To my fiancée, Jennifer, for her unending support and love
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# TABLE OF CONTENTS

**ACKNOWLEDGMENTS** ........................................................................................................ 4  
**LIST OF TABLES** ............................................................................................................... 7  
**LIST OF FIGURES** ............................................................................................................. 8  
**LIST OF ABBREVIATIONS** ............................................................................................... 9  
**ABSTRACT** .................................................................................................................... 10  

## CHAPTER

1 **INTRODUCTION** ........................................................................................................ 12  
   - The Periodontium in Health ............................................................................... 12  
   - Periodontal Tissue Destruction ....................................................................... 15  
   - Immunology of Periodontal Disease ................................................................ 16  
   - Toll-like Receptors ......................................................................................... 20  
   - Type 1 Diabetes and Periodontal Disease ...................................................... 22  

2 **MATERIALS AND METHODS** ................................................................................ 25  
   - Mouse Models .................................................................................................... 25  
   - Bacterial Growth ................................................................................................. 26  
   - Infection Model ................................................................................................. 26  
   - Alveolar Bone Loss .......................................................................................... 27  
   - Inflammatory Scoring ..................................................................................... 27  
   - Soluble Mediator Expression .......................................................................... 28  
   - Flow Cytometry ............................................................................................... 28  
   - Bacterial Colonization ..................................................................................... 29  

3 **RESULTS** ................................................................................................................. 30  
   - Epithelial Cell Specific Knockdown of MYD88 .............................................. 30  
   - Epithelial Cell Knockdown of MYD88 in B6 Mice Exacerbates Bone Loss in Periodontal Disease ................................................................. 30  
   - Epithelial Cell Knockdown of MYD88 in B6 Mice Exacerbates Inflammation in Periodontal Disease ................................................................. 31  
   - Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Expression of Pro-Inflammatory Cytokines and Reduces Expression of Anti-Inflammatory Cytokines in Periodontal Disease ......................................................... 31  
   - Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Colonization of P.g. and A.a. .................................................................................... 32
Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Prevalence of Th1 and Decreases Prevalence of Th17 and Treg in Periodontal disease .................. 32
Epithelial Cell Knockdown of MYD88 in NOD Mice Reduces Bone Loss in Periodontal Disease .................................................................................. 33
Epithelial Cell Knockdown of MYD88 in NOD Mice Reduces Inflammation in Periodontal Disease .................................................................................. 33
Epithelial Cell Knockdown of MYD88 in NOD Mice Increases Expression of Pro-Inflammatory Cytokines and Reduces Expression of Anti-Inflammatory Cytokines in Periodontal Disease .............................................. 34
Epithelial Cell Knockdown of MYD88 in NOD Mice Increases Colonization of P.g. and A.a.................................................................................................... 34
Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Prevalence of Th1 and Decreases Prevalence of Th17 and Treg in Periodontal disease ............ 35

4 DISCUSSION ........................................................................................................ 51
LIST OF REFERENCES ............................................................................................. 58
BIOGRAPHICAL SKETCH ........................................................................................ 61
# LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-1 Mouse Model</td>
<td>36</td>
</tr>
<tr>
<td>3-2 Experiment Timeline</td>
<td>37</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-------------</td>
</tr>
<tr>
<td>3-1</td>
<td>Murine oral epithelial cell knockdown of MYD88</td>
</tr>
<tr>
<td>3-2</td>
<td>Percentage of bone volume as measured by micro computed tomography</td>
</tr>
<tr>
<td>3-3</td>
<td>Inflammatory scores in the B6 mice assessed during histologic analysis of the left maxilla of each mouse</td>
</tr>
<tr>
<td>3-4</td>
<td>Pro-inflammatory and anti-inflammatory mediators in the B6 mice assessed with Luminex 200</td>
</tr>
<tr>
<td>3-5</td>
<td>IL17 Cytokine axis mediators in the B6 mice assessed with Luminex 200</td>
</tr>
<tr>
<td>3-6</td>
<td>16S levels of A.a. and P.g. measured by real time PCR</td>
</tr>
<tr>
<td>3-7</td>
<td>Immune cell profiles as measured by flow cytometry</td>
</tr>
<tr>
<td>3-8</td>
<td>Bone volume levels as measured by micro computed tomography</td>
</tr>
<tr>
<td>3-9</td>
<td>Inflammatory scores in the NOD mice assessed during histologic analysis of the left maxilla of each mouse</td>
</tr>
<tr>
<td>3-10</td>
<td>Inflammatory and anti-Inflammatory cytokine mediators in the NOD mice assessed with Luminex 200</td>
</tr>
<tr>
<td>3-11</td>
<td>IL17 Cytokine axis mediators in the NOD mice assessed with Luminex 200</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>A.a.</td>
<td>Aggregatibacter actinomycetemcomitans</td>
</tr>
<tr>
<td>CEJ</td>
<td>cement-enamel junction</td>
</tr>
<tr>
<td>K5</td>
<td>keratin 5</td>
</tr>
<tr>
<td>MMP</td>
<td>matrix metalloproteinases</td>
</tr>
<tr>
<td>MyD88</td>
<td>myeloid differentiation factor 88</td>
</tr>
<tr>
<td>NOD</td>
<td>non-obese diabetic mouse</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen associated molecular pattern</td>
</tr>
<tr>
<td>PDL</td>
<td>periodontal ligament</td>
</tr>
<tr>
<td>P.g.</td>
<td>Porphyromonas gingivalis</td>
</tr>
<tr>
<td>T1D</td>
<td>type 1 diabetes</td>
</tr>
<tr>
<td>TLR</td>
<td>toll-like receptors</td>
</tr>
</tbody>
</table>
The oral cavity serves as a gateway for invading pathogens, some of which may induce inflammatory responses that can lead to diseases such as periodontitis. The immune system has several mechanisms in place in order to restrict the entry of these pathogens. The primary defense is the barrier function of the epithelial cells. These epithelial cells also have the capacity to activate the immune system via TLR stimulation. These TLRs require the adapter protein MYD88 in order to activate their signaling cascade. Previous studies have shown that MYD88 knockdown in the oral epithelial cells of B6 mice leads to exacerbated inflammation and tissue destruction in periodontal disease.

T1D has a well-established bidirectional relationship with periodontal disease. The severity of each tends to increase the severity of the other. It has also been shown that T1Ds have dysregulation of their TLR function which leads to exaggerated expression of pro-inflammatory cytokines. In this study, the effects of MYD88 knockdown in T1D mice with periodontal disease were investigated. The results suggest that MYD88 knockdown leads to increased colonization of pathogenic bacteria.
This result would suggest increased disease severity, however, the MYD88 knockdown had an ameliorating effect on the tissue destruction and production of inflammatory mediators. The likely explanation for this phenomenon is due to the impaired functioning of the TLRs of the oral epithelial cells that mediate expression of pro-inflammatory cytokines. An important implication of this result is that it marks oral epithelial cell TLRs as a potential therapeutic target T1D patients with periodontal disease.
CHAPTER 1
INTRODUCTION

The Periodontium in Health

The periodontium serves as the foundation for teeth. It is comprised of the gingival tissues, periodontal ligament, cementum and alveolar bone (Nanci and Bosshardt, 2006). The primary role of the periodontium is to support the teeth during regular function. Additionally, it serves as a barrier against bacterial infection. In conditions of health, the periodontium is able to accomplish these tasks, withstanding trauma from function and repelling bacterial infiltration (Nanci and Bosshardt, 2006).

The normal range of dimensions for a periodontium in health are well established in the literature. Typically, the epithelial attachment to the tooth occurs approximately .67-1mm apical to the cementoenamel junction (CEJ). This epithelium is approximately 1mm in length. Moving apically down the surface of the tooth, the connective tissue is found next. This tissue is comprised primarily of collagen, fibroblasts, and ground substance. This ground substance is made up of water, glycosaminoglycans, proteoglycans, and glycoproteins. Similarly to the epithelial tissues, the connective tissue is approximately 1mm in length. These dimensions are averages found during a large cadaver study (Gargiulo et al., 1995). The sum of these dimensions described above represent what is known as the biologic width. Finally, the terminal portion of the connective tissue attachment is marked by the alveolar crest. From that point, moving apically towards the apex of the tooth, the alveolar bone and the surface of the tooth are joined by the periodontal ligament (PDL). The individual components of the periodontium will now be discussed in greater detail.
The gingiva is the periodontium’s first line of defense against infection. This is accomplished by it serving as a mechanical barrier to bacterial infiltration. In conditions of health, the gingiva forms a tight seal around the teeth, protecting the deeper layers of the periodontium from bacterial insult. However, this seal can be greatly compromised in an inflammatory state, which can be induced by bacterial biofilms or trauma. In periodontal disease, the barrier function of the gingiva is reduced to the point that bacterial invasion can occur readily and lead to the development of subgingival biofilms. Not only is this subgingival biofilm nearly impossible to remove with traditional oral hygiene methods, but over time, it can mineralize into calculus. This subgingival calculus acts as a gateway for additional bacterial invasion and further progression of the disease in an apical direction. This progression is likely to continue until the calculus is removed (Oshrain et al., 1971).

The periodontal ligament (PDL) acts as the link between the tooth and the alveolar bone. The PDL serves supportive, sensory, and remodeling functions. The primary function is to hold the tooth within the alveolar bone. Secondary functions include providing somatosensory information and nutrition to the local cells (Perera and Tonge, 1981). The width of the PDL can range from .15-.38mm. This width tends to taper towards the middle third of the root and expand as you move coronally and apically from there. The terminal ends of the PDL fibers are known as Sharpey’s fibers and they insert into the cementum on the tooth side and the periosteum on the bone side. Together, the cementum, alveolar bone, and PDL form what is known as the attachment apparatus. The PDL is composed primarily of water, approximately 70%. This is thought to aid in its ability to withstand the pressures generated under functional
and parafunctional habits. Within the PDL are progenitor cells that can differentiate into osteoblasts and provide maintenance to the surrounding alveolar bone.

The cementum of the tooth serves as one of the receiving ends of the Sharpey's fibers of the PDL. It is a specialized calcified substance that covers the roots of the teeth. It is less mineralized than both enamel and dentin, being approximately 45-50% composed of inorganic material, hydroxyapatite, and approximately 50-55% composed of water and organic material. This leads to it being slightly softer than enamel or dentin.

The alveolar bone housing the tooth serves as the other receiving end of the Sharpey's fibers of the PDL. It is a mineralized substance with its chief inorganic component being hydroxyapatite. Similarly to cementum, the levels of hydroxyapatite found in the alveolar bone are less than both enamel and dentin. By weight, it is composed of 60% inorganic, 25% organic material, and 15% water. The primary role of the alveolar bone is to support the teeth during function (Kornman et al., 1997). The forces produced during mastication can reach up to 600-750N and therefore, it is necessary for the bone and PDL to work together to distribute the forces appropriately. This is accomplished by allowing a small degree of movement due to the flexibility of the attachment apparatus (Kornman et al., 1997). Despite the PDL contributing to the management of the functional forces, the alveolar bone receives the vast majority of the forces. The result of long term periodontal disease can cause destruction of this bony housing and greatly compromise the support and stability of the teeth.
Periodontal Tissue Destruction

According to the data collected during the 2009 and 2010 NHANES studies, the prevalence of periodontal disease among adults aged 30 and older is 47.2%. This is broken down into mild (8.7%), moderate (30%), and severe (8.5%) cases. Among the adults aged 65 and older, 64% had either moderate or severe periodontal disease. Periodontal disease was demonstrated to be highest in men, Mexican Americans, individuals lacking a high school education, individuals below the Federal Poverty Level, and current smokers.

Periodontal disease can only be properly diagnosed by conducting both a clinical and radiographic examination. Clinical parameters that are gathered during this exam include: probing depth, bleeding on probing, recession, furcation involvement, and mobility. Typically, probing depth and recession are the most relevant of these measurements as far as diagnosing periodontal disease is concerned. In conditions of health, probing depths should fall in the range of 2-3mm with the probe not penetrating past the epithelial attachment (Anderson et al., 1991). Recession is a measurement of the distance between the free gingival margin and the cementoenamel junction (CEJ) of the tooth. Probing depth and recession values allow the attachment loss to be calculated by adding the two measurements together. Attachment loss is the extent of apical migration of the periodontium from its normal level and is the inevitable consequence of long-term periodontal disease. The attachment apparatus described above should begin approximately 1-2mm from the CEJ. This can be approximated radiographically by examining the level of the interproximal bone. Presently, the most common classification system is the one introduced during the International Workshop
for a Classification of Periodontal Diseases and Conditions (Armitage, 1999). This system is based on the amount of attachment loss and categorizes the severity of the disease as: 1-2mm Slight, 3-4mm Moderate, and >=5mm Severe. The disease may manifest as generalized (>30% of sites are involved) or localized (up to 30% of sites are involved). Furthermore, the type of disease can be further classified as chronic or aggressive (Armitage, 1999). Although there are many differences between chronic disease and aggressive disease, the key difference is the rate of destruction. Chronic periodontal disease tends to progress at a rate of approximately .2-.25mm per year, though there can be a wide range of variation among different individuals (Waerhaug, 1977). Additionally, chronic periodontal disease does not tend to follow any specific pattern of bone loss. Conversely, aggressive periodontal disease tends to progress approximately 3-4 times as quickly as chronic. Also, in the case of localized aggressive periodontal disease, the first molars and incisors tend to experience the greatest amount of destruction.

**Immunology of Periodontal Disease**

By far, the majority of cases of periodontal disease are a result of bacterial plaque and its byproducts producing a constant state of inflammation. A great deal of research has been conducted on whether specific species of bacteria have different effects on the establishment and progression of periodontal disease. In 1983, Socransky and Haffajee published a study that investigated the bacterial species present in periodontal disease via DNA checkerboard hybridization (Haffajee et al., 1983). Later, they associated specific bacterial species with various levels of disease severity and grouped them into colored groups. For example, the orange and red groups of bacteria were more strongly associated with the more severe forms of the
Among the various species identified with periodontal disease, Porphyromonas gingivalis (P.g.) and Aggregatibacter Actinomycetemcomitans (A.a.) have been strongly associated with chronic and aggressive periodontal disease (Socransky et al., 1998). P.g. is a member of the red group, while A.a. is a member of the green group. Both of these species have been shown to resist removal from the periodontium when subjected to non-surgical periodontal therapy (Saglie et al., 1982). The specifics of each of these species will now be addressed.

P.g. is a non-motile, gram negative, anaerobic bacteria that has been linked with various forms of periodontal disease. It has demonstrated the ability to invade human fibroblasts and epithelial cells and remain viable even in the presence of systemic antibiotics. Among the virulence factors that P.g. produces, the gingipains are one of the more relevant in regard to periodontal disease. These gingipains are cysteine rich proteinases which have demonstrated the ability to degrade the epithelial cell to cell junctions. The mechanism of this degradation is due to the epithelial cell’s response to the presence of P.g., which leads to proteolysis of adherins junction proteins and adhesion signaling molecules (Bosshardt and Lang, 2005). Recently, gingipains have been observed to disrupt the ICAM-1 dependent adhesion of neutrophils to epithelial cells. P.g. is also capable of inhibiting apoptosis in cells that it has invaded. This is accomplished by modifying the JAK/Stat pathway which regulate the mitochondrial apoptotic pathway. These are some of the ways in which P.g. is able to evade the immune system, in addition to its ability to invade and persist within human epithelial cells. These evasion mechanisms provide a biologically plausible basis for the ineffectiveness of non-surgical periodontal therapy on eradicating this microbe.
A.a. is a non-motile, gram-negative, facultative anaerobic bacteria. It has been strongly associated with localized aggressive periodontal disease, particularly the serotype b strain. Among its virulence factors are its production of leukotoxin. This leukotoxin is capable of killing neutrophils and monocytes. It is encoded by an operon belonging to the core genome of A.a.. Studies have revealed a correlation between high leukotoxicity and progression of periodontal tissue destruction and attachment loss (Tsai and Taichman, 1986). Similarly to P.g., A.a. is also capable of intracellular invasion of the gingival cells. This process was describe by Meyer in 1997. The entry process begins with A.a. being taken into the cell within a vacuole. From there, it is able to escape the vacuole, proliferate within the cell, and eventually spread to the adjacent cells. (Meyer et al., 1997). As in the case with P.g., the host immune system evasion mechanisms of A.a. can render non-surgical periodontal therapy insufficient in removing this pathogen.

The pathogenesis of periodontal disease was previous described by Page and Schroeder in 1976. The sequence of the disease is as follows: initial lesion, early lesion, established lesion, destructive lesion. Within 2-4 days of the accumulation of bacterial plaque, the initial lesion is formed. This is marked by a classic acute vasculitis in the surrounding tissues, which can lead to breakdown of the perivascular collagen. This may be a result of the release of chemotactic and antigenic substances released from the biofilm. After 4-10 days, the early lesion develops. This lesion is characterized by the recruitment of lymphocytes and other mononuclear cells by the host’s immune system. Additional observations include continued destruction of the adjacent connective tissue and pathologic alteration of the local fibroblasts. Within 2-3 weeks,
the early lesion will progress to the established lesion. A key distinction of this lesion is
the predominance of plasma cells without any significant bone loss. This lesion can
remain stable for months or even years before progressing to the final lesion, the
destructive lesion. In this lesion, plasma cells continue to predominate. The main
difference between the two lesions is that there is significant loss of alveolar bone and
PDL along with disruption of the tissue architecture with fibrosis. Gingivitis is
characterized by the initial, early, and established lesions. It is not until the destructive
lesion is reached that periodontitis has developed (Page and Schroeder, 1976).

The immune system responds to bacterial infiltration by releasing a host of
inflammatory mediators which are capable of producing tissue destruction in the
periodontium (Snyderman, 1973). Once the junctional epithelium has been breached,
bacteria are able to reach the deeper levels of the periodontium, such as the connective
tissue attachment and the PDL. Once they have penetrated this far, the bacterial
byproducts and toxins will induce an immune response from the host in the form of
cytokines, proteases, and prostaglandins (Snyderman, 1973). While this response is
capable of killing the invading bacteria or at least slowing their proliferation, it has the
unfortunate consequence of causing periodontal tissue destruction. This destruction
forms the basis for the tissue loss observed in periodontal disease (Kornman et al.,
1997). To further complicate matters, the rich vascular network of the periodontium
allows for rapid migration of the bacteria, their toxins, and the pro-inflammatory
mediators throughout the periodontium (Kornman et al., 1997). The subsequent release
of inflammatory mediators such as IL-1β and TNFα, as well as the matrix
metalloproteinases result in the tissue destruction associated with periodontal disease.
This process of destruction can occur rapidly in the case of aggressive forms of the disease or over a long period of time in the case of chronic disease. Specifically, this destruction is accomplished by a group of cells called osteoclasts. Osteoclasts are multinucleated cells approximately 150-200 microns in diameter. They play a key role in the maintenance and repair of bone, by performing a process known as bone resorption. This is accomplished by digesting the bone at a molecular via secreting acid and collagenase. Osteoclast progenitor cell differentiation into osteoclasts has been shown to be stimulated by TNFα (Kobayashi et al., 2000). IL-1 has also demonstrated to be a stimulant of osteoclast differentiation as well as activating existing osteoclasts (Kim et al., 2009).

The oral epithelial cells associated with the periodontium play a key role in the immunologic response to bacterial invasion. In addition to the barrier function they provide, there are various specialized cells within the epithelium. Among these are melanocytes, Merkel cells, and Langerhans cells. Most relevant to the immune system response are the Langerhans cells, a dendritic, antigen-presenting cell. In response to inflammation, the number of Langerhans cells can increase 2-10 fold, a result of cell migration from the adjacent connective tissue (DiFranco et al., 1985). It has been suggested that Langerhans cells serve the function of presenting antigens to T-cells during chronic periodontitis (DiFranco et al., 1985).

**Toll-like Receptors**

As previously discussed, periodontal disease invariably induces an immunologic response from the host. This response consists of both innate and adaptive immunity. Throughout all stages of the disease, the innate immune response is present. This
response is largely regulated by toll-like receptors (TLRs) (Mahanonda and Pichyangkul, 2007).

TLRs are membrane spanning proteins that are typically found in sentinel cells, such as macrophages and dendritic cells. They are able to recognize structurally conserved molecules that are present in microbes and upon recognition, activate an immune response. Some of the antigens recognized include lipopolysaccharide, flagellin, DNA, and RNA (Mahanonda and Pichyangkul, 2007). TLRs contain leucine-rich sections in their structure that correspond to certain pathogen-associated molecular patterns. These patterns allow the TLRs to recognize and respond to various bacterial byproducts (Mahanonda and Pichyangkul, 2007). This response is characterized by a signaling cascade in the immunologic cell housing the TLR. Within the periodontium, these cells include neutrophils, Langerhans cells, fibroblasts, epithelial cells, and endothelial cells.

The presence of commensal organisms in the oral cavity ensures that the TLRs on the gingival epithelial cells are constantly being activated. A balance of both the activation of an immune response and regulation of the immune response must be achieved to avoid an overproduction of inflammatory mediators. Once the epithelial layer has been breached by the bacteria, the TLRs on the cells within the deeper levels of the periodontium will become activated. This will lead to an exaggerated release of proinflammatory cytokines that may result in tissue destruction (Hans and Hans, 2011).

Among the various types of TLRs, TLR2 and TLR4 have been shown to be upregulated in the presence of bacteria within the periodontium, however, the specific cells involved remains unclear (Mochizuki et al., 2004) (Hirschfeld et al., 2001). Activate
of TLR2 and TLR4 triggers an upregulation of what are known as soluble mediators, such as cytokines, chemokines and growth factors (Abreu, 2003). Soluble mediators allow communication between the cells of the immune system and are responsible for positive and negative regulation of an immune response. These mediators are capable of initiating cell signaling pathways that result in the activation of T-cells and B-cells, which corresponds to the predominate immune system cells present during the established lesion described previously (Abreu, 2003). Chronic activate of these TLRs may result in a constant production of these soluble mediators and the subsequent tissue destruction that characterizes periodontal disease (Mahanonda and Pichyangkul, 2007).

**Type 1 Diabetes and Periodontal Disease**

Diabetes Mellitus refers to a group of metabolic diseases that result in a high level of blood sugar over an extended period of time. This can either be the result of the inability to produce insulin (Type 1) or the lack of response to insulin at the cellular level (Type 2). Diabetes affects more than 9% of the American population, totaling approximately 21 million individuals, with approximately 6 million of these individuals being undiagnosed (Mealey and Ocampo, 2007). Overall, the prevalence of diabetes is on the rise in the United States. Older individuals, Native Americans, Hispanics, and non-Hispanic blacks show a higher prevalence for the disease compared to younger, non-Hispanic whites. Furthermore, the incidence of diabetes is increasing annually. Approximately 1.3 million new cases of diabetes were diagnosed. This marked an increased incidence of 500,000 more cases compared to the incidence rate in 1998 of approximately 800,000. Both the rise in prevalence and incidence of diabetes are
directly proportional to the increased levels of obesity in the American population (Mealey and Ocampo, 2007).

An association between periodontal disease and diabetes was established in 1960 by Williams and Mahan (Williams and Mahan, 1960). Subsequent research has demonstrated a bidirectional relationship between the two diseases and diabetes has been well established as a risk factor for periodontal disease. On the other hand, periodontal disease has been shown to influence glycemic control depending on the severity of the disease (Preshaw et al., 2012). Furthermore, controlling the severity of the periodontal disease may also lead to an overall reduction in systemic inflammation that can result in the secondary complications of diabetes (Preshaw et al., 2012).

In Type 1 diabetes, a specific TLR-hyperinflammatory monocytic phenotype has been suggested as a mechanism for the exacerbated tissue destruction observed in diabetics with periodontal disease (Neiva et al., 2014). Oral epithelial cells have demonstrated dysregulated TLR function in Type 1 diabetics in the form of an exaggerated response. This response is due to a faulty induction of the regulatory mediators miR146a and miR155 in Type 1 diabetics (Neiva et al., 2014).

Research utilizing animal models has been critical to furthering our understanding of many diseases, diabetes included. Mouse models have been used extensively for decades and have aided us in the development of modern medicine. Mouse models have been used to study diabetes, particularly by using the autoimmune diabetes prone non-obese diabetic (NOD) mouse. The NOD mouse has been used to further explore the pathogenesis of Type 1 diabetes including the mechanism of the disease and identifying autoantigens (Thayer et al., 2010). In the NOD mouse, diabetes
rapidly develops after the infiltration of leukocytes into beta cells of the pancreas. This occurs at approximately 3 weeks of age. Several cells have been identified as playing key roles in the targeting and destruction of the beta cells and the pathogenesis is similar to that observed in humans with Type 1 diabetes. This makes the NOD mouse model an appropriate animal model to study the disease, though there are some key differences between human and mouse models, such as a resistance to ketoacidosis in mice (Atkinson and Leiter, 1999). Overall, the NOD mouse model has proven useful in studying the genes, polymorphisms, and genetic factors involved in Type 1 diabetes.
CHAPTER 2
MATERIALS AND METHODS

Mouse Models

C57Bl/6 Keratin CrePR mice (B6K5Cre) were backcrossed to the non-obese diabetic (NOD) mouse model using speed congenics until all satellite markers for the genetic background were of NOD origin. These mice are now referred to as NODK5Cre. NODK5Cre mice express a Cre recombinase progesterone receptor (PR) fusion protein whose expression is restricted by a Keratin 5 (K5) promoter. The CrePR is inducible by the progesterone antagonist RU486 (Sigma Aldrich, St. Louis, MO) but not by endogenous progesterone. Upon induction with RU486, CrePR translocates into the nucleus and excises DNA sequences flanked by Lox P sites. These mice were crossed with the NOD.Cg-Myd88tm1Defr/J (NOD.MyD88plox). These mice possess loxP sites on either side of exon 3 of the MyD88 gene. When these mutant mice are bred to NODK5Cre mice, offspring (NODK5Cre.MyD88plox) will have MyD88 deleted in epithelial cells upon the administration of RU486. Similarly, B6K5Cre were bred to B6. Myd88tm1Defr/J (B6.MyD88plox) and were used as controls for baseline periodontal disease as well as baseline measurements following MyD88 knockdown. Thus, 16 groups of mice (n=4/group) were utilized in this experimental design (Table 2-1). B6 and NOD mice of the indicated genotypes were either: 1) left uninduced and uninfected (U/U) (MyD88 sufficient; baseline measurements), 2) induced and left uninfected (I/U) (MyD88 deficient; effect of knockdown in the absence of infection), 3) left uninduced and infected (U/I) (MyD88 sufficient; baseline following infection), or 4) induced and infected (I/I) (MyD88 deficient; the effect of knockdown on response to infection). WT
(B6 and NON non-transgenic mice) were also used as a control for the effect of hormone (RU486) alone on parameters measured.

**Bacterial Growth**

All bacterial strains were grown under anaerobic conditions (85% N$_2$, 10% H$_2$, 5% CO$_2$) at 37°C in a Coy anaerobic chamber. All media (blood agar and liquid broth) were fully reduced for 24 to 48 h prior to inoculation with bacteria. Initially, *P. gingivalis* strain 381 was grown for 3 days on anaerobic 5% sheep blood agar plates (Remel, Lenexa, KS), and the resultant growth scraped from the agar surface using sterile cotton swabs and placed in in trypticase soy broth supplemented with yeast extract (1 mg ml$^{-1}$), haemin (5 µg ml$^{-1}$), and menadione (1 µg ml$^{-1}$) and allowed to culture anaerobically at 37°C until reaching an OD of 0.53. *A. actinomycetemcomitans* strain 29522 was grown in Trypticase Soy Broth supplemented with 0.6% yeast extract (TSB-YE) in a humidified, 10% CO$_2$ atmosphere, at 37°C until reaching an OD of 0.70.

**Infection Model**

*MyD88* in the oral epithelial cells was either induced or not to be knocked down. Induction of gene knockdown was achieved through the oral administration of 500µg of RU486 solubilized in 25uL of sesame oil (Publix, Gainesville, FL) for three consecutive days. After which all mice were lavaged with 0.12% chlorhexidine gluconate (3M, St. Paul, MN) for three days to decrease the amount of normal oral flora present. Infection consisted of an oral lavage with $2.5 \times 10^9$ *P. gingivalis* strain 381 and $2.5 \times 10^9$ *A. actinomycetemcomitans* strain 29522 (ATCC, Manassas, VA) resuspended in 2% low viscosity carboxy-methyl-cellulose (Sigma-Aldrich) on 4 consecutive days, every other week for 6 weeks. After which the soft tissues of the maxillae and mandibles were harvested to evaluate colonization by *P. gingivalis* and *A. actinomycetemcomitans* by
qPCR and soluble mediator expression by multiplex technology. In addition, the maxillae and mandibles were harvest for histology and bone morphometric analysis, Finally, the submandibular lymph nodes were harvested for flow cytometric analysis of T helper cells.

**Alveolar Bone Loss**

The structural properties of the mandible were evaluated using a desktop micro-computed tomography (µCT) imaging system (Brüker SkyScan 1173; Brüker microCT, Kontich, Belgium). Samples were scanned in saline with the following settings: 60 kV, 167-µA beam intensity, 0.5-mm aluminum filter, 0.7-degree rotation step, 4-frame averaging, 1090-ms integration time, 1024 × 1024 pixel matrix, and a 10-µm isotropic voxel dimension. After scanning, noise was removed from the images by eliminating disconnected objects smaller than 4 pixels in size. The volumes of interests (VOI) were identified and an appropriate and uniform threshold was applied to all specimens after comparing grayscale and binarized images in all groups. After thresholding, the bone volume/total volume (BV/TV; %), were quantified.

**Inflammatory Scoring**

The left maxilla of all mice were removed and fixed in 10% buffered formalin, decalcified, embedded and sectioned for histological analysis. 5-micron sections were deparafinzed in xylene (25min), followed by stepwise rehydration in 100% (5min) 95% (5min), and 70%(5min) ethanol. Rehydrated sections were then rinsed in distilled water (5min) followed by equilibration in PBS (5min). Sections were stained with hematoxylin and eosin (Sigma-Aldrich). Images were captured at 20x magnification and inflammation scored using PMN/mononuclear cell infiltration (0, no inflammatory cells; 1, minimal inflammation (scattered inflammatory cells close to the junctional epithelium);
2, moderate inflammation (numerous inflammatory cells in the gingival connective tissue); and 3, severe inflammation). In addition, changes in epithelial cell attachment, elongation and eruption were qualitatively assessed.

**Soluble Mediator Expression**

Tissues from the maxilla and mandible were homogenized using bead beating in cell extraction buffer (Life Technologies, Carlsbad, CA) prepared with a protease inhibitor cocktail (cOmplete, Roche, Basel, Switzerland) and PMSF protease inhibitor (Life Technologies, Carlsbad, CA). MILLIPLEX® Multiplex Assays (EMD Millipore, Billerica, MA) were used to probe resulting lysates for TNFα, IL6, IL8, TGFβ1, IL10, IFNγ, IL17A, IL23 and IL27, according to the manufacturer protocols. Briefly, tissue lysates were diluted 1:10 and cytokine capture-bead cocktails were incubated for 2 hours followed by incubation with biotin-labeled anti-cytokine for 1.5 hours and a 1:12.5 dilution of streptavidin-phycoerythrin (SAV-PE) for 30 min. All incubations were performed at room temperature in the dark while samples were gently shaken. Data was acquired on a Luminex 200® system running xPONENT® 3.1 software (Luminex, Austin, TX) and analyzed using a 5-paramater logistic spline-curve fitting method using Milliplex® Analyst V5.1 software (Vigene Tech, Carlisle, MA). Data are presented as pg/ml normalized to total protein as determined by BCA assay (Thermo Scientific Pierce, Waltham, MA).

**Flow Cytometry**

Submandibular lymph nodes were dissociated into FACS Buffer [1x PBS + 5% FBS + 0.372g EDTA]. prior to incubation with a fixable Live/Dead Yellow viability dye (Life Technologies) for 10 min at RT. Following Fc receptor blocking (BioLegend), surface staining was performed in FACS buffer interrogating expression of CD3, CD4, CD8,
CCR6, CXCR3, and FOXP3. FOXP3 Fix/Perm Buffer Set (BioLegend) was used for intracellular staining of FOXP3. All antibodies were used at manufacturer recommended concentrations. Fluorescence minus one (FMO) or isotype controls were used as controls. Data were acquired using a BD LSR Fortessa (BD Biosciences, Franklin Lakes, NJ) cytometer and analyzed using FlowJo data analysis software (FLOWJO LLC, Ashland, OR). Data are presented as percentage of parent populations as indicated.

**Bacterial Colonization**

On the first day of infection, prior to infection, and at the beginning of each week of infections, samples of the oral environment were taken with calcium alginate swabs (FisherScientific). After which the gDNA was isolated using a DNAeasy Kit (Qiagen) according to the manufacture's instructions. The gDNA was then probed for *P. gingivalis* 16S, *A. actinomycetemcomitans* 16S and total 16S using real time PCR. The percentage of *A. actinomycetemcomitans* 16S and *P. gingivalis* 16S within the total 16S compartment was calculated using the following formula: ct value of total 16S/ct value of *A. actinomycetemcomitans* or *P. gingivalis* 16S.
CHAPTER 3
RESULTS

Epithelial Cell Specific Knockdown of MYD88

The efficacy of a 3 day course of RU486 on inducing a knockdown of MYD88 in the oral epithelial cells was evaluated. This was accomplished by harvesting buccal tissue from the NODK5Cre.MyD88plox mice 7 days after the final administration of RU486. To serve as a control, tissues from NODK5Cre.MyD88plox that were sham treated were also harvested. Both sets of tissue samples were tested for the presence of MYD88 using immunohistochemistry. A lack of MYD88 specific immunofluorescence was noted in the RU486 treated group, but not in the sham group. This confirmed that that RU486 exposure was effective in knocking down MYD88 specifically in oral epithelial cells.

Epithelial Cell Knockdown of MYD88 in B6 Mice Exacerbates Bone Loss in Periodontal Disease

Bone loss was evaluated using a desktop micro–computed tomography (µCT) imaging system (Brüker SkyScan 1173; Brüker microCT, Kontich, Belgium). The volumes of interests (VOI) were identified and an appropriate and uniform threshold was applied to all specimens after comparing grayscale and binarized images in all groups. After thresholding, the bone volume/total volume (BV/TV; %), were quantified. B6 mice with MYD88 intact were compared to B6 mice with the knockdown of MYD88. Significantly greater reductions in bone volume were observed in the B6 group of mice with periodontal disease and the knockdown of MYD88 compared to the infected B6 mice with MYD88 intact (Figure 3-1).
Epithelial Cell Knockdown of MYD88 in B6 Mice Exacerbates Inflammation in Periodontal Disease

Inflammation was assessed by performing histologic analysis on the left maxilla of all B6 mice. Images were captured at 20x magnification and inflammation scored using PMN/mononuclear cell infiltration (0, no inflammatory cells; 1, minimal inflammation (scattered inflammatory cells close to the junctional epithelium); 2, moderate inflammation (numerous inflammatory cells in the gingival connective tissue); and 3, severe inflammation). In addition, changes in epithelial cell attachment, elongation and eruption were qualitatively assessed. In the groups of mice that were not infected, no inflammation was detected. Among the infected groups with MYD88 intact, there were no significant differences in inflammatory scores. The MYD88 knockdown group that was infected had significantly greater inflammatory scores compared to all other groups (Figure 3-3).

Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Expression of Pro-Inflammatory Cytokines and Reduces Expression of Anti-Inflammatory Cytokines in Periodontal Disease

The levels of both pro-inflammatory and anti-inflammatory cytokines were measured by homogenizing tissues from the maxilla and mandible and analyzing them with the Luminex 200® system running xPONENT® 3.1 software (Luminex, Austin, TX). They were analyzed using a 5-paramater logistic spline-curve fitting method using Milliplex® Analyst V5.1 software (Vigene Tech, Carlisle, MA). Levels of TNFα, IL6, IL8, TGFβ1, IL10, IFNγ, IL17A, IL23 and IL27 were measured. In the uninfected groups of mice, there were no significant differences between any of the inflammatory mediators measured. Similarly among the infected groups with MYD88 intact, no significant differences in the inflammatory mediators was observed. In the infected group with
MYD88 knocked down, significantly greater levels of TNFα, IL6, IL1β, IL17A, IL22, and IL23 were observed compared to all the other groups. In addition to this, significantly reduced levels of IL10, TGFβ1, and IL27 were measured in the infected group with MYD88 knocked down when compared to all the other groups (Figures 3-4 and 3-5).

**Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Colonization of P.g. and A.a.**

On the first day of infection, prior to infection, and at the beginning of each week of infections, samples of the oral environment were taken with calcium alginate swabs (FisherScientific). The samples were analyzed using real time PCR to measure the levels of P.g 16S and A.a 16S. The uninfected groups of mice had no detectable levels of P.g. 16S or A.a. 16S at any of the time points measured. Among the groups of infected mice with MYD88 intact, P.g. and A.a were both detected, however, no significant differences existed among the groups at any of the time points measured. In the infected group with MYD88 knocked down, significantly greater levels of P.g. and A.a. were detected compared to all groups at all time points measured (Figure 3-6).

**Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Prevalence of Th1 and Decreases Prevalence of Th17 and Treg in Periodontal disease**

The prevalence of both Th1 and the Th1/Th17 ratio (as measured by % of CD4+) was significantly greater in the B6 infected group with MYD88 knocked down compared to the other B6 infected groups. No significant differences were observed between the other infected B6 mice. The prevalence of both Th17 and Treg (as measured by % of CD4+) were significantly reduced in the B6 infected group with MYD88 knocked down compared to the other B6 infected groups. No significant differences were observed between the other infected B6 mice (Figure 3-7).
Epithelial Cell Knockdown of MYD88 in NOD Mice Reduces Bone Loss in Periodontal Disease

Bone volume loss of NOD mice with MYD88 intact were compared with that of NOD mice with MYD88 knocked down with micro computed tomography analysis as described previously. No significant differences were observed between the uninfected groups of mice. The infected groups of NOD mice with MYD88 intact also did not display any significant differences. Interestingly, when comparing the infected NOD group with MYD88 knocked down with the other infected groups, a significantly greater bone volume was present by the end of the study. This suggests that in conditions of T1D and periodontal disease, MYD88 may promote the destruction of periodontal tissues. Comparing the B6 uninduced, infected mice with the NOD uninduced, infected mice, the NOD group experienced significantly more bone loss. The B6 infected, MYD88 knock down group experienced significantly more bone volume loss compared to the NOD infected, MYD88 knock down group. This once again supports the idea that the presence of MYD88 in conditions of T1D and periodontal disease may promote an environment that produces periodontal tissue destruction (Figure 3-8).

Epithelial Cell Knockdown of MYD88 in NOD Mice Reduces Inflammation in Periodontal Disease

In the groups of mice that were not infected, no inflammation was detected. Among the infected groups of NOD mice with MYD88 intact, there were no significant differences in inflammatory scores. The NOD MYD88 knockdown group that was infected had significantly reduced inflammatory scores compared to all other infected NOD groups. The infected NOD group with MYD88 intact had significantly greater inflammatory scores compared to the infected B6 group with MYD88 intact. When comparing the infected B6 knock down group with the infected NOD knock down group,
it was observed that the NOD mouse had significantly lower inflammatory scores. (Figure 3-9).

**Epithelial Cell Knockdown of MYD88 in NOD Mice Increases Expression of Pro-Inflammatory Cytokines and Reduces Expression of Anti-Inflammatory Cytokines in Periodontal Disease**

In the uninfected groups of mice, there were no significant differences between any of the inflammatory mediators measured. Similarly among the infected groups with MYD88 intact, no significant differences in the inflammatory mediators was observed. In the infected group with MYD88 knocked down, significantly reduced levels of TNFα, IL6, IL1β, IL17A, IL22, and IL23 were observed compared to all the other groups. Interestingly, among the anti-inflammatory cytokines, IL10, TGFβ1, and IL27, there were no significantly differences between any of the infected groups, including the MYD88 knock down group. This suggests that while the pro-inflammatory mediators seem to be at least partially under control of TLR function, the anti-inflammatory mediators measured appear to be expressed independently of it. (Figures 3-10 and 3-11).

**Epithelial Cell Knockdown of MYD88 in NOD Mice Increases Colonization of P.g. and A.a.**

The uninfected groups of mice had no detectable levels of P.g. 16S or A.a. 16S at any of the time points measured. Among the groups of infected mice with MYD88 intact, P.g. and A.a were both detected, however, no significant differences existed among the groups at any of the time points measured. In the infected group with MYD88 knocked down, significantly greater levels of P.g. and A.a. were detected compared to all groups at all time points measured (Figure 3-12).
Epithelial Cell Knockdown of MYD88 in B6 Mice Increases Prevalence of Th1 and Decreases Prevalence of Th17 and Treg in Periodontal disease

The prevalence of both Th1 and the Th1/Th17 ratio (as measured by % of CD4+) was significantly lower in the NOD infected group with MYD88 knocked down compared to the other NOD infected groups. No significant differences were observed among the other infected NOD mice. The prevalence of Th17 (as measured by % of CD4+) was significantly greater the NOD infected group with MYD88 knocked down compared to the other NOD infected groups. No significant differences in prevalence of Treg (as measured by % of CD4+) were observed among any of the infected groups of mice. This suggests that in diabetic, periodontally diseased mice, MYD88 does not regulate the production of Treg (Figure 3-13).
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Table 3-2. Experiment timeline

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<td>outcome measures</td>
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Figure 3-1. Murine oral epithelial cell knockdown of MYD88

Immunohistochemistry of the buccal tissues from NOD$^{K5\text{Cre}}$.MyD88plox 7 days following exposure to (A) Sham and (B) RU486. Anti-MYD88 (red) and nuclear stain (blue). The lack of expression in the RU486 group is evident in (B)
Figure 3-2. Percentage of bone volume as measured by micro computed tomography
Figure 3-3. Inflammatory scores in the B6 mice assessed during histologic analysis of the left maxilla of each mouse

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Figure 3-4. Pro-inflammatory and anti-inflammatory mediators in the B6 mice assessed with Luminex 200
Figure 3-5. IL17 Cytokine axis mediators in the B6 mice assessed with Luminex 200
Figure 3-6. 16S levels of A.a. and P.g. measured by real time PCR
Figure 3-7. Immune cell profiles as measured by flow cytometry
Figure 3-8. Bone volume levels as measured by micro computed tomography.

A. A comparison of the uninduced, infected B6 and NOD groups.  B. Bone loss of the various NOD mice groups.  C. A comparison of the induced, infected B6 and NOD groups.
Figure 3-9. Inflammatory scores in the NOD mice assessed during histologic analysis of the left maxilla of each mouse.

A. Effect of T1D on Inflammatory Score B. Affect of MyD88 in T1D on Inflammatory Score C. Difference in MyD88 affect on Inflammatory Score between health and T1D
Figure 3-10. Inflammatory and anti-Inflammatory cytokine mediators in the NOD mice assessed with Luminex 200.
Figure 3-11. IL17 Cytokine axis mediators in the NOD mice assessed with Luminex 200.
Figure 3-12. Colonization in T1D as measured by real time PCR
Figure 3-13. Immune cell profiles in T1D
Periodontal disease is a result of bacterial induced inflammation in a susceptible host. The resultant inflammation produces an environment that favors the loss of periodontal hard and soft tissues. Microbes are initially detected by pattern recognition receptors (PRRs) on cells of the innate immune system. One such class of PRRs are the toll-like receptors (TLRs). TLRs are able to detect highly conserved pattern associated molecular patterns (PAMPs) and then mount an immunologic response. TLR are able to recognize both commensal and invading microbes and either upregulate the production of pro-inflammatory cytokines in the presence of harmful microbes, or induce inhibition of the immune system in the presence of commensal microbes. TLRs are expressed on numerous types of cells throughout the body. Interestingly, they are not restricted to cells of the immune system and are expressed on a variety of non-immune cells, including those of the periodontium (Mahanonda and Pichyangkul, 2007). Of the cells of the periodontium that express TLRs, those of the epithelium act as the first line of defense against microbial invasion. These epithelial cell TLR will experience continuous stimulation by the commensal organisms and in fact, it has been demonstrated that this activation by commensal microbes is critical for the maintenance of oral health (Hatakeyama et al., 2003). Because they are involved in both upregulating and suppressing the immunologic response to microbial detection, TLRs of the epithelial cells play an important role in maintaining the balance of pro-inflammatory and anti-inflammatory cells. The absence of their function, as
demonstrated previously in our laboratory by targeted knockdown of MYD88 in oral epithelial cells, leads to exacerbated periodontal disease (unpublished data).

The relationship between diabetes and periodontal disease has been known for decades and was first presented in the literature by Williams and Mahan in 1960 (Williams and Mahan, 1960). Further investigation into the matter yielded the knowledge that this relationship is actually bi-directional, i.e. the severity of each disease can exacerbate the severity of the other. On the other hand, improvement of one disease tends to ameliorate the severity of the other. For example, successful treatment of periodontal disease has been shown to produce improvements in glycemic control and help reduce the systemic inflammation that results in some of the secondary complications of diabetes (Preshaw et al., 2012). Another example of the bi-directional nature of the relationship is the increased periodontal tissue destruction that diabetes can help promote. Specifically, in type I diabetes, aberrant activation of TLRs on oral epithelial cells has been demonstrated, leading to an overall increase in the expression of pro-inflammatory cytokines (Neiva et al., 2014).

Our original hypothesis predicted that we would observe significantly more tissue destruction under conditions of type I diabetes, periodontal disease, and a knockdown of MYD88. The results demonstrated quite the opposite. The NOD knockdown group with periodontal disease experienced the least amount of bone loss compared to any of the other infected groups. Furthermore, the knockdown group also had significantly lower levels of the pro-inflammatory mediators that were measured compared to all of the other infected groups. A possible explanation for this phenomenon could be that the aberrant activation of oral epithelial cell TLRs that we know can occur in type I diabetes
was prevented by disabling TLR dependent signaling via the knockdown of MYD88. This could lead to an overall decrease in the levels of pro-inflammatory cytokines and subsequently less periodontal tissue destruction. It is important to note that while the knockdown of MYD88 in the oral epithelial cells restricted the TLR mediated expression of these pro-inflammatory cytokines, the other immune cells that were not targeted with the knockdown still retained their function. This explains the observed levels of tissue destruction and pro-inflammatory cytokine expression.

Our results indicated that there were no significant differences in the levels of colonization of \textit{A.a} or \textit{P.g.} when comparing the B6 and NOD mouse models when MYD88 was intact. This suggests that NOD mice are not any more susceptible to colonization of periodontal pathogens compared to their non-diabetic counterparts. In the MYD88 knockdown, infected groups, both B6 and NOD mice, significantly greater levels of colonization of both \textit{A.a} and \textit{P.g.} were observed compared to the groups with MYD88 intact. This suggests that in both the NOD and B6 models, MYD88 plays a protective role against bacterial colonization. The exact mechanism of this protective aspect was not determined in this study, however, it is plausible that it is a result of a reduction of beta defensin expression. Defensins are cysteine rich cationic proteins that serve a protective role against microbes such as bacteria and fungi. They are found in every mammalian species explored to date. Their mechanism of action is to disrupt the cell membrane of the invading microbe and causing membrane depolarization and cell lysis. Previous studies have indicated that defensing production is mediated by TLR signaling (Beckloff and Diamond, 2008; Diamond and Bevins, 1998; Diamond and Ryan, 2011; Yount et al., 1999). Therefore, it is biologically plausible that he
knockdown of MYD88 could cause increased proliferation due to the expected decline in defensin prevalence. The resultant reduction in tissue destruction in the NOD knockdown group despite the higher prevalence of pathogenic bacteria lends support to the concept that bacterial colonization is necessary but not sufficient to produce periodontal disease. The host’s response, in this case in the form of pro-inflammatory mediators, is also a required component in establishing the disease.

The expression of the pro-inflammatory mediators measured in this study were coincident with those of the bone volume loss. The mediators measured were those of the IL17 axis has been heavily implicated in the progression of autoimmune and inflammatory diseases, including periodontitis. The IL17 axis is made up of several inducers and inhibitors of the inflammatory response. For example, IL17 is responsible for recruiting neutrophils when acting alone. However, in the presence of IL22 it becomes a potent inducer of several inflammatory mediators, such as TNFα, IL6, IL8, and MMPs. Additionally, more recent data shows that IL17 may be involved in bone remodeling via activating RANKL and inhibiting expression of osteoprotegerin in PDL cells. This suggests that IL17 may play a key role in the tissue destruction we observe in periodontal disease (Miyazaki et al., 2008). IL17 is primarily regulated by IL23 and IL27, with IL23 upregulating its induction and IL27 downregulating its induction. IL27 is also an important regulator of T-helper cell differentiation. In fact, its absence can lead to an exaggerated production of various pro-inflammatory cytokines, resulting in severe inflammation (Miyazaki et al., 2008). The infected NOD knockdown group had significantly less expression of the cytokines TNFα, IL6, IL1β, IL17A, IL22, and IL23 compared to the other infected groups. At first, this observation may appear to be an
overall reduction in the immunologic response due to the restriction of TLR activity. However, when studying the levels of the anti-inflammatory mediators IL10, TGFβ1, and IL27, this does not seem to be the case. There were no significant differences in the expression of IL10, TGFβ1, and IL27 among any of the infected groups. This finding suggests that only the pro-inflammatory mediators measured in this study are under control of epithelial cell TLRs, not the anti-inflammatory ones.

Additionally, the levels of the T helper cells, Th1, Th17, Treg, and the ratio of Th1/Th17 were assessed by flow cytometry. T-helper cells are an important part of the immune system and play a role in suppressing or regulating immune responses. Th1 cells are involved in the immune response to intracellular bacteria and protozoa, which is particularly relevant to the P.g. used in this study. TH17 cells are involved in producing IL17. Depending on the inducing cytokines that are present, they can differentiate into either protective, non-pathogenic cells or pro-inflammatory pathogenic cells. The protective version of TH17, called T17reg, is induced with IL6 and TGFβ, while the pathogenic form is induced by IL23 and IL1β. Recent evidence suggests that TH17 can play a major role in osteoclast differentiation through cell-cell contact with osteoclast precursors (Fumoto et al., 2014), (Won et al., 2011). Treg cells, which were formerly known as suppressor Tcells, are modulators of the immune system. They are responsible for maintaining tolerance to self-antigens. Treg cells are also responsible for shutting down the immune response after the invading pathogen has been eliminated (Shevach, 2000). In our study, the B6, infected, MYD88 knockdown group, had levels of Th1 and a ratio of Th1/Th17 that were significantly greater than the B6 infected groups with MYD88 intact. Conversely, the levels of Th17 and Treg were
significantly lower in the MYD88 knockdown group. This increase in the levels of Th1 and the resulting Th1/Th17 ratio is likely due to the increased expression of the pro-inflammatory cytokines observed in the B6 knockdown group. Interestingly, in the NOD, infected, knockdown group, the levels of Th1 and the ratio of Th1/Th17 were significantly lower compared to any of the other NOD infected groups with MYD88 intact. Additionally, the levels of Th17 were significantly higher in the knockdown group. No significant differences were noted in the levels of Treg among any of the infected groups. This data suggests that the reduced levels of pro-inflammatory cytokines observed in the NOD knockdown group led to a reduction in the prevalence of Th1 and as a consequence, the ratio of Th1/Th17.

An analysis of the data collected in this study suggests that one of the key mediators of the exacerbated inflammation and tissue destruction observed in periodontal disease is the IL17 axis. Our results demonstrated that the infected, NOD, knockdown group had significantly less expression of IL17 and its inducers compared to the other NOD infected groups. This can be explained by looking back to what we know about the TLR dysregulation in T1ds (Neiva et al., 2014). In T1ds, the TLRs can produce exaggerated responses when encountering a PAMP that it recognizes that leads to increased levels of pro-inflammatory cytokines. Therefore, it is biologically plausible that disabling the TLRs of the epithelial cells may prevent this exaggerated response in at least a portion of the cells responsible for producing these cytokines. Interestingly, the inhibitors of the IL17 axis appear to be unaffected by the MYD88 knockdown, suggesting that they are not under control of oral epithelial TLRs. These reduced levels of the pro-inflammatory cytokines are responsible for the significantly
reduced amount of bone loss and histologic inflammation in spite of increased bacterial colonization.

In conclusion, T1D associated exacerbation of periodontal disease appears to be related to dysregulation of the TH17 axis, which is at least partially controlled by oral epithelial TLRs. This marks oral epithelial TLRs as a potential target for host modulation during periodontal therapy in T1D patients.
LIST OF REFERENCES


BIOGRAPHICAL SKETCH

Dr. Todd M. Jenny studied computer science at the University of California, Irvine, where he graduated in 2006. After which, he enrolled in a health professions post-baccalaureate program at California State University, Fullerton. He then attended dental school at the University of California, Los Angeles, where he graduated in 2013. Dr. Jenny completed his training in periodontology at the University of Florida in 2016.