01$; Mann-Whitney Test)

Chapter 4 Future directions

Summary and Future directions in Health, Dysbiosis and Disease

In the previous chapters, I have shown that commensal bacteria have a function in host innate immune status in healthy periodontal tissue by influencing migration of neutrophils and induction of associated neutrophil chemokines. I have also shown in another animal model of disease that disruption of the commensal polymicrobial community by *P. gingivalis* or its purified LPS results in dysbiosis and disease. These results demonstrate that commensal bacteria can modulate both beneficial and destructive host responses. Recently, it has been shown in a mouse gavage model of disease that *P. gingivalis* can disrupt the commensal polymicrobial community creating a dysbiosis similar to that observed in the rabbit (Hasturk *et al.*, 2007). These new findings, combined with the work presented here challenge the notion of the traditional disease-by-pathogen causal relationship. Rather, the 'pathogen' appears to disrupt the innate host defense system, which in turn, causes dysbiosis to the commensal bacteria that ultimately results in disease. In the following pages, I will explore possible future studies of oral polymicrobial-host interactions to clarify their function in both health and disease; due to the knowledge obtained in chapter 2, the first section will highlight specific examples of future work and methods however, very little is understood about dysbiosis and disease in the oral cavity and therefore the subsequent section on will be more of a discussion about how future endeavors could be explored

Section One: Commensal Bacteria and Neutrophils in Health

Neutrophils are required for oral health as evidenced by people with neutropenia (and other neutrophil disorders) who are predisposed to periodontal disease yet they can also contribute to disease (Nussbaum *et al.*, 2011). In chapter two, commensal bacteria were shown to be responsible for an influx of neutrophils in healthy tissues however, the collaborative study mentioned above (Hajishengallis *et al.*, 2011) suggested that P. gingivalis caused dysbiosis of commensal bacteria by the subversion of the host immune system; likely affecting the ability of neutrophils to kill invading bacteria facilitated the outgrowth of all commensal communities and ultimately caused disease (Hajishengallis *et al.*, 2012). Due to the pervasive requirement of neutrophils in health and disease it is imperative to understand the regulatory components of commensal activation of neutrophils in health.

In Chapter two, we explored two different ligands for CXCR2, CXCL1 and CXCL2 and found that while both are expressed constitutively, one, CXCL2 is increased by exposure to oral commensal bacteria; this results in an increase of neutrophils into the junctional epithelium. Additionally, CXCR2 is required for neutrophil homing into these same tissues. While we now know that commensal bacteria contribute, at least in part to neutrophils found in the gingiva, there are questions that follow. Are there other CXCR2 ligands present in the gingival tissues that participate in neutrophil homing? Are all or a select few of the commensal bacteria responsible for the induction of additional CXCR2 ligands? What cells in the gingival tissues are responsible for expression of neutrophil chemokines? Are they the same cells that are responsible for the constitutive expression of ligands found in germ-free conditions? Several different approaches can be utilized to better understand the mechanisms of cytokine expression required for neutrophil homing into the periodontium.

CXCR2 ligands

There are several different ligands known to bind CXCR2 and although originally thought to be redundant, more recent studies have shown these ligands to be both temporally and spatially expressed in a variety of mice in homeostasis, inflammation and during bacterial infection. In a demonstration of non-redundancy, three of the most well studied CXCR2 ligands, CXCL1 (KC), CXCL2 (MIP2) and CXCL5 (LIX) have been shown to have different affinities for the CXCR2 receptor; this creates a hierarchy of neutrophil chemotaxis and activation (Ritzman *et al.*, 2010, Rovai *et al.*, 1998). Further, these three ligands differ in kinetics of expression, both temporally and spatially in a mouse model of sepsis, which could be indicative of functional differences between ligands (Mei *et al.*, 2012). In an experimental mouse model of Lyme-associated arthritis, all three ligands are increased in their expression however; the depletion of just CXCL2 caused mice to be resistant to associated disease (Hernandez *et al.*, 2011). Lastly, CXCL5 is responsible for the regulation of circulatory neutrophil homeostasis in the intestine but not lung (Tester *et al.*, 2007). These data demonstrate that although these chemokines signal through the same receptor, their biological functions may be very different.

To date, very little is known about neutrophil homing in the periodontium. In addition to data presented in the previous chapter, another research group examined contribution of CXCR2 ligand, CXCL5 in periodontitis; they observed that MMP8 (a neutrophil collagenase) deficient mice gavaged with *P. gingivalis* had significantly reduced CXCL5 expression (Hernandez *et al.*, 2011). It was proposed that the disease severity seen in the MMP8 deficient mice was conceivably due to the reduced capacity of MMP8 to cleave CXCL5 and allow the more active form of peptide to sequester neutrophils into the gingival papilla (located in the interdental spaces between teeth). This is potentially another mechanism by which neutrophil homing exists

in the periodontium. Together these data suggest that there may exist several CXCR2 chemokine ligands in the periodontium that contribute to neutrophil recruitment and activity.

Future studies will need to address other CXCR2 ligands in the gingival tissues. In an effort to address this issue, we looked in the junctional epithelium for CXCL5 expression but were unsuccessful. As a control for the antibody, we stained the interdental gingival papilla as previously published (Hernandez *et al.*, 2011) and found a very strong staining pattern. It appears that localized expression of CXCL5 is specific to the interdental area. We are now working on a more thorough approach that involves staining sequential slides through one entire tooth that would include junctional epithelium (both buccal and lingual) as well as gingival papilli (both mesial and distal) for the major CXCR2 ligands (CXCL1, 2, 5 and 7) and neutrophils in both germ free and SPF animals. This approach will aim to elucidate spatial expression of the major CXCR2 ligands and neutrophils with regard to commensal bacteria.

Oral bacteria: who modulates neutrophil migration and CXCR2 ligand expression?

There are many documented examples of commensal bacteria inducing innate host defense systems; one such organism is *Bacteroides thetaiotaomicron* that inhabits the human gut. Within this niche, *B. thetaiotaomicron* induces angiogenesis that increases the capillary network within the intestinal tissues, which allows more blood and nutrients to flow into tissues. Most of our knowledge of commensalism comes from the studies of the intestinal system. A key contribution of studies using germ free mice has been the elucidation of particular bacteria with regard to normal intestinal development and function (Falk *et al.*, 1998, Gordon *et al.*, 1971, Hooper *et al.*, 2001, Komatsu *et al.*, 2000, Macpherson *et al.*, 2004, Rakoff-Nahoum *et al.*, 2004, Stappenbeck *et al.*, 2002, Umesaki *et al.*, 2000, Xu *et al.*, 2003). Indeed, it has been revealed

that intestinal bacteria are recognized by TLRs and contribute to intestinal epithelial homeostasis through the MyD88-pathway (Cebra, 1999).

In a similar manor, I was able to identify that oral commensal bacteria required MyD88- for the increase in CXCL2 and neutrophils into oral tissues. Ideally, we would continue this work and identify one or a group of bacteria responsible for this observation. The SPF mice that we used in the study had a very simple oral microbiota that has been fully characterized and isolated by culture (Hajishengallis *et al.*, 2011). We have also determined in a preliminary co-caging experiment where GF mice co-caged with SPF mice for greater than two months obtained similar bacteria and also acquired similar numbers of neutrophils when compared to WT-SPF mice. The bacteria identified consist of species from Streptococcus, Enterococcus, *Escherichia coli*, Lactobacillus, Staphylococcus and Propionbacterium; from these groups, the isolated pure cultures obtained from the SPF characterization study will be used in add-back experiments in germ free mice. The bacteria will be applied as single species by the gavage Baker model of infection (Wolff *et al.*, 1985). In the event that none of the individual species may result in modulation of host neutrophil migration or CXCR2 ligand expression; combinations of different genera will be combined and applied to the mouse model to determine the necessary community that is necessary to obtain normal neutrophil trafficking. Confirmation and quantification of gavaged species will be determined by CFUs.

Mechanism of host induction to neutrophil migration

As stated previously, the periodontium is protected mainly by the presence of neutrophils in the gingival crevice where bacteria accumulate; the neutrophils serving to protect the tissues from microbial attack. The host mechanisms involved in neutrophil homing into the periodontium are not completely understood. A series of knockout mice deficient in responding

to microbial signals either through the TLR family (Kawai *et al.*, 2005) or inflammasome mediated mechanisms (Henao-Mejia *et al.*, 2012) can be utilized to identify host pathways involved. Using the methods explored above (IHC, *in situ* hybridization of RNA and co-localization of neutrophils, CXCR2 ligands, TLR and IL-1bR) on gingival samples from MyD88KO, TLR2KO, TLR4KO, TLR2+4DKO, caspase-1KO and a triple knockout TLR2/4 and caspase-1 will cover possible pathways involved in bacterial induction of host tissues.

Differences in neutrophil enumeration and expression of CXCR2 ligands in the junctional epithelium should be anticipated between the various groups of knockout mice. As seen in the MyD88ko; this receptor co-adapter protein is required for all TLRs except TLR3 and TLR4, which can also use the TRIF co-adapter as well as three of the IL-1 receptors (Kawai *et al.*, 2005). Therefore, the results of a study like this will further define neutrophil migration and CXCR2 ligand expression by determining if the TLR2 and/or TLR4 pathways directly contribute to neutrophil migration or CXCR2 ligand expression. In addition, caspase-1ko mice will determine if IL-1b contributes to neutrophil migration or CXCR2 ligand expression by inhibiting the inflammasome mediated processing of the IL-1b precursor (Henao-Mejia *et al.*, 2012). The inclusion of the caspase-1ko is particularly relevant for two reasons; it has been recently shown that the intestinal bacteria of caspase-1KO mice are significantly altered (Elinav *et al.*, 2011) and therefore it will be interesting if inflammasome sensing in the periodontium will result in modulation of neutrophil migration or CXCR2 ligand expression. Second, if it is found that IL-1b contributes to neutrophil migration and CXCR2 ligand expression, this will indicate that bacteria influence an indirect pathway for modulation of host defense. The comparison of neutrophil migration and CXCR2 chemokine ligands in this series of knockout mice will determine both the contribution of direct or Il-1b indirect activation pathways. Specifically,

commensal bacteria may activate neutrophil migration through direct interactions with junctional epithelial cells or indirectly through activation of monocytes and/or neutrophils that results in secretion of IL-1b which then induce CXCR2 ligand expression from neighboring epithelium. These data combined with the co-expression experiments examining CXCR2 ligands, TLR2, TLR4 and IL-1R expression will provide evidence for direct, indirect or both methods of gingival cell expression of chemokine ligands.

Finally, the studies proposed above will provide insights into understanding key protective periodontal processes. The focus of analysis is centered on neutrophil migration and CXCR2 chemokine ligand expression since these have been shown to be required for healthy periodontal homeostasis. The final analysis of IHC and *in situ* hybridization of RNA of neutrophils and CXCR2 ligands as well as examination of co-localization of TLRs, IL-1bR and CXCR2 ligands will provide information on the contribution of individual bacterial species on neutrophil transit in the periodontium. The discovery of other CXCR2 ligands that may participate in neutrophil homing, as well as the identification of cells and commensal bacteria responsible for the induction of additional CXCR2 ligands will further the current understanding of host homeostatic mechanisms, specifically in the periodontium where neutrophil transit is a major protection mechanism. This information will significantly improve our appreciation of commensalism in the oral cavity and may ultimately lead to novel diagnostic and therapeutic intervention strategies.

Section Two: Polymicrobial communities in Dysbiosis and Disease

Dysbiosis of commensal bacteria has been shown to result in disease and is now recognized as a property of the microbiota in several complex gastrointestinal chronic diseases

(Frank *et al.*, 2011). The most persuasive evidence comes from studies that utilize the transplantation of disease-associated (gut) microbiota into healthy germ free animals and demonstrate a reproducible disease phenotype; such has been demonstrated for conditions including adiposity, metabolic syndrome and colitis (Spor *et al.*, 2011). Progress in the understanding of the involvement in microbial dysbiosis with regard to periodontal disease has trailed behind progress in other areas of gastrointestinal studies. This is partly due to issues of obtaining suitable animal models for periodontal disease that would allow the study of total microbiota influence on disease in a well-characterized genetic background. Further confounding this issue, are the findings from recent high throughput studies that do not require culture techniques; characterization of the human oral microbiota has revealed a more diverse microbiota than previously recognized, including some un-cultivated species that are highly associated with disease (Keijser *et al.*, 2008, Wade, 2011, Zaura *et al.*, 2009). The widely accepted belief is that disease results from the interaction between a microbial challenge and a deregulated host immune response. Through numerous studies, it is evident that the oral microbiota changes extensively in composition during the transition from health to disease as we saw in chapter three from the ligature model in the rabbit revealed that different agents (whole bacteria or purified LPS) produced distinctive changes to oral bacteria and all animals developed similar disease. However, it is unknown if the changing microbial community is a consequence or cause of disease.

The subversion of host immunity is a probable component for dysbiosis. Extensive compositional analysis of dental biofilms have identified potential periopathogens, designated the ‘red’ complex (Socransky *et al.*, 1998). These bacteria have a shared characteristic in their ability to subvert the host immune response. This has led to the speculation that these bacteria

may be able to disrupt periodontal innate defense functions that would otherwise control and maintain the commensal bacteria, allowing an uncontrolled environment beneficial for the overgrowth of all oral microbiota (Darveau, 2010). In one example, it was shown that *P. gingivalis* caused the disruption and overgrowth of commensal bacteria in SPF mice by the subversion of the complement system. C5aR and C3aR knockout mice were inoculated with *P. gingivalis* but did not result in the accelerated bone loss seen in SPF mice. Further, these knockout mice had more bone than the SPF uninfected mice, similar to the findings in germ free mice indicating a component in the complement system is responsible for the commensal-associated bone loss seen in SPF mice (Hajishengallis *et al.*, 2011). Interestingly, *P. gingivalis* has a protease that acts like the C5a convertase that can result in a population of neutrophils with a reduced capacity to kill invading bacteria (Hajishengallis *et al.*, 2012). This subversive quality of *P. gingivalis* could alter host function and allow the outgrowth of oral microbiota and cause disease.

A dysbiotic oral commensal profile may be responsible for disease progression. Dysbiotic commensal profiles have been shown to cause similar phenotypes from host to recipient using transplantation studies. For example, in the gut microbiota, transplantation of microbiota from obese mice has been shown to cause twice the weight gain in germ free mice than lean donors (Turnbaugh *et al.*, 2006). Although no such studies have been performed in the mouth, the oral commensal bacteria have been shown to participate in disease-associated bone loss. A recent study (Hajishengallis *et al.*, 2011) showed that despite low colonization of *P. gingivalis* (0.1% of total microbiome), exposure to *P. gingivalis* caused distinct changes to the microbiota and significant increase to the total culturable oral commensal bacterial. The resulting overgrowth of bacteria resulted in accelerated periodontal bone loss that was not seen in germ free animals

inoculated with *P. gingivalis*. The results of this study describe a distinct role for commensal bacteria in disease. Although it is unknown if disease and dysbiosis occurred from the high exposure of the *P. gingivalis* inoculum or the low level of colonization, it is clear that *P. gingivalis* induced dysbiosis that caused disease. The results detailed in chapter three also show that the dysbiosis and disease occurred from each treatment (whole bacteria or purified LPS) regardless of *P. gingivalis* colonization. It is possible that the dysbiotic microbial communities formed by the application of different agents (LPS) or different bacteria ('periopathogens') are the cause of disease. Therefore, studies that utilize microbiome transplantation into germ free animals are needed to explore pathologies of dysbiotic oral microbiota.

The presence of a periopathogen is not a requirement for dysbiosis and disease; genetic traits also influence health or susceptibility to disease and can be a factor in shaping the commensal microbiome in both humans (Benson *et al.*, 2010) and knock-out mice. For example, mice deficient in a receptor generally known for the recognition of Gram-positive bacteria, TLR2 have a defect in insulin resistance. This phenomenon has been linked to their gut microbiota, which has been shown to be very different from wild type mice (Caricilli *et al.*, 2011). The transplantation of fecal bacteria from TLR2-deficient mice into wild type mice can cause the same phenotype of insulin resistance suggesting that the dysbiotic microbiota are the cause of insulin dysfunction. It was discovered in 2000 that laboratory mice of different backgrounds were either susceptible or resistant to periodontal disease (Baker *et al.*, 2000). Further examination of these animals revealed a tractable genetic basis that could be exploited to identify loci important in host susceptibility or resistance. Additionally, mice with genetic susceptibility or resistance also show altered microbiota in the oral cavity. The plaque harvested from mice examined above each had a unique profile of oral bacteria. It is possible that the commensal

bacteria from genetically altered mice that are susceptible or protected from periodontal disease are a contributing element of health or disease and could be used to examine dysbiosis in future studies. To understand what role the oral commensal microbiota play during health and disease, further examination of the effect of genotype on oral microbiome selection is needed.

In fact much more work is needed to better understand commensal influence on disease; first would be to utilize high throughput sequencing to fully characterize the oral microbiota in the mouse. Although some characterization studies have been done, it has been found through unpublished work that the microbiota of the mouse can change depending on strain background or if the mice were grown in different facilities or purchased from different suppliers. Once a mouse colony has been established and kept in the same facility, the oral microbiota appears to remain highly stable (unpublished). After characterization studies on the stable microbiota are complete, microbiome transplantation studies can follow using germ free mice. For example, to further define the *P. gingivalis* gavage-model of disease, it will be important to determine if the altered plaque with low *P. gingivalis* colonization is the cause of disease instead of the *P. gingivalis* bacterium. To address this, transplantation of dysbiotic or altered plaque can be transferred into germ free mice to determine if similar disease phenotype is observed in the absence of *P. gingivalis*. In summary, the characterization of the microbiota in an established mouse colony is necessary to create a foundation for experiments that utilize the transplantation of disease-associated (oral) microbiota into healthy germ free animals to demonstrate a reproducible disease phenotype; these will be important studies to advance the understanding of the involvement in microbial dysbiosis to periodontal disease.

In closing, future studies to elucidate the role of commensal bacteria in health as well as disease will be paramount for a better understanding of periodontal disease. As we have seen, the

commensal bacteria appear to play an important part in the regulatory process of neutrophil migration in health even though it comes as a cost to the host by a slow progression of bone loss; conversely, dysbiosis of the commensal community is a likely cause of accelerated bone loss in periodontal disease. The new paradigm of thinking about periopathogens as ‘keystone’ species in dysbiosis, instead of pathogens that cause disease directly is already having an effect on potential treatments for periodontal disease. For example, it was shown that P. gingivalis caused commensal dysbiosis and overgrowth, which led to disease by commensal bacteria (Hajishengallis *et al.*, 2011). This effect was abolished in germ free and complement receptor knockout mice. Due to these recent findings, the authors decided to try a proof of concept; in their very recent study, they show that treatment of periodontal disease using a C5aR antagonist abrogated bone loss in SPF mice (Abe *et al.*, 2012). Other approaches are also being employed to treat microbial dysbiosis such as the application of probiotics, which have shown some success in both animals and humans (Berezow *et al.*). These are early studies that have yet to show clinical efficacy however, it is evident that treatment regimes are changing with a new appreciation for the effect of commensal bacteria in periodontal tissues.

Chapter 5: Materials and methods

Experimental procedures for Germ free, SPF animals and immunohistochemistry of neutorphils and CXCR2 ligands:

Animal (Mouse) resource:

All animal procedures described in this study were approved by the institutional animal care and use committees, in compliance with established federal and state policies. Germ-free (GF) C3H/Orl mice (Charles River Laboratories International) or CXCR2-/- mice on BALB/c genetic background (The Jackson Laboratory) were maintained in isolators at the Royal Veterinary College, University of London. The sterility of GF animals was examined by aerobic and anaerobic culture of oral swabs and fecal pellets on nonselective media and by PCR using universal 16S primers. Specific-pathogen-free (SPF) mice (below) were maintained in individually ventilated cages at the animal care facilities of Queen Mary University of London. Original GF mice were divided; half were raised and propagated in conventional cages, thus creating the SPF. MyD88KO mice and wild-type littermate controls (C57BL/6) mice were raised under specific-pathogen-free conditions in individually ventilated cages at the animal care facility of the University of Louisville.

Histology (Mice)

All mice were sacrificed between 12 and 14-weeks of age. 5 mice per group were dissected and mandibles and maxillas were prepared for immunohistochemistry. Tissues were fixed in Bouin's solution for 24 h, rinsed with 70% ethanol and demineralized (for post-27 dpc tissues) in acetic

acid/formalin/sodium chloride solution. Tissues were processed according to standard histological procedures and embedded in paraffin. Each tooth was sectioned serially in a buccolingual (frontal) orientation (6mm) using a microtome and mounted as numbered serial sections on charged glass slides. This resulted in approximately 100 sections/tooth. High-resolution digital images were captured using Metavue software (Molecular Devices, Sunnyvale, Calif., USA) with a SPOT CCD camera (Diagnostic Instruments, Sterling Heights, Mich., USA).

Immunohistochemistry (IHC) (Mice)

IHC was performed on mouse mandible and maxilla tissues. Every fourth section of serially sectioned tooth was stained resulting in approximately 25 stained sections/tooth for each primary antibody. Tissues were deparaffinized in xylene and rehydrated using decreased graded dilutions of ethanol. Tissue specimens were blocked by incubation in 1.5% H_20_2 in methanol solution for 30 minutes. Primary antibodies (neutrophil elastase (Santa Cruz Biotechnology, sc-71674), KC (abcam, ab17882) and MIP2 (abcam ab9950) were used with biotinylated secondary antibodies against rabbit primary antibodies, as appropriate, and slides were developed using a 3-amino-9-ethylcarbazole substrate kit The Abcam IHC-P staining kits were used for immunohistochemical staining. Positive controls included staining in WT tissues, where immunolocalization of target proteins were well characterized. Negative controls were performed lacking a primary antibody.

ELISA

Oral tissues were extracted by previous published method (Dixon *et al.*, 2004). Extracted tissues were dissected from mandible and maxilla, stored in RNAlater (Qaigen) as per manufacturer instructions. The tissues were then sonicated with microtip in PBS on ice for 15s on, 15s off for total of 3min on. Serial dilutions of the supernatents were made on each ELISA and BioRad protein assay (500-0001). The ELISAs employed were as follows: MIP2, R&D Systems Cat

Num MM200; TNF-α, eBioscience 88-7324-22; IL-1β, ThermoScientific 1858003; IFN-γ, eBioscience 88-8314-22. All ELISA kits were used as per manufacturer directions.

Statistical Analyses

IHC$_{neutrophils}$

Initial experiments revealed there were no significant differences between mandibular and maxillary teeth within each group ($p<0.001$, random mixed model test). Therefore, quantitative analysis of neutrophil numbers in the junctional epithelium of GF and SPF mice was performed by obtaining sections surrounding either the maxillary first molar or mandibular first molar in each mouse. Individual neutrophils were counted in each section and totaled for each mouse. The differences between WT and KO groups were tested in PRISM by the parametric Student t test and nonparametric Mann-Whitney test.

IHC$_{KC\ and\ MIP2}$

4 mice per group were examined by serial sectioning, 100 sections/group. Each slide was blindly rated 0-3 for intensity. Semi-quantitative analysis for staining intensity was performed using multilevel logistic regression.

Experimental procedures for Rabbit model of periodontal disease:

Animal model

The study was approved by Boston University Medical Center (BUMC) Institutional Animal Care and Use Committee before study initiation (IACUC protocol no. AN-13948). In addition, BUMC Institutional Bio- hazard Committee (IBC) approved the use of *P. gingivalis* in this animal model to induce periodontal disease (IBC protocol no. 06-016). (Hasturk *et al.*, 2007)

New Zealand White rabbits (24 males, 3.5– 4.0 kg) were purchased from Pine Acre Rabbit Farms (PARF), kept in individual cages, received water ad libitum, and fed standard rabbit chow at the Laboratory Animal Science Center at BUMC.

Bacteria growth and gel preparation

P. gingivalis (strain A7436) was grown as previously described (Hasturk *et al.*, 2007). In brief, bacteria were cultured on agar plates containing trypticase soy agar supplemented with 0.5% (w/v) yeast extract (Invitrogen Life Technolo- gies), 5% defibrinated sheep RBC, 5 μg of hemin, and 1 μg/ml vitamin K (Sigma-Aldrich). Plates were incubated for 3 days at 37°C in jars anaerobically maintained through palladium-catalyzed hydrogen/carbon dioxide envelopes (GasPak Plus; BD Microbiology Systems). Colonies were ran- domly selected and anaerobically cultured overnight at 37°C in Schaedler's broth supplemented with vitamin K and hemin. Bacterial numbers were spectrophotometrically determined at 600 nm, adjusted to 10^9 CFU (0.8 OD) or 35ng of LPS and mixed with carboxymethylcellulose to form a thick slurry, which was applied topically to the ligated teeth every other day for 6 wk of the experiment.

P. gingivalis-induced *periodontitis*

Periodontitis in rabbits was used to monitor inflammatory events associated with bone disorders and the actions of phosphatase mutants and purified LPS. Periodontitis was induced and established in all animals for a 6-wk period using a previously established protocol (Hasturk *et al.*, 2007). A 3-0 silk suture (ligature) was placed around the second premolar of both mandibular quadrants un- der general anesthesia (40 mg/kg ketamine, Ketaset (Fort Dodge Animal Health) and 5 mg/kg xylazine, Anased (Ben Venue Laboratories) injections). The slurry containing *P.*

gingivalis, phosphatase mutants or purified LPS (35ng) was topically applied to the ligatures on Monday, Wednesday, and Friday over a 6-wk period to induce periodontal inflammation. The sutures were checked at every application, and lost or loose sutures were replaced. (Hasturk *et al.*, 2007)

At the end of the 6-wk period, animals were euthanized using an overdose of pentobarbital (120 mg/kg Euthanasia-5 solution; Veterinary Laboratories) according to the approved protocol of IACUC.

Morphometric analysis

After sacrificing the animals, the mandible was dissected free of muscle and soft tissue, keeping the attached gingiva intact. The mandible was split into two halves from the midline between the central incisors. Half was taken for morphometric analysis of bone loss, and the other half was used for histological evaluation of periodontitis. For morphometric analysis, the sectioned mandible was defleshed by immersion in 10% hydrogen peroxide followed by careful soft tissue removal, washed with distilled water, air- dried and stained with methylene blue for visual distinction between the tooth and bone. The bone level around the second premolar was measured directly using a 0.5 mm calibrated periodontal probe. The bone level was also quantified using Image Analysis (Image-Pro Plus 4.0; Media Cyber- netics). Radiographs were taken with a digital x-ray (Schick Technol- ogies). To quantify bone loss, the length of the tooth from cusp tip to the apex of the root was measured, as was the length of the tooth structure outside the bone that was measured from the cusp tip to the coronal extent of the proximal bone. From this, the individual percent- ages of the teeth within the bone were calculated. Bone values are expressed as the percentage of bone loss. (Hasturk *et al.*, 2007)

In addition, the soft tissue (pocket) depth and infrabony defect depth were measured in all groups using a 0.5 mm calibrated periodontal probe. The tip of the tooth at the measured site was used as the reference point for these measurements. Furthermore, tooth mobility was also calculated using Muhleman's mobility index (43) as follows: 0 = no mobility; 1 = >0.5 and <1 mm mobility buccolingually; 2 = >1 mm but <2 mm mobility buccolingually; 3 = >2 mm mobility buccolingually; 3+ = both vertical and buccolingual mobility.

Qualitative histological evaluations

Half of the mandible was immersed in 10 volumes of Immunocal (Decal Corporation) and the solution was replaced every 24 h for 2 wk. Decalcification was confirmed by serial radiographs, which were taken every other day. After the decalcification, the tissues were rinsed for 1–3 min in run- ning water, placed in Cal-Arrest (Decal Corporation) to bring tissues to neutral pH, which enhanced embedding and staining characteristics, and to terminate further decalcification. Tissues were kept in this solution for 2–3 min, rinsed again in flowing deionized water for at least 3 min and placed in formalin for ~24 h before embedding in paraffin. Thin sections (5 μm) were cut and either stained with H&E for light microscopy and identification of the cellular composition of inflammatory infiltrates or with tartrate-resistant acid phosphatase (TRAP) to examine osteoclastic activity (Hasturk *et al.*, 2007). For qualitative assessments, three areas were analyzed on each tooth corresponding to the coronal, middle, and apical third of the root. In addition, some of the sections per animal were stained with Masson's trichrome to detect whether new collagen depositions and new bone formation were detectable following treatment (Hasturk *et al.*, 2007). Furthermore, some of the sections of each of the groups were stained with osteocalcin as a measure of osteoblastic activity and new bone formation.

For un-decalcified sections, some of the harvested blocks were placed in 4% formalin/1% CaCl2 fixative (Hasturk *et al.*, 2007). The specimens were dehydrated and embedded in methylmethacrylate. Eight to ten un-decalcified sections of ~10 μm in thickness were obtained from each specimen using a slow- speed diamond saw with coolant and stained superficially with Van Gieson's trichrome. Sections were analyzed for qualitative histological findings.

Quantitative histomorphometry

To quantify the changes in bone, the mean value (±SD) of the linear distance and the area of bone loss were calculated for each group. Previously developed measurement technique (Hasturk *et al.*, 2007)was used to calculate the bone changes at three different sections of the root using the ProImage software. The linear measurements were made at three levels each corresponding to one third of the root and alveolar bone interface: crestal, mid, and apical. Linear distance is reported as the distance from the base of the epithelium to the alveolar crest border at the three chosen levels, the apical, middle, and the coronal third of the root and is expressed as the difference between treated and untreated sites. Likewise, area measurements were presented as the difference between the treated and untreated total area.

In addition, osteoclastogenesis was examined in all TRAP-stained sections by calculating the osteoclasts in affected areas. The total number of osteoclasts at the surface of the bone was compared between the groups.

Microbial sampling

Microbial dental plaque was sampled at baseline at 6 and 12 wk using paper points. The area was isolated to prevent saliva contamination, air-dried and 30 s samples were collected using sterile

paper points according to previously reported methods (Hasturk *et al.*, 2007). Each sample was placed in an individual Eppendorf tube containing 0.15 ml TE (10 mM Tris-HCl, 1 mM EDTA (pH 7.6)) and 0.5 M NaOH was added for stabilization. Twentynine species representing periodontal organisms including *P. gingivalis, Acti- nobacillus actinomycetemcomitans, Actinomyces odontolyticus, Actinomy- ces viscosus, Actinomyces israelii, Peptostreptococcus micros, Prevotella intermedia, Prevotella nigrescens, Capnocytophaga curva, Capnocyto- phaga rectus, Streptococcus oralis, Streptococcus intermedius, Tannerella forsythensis, Treponema denticola, Eikenella corrodens, Fusobacterium nucleatum* subsp. *vincenti, Escherichia coli, Campylobacter concisus, Capnocytophaga sputigena, Prevotella bivia, Selenomonad noxia, Veillonella parvula, Capnocytophaga ochracea, Filifactor alocis, Actinomyces naeslundii, Lactobacillus acidophilus, Eubacterium saphenum* and *Streptococcus sanguis* were investigated in each plaque sample using the checkerboard DNA-DNA hybridization technique (49). Evaluation of the chemiluminescent signals is performed by radiographic detection, comparing the obtained signals with the signals generated by pooled standard comparisons of 0, 10^3 bacteria and 10^6 bacteria of each of the 29 species.

Statistical analysis

Mean values for linear and area measurements were used to determine the changes in bone level. In addition, TRAP plus stained cell counts were calculated to detect the osteoclastogenesis.

Experimental procedures for phosphatase phenotypic analyses:

Bacterial growth conditions

P. gingivalis strain ATCC 33277 was obtained from our stock collection. Bacteria were grown in TYHK medium consisting of trypticase soy broth (30 g l−1) (Becton Dickinson, Sparks, MD), yeast extract 5 g l−1 (Becton Dickinson, Sparks, MD), and vitamin K3 (menadione) (Sigma-Aldrich, St. Louis, MO). The basal TYHK medium was sterilized by autoclaving, followed by the addition of filter-sterilized hemin (Sigma-Aldrich, St. Louis, MO) to the desired final concentration of either (1 μg ml−1) or (10 μg ml−1) as indicated in the text and figure legends. Cultures were grown in an anaerobic growth chamber (5% H_2, 5% CO_2, 90% N_2) and maintained at 37°C on TYHK-agar plates.

Gene Deletions in P. gingivalis 33277

The genomic nucleotide sequences encoding the putative lipid A 1-phosphatase, PG1773, and the putative lipid A 4'-phosphatase, PG1587, were obtained from searches of the annotated *P. gingivalis* W83 genome at The Comprehensive Microbial Resource (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi). Gene deletions were created by introducing either a tetracycline resistance cassette (*tetQ*) in place of the coding region for PG1773 or an erythromycin resistance cassette (*ermF/AM*) in place of the coding region for PG1587. Polymerase chain reaction (PCR) amplification of genomic DNA from *P. gingivalis* A7436 was performed using primer sets designed against the W83 sequence to amplify 1000 base-pairs upstream and 1000 base-pairs downstream from the regions adjacent to the PG1773 and PG1587 coding regions respectively. The amplified 5' and 3' flanking regions for PG1773 and PG1587 respectively, were co-ligated with the *tetQ* and *ermF/AM* cassettes respectively into pcDNA3.1(−) to generate the gene disruption plasmids, p1773 5'flank:tetQ:3'flank and p1587 5'flank:erm:3'flank. *P. gingivalis* 33277 deficient in either PG1587 (1587KO) or PG1773

(1773KO) was generated by introducing either p1587 5'flank:erm:3'flank or p1773 5'flank:tetQ:3'flank into *P. gingivalis* 33277 by electroporation in a GenePulser Xcell (BioRad, Hercules, CA). Bacteria were plated on TYHK/agar plates containing the appropriate selective medium, which included either erythromycin (5 μg ml−1) or tetracycline (1 μg ml−1) and incubated anaerobically. One week later, colonies were selected for characterization. Loss of the PG1587 and PG1773 coding sequences were confirmed in all clones by PCR analyses using primers designed to detect the coding sequences. (Coats *et al.*, 2009)

Isolation of LPS and Lipid A

Bacteria were cultured for 48 hours in TYHK medium containing hemin at a concentration of either (1 μg ml−1) or (10 μg ml−1). LPS was isolated using a modified version of the Tri-reagent protocol for LPS isolation as previously described (Al-Qutub *et al.*, 2006). To generate lipid A, dried LPS samples were resuspended in 10 mM sodium acetate [pH 4.5] containing 1% sodium dodecyl sulfate (w/v). The solution was heated 100°C for 1 hour followed by lyophilization overnight. The resulting lipid A pellets washed once in ice-cold 95% ethanol containing 0.02 N HCl, three times in 95% ethanol, followed by a final extraction with 1160 μl of chloroform-methanol-water (1:1:0.9, v:v:v) to remove residual carbohydrate contaminants. The chloroform layer containing the lipid A was dried and used for MALDI-TOF MS or MALDI-TOF/TOF tandem MS analyses.

MALDI-TOF MS Analyses

For MALDI-TOF MS analyses, lipid A samples were dissolved in 10 μl of a mixture of 5-chloro-2-mercaptobenzothiazole (20 mg ml−1) in chloroform/methanol 1:1 (v/v) and 0.5 μl of each sample was analyzed in both positive and negative ion modes on an AutoFlex Analyzer

(Bruker Daltonics). Data were acquired with a 50 Hz repletion rate and up to 3000 shots were accumulated for each spectrum. Instrument calibration and all other tuning parameters were optimized using HP Calmix (Sigma-Aldrich, St. Louis, MO). Data was acquired and processed using flexAnalysis software (Bruker Daltonics).(Coats *et al.*, 2009)

HEK293 TLR4 Activation Assays

HEK293 cells were plated in 96-well plates at a density of 4 × 104 cells per well and transfected the following day with plasmids bearing firefly luciferase, *Renilla* luciferase, human TLR4 and MD-2 by a standard calcium phosphate precipitation method as described previously (Coats *et al.*, 2005) The test wells were stimulated in triplicate for 4 hours at 37°C with the indicated doses of LPS or intact bacteria that had been suspended by vortexing in DMEM containing 10% human serum. Following stimulation, the HEK293 cells were rinsed with phosphate-buffered saline and lysed with 50 µl of passive lysis buffer (Promega, Madison, WI). Luciferase activity was measured using the Dual Luciferase Assay Reporter System (Promega, Madison, WI). Data are expressed as fold increase of NF-κB-activity, which represents the ratio of NF-κB-dependent fire-fly luciferase activity to β-Actin promoter-dependent *Renilla* luciferase activity.(Coats *et al.*, 2009)

Polymyxin B Sensitivity Assays

Overnight cultures of Wt *P. gingivalis* A7436 and its isogenic putative phosphatase mutants were grown in THYK media containing hemin (1 µg ml−1) or (10 µg ml−1) as indicated in the text. Liquid cultures that had been grown for 24 hours in an anaerobic growth chamber were diluted to a starting optical density (OD600) of 1.0, which represents approximately 1 × 109 cfu/ml for all of the strains examined (data not shown). Subsequently, 10−3 to 10−8 dilutions of each strain

were plated on TYHK-agar plates containing PMB (0, 5, and 200 μg ml−1). After 8–10 days of incubation in an anaerobic chamber, the resulting colonies were counted to determine the viability at the different PMB treatments. The results for each strain were plotted as percent survival, which was derived from the ratio of the number of colonies detected on the experimental plates containing polymyxin B (5 or 200 μg ml−1) to the number of colonies detected on the control plates that did not contain polymyxin B. (Coats *et al.*, 2009)

Statistical Analyses

Data were analyzed by two-tailed unpaired t tests (GraphPad Prism) where indicated. $P < 0.05$ was considered indicative of statistical significance.

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