Title

Molecular Mechanisms of T. denticola Induced Tissue Destruction in Human Periodontal Ligament Cells

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Molecular Mechanisms of T. denticola Induced Tissue Destruction in Human Periodontal Ligament Cells

by
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1.1.2 Contributions

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Molecular Mechanisms of *Treponema denticola* Induced Tissue Destruction in Human Periodontal Ligament Fibroblast Cells

Sean Thomas Ganther

ABSTRACT

Periodontal disease (PD) is a complex infection driven by a myriad of bacterial species interacting with host tissues, triggering the release of pro-inflammatory cytokines, chemokines, and tissue-remodeling enzymes like Matrix Metalloproteinases (MMP), culminating in periodontal tissue degradation. Despite clinical intervention, severe periodontitis patients exhibit a persistent pro-inflammatory state, perpetuating tissue destruction. *Treponema denticola* (T. denticola), an oral anaerobic bacteria, notably abundant in advanced lesions, harbors potent and abundantly expressed virulence factors, including the chymotrypsin-like protease complex (CTLP or dentilisin), implicated in various pathogenic activities like adhesion, ECM degradation, and MMP activation. Our research has shown elevated *T. denticola* DNA levels correlated with increased MMP2 mRNA expression. Short-term exposure of human periodontal ligament fibroblast cells (hPDL) to wild-type *T. denticola* bacteria led to prolonged upregulation of the MMP-2-MMP-14-TIMP-2 activation axis for up to 12 days *in Vitro*. Despite extensive protein-level studies, direct links between *T. denticola* protease activity and periodontal tissue destruction at transcriptional or epigenetic levels remain scarce. Chemically characterized as a tri-acylated lipoprotein, dentilisin more than likely engages with TLR2-dependent mechanisms. Thus, based on literature and current data from our laboratory, we hypothesize that *T. denticola* exploits dentilisin to activate a TLR2-dependent pathway, inducing the upregulation of tissue-destructive genes in hPDL cells. This work sheds light on the specific interactions between human periodontal ligament cells and *T. denticola*, elucidating the transcriptional drivers of periodontal disease chronicity using an array of molecular techniques and high-throughput sequencing methods, revealing potential therapeutic targets beyond antibiotics.

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LIST OF ABBREVIATIONS

Abbreviation	Full Name
T. denticola or Td	Treponema Denticola
P. gingivalis	Porphyromonas gingivalis
PD	Periodontal Disease
hPDL	Human Periodontal Ligament Cells
MMP	Matrix Metalloproteinases
ECM	Extracellular Matrix
qRT-PCR	Quantitative Real Time Polymerase Chain Reaction
PRR	Pattern Recognition Receptor
TLR	Toll Like Receptor
A. *actinomycetemcomitans*	*Aggregatibacter actinomycetemcomitans*
CAL	Clinical Attachment Loss
F. nucleatum	*Fusobacteriu. Nucleatum*
FH	Factor H
FHhB	Factor H Binding Protein B
PAMPS	Pathogen Associated Microbial Patterns Molecules
DAMPS	Damage Associated Molecular Patterns Molecules

INTRODUCTION

Periodontitis or Periodontal Disease is characterized as a chronic oral inflammatory disease that compromises the integrity of the tooth-supporting tissues, which include the gingiva, periodontal ligament and alveolar bone, and are collectively known as the periodontium. In its severe form, which afflicts 11% of adults in the United States, periodontitis may cause tooth loss, and also affect systemic health by increasing the patients' risk for diabetes (ref), atherosclerosis, adverse pregnancy outcomes, rheumatoid arthritis, aspiration pneumonia and cancer[1-8]. Additionally, patients with chronic forms of periodontal disease are left with a non-resolving pro-inflammatory transcriptional profile throughout the periodontium, even after clinical intervention, leading to tissue-destruction and tooth loss[9-11]. This suggests that previously uncharacterized cellular and molecular mechanisms underlying periodontal disease pathophysiology may explain why many patients do not respond to the conventional treatment schema.

The periodontal ligament (PDL) has two primary functions: 1) to absorb and respond to mechanical stresses, and 2) to provide vascular supply and nutrients to the cementum, alveolar bone and the PDL itself. Osteoblasts, osteoclasts, cementoblasts, fibroblasts, and endothelial cells make up the PDL and reside on the surface of the lamina dura and endosteal surfaces of the alveolar bone and cementum[12-15]. Loss of the PDL increases tooth mobility and the amount of irreversible clinical attachment loss (CAL) which is a key clinical indicator for staging and grading the diagnosis of periodontal disease. Additionally, loss of the PDL diminishes the ability for the periodontium to replenish itself through regenerative pathways. The most prominent cell type within the PDL is the human periodontal ligament (hPDL) fibroblast , which specializes in mechanosensing and tissue remodeling through robust expression of various hydrolytic enzymes, such as matrix metalloproteinases (MMPs)[12, 16]. These cells also function as immune-like cells demarcated by the production of inflammatory cytokines, chemokines and expression of pattern recognition receptors (PRRs), such as toll-like receptors

(TLRs), which are responsible for monitoring the local environment for signs of danger in the form of highly conserved microbial molecules present during periodontal disease[17-20].

When TLR signaling is left unchecked, downstream genes, such as MMPs can become significantly upregulated and constitutively activated, thereby contributing to the overall destruction of the periodontium by degrading extracellular matrix (ECM) proteins, such as collagen and fibronectin[21-27]. Although the role of MMPs has been primarily ascribed to turnover of the ECM, the great number of new substrates discovered for MMPs in the last few years suggest they are capable of regulating many signaling pathways, cell behaviors and diseases through novel mechanisms[28]. Therefore, PDL cells likely play a significant role in initiating and exacerbating tissue degradation and suggest that pharmacological anti-inflammatory treatment of periodontal disease should target dysregulated MMP activity and levels in the PDL tissues.

The trigger for the initiation of disease is the presence of complex microbial biofilms[29-31] that colonize the sulcular regions between the tooth surface and the gingival margin through specific adherence interactions and accumulation due to architectural changes in the sulcus (i.e. attachment loss and pocket formation). From these observations, it is evident that addressing the pathogens associated with periodontitis is crucial for effective resolution of the condition. The current tripartite model of treatment planning underscores the importance of managing periodontitis and bacterial load through phase I and II periodontal therapy[32]. Phase I treatment primarily focuses on reducing bacterial infection through mechanical debridement of calculus and the use of broad-spectrum antibiotics such as azithromycin, doxycycline, tetracycline, moxifloxacin, and amoxicillin/metronidazole. In contrast, Phase II periodontal therapy aims to eliminate diseased tissues by enhancing access for debridement, often through procedures like gingivectomy and gingivoplasty. While Phase II approaches effectively remove infected tissue,

they may also involve adjunctive drug therapy such as sub-antimicrobial dose doxycycline and local antibiotic delivery techniques like Atridox, PerioChip, and Arrestin.

Combination antibiotic therapy, particularly amoxicillin and metronidazole, is sometimes employed as an adjunctive therapy during Phase II treatment due to its broad-spectrum antibacterial effects[33]. Studies have shown that high-dose combination therapy can lead to short-term reductions in pocket depth and clinical attachment loss in aggressive periodontitis[34-36]. However, without effective bacterial control, these improvements may not be sustained, as relapse can occur[37].

Moreover, consistent intracellular infection may decrease susceptibility to antimicrobials, potentially contributing to higher rates of treatment failure[38]. These findings highlight the need for further research to address the growing evidence that periodontal disease pathogens can persist both intracellularly and extracellularly within periodontal tissues. Additionally, there is a need for therapies targeting these infections with concentrations that effectively remove the microbial load without promoting the development of resistant strains. Lastly, investigation is warranted to determine whether infected tissues can be salvaged after microbial load reduction, addressing both intracellular and extracellular infections.

Identifying a promising antibiotic for periodontal treatment presents a significant challenge due to variations in bacterial susceptibility among species. For instance, a study demonstrated that doxycycline and clindamycin, even at a concentration 10-fold higher than the minimum inhibitory concentration (MIC), had no effect on killing *Porphyromonas gingivalis* (*P. gingivalis*), while metronidazole was effective[39]. Conversely, under the same experimental conditions, intracellular *Aggregatibacter actinomycetemcomitans* (*A. actinomycetemcomitans*) was effectively eliminated by Azithromycin[39]. Additionally, Beikler et al. (2004) highlighted the

complexity of microbial profiles in periodontitis patients as means of prescribing bacteria specific regimens[40]. They identified nine major pathogen complexes (PCs) in 73.4% of patients and 38 minor PCs in 26.6% of patients. The study concluded that at least 46 different combinations of assessed periodontal pathogens exist on average, necessitating potentially 10 different antibiotic regimens to target these pathogen complexes specifically.

Moreover, exploring drugs targeted against the epigenetic machinery offers promising avenues outside of the conventional antibiotic infrastructure. These drugs act on host modulation pathways, presenting potential benefits for patients unresponsive to current adjunct regimens. For example, compounds like JQ1 or I-BET151 have shown efficacy in treating infected gingival epithelial cells or in a disease context with diseases that exhibit similar chronic progression profiles, such as Juvenile Idiopathic Arthritis[41, 42]. Such host modulation therapies could revolutionize periodontal treatment by addressing underlying host factors contributing to disease progression. To advance periodontal therapy, a deeper understanding of microbial-host interactions at the genetic and epigenetic levels is imperative. This knowledge will not only inform the development of more targeted therapies but also enhance our ability to tailor treatment strategies to individual patient needs.

The characteristics of microbiological progression from periodontal health to gingivitis (e.g. chronic inflammation of the gingival tissue without tissue destruction), and eventually to periodontal disease are vast and complicated. It has been estimated that nearly 700 bacterial taxa, phylotypes and species, which show some structural organization in the biofilms[30, 43-45], can colonize the oral cavity of humans, although it remains unclear how this multitude of bacteria compete, coexist, and／or synergize to initiate this chronic disease process. Seminal studies of Socransky and Haffajee and colleagues catalogued and stratified the microbiota into groups or complexes, representing bacterial consortia that appear to occur together and that are associated with the biofilms of gingival health, gingivitis and periodontitis[46]. Among microbial

complexes, the first, such complex that has been related to disease is the orange complex consisting of anaerobic gram-negative species such as *Prevotella intermedia, Prevotella nigrescens, Prevotella micros*, and *Fusobacterium* nucleatum, which during disease progression shifts toward a red-complex consisting of *Tannerella forsythia, Treponema denticola*, and *Porphyromonas gingivalis[43, 47, 48]*. Interactions between bacteria, viruses and host cells have been associated with shifts from health to disease in oral tissues as well[49-51]. The virulence potential and determinants of these commensal opportunists including requirements for adherence, sustenance (i.e. accessing required nutritional materials for growth and replication), and evasion of host responses are still largely unexplored.

While a large focus has been placed on species such as *Porphyromonas gingivalis*, *Fusbacterium nucleatum*, *and Aggrigatibacter actinomycetemcomitans*, recent metagenomic and metatranscriptomic studies have implicated the gram negative-oral spirochete, *T*. *denticola* with advanced or aggressive forms of periodontal disease and recurrence of disease[31, 52]. Greatly increased numbers of *T*. *denticola* levels in periodontal disease vs health has been well-documented in the literature for >40 years[53-55]. Our lab has readily demonstrated that *T*. *denticola* was ~15-fold higher in the subgingival biofilm of periodontal lesions[56]. Additionally, elevated *T*. *denticola* biofilm levels combined with elevated MMP levels in host tissues display robust combinatorial characteristics in predicting advanced periodontal disease severity[56, 57]. Further, Lee *et al*. and colleagues demonstrated that *T*. *denticola* infection was sufficient to induce alveolar bone resorption in a mouse model of periodontal disease[58]. Thus, clinical data regarding the increased presence of *T*. *denticola* in periodontal lesions, together with basic and *in vivo* studies involving the role of *T*. *denticola* products suggest that it plays a pivotal role in driving periodontal disease progression.

Among the various *T. denticola* effector molecules that have been described, its acylated chymotrypsin-like protease complex (CTLP), more recently called dentilisin, is a major virulence factor which facilitates numerous cytopathic effects that align with periodontal disease pathophysiology. A few examples including adhesion, degradation of endogenous ECM-substrates[59], tissue penetration[60], complement evasion[61, 62], ectopic activation of host MMPs[63, 64] and degradation of host chemokines and cytokines, such as IL-1*β* and IL-6, primarily due to its potent proteolytic activity[65].

As in other spirochetes, conserved lipid moieties of the protease complex recognized by host TLR2 receptor complexes may contribute to activation of innate immune responses[66]. Because predominant host responses to lipoproteins are believed to be to their lipid moieties, most studies have focused on diacylated lipopeptide, Pam2CSK4, and triacylated lipopeptide, Pam3CSK4, which mimic bacterial lipoproteins for their potent immunostimulatory and osteoclastogenic activities by preferentially activating TLR2-dependent pathways[67, 68]. Recent studies have demonstrated that synthetic di- and tri-acylated lipopeptides which preferentially activate TLR2/6 and TLR2/1-dependent pathways respectively, are sufficient to induce alveolar bone loss in mice[69], broadening the avenues of investigation into the role of lipoproteins underpinning the pathogenesis of periodontal disease. However, studies which utilize endogenously expressed bacterial lipopeptides are greatly lacking.

While a clear role for dentilisin in the context of periodontal disease has been delineated at the protein level, its ability to influence host-cell functions in a gene-centric context has yet to be characterized. Thus, the aim of this study was to determine the extent to which *T. denticola* and its highly expressed acylated dentilisin protease complex influence the transcriptional regulation of MMPs through TLR2-dependent pathways in hPDL cells.

Chapter 1.1: ABSTRACT

Treponema denticola (*T. denticola*), a spirochete bacterium, plays a significant role in periodontal disease despite existing at low levels in commensal biofilms Its ability to manipulate the host immune response rather than induce direct inflammation distinguishes *T. denticola* as a master evader of host defenses[53].

Moreover, *T. denticola*'s role is further highlighted through its synergistic interactions with *Porphyromonas gingivalis* (*P. gingivalis*), another keystone pathogen in periodontal disease. Although *P. gingivalis* and *T. denticola* comprise a minor portion of subgingival biofilms in healthy patients (<0.01%), they significantly impact the inflammatory milieu. Isobutyric acid production by *P. gingivalis* stimulates *T. denticola* growth, while *T. denticola* reciprocates by producing succinic acid that promotes *P. gingivalis* growth[70]. Upon contact with *T. denticola*, *P. gingivalis* upregulates the expression of adhesins and proteases, such as its potently expressed gingipains[71]. These interactions underscore the complex dynamics within the dysbiotic periodontal community and the critical role of *T. denticola* in subverting host defenses and concomitantly promoting disease progression by increasing the proliferative rate of other disease associated pathogens.

T. denticola's interaction with the host's innate immune system is pivotal in the progression of periodontal disease. Our study demonstrated *T. denticola's* ability to avoid the activation of transcription factors like NF-κB and NFATc2, essential for inflammatory responses, indicating a nuanced regulatory mechanism that *T. denticola* employs to modulate host immunity effectively. Short-term exposure of hPDL cells to *T. denticola* bacteria induces significant shifts in gene profiles, but our study demonstrated that *T. denticola* suppresses the upregulation of multiple cytokines and chemokines at the transcriptional level.

Our findings provide a better understanding of the molecular interactions between *T. denticola* and oral cells. The pathogen's ability to suppress pro-inflammatory cytokine production and inhibit key transcription factors such as NF-κB and NFATc2 highlights its sophisticated strategies to evade the host immune response. These insights contribute to our understanding of periodontal disease pathogenesis and underscore the need for therapeutic interventions targeting these bacterial evasion mechanisms.

Chapter 1.2: INTRODUCTION

The dysbiotic periodontal community faces a challenging paradox: while these bacteria must evade immune-mediated destruction, they also rely on inflammation to obtain nutrients from tissue degradation, including collagen peptides and hem-containing compounds[72]. As a result, conditions lead to a dysbiotic subgingival biofilm changing the overall biomass, virulence factor output, synergistic effects and the ability to subvert the normal immune host-response. For example, the gram-negative asaccharolytic bacterium *Porphorymonas gingivalis* (*P. gingivalis)* comprises a rather small portion of subgingival biofilms in healthy patients (<0.01%) and has long been associated with human periodontitis. Similarly, *T. denticola* exists at a relatively similar level in commensal biofilms[56]. The ability of the low-abundant *P. gingivalis* to instigate inflammatory disease through community-wide supportive effects has prompted its designation as a keystone pathogen in tandem with *T. denticola* through direct interactions. For example, isobutyric acid production by *P. gingivalis* stimulates the growth of *Treponema denticola*, and reciprocally *T. denticola* produces succinic acid that promotes *P. gingivalis* growth[73]. Moreover, upon contact with *T. denticola*, *P. gingivalis* upregulates the expression of adhesins and proteases such as its potently expressed gingipains[74]. Consistent with these *in vitro* findings, *P. gingivalis* and *T. denticola* are synergistically pathogenic *in vivo*[75].

The virulence credentials of *T. denticola* are more consistent with its being a manipulator of the host response rather than a potent inducer of inflammation, an activity normally associated with a bacterium involved in an inflammatory disease[76]. Specifically, by subverting innate immune signaling including the crosstalk between complement and Toll-like receptors (TLR)[61, 76-78]. Notably, Brisette et al. reported that key genes involved in innate immune responses, such as those encoding pro-inflammatory cytokines (e.g., IL-8, IL-6) and chemokines (e.g., ICAM-1, HβD-2), were not induced by *T. denticola[62]*. Despite this, the authors demonstrate that TLR2

signaling remained active upon stimulation. Additionally, Shin and colleagues reported that *T. denticola* suppresses human beta-defensin 3 through the activation of the TLR2-MAPK signaling axis[79, 80].

T. denticola thrives within the complex microenvironment of the gingival crevicular fluid (GCF), a crucial anatomical niche surrounding the teeth. GCF, comprising a blend of serum and local tissue exudate, serves as a dynamic reservoir of immune effectors pivotal in the host's defense against microbial invaders[81]. Notably, GCF collected from patients afflicted with periodontal disease manifests a rich array of immune mediators, including activated complement, C-reactive protein (CRP), pro-inflammatory cytokines, and IgA and IgM antibodies[79]. The presence of such potent immune components underscores the need for *T. denticola* to possess robust evasion mechanisms to survive in this hostile milieu. Among its armamentarium, dentilisin emerges as a multifaceted surface-expressed protease complex renowned for its proteolytic prowess and its capability to dismantle crucial complement components, notably Factor H (FH). FH, a prominent 155 kDa serum glycoprotein, plays a pivotal role in regulating the alternative complement cascade, acting as a potent defender against invading pathogens. However, *T. denticola's* deployment of dentilisin disrupts this delicate balance, leading to the degradation of FH and subsequent dysregulation of the host complement system[61, 76]. Furthermore, the surface protein FhbB serves as a critical player in *T. denticola's* evasion repertoire by binding to human FH, thereby facilitating the sequestration of this glycoprotein[61, 82]. This intricate interplay exacerbates the inhibition of complement-mediated killing, further enhancing the bacterial survival within the host. These mechanisms underscore the sophisticated strategies employed by *T. denticola* to evade immune surveillance and thrive in the periodontal niche. Understanding these evasion tactics is imperative for devising targeted therapeutic interventions to combat *T. denticola*-associated periodontal diseases.

T. denticola is frequently found in close proximity to and within the periodontium, contributing significantly to the inflammatory milieu associated with periodontal infections. The presence of *T. denticola* has been intricately linked to the initiation and progression of periodontal disease, where its interaction with the host's innate immune system plays a crucial role. Indeed, mounting evidence suggests that the host's innate immune response to *T. denticola* serves as a pivotal determinant in the pathology of periodontal disease. This response is characterized by the regulation of inflammatory cytokines, which orchestrate gingival inflammation and alveolar bone loss, hallmark features of periodontal infection. Understanding the intricate interplay between *T. denticola* and the host immune system is essential for unraveling the mechanisms underlying periodontal disease progression and developing targeted therapeutic interventions to mitigate its deleterious effects on periodontal tissues.

Toll-like receptors (TLRs), a type of pattern recognition receptor (PRR), have been intensively investigated in recent years. They are believed to serve as a bridge between innate and adaptive immunity[83-85]. To date, ten TLRs (TLR1 to TLR10) have been identified in humans. Each TLR resides in a specific part of the cell and is capable of sensing distinct pathogen-associated microbial patterns (PAMPs). Based on the composition of PAMPs that they recognize, TLRs can be classified into two categories. TLR1, TLR2, TLR4, TLR5, TLR6, and TLR10 reside in the cell membrane and can sense PAMPs that are mainly composed of lipids or proteins. In contrast, TLR3, TLR7, TLR8, and TLR9 are found in the endosomal membranes and can sense PAMPs that consist primarily of nucleic acids[84, 85]. When a TLR-mediated MyD88-dependent pathway is activated by specific ligands, transcription factors NF-κB and AP-1 can be activated through either of the two canonical pathways leading to the upregulation of pro or anti-inflammatory genes[86].

Oral treponemes, including *T. denticola*, interact with human gingival epithelial cells through Toll-like receptor 2 (TLR2) signaling pathways, a key component of the innate immune system [23]. Studies have demonstrated that TLR2 serves as the primary receptor for recognizing specific molecular components of treponemal outer membranes, including glycolipids and lipoteichoic acid [24]. Moreover, investigations utilizing human embryonic kidney (HEK) cell lines transfected with TLR2 have revealed that *T. denticola* cell walls possess the ability to activate TLR2-mediated immune responses [25]. Additionally, *T. denticola* cells, along with their major outer sheath protein (MSP), have been found to stimulate murine macrophages, further implicating the pathogen's involvement in innate immune activation [26]. Despite the identification of TLR2 as a key mediator in *T. denticola*-induced immune responses, the specific pathogen-associated molecular patterns (PAMPs) responsible for activating TLR2 and triggering innate immune activation remain unresolved.

As we delve into the intricate interactions between *T. denticola* and human periodontal ligament fibroblast (hPDL) cells, we aim to shed light on the underlying molecular mechanisms driving periodontal tissue dysregulation and inflammation. A central hypothesis guiding this chapter is that short-term exposure of hPDL cells to wild-type *T. denticola* will induce significant shifts in the gene profile and enrich pathways associated with pro-inflammation and tissue destruction. Previous studies have demonstrated that *T. denticola* is capable of inducing the production of various pro-inflammatory cytokines, including interleukin-1ß (IL-1ß), IL-6, IL-8, and TNF-α, from various cell types, such as gingival epithelial cells[87], gingival fibroblasts[88], and human macrophages[89]. However, the specific molecule(s) of *T. denticola* responsible for these effects remain elusive, and investigations focusing on the dysregulation of host gene expression in PDL tissues in response to *T. denticola* exposure are notably lacking in the literature. Therefore, our study aims to fill this critical gap in knowledge by elucidating the gene expression changes induced by *T. denticola* in hPDL cells and unraveling the molecular pathways

underlying its pathogenicity in the periodontal microenvironment. By unraveling these intricate host-pathogen interactions, we aim to provide valuable insights into the pathogenesis of periodontal diseases and identify potential therapeutic targets for their management and treatment.

Chapter 1.3: RESULTS

1.3.1 T. denticola Suppresses The Upregulation of Multiple Cytokines and Chemokines At The Transcriptional Level in hPDL Cells

We aimed to comprehensively understand the influence of *T. denticola* on host transcriptomes in human periodontal ligament (hPDL) cells. We challenged three healthy patient replicates of hPDL cells with wild-type *T. denticola* (Td-WT) for 2 hours, followed by incubation for an additional 3 or 22 hours (5 and 24 hours, respectively), before extracting total RNA for sequencing. Unchallenged hPDL cells served as a negative control. Hierarchical clustering analysis was used to determine similarity of transcriptome profiles based on differential expression as a heatmap in **(Figure 1.3.1A).** A Red (Upregulation) to blue (Downregulation) color gradient of heatmap represents normalized gene expression as row Z-scores. While the 5-hour incubation group clustered with the control group, the 24-hour incubation group clustered away from the control group illustrating *T. denticola's* ability to mediate large-scale transcriptional changes within 24-hours after infection.

The results, illustrated in **Figure 1.3.1B**, revealed significant alterations in gene expression profiles visualized by a volcano plot using normalized FPKM values of differentially expressed genes. To ensure robust analysis, we examined genes that met our cutoff criteria, including a significant difference (-log10 Adjusted p-value of ≤2) and significant differential expression (-log2 of ≤2).

TRIM9, identified as the most statistically significant upregulated gene, plays a pivotal role in innate and adaptive immunity[90]. Specifically, these proteins contribute to intracellular anti-viral restriction in the dendrites of neurons[90]. TRIM E3 ligases, including TRIM9, regulate IFN

production and signaling, modulate NF-κB induction, and orchestrate multiple levels of immune responses, from pattern recognition receptor (PRR)-mediated pathogen-associated molecular pattern (PAMP) recognition to the degradation of inhibitors[90, 91]. Furthermore, our analysis revealed significant downregulation of numerous genes associated with the pro-inflammatory response. Among these were Stanniocalcin (STC), Interleukin-1 receptor 1 (IL1R1), Interleukin-6 (IL-6), Prostaglandin-endoperoxide synthase 2 (PTGS2), CXC motif chemokine ligand 1 (CXCL1), Interleukin-8 (IL-8), Interleukin-7 receptor (IL7R), CXC motif chemokine ligand 3 (CXCL3), and Tumor necrosis factor α-induced protein 6 (TNFAIP6). Interestingly, our analysis did not identify any upregulated genes associated with either anti-inflammatory or pro-inflammatory responses, suggesting a nuanced regulatory mechanism underlying the host response to *T. denticola* challenge.

1.3.2 Inhibition of Transcription Factor NF-kB Activation/Translocation

Activation of NF-κB and its subsequent nuclear translocation represent key events in activation triggered by pathogen-associated (PAMPs) or damage-associated (DAMPs) molecular patterns through pattern recognition receptor (PRR) pathways[92]. In its quiescent state, NF-κB is sequestered in the cytoplasm by members of the Inhibitor of NF-κB (IκB) family, which effectively mask NF-κB's nuclear translocation signal, thereby maintaining the transcription factor in an inactive form within the cytoplasm[92]. However, upon cellular stimulation induced by pathogens or stress signals, IκB undergoes phosphorylation by the IκB kinase complex (IKK), leading to ubiquitin-mediated degradation of IκB by the 26S proteasome[92, 93]. Consequently, NF-κB is liberated from the cytoplasmic inhibitory complex, undergoing further activation through post-translational modifications[94]. This activated NF-κB then translocates into the nucleus, where it forms dimers and binds to κB sites present at target gene regulatory loci. This binding facilitates transcriptional activation through the recruitment of co-activators and

co-repressors, orchestrating a cascade of downstream gene expression events[95]. To investigate whether NF-κB plays a role in mediating host responses to *T. denticola*, hPDL cells were challenged with purified dentilisin, wild-type *T. denticola*, or an isogenic Td-CF522 mutant lacking the prtP subunit of dentilisin, which contains the catalytic activity of the complex. Immunofluorescence analysis was performed using a rabbit polyclonal anti-NF-κB (P65/RelA) primary antibody, followed by secondary antibody conjugated to Alexa 488 fluorophore, and nuclear staining using Hoescht 33342. Confocal microscopy imaging and analysis using Fiji software revealed that compared to the control group of hPDL cells without challenge or stimulation, no colocalization of NF-κB with *T. denticola* live bacteria or purified dentilisin was observed in any of the experimental groups **(Figure 1.3.2).** Subsequently, using the same experimental schema, *IL-6*, *IL-1β* and *NF-kB* mRNA expression levels were assessed using qRT-PCR which also revealed no statistically significant upregulation as well **(Supplemental Figure).** These findings suggest that while NF-κB is prominently expressed in the cytoplasm, neither *T. denticola* live bacteria nor purified dentilisin are capable of activating the transcription factor and inducing its translocation into the nucleus in human periodontal ligament fibroblast cells.

1.3.3 Inhibition of Transcription Factor NFATc2 Activation/Translocation

Upon challenging hPDL cells with wild-type *T. denticola*, Nuclear factor of Activated T-Cells activated by calcineurin 2 (NFATc2, also called NFAT1) emerged as the most significantly upregulated transcription factor **(Figure 1.3.3A).** NFAT1, initially described nearly three decades ago, serves as a Ca2+/calcineurin-regulated transcription factor primarily in T-cells[96, 97]. Over the years, extensive research has elucidated the regulation and physiological functions of various NFAT homologs across the immune system and other tissues. A distinctive feature of NFAT is its modulation by Ca2+ and the Ca2+/calmodulin-dependent serine phosphatase

calcineurin[96]. In resting cells, NFAT proteins are phosphorylated and reside in the cytoplasm[98]. Upon cellular stimulation, they undergo dephosphorylation by calcineurin, translocate to the nucleus, and activate transcription, thus establishing a direct link between intracellular Ca2+ signaling and gene expression[96-98]. NFAT1 activation has been linked to the upregulation of numerous genes associated with bacterial clearance, stress response, and cell survival[96, 97, 99]. Osorio et al. 2015 demonstrated that sublethal hydrogen peroxide (H2O2) exposure is sufficient to induce calcium (Ca2+)-dependent NF-κB signaling, accompanied by an increase in matrix metalloproteinase 2 gelatinolytic activity in human periodontal ligament cells[100]. Notably, numerous studies have shown that Toll-like receptor 2 (TLR2) activation induces the production of pro-inflammatory cytokines through TLR2-induced calcium-dependent mechanisms[101, 102].

Building upon this knowledge, hPDL cells were challenged or stimulated with purified dentilisin or wild-type *T. denticola*. Following fixation and staining, polyclonal primary antibodies against NFATc2 were utilized, followed by incubation with a secondary goat antibody conjugated to an Alexa 594 fluorophore, along with Hoescht 33342 for nuclear staining. Similar to NF-κB, NFATc2 exhibited a failure to activate or translocate into the nucleus across all experimental groups compared to the control group without challenge or stimulation **(Figure 1.3.3B)**. These findings suggest that neither dentilisin nor wild-type *T. denticola* induces NFATc2 activation or nuclear translocation in hPDL cells under the conditions tested.

Chapter 1.4: DISCUSSION

Our study aimed to elucidate the impact of *T. denticola* on the transcriptomic landscape of human periodontal ligament (hPDL) cells. By exposing hPDL cells from healthy donors to wild-type *T. denticola* (Td-WT) for varying durations (5 and 24 hours), we were able to observe significant transcriptional changes. Hierarchical clustering analysis, visualized through a heatmap **(Figure 1.3.1A),** indicated that the 24-hour exposure to *Td-WT* caused substantial deviations in gene expression compared to the control group, highlighting the pathogen's ability to alter host cellular functions after a short infection period and that significant gene dysregulation is established in as little as 24-hours.

The volcano plot analysis demonstrated significant alterations in gene expression, with *TRIM9* emerging as the most prominently upregulated gene **(Figure 1.3.1B**). TRIM9, part of the TRIM E3 ligases family, plays a crucial role in modulating innate and adaptive immune responses, particularly in antiviral defense mechanisms[90]. Conversely, several genes associated with pro- and anti-inflammatory responses, including Stanniocalcin (STC)[103], Interleukin-1 receptor 1 (IL1R1)[104], Interleukin-6 (IL-6)[105], Prostaglandin-endoperoxide synthase 2 (PTGS2)[106], CXC motif chemokine ligand 1 (CXCL1)[107], Interleukin-8 (IL-8)[108], Interleukin-7 receptor (IL7R)[109], CXC motif chemokine ligand 3 (CXCL3)[110], and Tumor necrosis factor α-induced protein 6 (TNFAIP6)[111], were significantly downregulated **(Figure 1.3.1B**). This suppression points to a potential strategy employed by *T. denticola* to evade the host immune response by dampening inflammatory signaling pathways. Interestingly, our findings did not indicate any upregulation of genes associated with anti-inflammatory responses, suggesting a complex regulatory mechanism that *T. denticola* employs effectively modulate host immunity. These observations are consistent with previous studies showing that *T. denticola* does not induce canonical pro-inflammatory associated genes to create a favorable niche for their survival and proliferation[62].

NF-κB is a critical transcription factor involved in the regulation of immune responses, inflammation, and cell survival[95, 112, 113]. Typically, NF-κB is held inactive in the cytoplasm by IκB proteins, which are degraded upon cellular stimulation, allowing NF-κB to translocate into the nucleus and initiate transcription of target genes[94]. Our study investigated whether *T. denticola* or its dentilisin protease could activate NF-κB in hPDL cells. Immunofluorescence analysis and confocal microscopy revealed no colocalization of NF-κB with *T. denticola* or purified dentilisin. This was corroborated by qRT-PCR results showing no significant upregulation of IL-6, IL-1β, or NF-κB mRNA expression in the experimental groups compared to controls (**Supplemental Figure 1.7.1**). These findings suggest that *T. denticola* does not activate NF-κB in hPDL cells, thereby avoiding the initiation of an inflammatory response that could potentially hinder its colonization and persistence in the periodontal niche.

NFATc2, also known as NFAT1, is a transcription factor primarily regulated by calcium signaling and calcineurin-mediated dephosphorylation. NFAT1 activation is crucial for the transcription of genes involved in immune responses and cell survival, often in response to bacterial infections and other stress signals. Our study showed that NFATc2 was the most significantly upregulated transcription factor in hPDL cells challenged with *T. denticola* **(Figure 1.3.2.C)**. However, similar to NF-κB, immunofluorescence analysis indicated no translocation of NFATc2 into the nucleus upon exposure to *T. denticola* or dentilisin (**Figure 1.3.2.B**). This lack of nuclear translocation suggests that *T. denticola* does not activate the NFATc2 pathway under the conditions tested.

Studies on gingival fibroblast cells have shown how disruptions in calcium signaling upon exposure to *T. denticola* can occur. As the outer membrane (OM) of *T. denticola* disrupts actin-dependent processes that normally require precise control of intracellular calcium, Ko and colleagues studied the effects of *T. denticola* derived OM extract on internal calcium release, ligand-gated and calcium release-activated calcium channels, and related mechanosensitive cation fluxes in human gingival fibroblasts (HGF)[114]. The *T. denticola* OM inhibitory activity

was eliminated by heating the OM extract to 60°C and by boiling but not by phenylmethylsulfonyl fluoride treatment. Thus, nonlipopolysaccharide, nonchymotrypsin, heat-sensitive protein(s) in *T. denticola* OM can evidently inhibit both the release of calcium from internal stores and the uptake of calcium through the plasma membrane, possibly by interference with calcium release-activated channels highlighting a broader strategy of immune evasion[114]. It is possible that this impairment of intracellular calcium signaling is also influencing the inhibition of transcription factor NFATc2 as well.

The findings from this study provide a comprehensive understanding of the molecular interactions between *T. denticola* and hPDL cells. The pathogen's ability to suppress pro-inflammatory cytokine production and inhibit key transcription factors such as NF-κB and NFATc2 highlights its sophisticated strategies to evade the host immune response. These insights contribute to our understanding of periodontal disease pathogenesis and underscore the need for therapeutic interventions targeting these bacterial evasion mechanisms.

Chapter 1.5: MATERIALS AND METHODS

Human Subjects IRB Approval: Approval to conduct human subjects' research was obtained from the University of California San Francisco Institutional Review Board (# 16-20204; reference #227030).

Human PDL Cell Cultures: As described previously, the primary culture of PDL cells was conducted via the direct cell outgrowth method by isolating cells from the PDL tissue around the middle third of extracted healthy human teeth[115, 116]. Cells were maintained in minimal essential medium-α (MEM-α) augmented with 10% fetal bovine serum, 1% penicillin/streptomycin (P/S), and 1% amphotericin B (Gibco, Grand Island, NY, USA) in a humid atmosphere with 95% air and 5% CO2 at 37 °C. Cell outgrowths were passaged when they reached confluency. Cells passaged three to six times were used for experimentation and validated by morphology. Protocols involving the collection and use of human teeth and PDL cells/tissue were approved by the Health Sciences Institutional Review Board of the University of California, San Francisco.

Anaerobic Bacterial Cultures: *T. denticola* ATCC 35405 (Td-WT) and its isogenic mutant, Td-CF522 lacking the PrtP and PrcB subunits of dentilisin[117] were grown in tanks filled with an anaerobic gas mixture at 37 °C in Oral Treponeme Enrichment Broth (Anaerobe Systems, Morgan Hill, CA). *Veilonella parvula* ATCC 10790 was grown under the same anaerobic conditions as *T. denticola* in brain heart infusion media supplemented with 0.075% sodium thioglycolate, 0.1% Tween 80 and 1% of 85% lactic acid and pH adjusted to 6.5-6.6 using 1 M NaOH. Purity of cultures was confirmed by periodic plating prior to use in experiments or 16S sequencing.

Bacterial Challenging of hPDL Cells: hPDL cells were prepared in MEM-α media with 10% FBS and 1% PenStrep and 1% amphotericin B. As described previously[56], the bacteria in broth culture were collected by centrifugation at ~4000 RPM for 15 minutes and resuspended in MEM-α with 10% FBS (No antibiotic and phenol red) to an optical density of 0.1 at 600 nm, such that the cellular density was approximately $2.4x10^8$ cells/ml. *T. denticola,* Td-CF522 or *V. parvula* in FBS supplemented and antibiotic-free MEM-α was added to the test groups of hPDL cells at a multiplicity of infection (MOI) of 50, whereas only supplemented MEM-α was added to the control group. All groups were then incubated for 2 hr at 37 °C in 5% CO2-containing air. After the 2-hr challenge, hPDL cells were washed three times with PBS and incubated for an additional 22-hours in antibiotic-free MEM-α supplemented with 10% FBS.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR): The total RNA was extracted and purified using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions and quantified using a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific, Rockford, IL, USA). Reverse transcription of the RNA into cDNA was then performed using the SuperScript III cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA). All cDNA samples were diluted to 5 ng/mL working concentrations and stored at -20°C. cDNA was then amplified by qRT-PCR using primer sequences (Listed below) and PowerUp SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA, USA) on a QuantStudio 3 platform (Applied Biosystems, Carlsbad, CA, USA). The relative expression levels of target genes were plotted as fold change compared with untreated or negative controls. The $2^{-\Delta\Delta}$CT method was used to determine relative change in target gene expression and gene expression was normalized against GAPDH expression.

RNA-Seq: RNA was extracted from ~900,000 hPDL cells challenged with wild-type *T. denticola* using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The quality of the extracted RNA was assessed using a Nanodrop UV-Vis Spectrophotometer and by calculating the percentage of RNA fragments with size > 200 bp (DV200) using an Agilent 2100 Bioanalyzer. The RNA was used for first and second strand synthesis, polyA tail bead capture, and sequencing adapter ligation using a TruSeq RNA Library Prep Kit v2 (Illumina, San Diego,CA). Libraries were sent to Novogene genomic services (Davis, CA, USA) for paired-end sequencing on a HiSeq 4000 instrument (150 bp paired-end reads). Raw reads were aligned to hg38 using STAR[118] and transcript quantification was performed in a strand-specific manner using RSEM[119] with the GENCODE 29[120] annotation. DESeq2[121] was utilized to perform differential expression analysis using R statistical open source software. The mean gene expression across all replicates was used for further downstream analysis.

Confocal Microscopy: Approximately 80,000 hPDL cells were seeded into 4-well glass bottom wells each to achieve approximately 90% confluency. Cells were challenged with purified dentilisin, wild-type *T. denticola* or isogenic Td-CF522 mutant bacteria as described above. Cells were washed with PBS before being fixed using 4% paraformaldehyde for 10 minutes at room temperature. Next, cells were incubated for 20 minutes with 0.1 M Glycine in PBS, washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 2 minutes at room temperature. Cells were incubated in a 10% serum/PBS blocking buffer for 5 minutes at room temperature followed by a 60-minute incubation in a rabbit sourced polyclonal anti-Sp1primary antibody diluted 1:500 in 10% serum/PBS solution. Next, cells were washed 3 times with PBS followed by incubation in 10% serum/PBS blocking buffer for 1 minute at room temperature followed by incubation in anti-rabbit, mouse secondary antibody conjugated to Alexa 488 fluorophore for 30 minutes at 37°C (1:3000 dilution) and Hoescht 33342 nuclear staining

(1:2000 Dilution) in 10% serum/PBS solution. Cells were washed 3 times with PBS, imaged using an SP8 confocal microscope (Leica, Wetzlar, Germany).

Chapter 1.6: FIGURES

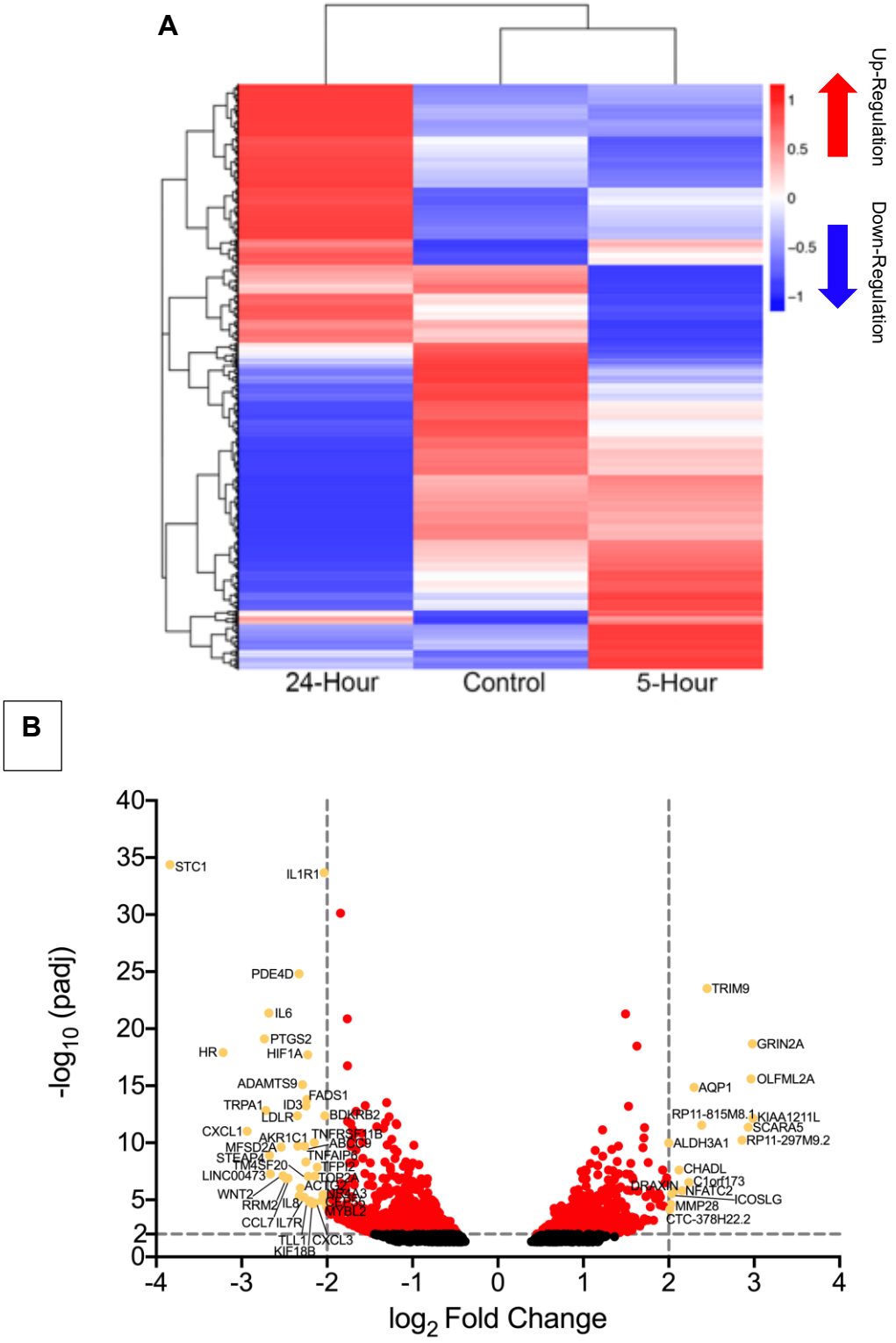


Figure 1.3.1 Differential expression analysis of *T. denticola* stimulated hPDL cells. A. Hierarchical clustering analysis of *T. denticola* challenged hPDL Cells. Total RNA was extracted from healthy patient-derived hPDL cells challenged (Figure caption continued on next page)

(Figure caption continued from previous page) with Td-WT bacteria at a MOI of 50 for 2-hours in media free of supplements followed by 3 and 22-hours in media supplemented with 10% FBS, 1% Pen Strep and 1% Amphotericin B. Mean FPKM values were used for downstream analysis (n = 3 patient replicates). Hierarchical clustering analysis was used to determine similarity of transcriptome profiles based on differential expression as a heatmap. Red (Upregulation) to blue (Downregulation) color gradient of heatmap represents normalized gene expression as row Z-scores. B. Differential Expression of T. denticola Challenged hPDL Cells. Total RNA was extracted from healthy patient-derived hPDL cells challenged with Td-WT bacteria at a MOI of 50 for 2-hours in media free of supplements followed by 22-hours in media supplemented with 10% FBS, 1% Pen Strep and 1% Amphotericin B. Mean FPKM values were used for downstream analysis (n = 3 patient replicates). Differentially expressed genes were visualized as a volcano plot. Genes with an adjusted p value greater than or equal to 2 are indicated in red and genes with a differential expression of 2-fold or more are indicated in yellow. Statistical significance was assessed using a Kolmogorov-Smirnov test followed Benjamini-Hochberg correction ($p<0.05$).

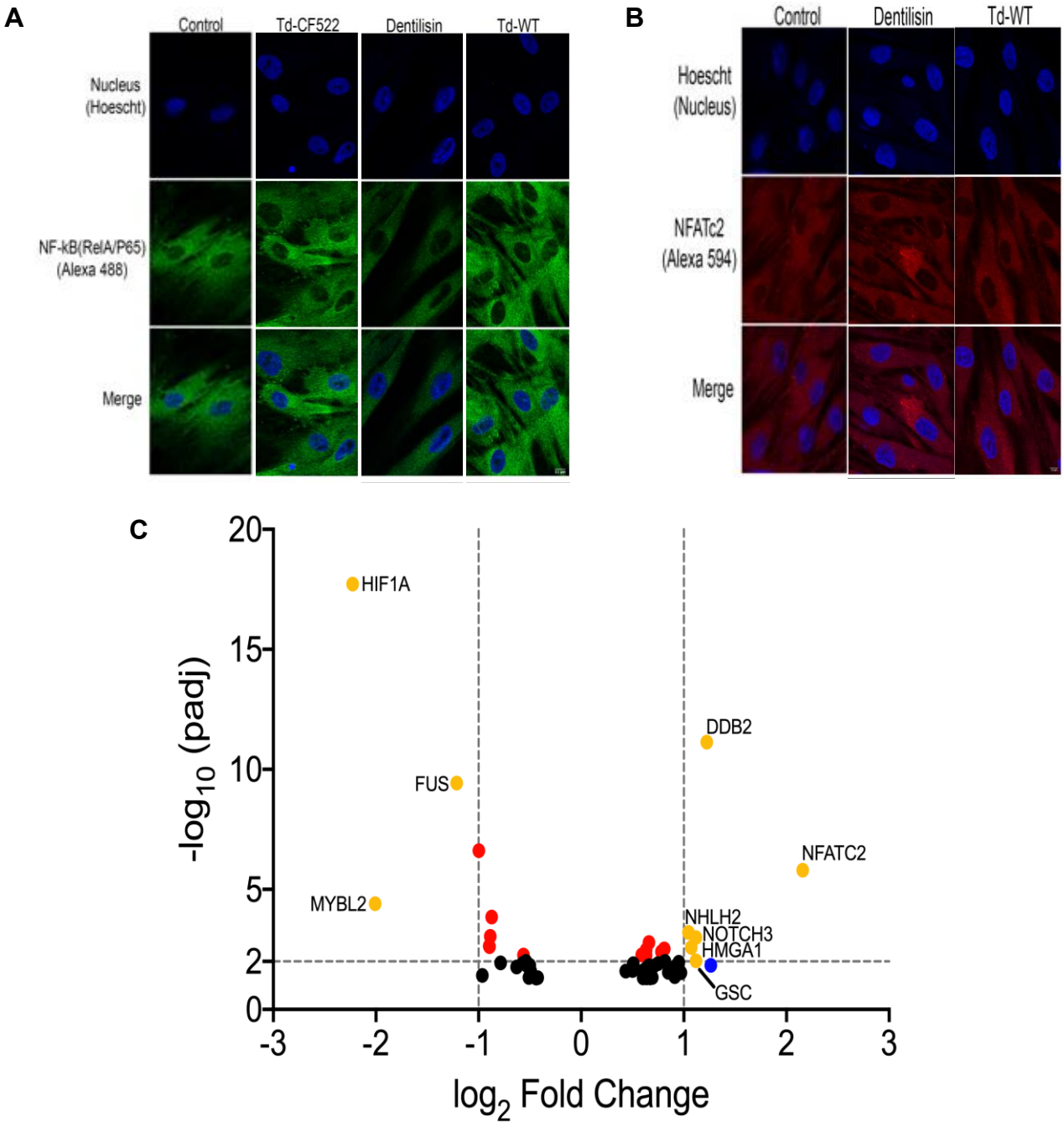
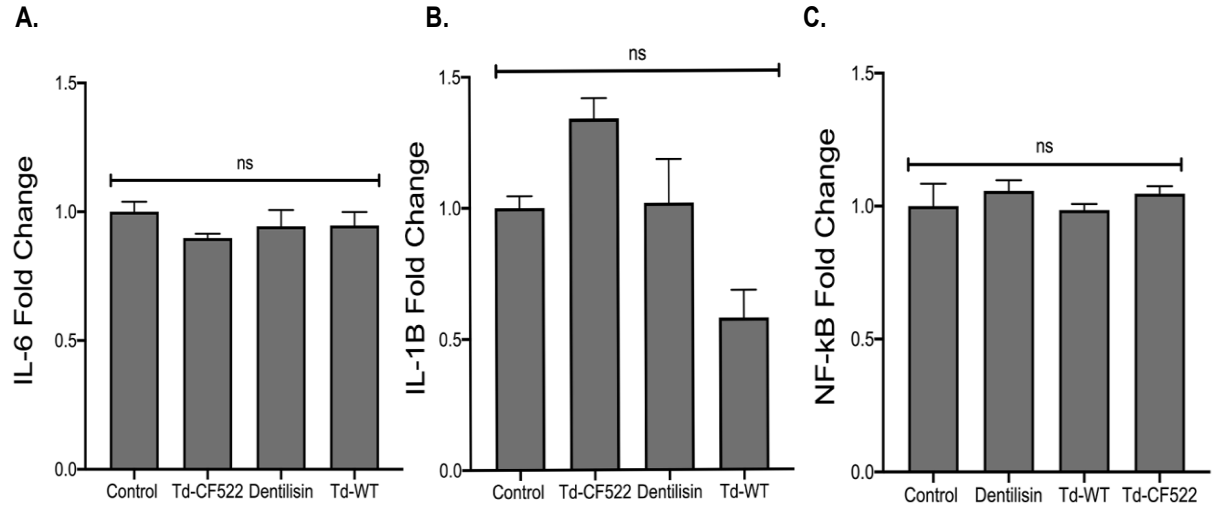


Figure 1.3.2. *T. denticola* challenge inhibits the activation of transcription factors NF-kB and NFATc2 in hPDL cells. 5 Healthy patient replicates of hPDL cells were challenged with *Td-WT* and isogenic *Td-CF522* bacteria at an MOI of 50 or 1ug/mL of *T. denticola* expressed dentilisin as previously described. Nuclei were stained with Hoescht 33342 (Blue), while protein targets were stained with antibodies against A) RelA/P65 (Alexa 488) or B) NFATc2 (Alexa 594) and subjected to confocal microscopy. Scale bar represents 10 μm. C) Volcano plot of differentially expressed transcription factors from RNA-Seq data of *T. denticola* challenged hPDL Cells for 24-hours. DESeq2 was utilized to perform differential expression analysis using R statistical open source software. The mean gene expression across all replicates was used for further downstream analysis.

Chapter 1.7: SUPPLEMENTAL FIGURES



Supplemental Figure 1.7.1 mRNA expression of IL-6, IL-1B and NF-kB in T. denticola challenged hPDL cells. RNA was isolated from PDL cells using the RNeasy Mini kit (Qiagen, Valencia, CA), reverse transcribed to cDNA using SuperScript Vilo cDNA Synthesis Kit and amplified by qRT-PCR using gene-specific primers for A) *IL-6* B) IL-1B, C) NF-kB all normalized to GAPDH expression. Cycle threshold values of the genes of interest and the quantitative gene expression levels normalized to GAPDH for each experimental sample were determined and compared with that of unchallenged control samples.

Chapter 2: Toll-Like Receptor-Dentilisin Host Signaling and Tissue-Destructive Gene Regulation

Chapter 2.1: ABSTRACT

Host-expressed Toll-Like Receptor 2 (TLR2) senses a variety of bacterial ligands including acylated lipoproteins such as the *T. denticola* expressed protease dentilisin. Dentilisin is a surface-expressed protease complex comprised of three lipoproteins that has been implicated as a virulence factor in periodontal disease, primarily due to its proteolytic activity. While the role of acylated bacterial components in induction of inflammation is well-studied, little attention has been given to the potential role of the acylated nature of dentilisin and its influence on host signaling cascades during periodontal infections.

The purpose of this study was to test the hypothesis that *T. denticola* dentilisin activates a TLR2-dependent mechanism, leading to upregulation of tissue-destructive genes in periodontal tissue. RNA-sequencing of periodontal ligament cells challenged with *T. denticola* bacteria revealed significant upregulation of genes associated with extracellular matrix organization and degradation including potentially tissue-specific inducible MMPs, such as MMP-28, that may play novel roles in modulating host immune responses that have yet to be characterized within the context of periodontal disease. The Gram-negative oral commensal, *Veillonella parvula and* while *Porphyromonas gingivalis* failed to induce all MMP targets except MMP-28. *T.denticola*-induced upregulation of MMPs was mediated via TLR2 and MyD88 activation, since knockdown of expression of either abrogated these effects. Challenge with purified dentilisin upregulated all MMP targets while a protease-deficient dentilisin isogenic *T. denticola* mutant had no effect.

Finally, *T. denticola*-mediated activation of TLR2/MyD88 lead to the nuclear translocation of the transcription factor Sp1, which was shown to be a critical regulator of all *T. denticola*-dependent MMP expression in hPDL cells. Taken together, these data suggest that *T. denticola* dentilisin stimulates tissue-destructive cellular processes in a TLR2/MyD88/Sp1-dependent fashion.

Chapter 2.2 INTRODUCTION

In its severe form, patients are left with a non-resolving pro-inflammatory transcriptional profile throughout the periodontium, even after clinical intervention, leading to tissue-destruction and tooth loss[9-11]. This suggest that previously uncharacterized cellular and molecular mechanisms underlying periodontal disease pathophysiology may explain why many patients do not respond to the conventional treatment schema.

Human periodontal ligament fibroblasts (hPDL) which specialize in mechanosensing and tissue remodeling through robust expression of various hydrolytic enzymes such as matrix metalloproteinases (MMPs)[12, 16] also function as immune-like cells. This primarily demarcated by the production of inflammatory cytokines, chemokines and expression of pattern recognition receptors (PRRs), such as toll-like receptors (TLRs), which are responsible for monitoring the local environment for signs of danger in the form of highly conserved microbial molecules present during periodontal disease [18, 19, 122, 123]. When TLR signaling is left unchecked, downstream genes, such as MMPs can become significantly upregulated and constitutively activated, thereby contributing to the overall destruction of the periodontium by degrading extracellular matrix (ECM) proteins, such as collagen and fibronectin[16, 22, 25, 27, 124, 125]. Although the role of MMPs has been primarily ascribed to turnover of the ECM, the great number of new substrates discovered for MMPs in the last few years suggest they are capable of regulating many signaling pathways, cell behaviors and diseases through novel mechanisms[28]. Therefore, PDL cells likely play a significant role in initiating and exacerbating tissue degradation and suggest that pharmacological anti-inflammatory treatment of periodontal disease should target dysregulated MMPs activity and levels in the PDL tissues.

Among the various *T. denticola* effector molecules that have been described, its acylated chymotrypsin-like protease complex (CTLP), more recently called dentilisin, is a major virulence factor which facilitates numerous cytopathic effects that align with periodontal disease pathophysiology[126-128]. A few examples including adhesion, degradation of endogenous ECM-substrates[59], tissue penetration[60], complement evasion[61], ectopic activation of host MMPs[63] and degradation of host chemokines and cytokines, such as IL-1β and IL-6, primarily due to its potent proteolytic activity[65]. However, as in other spirochetes, conserved lipid moieties of the protease complex recognized by host TLR2 receptor complexes may contribute to activation of innate immune responses[66]. Because predominant host responses to lipoproteins are believed to be to their lipid moieties, most studies have focused on diacylated lipopeptide, Pam2CSK4, and triacylated lipopeptide, Pam3CSK4, which mimic bacterial lipoproteins for their potent immunostimulatory and osteoclastogenic activities by preferentially activating TLR2-dependent pathways[66-68]. Recent studies have demonstrated that synthetic di- and tri-acylated lipopeptides which preferentially activate TLR2/6 and TLR2/1-dependent pathways respectively, are sufficient to induce alveolar bone loss in mice[67, 69], broadening the avenues of investigation into the role of lipoproteins underpinning the pathogenesis of periodontal disease. However, studies which utilize endogenously expressed bacterial lipopeptides are greatly lacking.

While a clear role for dentilisin in the context of periodontal disease has been delineated at the protein level, its ability to influence host ECM metabolism in a gene-centric context has yet to be characterized. Thus, the aim of this study was to determine the extent to which *T. denticola* and its highly expressed acylated dentilisin protease complex influence the transcriptional regulation of MMPs through TLR2-dependent pathways in hPDL cells.

Chapter 2.3: RESULTS

2.3.1. T. denticola upregulates genes associated with ECM and degradation in hPDL cells

For the gene ontology analysis, we utilized the mean FPKM values for each gene across experimental groups from the previously described hPDL-Wild-Type *T.denticola* RNA-Seq experiment. Gene clusters at or above a 0.05 adjusted p-value were delineated as statically significant increases. ECM (~6-fold change), collagen degradation (~2-fold change) and degradation of the ECM (~2-fold change) amongst the top 20 significantly enriched biological processes **(Figure 2.3.1A)**. KEGG pathway analysis revealed ECM-Receptor (~4-fold change), Notch (~2-fold change), endocytosis (~2-fold change) and protein digestion (~2-fold change) signaling amongst the top 20 enriched signaling pathways upon *T. denticola* challenge **(Figure 2.3.1B)**. Downregulated GO and KEGG Terms can be found in (**Supplemental Figure 2.7.1**). Interestingly, membrane-type matrix metalloproteinase 4 (MMP-17), Stromelysin-3 (MMP-11) and Epilysin (MMP-28) were consistently clustered with various GO terms associated with ECM remodeling and catabolism and have not been associated with periodontal disease to date. Although RNA-seq analysis revealed increases in MMP-2 and MMP-14 read counts, they were not found to be statistically significant. Both genes, however, have been found to be chronically upregulated in hPDL fibroblasts upon *T. denticola* challenge for up to 12 days *in vitro* [56]. Additionally, while the primary functions of these enzymes has been associated with tissue turnover, both MMP-2 and MMP-14 participate in the active degradation of various cytokines [129], chemokines [129], ECM proteins [130] and various intracellular substrates[28, 131, 132] expressed by hPDL cells expressed during periodontal disease infections, delineating their potentially important role in driving periodontal induced tissue destruction. Next, we sought to determine if this upregulation of tissue destructive genes is common among pathogens and commensal bacteria.

2.3.2. MMPs are not upregulated by commensal oral Gram-negative *Veillonella parvula*

To determine if the induction of these MMPs was specific to *T. denticola*, we utilized *Veillonella parvula,* a Gram-negative anaerobic oral commensal that is prevalent in the gut and oral cavity and is not associated with oral diseases[133]. hPDL cells were challenged with wild-type *T. denticola* or *V. parvula* bacteria for 2 hours at 50 MOI followed by a 22-hour incubation period in α-MEM media free of antibiotics and supplemented with 10% FBS. *V. parvula* challenge did not induce the upregulation of MMPs *2*, *11*, *14*, *17* or *28* **(Figure 2.3.2).** By contrast, *T. denticola* elicited a ~2-fold upregulation or more of all target MMP genes **(Figure 2.3.2)**

2.3.3. T. denticola dentilisin mediates the upregulation of various MMPs in hPDL cells

Among the various TLR2 stimulatory bacterial ligands, lipoproteins are considered as a major virulence factor because of their strong immunostimulating capacity[68, 134, 135]. Interestingly, while differential innate immune responses have been reported depending on the acylation status and tissue type[135-137], both di- and triacylated synthetic lipopeptides have demonstrated the ability to sufficiently drive alveolar and long bone resorption in mice through TLR2/MyD88 dependent mechanisms[67, 69]. We investigated whether purified dentilisin can drive MMP gene regulation in hPDL cells and the results can be seen in **Figure 2.3.3**. hPDL cells were challenged with dentilisin purified from *T. denticola* ATCC 35405 at a final concentration of 1 μg/mL for 2-hours in MEM- media supplemented 1% P/S. Enzymatic activity of dentilisin protein was verified by collecting conditioned media from dentilisin stimulated hPDL cells as described above and analyzing samples using gelatin zymography **(Supplemental Figure 2.7.2)**. While dentilisin enzymatic activity (98 kDa) increased in a dose dependent manner, MMP-2 enzymatic activity (72 kDa) concomitantly increased in a statistically significant manner (~2.0-fold) across all 3 experimental conditions.

Total RNA from hPDL cells (unchallenged and challenged with *T. denticola* strains or purified dentilisin) was extracted, processed for cDNA synthesis and rendered to RT-qPCR. Cells stimulated with purified dentilisin resulted in statistically significant increases in all MMP targets with MMP-17 being the most responsive **(Figure 2.3.3.)**. Similarly, hPDL cells challenged with *T. denticola* 35405 (Td-WT) resulted in statistically significant increases across all MMP targets (~2-2.5-fold) **(Figure 2.3.3.)**. By contrast, hPDL cells challenged with dentilisin-deficient *T. denticola* (Td-CF522) resulted in no statistically significant upregulation across all MMP targets compared to the control group **(Fig 2.3.3).**

2.3.4. shRNA knockdown of *TLR2* inhibits *T. denticola*-induced upregulation of *MMPs 2, 14 17 and 28* in hPDL cells while synergistically increasing MMP 11 expression

Thus, after determining dentilisin plays an important role as a regulator of MMP expression in host hPDL cells, we sought to establish a direct link between TLR2 activation and MMP gene regulation. TLRs, particularly *TLR2*, and to a much lesser extent *TLR4*, regulate important immune responses to periodontal bacteria *in vivo* and *in vitro*[138-145]. TLR2 has been identified as a receptor for an array of ligands comprising *T. denticola* bacteria, including lipoteichoic acid, beta barrel proteins, and acylated lipopeptides, such as dentilisin[146, 147]. Thus, to examine the role of TLR2 and MMP transcriptional regulation in hPDL cells, we generated a primary hPDL *TLR2* knockdown cell line using shRNA targeted against the TLR2 gene and transduced using lentiviral particles. Basal *TLR2* expression was reduced ~60% across 3 clonal replicates as validated using RT-qPCR; reduction was found to be statistically significant using an unpaired t-test **(Figure 2.3.4).** Next, *TLR2* knockdown cells were challenged with either purified dentilisin or wild-type *T. denticola* bacteria. *MMP 2, 11, 14, 17 and 28* expression increased upon stimulation with both purified dentilisin and Td-WT in the shRNA control group **(Figures 2.3.4B-2.3.4F)**. Compared with hPDL cells transfected with the scrambled shRNA control, induction of expression

of MMPs 2, 14, 17 and 28 was suppressed in *TLR2*-deficient hPDL cells challenged with *T. denticola* or dentilisin, while MMP 11 was significantly upregulated in *T. denticola*-challenged *TLR2*-deficient hPDL cells. After determining TLR2 activation plays an integral role in the regulation of MMP expression, we next sought out to determine which downstream mediator is required to propagate this signal.

2.3.5 shRNA knockdown of *MyD88* inhibits *T. denticola*-stimulated upregulation of *MMPs 2, 11, 14, 17 and 28* in hPDL cells.

All TLRs signal through MyD88 with the exception of TLR3 and TLR4 which utilize TIRAP[148]. However, numerous recent reports suggest that MyD88-independent TLR2 pathways may also contribute to periodontal disease progression[149]. Thus, to determine if *T. denticola*-induced TLR2 activation requires MyD88 to regulate targeted MMPs, we generated an hPDL *MyD88* knockdown cell line, achieving approximately ~60% reduction in *MyD88* basal expression across 3 validated clonal replicates **(Figure 2.3.5A)**. *MyD88*-deficient hPDL cells were stimulated with either Td-WT or purified dentilisin as described above. In the negative shRNA control group, all MMP targets showed increased mRNA levels following challenge with both Td-WT and dentilisin **(Figures 2.3.5B-2.3.5F)**. By contrast, *MyD88*-deficient cells challenged with Td-WT bacteria and purified dentilisin revealed unanimous depression of all MMP targets as compared to the respective scramble shRNA control samples **(Figure 2.3.5B-5F)**.

2.3.6 shRNA knockdown of TLR2/MyD88-dependent signaling inhibits nuclear translocation of transcription factor Sp1 in hPDL cells stimulated with *T. denticola* or *dentilisin*.

One of the many downstream targets of TLR/MyD88 signaling is the transcription factor Specificity Protein 1 (Sp1). Sp1 regulates a variety of genes associated with cell cycle, pro-inflammation and tissue-destruction, including *MMPs 2, 11, 14 17 and 28.* Interestingly, Larsson and colleagues have readily demonstrated that mRNA levels of Sp1 are consistently upregulated at periodontally involved sites in humans[150]. Thus, to determine if *T. denticola* influences Sp1 expression in the PDL, hPDL cells were challenged with Td-WT and Td-CF522 bacteria at a multiplicity of infection of 50 for 2-hours followed by a 22-hour incubation period in media. Cells were collected and used for western blot analysis utilizing a monoclonal antibody against total Sp1 protein. Total Sp1 protein expression levels increased in the groups challenged with Td-WT at 50 MOI compared to controls and were statistically significant **(Figure 2.3.6)**. By contrast, challenge with Td-CF522 failed to recapitulate this effect **(Figure 2.3.6)**.

Transcription factors are activated post-translationally by TLR signaling, often at the step of nuclear translocation leading to the induction of the primary response genes[151]. After finding that *T. denticola* increases total Sp1 protein expression, we investigated whether *T. denticola or* dentilisin are able to induce nuclear translocation of Total Sp1 via TLR2 and MyD88 activation in hPDL cells and the results can be seen on **Figures 2.3.7 and 2.3.8**. *TLR2*, *MyD88*-deficient and shRNA control cells were stimulated with purified dentilisin, Td-WT or Td-CF522 as previously described. Protein localization was assessed using primary antibodies against Sp1 and a secondary antibody conjugated to an Alexa 488 fluorophore and subjected to confocal microscopy. shRNA control hPDL cells challenged with Td-CF522 resulted in an increased Sp1 protein signal compared to the control group but remained localized to the cytoplasm **(Figure**

2.3.7A). In contrast, shRNA control hPDL cells stimulated with purified dentilisin and Td-WT bacteria resulted in an increased Sp1 signal and translocation to the nucleus **(Figure 2.3.7A)**. hPDL cells deficient in *TLR2* and *MyD88* and challenged with the Td-CF522 resulted in similar results as the shRNA control group **(Figures 2.3.7 and 2.3.8)**. However, when challenged or stimulated with purified dentilisin and Td-WT bacteria, both *TLR2* and *MyD88* deficient cells inhibited the translocation of Sp1 into the nucleus **(Figures 2.3.7 and 2.3.38A)**. Thus, after determining both TLR2 and MyD88 play an integral role in the translocation of Sp1 in our system, we sought out to determine if Sp1 is required or sufficient to regulate target MMPs.

2.3.7 Knockdown of transcription factor Sp1 inhibits *T. denticola*/dentilisin-stimulated upregulation of MMPs 2, 11, 14, 17 and 28 in hPDL cells.

Promoter characterization and luciferase promoter activity assays have identified Sp1 as a regulator of most MMPs and TLRs, through interactions with other transcription factors and cis-regulatory elements such as distal enhancers[152-157]. Thus, it is possible that Sp1 may function as a critical regulator in response to *T. denticola* challenge. To answer this question, shRNA vectors against the *Sp1* gene were used to transduce healthy patient-derived hPDL cells using lentiviral particles as previously described. Cells transfected with scrambled shRNA were used as a negative control. Average basal Sp1 mRNA levels were decreased ~40% across three clones as assessed using RT-qPCR **(Figure 2.3.9A)**. These shRNA control and Sp1 deficient hPDL cells were then challenged with purified dentilisin at 1 ug/mL concentration or Td-WT bacteria at a MOI of 50 followed by RNA isolation and RT-qPCR as previously described. Control shRNA hPDL cells stimulated or challenged with purified dentilisin and Td-WT bacteria resulted in the statistically significant upregulation of MMPs 2, 11, 14, 17 and 28 **(Figures 2.3.9B-2.3.3.9F).** By contrast, Sp1 deficient hPDL cells under the same conditions suppressed the upregulation of all MMP targets **(Figures 2.3.9B-2.3.9F).**

Chapter 2.4: DISCUSSION

Data from this study extends our earlier reports that short term exposure to dentilisin leads to sustained upregulation of tissue destructive processes at both the transcriptional and translational levels. Specifically, we demonstrated that *T. denticola* surface-expressed dentilisin plays a direct role in activating TLR2/MyD88-dependent pathways resulting in the subsequent upregulation of MMPs in hPDL cells. Additionally, we have identified the transcription factor Sp1 as an important downstream target that is activated as a result of TLR2/MyD88 activation, leading to its translocation into the nucleus and mediating the upregulation of various MMPs **(Figure 2.3.1).** Our RNA-Seq analysis revealed specific gene expression patterns that are associated with distinct pathologic features presented clinically[25], reported in *in vivo* studies [158] and previous studies from our lab [56, 63]. Additionally, our present data expands on this finding demonstrating *T. denticola's* ability to drive a tissue-destructive gene profile after a brief exposure. While the 5-hour incubation group clustered with the control group, the 24-hour incubation group clustered away from the control group illustrating *T. denticola's* ability to mediate large-scale transcriptional changes within 24-hours after infection **(Figure 2.3.1B)**.

PDLs insert into the cementum of the tooth root through Sharpey's fibers which are primarily comprised of fibrillar collagen [14, 159]. While most collagen is degraded by collagenases such as MMP-1, MMP-8 and MMP-13, MMP-2 (gelatinase B) and MMP-14 are the predominately expressed species in human PDLs and they are able to actively degrade fibrillar collagen [160, 161]. MMP proteolytic cascades can also lead to widespread periodontal tissue destruction due to cooperative MMP activation [21]. A myriad of MMP-14 substrates have been identified including complement components [162] and fibrinogens [163] as well as being a potent activator of other MMPs such as pro-enzymatic MMP-2 [21]. Additionally, both MMP-2 and MMP-14 participate in the active degradation of various cytokine and chemokine substrates expressed by hPDL including IL-1β, IL-6, TNF-α, CXCL12, IL-8 and MCP protein family [129].

Lastly, although MMPs are classically viewed as extracellular and localized to the cell surface, they have also been found in every cellular compartment [28]. Compelling evidence has revealed MMP-14 moonlighting as a transcription factor independent of its catalytic activity, regulating >100 genes associated with inflammation and bacterial clearance[23, 28]. Therefore, it is possible that MMP-14 regulates other MMPs or initiate a forward feed-back loop to regulate itself. Taken together, these data suggest that dysregulation of MMP-2 and MMP-14 play a key role in modulating numerous host responses during periodontal infections.

While MMPs 2 and 14 have been linked to periodontal disease progression, MMPs 11, 17 and 28 have yet to be described in this disease context [22]. Our gene ontology analysis places all within the various collagen and ECM catabolic processes (**Figure 2.3.1C**). Similar to many other MMPs, clear roles for MMP-17 (also called MT4-MMP) have been described within the context of cancer [164]. However, identifying a specific role in tissue homeostasis and in other diseases has been challenging. The GPI- anchored MMP-17 has minimal catalytic activity against ECM substrates only showing affinity for fibrin/fibrinogen, gelatin and pro-TNF-alpha ligands [164, 165]. To date, analysis of MMP-17 null mice has revealed a weak phenotype associated with water retention that is regulated by processes in the hypothalmus [166]. Additionally, MMP-17 mediates C-terminal processing of ADAMTS4, one of the aggrecanases that are thought to play a role in arthritis [167]. Pro-MMP-11 (also called Stromelysin-3) contains a unique pro-peptide motif for intracellular calcium activation by furin and has also demonstrated rather restricted substrate specificity [168, 169]. Identified MMP-11 ECM substrates include the laminin receptor, fibronectin, elastin and the native alpha3 chain of collagen [170]. All of these are constituent ECM proteins found to be upregulated by *T. denticola* challenge **(Figure 2.3.1C)**, highlighting MMP-11 as a potentially unique response regulator underpinning periodontal disease progression.

MMP-28 overexpression *in vitro* lead to increased *MMP-2* and *MMP-14* mRNA levels, constitutive MMP-2 activation, and revealed e-cadherin as a high-affinity substrate for proteasomal degradation in lung carcinoma cells [171, 172]. Our data aligned with these findings as *MMP-28* was the most upregulated MMP (~2-fold change) within the proteinaceous ECM gene ontology term and was not expressed in control groups, suggesting that MMP-28 may play a multifaceted role in exacerbating *MMP-2* and *MMP-14* mRNA upregulation while directly contributing to their constitutive activation. Taken together, the multi-dimensionality of MMPs and their varied localizations highlight their role as integral host factors that, in addition to modulating homeostatic tissue turnover, also respond to bacterial challenge through a web of complex interactions.

Spirochetes express a wide range of novel and structurally diverse lipoproteins that help define how these unique microbes interact with their environments [173, 174]. All lipoproteins are acylated on the N-terminal cysteine residue, and many spirochete lipoproteins are localized to the outer membrane [68]. Compelling reports have demonstrated the importance of specific lipoprotein acyl groups as drivers of both pro and anti-inflammatory cytokine responses in various experimental infection models over the last few decades [137, 173]. However, thorough characterization of acylation of *T. denticola* lipoproteins including dentilisin has yet to be done. Therefore, due to the availability and access to numerous other *T. denticola* dentilisin isogenic mutants, we decided to investigate the role of dentilisin proteolyitic activity in the context of host gene regulation, an area which has yet to be explored in oral tissues. Of the various available dentilisin mutants, we chose to utilize CF522 which expresses PrcA (one of the three lipoproteins of the dentilisin complex), but lacks PrtP, the actual protease. Planned future studies will include an otherwise native “catalytically dead” isogenic *T. denticola* dentilisin mutant strain carrying single base changes in each of the triad of PrtP catalytic residues (Asp^{203}, His^{258}, and Ser^{447}).

In our study, stimulation with purified dentilisin lead to the upregulation of *MMPs 2, 11, 14, 17* and *28*. By contrast, challenging hPDL cells with isogenic Td-CF522 (which lacks the proteolytic PrtP subunit) [117] had no effect **(Figure 2.3.4)**. Thus, while we originally hypothesized dentilisin lipid moieties to be the causative drivers of TLR2 activation, our data suggest dentilisin protease activity may be an integral factor responsible for the induction of various MMPs in oral tissues. However, the exact mechanism of action remains to be determined. We plan to pursue the issue of relative contributions of dentilisin acylation and proteolytic activity in future studies.

Although both TLR2 and TLR4 have been implicated in periodontal disease progression, various studies utilizing knockout mice and *in vitro* systems suggest that TLR2 plays the most direct role is in sensing various components produced by periodontal pathogens that drive alveolar bone destruction [87, 141, 158, 175]. However, the precise roles, whether protective or destructive, played by TLR2 in periodontal inflammation and disease are poorly understood, particularly in the context of *T. denticola* infection. Once a bacterial ligand binds, a conformational change is thought to occur that brings the two Toll/interleukin-1 (IL-1) receptor (TIR) domains on the cytosolic face of each receptor into closer proximity, creating a new platform on which to build a signaling complex through various adaptor molecules [176]. Thus, availability of intracellular adaptors can accommodate rather distinct signaling patterns as a result of TLR activation [147]. Because MyD88 is the predominant mediator for almost all TLRs with the exception of TLR3 and TLR4, we sought out to determine its role in regulating MMP expression in PDL tissues first.

MyD88 deficient hPDL cells stimulated with Td-WT or purified dentilisin resulted in universal suppression of all MMP targets compared to the shRNA control group **(Figure 2.3.5)**. Interestingly, while shRNA knockdown of MyD88 resulted in a statistically significant downregulation of MMP-2 in the negative control group **(Figure 2.3.5B)**, this was not observed for any other MMP targets as well as in any TLR2 knockdown groups. Similarly, TLR2

deficiency (in hPDL cells under the same experimental conditions) was sufficient to suppress all MMP targets with the exception of MMP-11 **(Figure 2.3.4)**. Thus, our data suggests that *T. denticola* utilizes a TLR2/MyD88-dependent pathway to regulate MMPs 2, 14, 17 and 28 in PDL tissues. A possible explanation for this differential response is that MMP-11 may be more highly regulated by an alternative pathway that is indirectly activated as a result of *T. denticola* infection or treatment with dentilisin.

ECM degradation is a key event in the early stages of periodontal disease, generating fragments that can act as danger-associated molecular patterns (DAMPs) such as fragmented aggrecan, fibronectin, biglycan, decorin and low molecular weight hyaluronic acid [177]. Our lab has readily demonstrated that dentilisin mediates MMP-2-dependent degradation of Fibronectin in patient derived hPDL cell cultures [59], with specific fragments (i.e. 29, 40, 68 and 120 kDa) capable of modulating various cellular processes including apoptosis, proliferation and proteinase expression through integrin signaling [178-182]. Dongsheng and colleagues reported that fibronectin was able to activate TLR2/4 to stimulate an innate immune response in macrophages through cross-talk between TLR2, TLR4 and integrin signaling, leading to upregulation of pro-inflammatory genes [183]. Interestingly, while the upregulation of integrins α10 and β4 associated with collagen and laminin interactions were detected, integrins responsible for sensing full-length or fragmented fibronectin did not change. These data suggest that *T. denticola* dentilisin-induced fibronectin fragmentation may play a role in activating and amplifying TLR2-dependent signaling resulting in the differential expression of MMPs.

Several other pathways modulate TLR-signaling, fostering tissue-specific outcomes. Synergistic cooperation between the Notch pathway and acute TLR-induced signals in the activation of canonical and non-canonical Notch target genes has been characterized as an integral pathway governing the regulation of innate and adaptive immunity[184-187]. Consistent with this, our

KEGG pathway analysis revealed Notch signaling (~2-fold change) as the second most enriched pathway resulting from *T. denticola* challenge in hPDL cells 22-hours post-challenge **(Figure 2.3.1D)**. However, mechanisms by which TLRs induce Notch ligand expression and indirectly activate Notch signaling are not well understood and are in many cases, contradictory [188-190].

Although inflammatory pathways include a core set of genes that are activated in response to inflammatory stimuli, they differ extensively depending on the cell and tissue type and on the intensity of the trigger[151]. Examples include nuclear factor-κB (NF-κB)[151, 191], interferon-regulatory factor (IRF) proteins[192] and Kruppel-Like family transcription factors such as Sp1 [193, 194]. While increased Sp1 levels have been implicated as a factor in periodontal disease progression in previous studies, a thorough characterization of its role in PDL tissues has not been reported. Many MMPs, including MMPs 2, 11, 14, 17 and 28, have multiple GC boxes in their proximal promoters that bind Sp1 and potentially other GC-binding proteins [156]. For example, the Sp1 binding site in the human MMP-14 promoter is crucial in maintaining expression of this gene, since introducing mutations reduces promoter activity by approximately 90% [195]. Sp1 has also demonstrated the capacity to affect cis-regulatory elements that are highly susceptible to cell stimulation[196], potentially explaining how constitutively expressed MMPs (such as MMP-2 and MMP-14) are still subject to upregulation upon *T. denticola* stimulation. Swingler et al. 2010 reported that Sp1 transcription factors are acetylated in response to HDAC inhibitors, bind to the conserved GT-box of the MMP28 promoter, potentially working in conjunction with p300/CREB to upregulate mRNA expression [153].
When healthy hPDL cells were challenged with wild-type *T. denticola*, total Sp1 protein expression increased compared to the control group **(Figure 2.3.6A)**. In contrast, this effect was inhibited in hPDL cells challenged with isogenic mutant Td-CF522 **(Figure 2.3.6B)**. This led us to determine if this increase in Sp1 protein expression was combined with increased Sp1

translocation to the nucleus. To answer this question, TLR2- and MyD88-knockdown patient-derived hPDL cells lines were generated and challenged with wild-type *T. denticola*, Td-CF522 and purified dentilisin. shRNA-control cells stimulated with dentilisin and challenged with Td-WT resulted in Sp1 protein localizing to the nucleus, while TLR2 and MyD88 suppression under the same conditions inhibited this effect **(Figures 2.3.7 and 2.3.8)**. Interestingly, a slight increase in cytoplasmic Sp1 was noted in shRNA control, *TLR2* and *MyD88* knockdown groups when challenged with the Td-CF522 isogenic mutant, suggesting that other factors expressed by *T. denticola* may influence Sp1 expression, whereas dentilisin preferentially regulates its post-translational activation. While shRNA-control hPDL cells stimulated with Td-WT and dentilisin resulted in the upregulation of MMPs 2, 11, 14, 17 and 28, this effect was suppressed in Sp1-deficient hPDL cells under the same conditions **(Figure 2.3.9).** Taken together, these data suggest that the transcription factor Sp1 is an important downstream early response gene that is post-translationally activated as a consequence of TLR2/MyD88 activation, mediating *T. denticola*-induced upregulation of several MMPs in hPDL cells.

This study has potential limitations, most of which we intend to address in future studies. Because our samples are generated from a small number of human patient tissues, genetic variation may lead to heavily variegated responses. Due to a low sample size, our RNA-Seq in particular may be subject to read count bias and confounding that may have influenced our results. The Pearson's correlation reflects the linear relationship between two variables accounting for differences in their mean and SD. The more similar the expression profiles for all transcripts are between two samples, the higher the correlation coefficient will be. As shown in (**Supplemental Figure 2.7.3)**, our samples clustered well, suggesting that dispersion effects are low across the 3 biological replicates. In addition, comprehensive analyses have shown that data sets with very low dispersions, a power of 0.8 is easily reached with very low sample size and sequencing depth[197]. While our results provide a strong support for mechanisms of action

underpinning periodontal disease, other systemic factors could potentially modulate true phenotypic effects. As such, simulation of similar experimental conditions in an animal model in planned future studies.

Studies that focus on the effects of unchecked TLR signaling and its contributions to dysregulation of tissue destructive genes in periodontal ligament connective tissues are lacking. Considering the limitations of the study, to the best of our knowledge this study is the first associating dentilisin with TLR2 activation using an *in vivo* isogenic mutant bacteria in conjunction with purified product from its parent strain. Additionally, we also identified potentially tissue-specific inducible MMPs that that may play novel roles in modulating host immune responses that have yet to be characterized in the context of periodontal disease.

Chapter 2.5: MATERIALS AND METHODS

Human Subjects IRB Approval: Approval to conduct human subjects' research, including protocols for the collection and use of human teeth and PDL tissue was obtained from the University of California San Francisco Institutional Review Board (#16-20204; reference #227030). Consent was not obtained due to anonymity of the samples.

Human PDL Cell Cultures: As described previously, the primary culture of PDL cells was conducted via the direct cell outgrowth method by isolating cells from the PDL tissue around the middle third of extracted healthy human teeth[115, 116]. Cells were maintained in Minimum Eagle Medium-α (MEM-α) (Gibco, USA) augmented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin/streptomycin (P/S), and 1% amphotericin B (Gibco, USA) in a Steri Cycle 370 incubator (Thermo-Fisher Scientific, USA) with a humid atmosphere containing 95% air and 5% CO_2 at 37 °C. Cell outgrowths were passaged before reaching confluency. Only cells passage three to seven were used for experimentation. Cells were validated by morphological assessment and gene expression of confident biomarkers such as *Periostin*.

Anaerobic Bacterial Cultures: *T. denticola* ATCC 35405 (Td-WT) and its isogenic Td-CF522 dentilisin-deficient mutant Td-CF522 were grow in in Oral Treponeme Enrichment Broth (OTEB; Anaerobe Systems, USA) at 37 °C) grown in tanks filled with an anaerobic gas mixture [198]. *Veilonella parvula* ATCC 10790 was grown under the same anaerobic conditions as *T. denticola* in brain heart infusion (BHI) media supplemented with 0.075% sodium thioglycolate, 0.1% Tween 80 and 1% of 85% lactic acid and pH adjusted to 6.5-6.6 using 1 M NaOH.

Purification of *T. denticola* dentilisin: The dentilisin protease complex was purified from the detergent phase of Triton X-114 extracts of an isogenic *T. denticola* mutant that lacks Msp ,the

dominant outer membrane protein, as described previously. Briefly, following overnight Triton X-114 treatment, cells were pelleted, the supernatants were phase-separated, the detergent phase was re-extracted twice, precipitated in acetone, then subjected to preparative SDS-PAGE electrophoresis. Fractions of interest, eluted in detergent-free buffer, were concentrated by ultrafiltration, subjected to buffer exchange into PBS containing 0.1% CHAPS (3-[(-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate) and concentrated again. Purified [59]. Aliquots of purified dentilisin were stored at -80˚C. Activity of purified dentilisin samples was monitored periodically, using the chromogenic substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPFNA) as described previously[128].

Bacterial Challenging of hPDL Cells: ~$9x10^5$ healthy hPDL cells were seeded on 6 cm plates. On the next day, these cells were challenged with either *T. denticola,* Td-CF522 or *V. parvula*, as previously described by Ateia et al.[56]. Briefly, the bacteria were centrifuged at 4000 RPM for 15 minutes and the supernatant was removed. The pelleted bacterial cells were then resuspended in antibiotic-free MEM-α (without phenol red) at an optical density (OD) of 0.1 at 600 nm using a Spectramax M2 microplate spectrophotometer (Molecular Devices, USA). It has been previously established that an OD of 0.1 at 600nm is equivalent to $2.4x10^8$ CFU/ml for *T. denticola[198]* and $2x10^8$ CFU/mL for *V. parvula* strains[199]. Next, hPDL cells were challenged with the bacteria at 50 multiplicity of infection (MOI), whereas the control group was challenged with antibiotic-free MEM-α. Next, the cells were incubated for 2h at 37 °C and 5% CO_2, washed twice with Phosphate Buffer Saline (PBS) (Gibco, USA) and incubated again at 37 °C and 5% CO_2 overnight in antibiotic-free MEM-α. On the next day, cells were either harvested for RNA isolation, generation of cell lysates and stored at -80 °C for further investigations.

RNA-Seq: RNA was extracted from ~9.5x10^5 hPDL cells challenged with wild-type *T. denticola* (as described above using a RNeasy Mini Kit and following the manufacturers protocol (Qiagen, Germany). The quality of the extracted RNA was assessed using a Nanodrop UV-Vis Spectrophotometer (Thermo-Fisher Scientific, USA) and by calculating the percentage of RNA fragments with size > 200 bp (DV200) using an Agilent 2100 Bioanalyzer. The RNA was used for first and second strand synthesis, polyA tail bead capture, and sequencing adapter ligation using a TruSeq RNA Library Prep Kit v2 (Illumina, USA). Libraries were sent to Novogene Genomic Services (Davis, USA) for paired-end sequencing on a HiSeq 4000 instrument (150 bp paired-end reads) (Illumina, USA) and analysis. The mean gene expression across all replicates were used for data visualization. Analysis was conducted using the opensource statistical software called R [200] and figures were produced using the Grammar of Graphics Plot 2 (GGplot2) [201] software package.

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR): Total hPDL RNA was extracted and purified using the RNeasy mini kit (Cat# 74134, Qiagen, Germany) according to the manufacturer's instructions and quantified using a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific, USA). Reverse transcription of the RNA into cDNA was then performed using the SuperScript III cDNA synthesis kit (Cat #11754050, Invitrogen, USA). Samples were ran on a Bio-Rad MyCycler Thermal Cycler according to manufacturers protocol. All cDNA samples were diluted to 5 ng/mL working concentrations and stored at -20°C. cDNA samples were probed for target gene expression via RT-qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems, USA) and primer sequences (Table 1) on a QuantStudio 3 platform (Applied Biosystems, USA). The relative expression levels of target genes were plotted as fold change compared with untreated or negative controls. The $2^{-\Delta\Delta}$CT method was used to

determine relative change in target gene expression, and gene expression was normalized against GAPDH expression.

Table 1. RT-qPCR forward and revers primer pairs

Gene	Forward Primer	Reverse Primer
GAPDH	5'-TTGAGGTCAATGAAGGGGTC-3'	5'-GAAGGTGAAGGTCGGAGTCA-3'
MMP-2	5'-TTTCCATTCCGCTTCCAGGGCAC-3'	5'- TCGCACACCACATCTTTCCGTCACT-3'
MMP-11	5' CGATGCTGCTGCTGCTGCTCCAG-3'	5'-TGGCGTCACATCGCTCCATACCTTTAG-3'
MMP-14	5' TGAGGATCTGAATGGAAATGAC-3'	5'-CATAAAGTTGCTGGATGCCC-3'
MMP-17	5-CACCAAGTGGAACAAGAGGAACCT-3'	5-TGGTAGTACGGCCGCATGATGGAGTGTGCA-3'
MMP-28	5'-CACCTCCACTCGATTCAGCG-3'	5'-AAAGCGTTTCTTACGCCTCA-3'
MyD88	5'-TCTCTGTTCTT GAACGTGCGGACA-3'	5'-TTTGGCAATCCTCCTCA ATGCTGG-3'
Periostin	5′-CCAGCAGACACACCTGTTGG-3′	5′-CCTTGAACTTTTTTGTTGGC-3′
Sp1	5′-TAATGGTGGTGGTGCCTTT-3′	5′-GAGATGATCTGCCAGCCATT-3′
TLR2	5'-ATCCTCCAATCAGGCTTCTCT-3'	5'-ACACCTCTGTAGGTCACTGTTG-3'

Stimulation of hPDL Cells Using Purified Dentilisin: A BCA protein assay kit (CAT # 23227 Thermo Scientific, USA) was used to determine purified dentilisin sample concentrations as described above. Assessment of enzymatic activity was determined using gelatin zymography as described below. ~$9.5x10^5$ cells were seeded on 6 cm plates. Purified dentilisin was added to

MEM-α media with no serum and 1% P/S at a final concentration of 1 μg/mL and added to healthy hPDL cell cultures for 2-hours. The treated cells were washed with PBS and incubated overnight in MEM-α media free of FBS and supplemented with 1% P/S. Next, the Supernatant (conditioned media) was collected and total RNA was extracted using an RNeasy Mini kit (Cat# 74134, Qiagen, Germany). The resulting samples were stored at −80°C until analyzed further in downstream applications.

Gelatin Zymography: Supernatant collected from dentilisin stimulated hPDL cells were quantified and normalized to an albumin standard using the Pierce BCA protein assay kit (Cat# 23225, Thermo Scientific, USA) according to the manufacturers protocol. Equivalent protein concentrations from samples were mixed with nonreducing sample buffer (0.25 M tris-base, 40% glycerol, 0.8% sodium dodecyl sulfate (SDS), and 0.05% bromophenol blue stain in distilled deionized water/ddH2O at pH 6.8) and loaded into 8% polyacrylamide gels that were co-polymerized with 0.4% SDS and 0.2% gelatin. Samples were electrophoretically resolved on gelatin-containing gels at 125 V for 110 min at 4 °C using a Power Pack supply (Bio-Rad, USA) Next, gels were washed in a series of buffers to facilitate re-constitution of endogenous protein and stained with Coomassie Blue as previously described[56]. Briefly, Gels were then washed twice for 15 min under continuous agitation using renaturation/ washing buffer (2.5% v/v Triton-X100 and 0.05 M Tris-base in ddH2O at pH 7.5) to eliminate SDS and promote the renaturation of MMP enzymes. Subsequently, gels were incubated in developing/incubation buffer (0.05MTris-base, 0.15Msodium chloride, 0.01Mcalcium chloride, and 0.02% sodium azide in ddH2O at pH 7.5) for 30 min under agitation, and then the buffer was replaced and gels incubated for 16–20 hr at 37 °C. After that, gels were stained using filtered Coomassie Brilliant blue stain for 1 or 2 hr under agitation. Destaining of the gels was performed using amethanol/acetic acid destaining buffer (40% methanol and 10% acetic acid in ddH2O) until the

bands on the gel appeared clear. Zymograms were scanned using an Hp Officejet 6700, and the densitometry of gelatinolytic activity represented by the clear bands was analyzed using Fiji software [202]. Brightness and contrast levels of zymogram images were slightly adjusted for publication only.

Lentiviral shRNA Knockdown: ~6x10^4 hPDL cells were seeded on 96-well plates. 48 hours later, these cells were infected with either TLR2, MyD88, or Sp1 targeted short hairpin RNA (shRNA) constructs via lentiviral particles according to the kits instructions (Cat# sc-40256-V, Cat# sc-44313-V and Cat# sc-29487-V, Santa Cruz Biotechnology, USA,). Control samples were transduced using nonspecific scrambled shRNA control constructs (Cat# sc-108060, Santa Cruz Biotechnology, USA). hPDL cells were concomitantly permeabilized using Polybrene reagent at a final concentration of 5 µg/mL (EMD Millipore, USA). Approximately 10 hours after infection, cells were washed and incubated overnight in complete MEM-α media supplemented with 10% FBS, 1% PenStrep and 1% Amphotericin B. A pol III promoter drives expression of a silencing cassette containing a puromycin resistance gene used for selection [203]. As a result, a short hairpin RNA complex is exported into the cytoplasm, processed by Dicer and assembled into the RISC complex [203]. Degradation of targeted mRNA transcripts is mediated by unwinding the siRNA duplex leading to a stable reduction in gene expression. Selection of clones with successfully integrated knockdown constructs was accomplished by treating infected hPDL cells with puromycin (Calbiochem, USA) at a final concentration of 5 µg/mL for 24-hours in supplemented MEM- α media. This media was then exchanged for supplemented MEM-α media containing 1 µg/mL puromycin until single cell colonies were isolated. Finally, cell colonies were expanded and validated via RT-qPCR and/or Western Blot.

Western Blot Analyses: Cell lysates generated from shRNA lentiviral infection were normalized to an albumin standard as described above. These samples were then subjected to

SDS-PAGE 4–12% polyacrylamide gels (Invitrogen, USA) and transferred to polyvinylidene difluoride (PVDF) membranes (EMD Millipore, USA) using a Turbo Blot transfer system and PowerPac Basic power supply (Bio-Rad, USA). The membranes were exposed to a rabbit polyclonal anti-Sp1 (Cat# PA5-29165, Invitrogen, Carlsbad, CA, USA) primary antibody diluted 1:500 in Tris-buffered saline, 0.1% Tween 20 (TBST) solution overnight at 4°C followed by a horseradish peroxidase-conjugated mouse anti-rabbit IgG secondary antibody (Cat# sc-235, Santa Cruz, Dallas, TX, USA) diluted 1:3000 in TBST for 1 hour at room temperature with agitation. Western blots using anti-GAPDH antibodies (Cat# sc-32233, Santa Cruz Biotechnology, USA) were used to confirm equal protein loading. Blots were developed using the Super Signal West Pico kit (ThermoFischer Scientific, USA), scanned for digitization using an Officejet 6700 scanner (Hewlett-Packard, USA) and analyzed using Fiji software[202] .

Confocal Microscopy: Approximately $8x10^4$ hPDL cells were seeded into 4-well glass bottom wells. Cells were challenged with purified dentilisin, wild-type *T. denticola* or isogenic Td-CF522 mutant bacteria as described above. Cells were washed with PBS before being fixed using 4% paraformaldehyde for 10 minutes at room temperature. Next, cells were incubated for 20 minutes with 0.1 M glycine in PBS, washed with PBS and permeabilized with 0.2% Triton X-100 in PBS for 2 minutes at room temperature. Cells were incubated in a 10% serum/PBS blocking buffer for 5 minutes at room temperature followed by a 60-minute incubation in a rabbit sourced polyclonal anti-Sp1primary antibody diluted 1:500 in 10% serum/PBS solution. Next, cells were washed 3 times with PBS followed by incubation in 10% serum/PBS blocking buffer for 1 minute at room temperature followed by incubation in anti-rabbit, mouse secondary antibody conjugated to Alexa 488 fluorophore for 30 minutes at 37°C (1:3000 dilution) and Hoescht 33342 nuclear staining (1:2000 Dilution) in 10% serum/PBS solution. Finally, cells were washed 3 times with PBS, imaged using an SP8 confocal microscope (Leica, Germany) and analyzed using Fiji [202].

Statistical Analysis: Statistical analysis of differentially expressed genes in RNA-Seq data was assessed using a Kolmogorov-Smirnov test followed by Benjamini-Hochberg correction (p<0.05) using R open source software[200]. All other data was analyzed using GraphPad Prism Version 8 software (San Diego, USA). Results were evaluated by a one-way ANOVA when comparing more than two groups with a single independent variable while a two-way ANOVA was used to compare more than two groups with 2 independent variables. Western blot and gelatin zymography data was analyzed using a paired or unpaired t-test, respectively.

Chapter 2.6: FIGURES

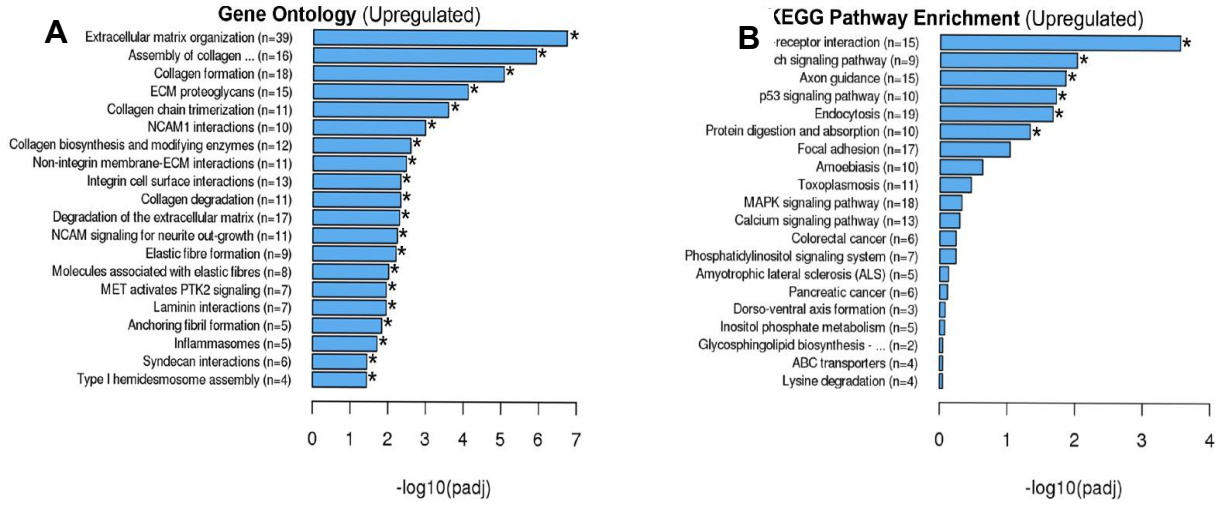


Figure 2.3.1 Gene Ontology and KEGG Pathway Enrichment Analysis of *T. denticola* Infected hPDL Cells. Total RNA was extracted from healthy patient-derived hPDL cells challenged with Td-WT bacteria at a MOI of 50 for 2-hours in media free of supplements followed by 3 and 22-hours in media supplemented with 10% FBS, 1% Pen Strep and 1% Amphotericin B. Mean FPKM values were used for downstream analysis (n = 3 patient replicates). A) Top 20 enriched Gene Ontology terms of hPDL cells challenged for 2-hours followed by a 22-hour incubation using the Reactome nomenclature. Statistical significance was assessed using a Kolmogorov-Smirnov test followed by Benjamini-Hochberg correction ($p<0.05$). B) Top 20 enriched signaling pathways of hPDL cells challenged for 2-hours followed by a 22-hour incubation using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Statistical significance was assessed using a Kolmogorov-Smirnov test followed Benjamini-Hochberg correction ($p<0.05$).

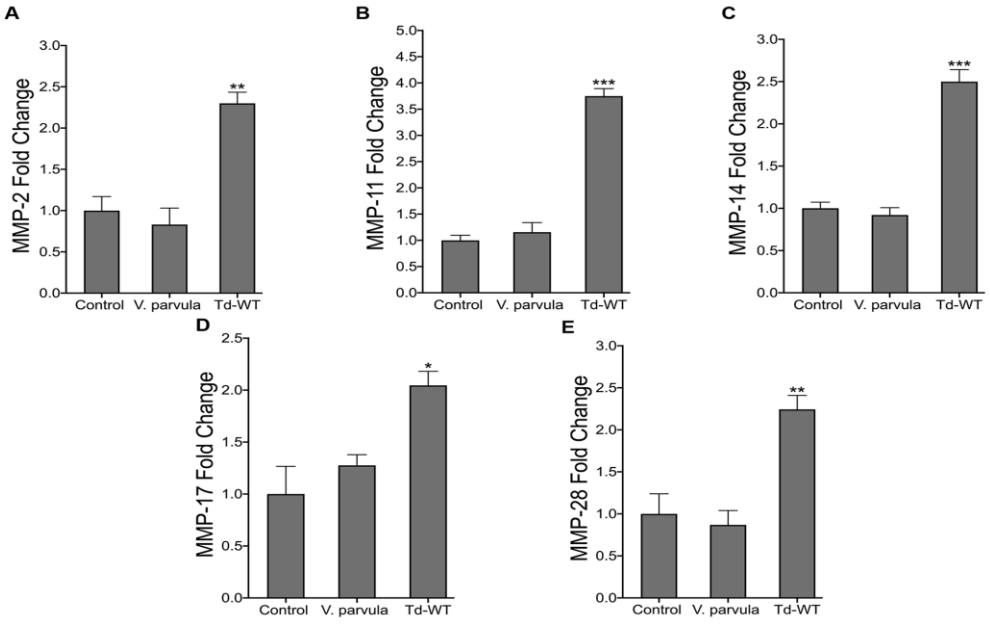


Figure 2.3.2 *T.denticola* upregulates MMPs in hPDL cells while *V. parvula* does not. (A-E) RT-qPCR for *MMP-2*, *MMP-11*, *MMP-14*, *MMP-17 and MMP-28* mRNA expression of healthy hPDL cells challenged with *V. parvula (*ATCC 10790) and Td-WT bacteria at a MOI of 50 for 2-hours, followed by a 22-hour incubation in media supplemented with 10% FBS and 1% Pen/Strep and 1% Amphotericin B. Expression of each gene was normalized to that of *GAPDH*. Statistical significance was determined using a One-Way ANOVA followed by Post-Hoc Tukey's multiple comparisons. Bars represent mean ± SEM (n = 5). **p* < .05 versus control. ***p* < .01 versus control. ****p* < .001 versus control.

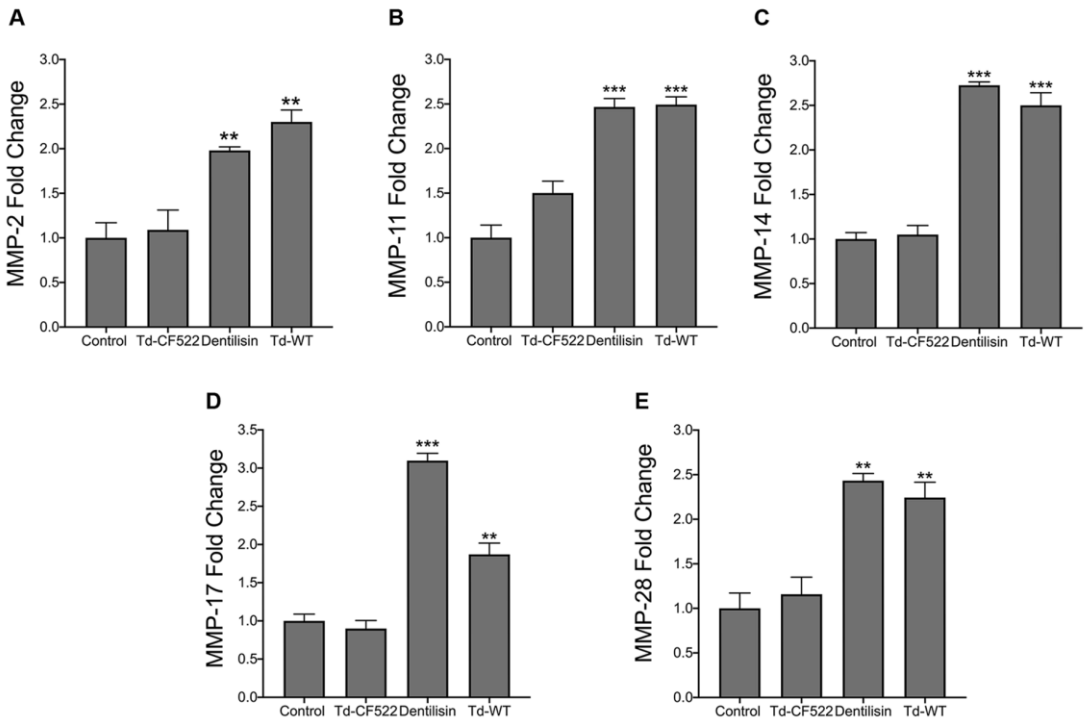


Figure 2.3.3 *T. denticola* surface-expressed dentilisin mediates the upregulation of MMP 2, 11, 14, 17 and 28 mRNA levels in hPDL cells. (A-E) RT-qPCR for MMP-2, MMP-11, MMP-14, MMP-17 and MMP-28 mRNA expression of healthy hPDL cells challenged with isogenic Td-CF522 bacteria, Td-WT bacteria and purified dentilisin. Cells were stimulated at an MOI of 50 and a final concentration of of 1 µg/mL. Cells were challenged for 2-hours in alpha-MEM media supplemented with 10% FBS followed by a 22-hour incubation in alpha-MEM media with 10% FBS, 1% PenStrep and 1% Amphotericin B. The expression of each gene was normalized to that of GAPDH. Statistical significance was determined using a One-Way ANOVA followed by Tukey's Post-Hoc multiple comparisons. Bars represent mean ± SEM (n = 4). **p < .01 versus control. ***p < .001 versus control.

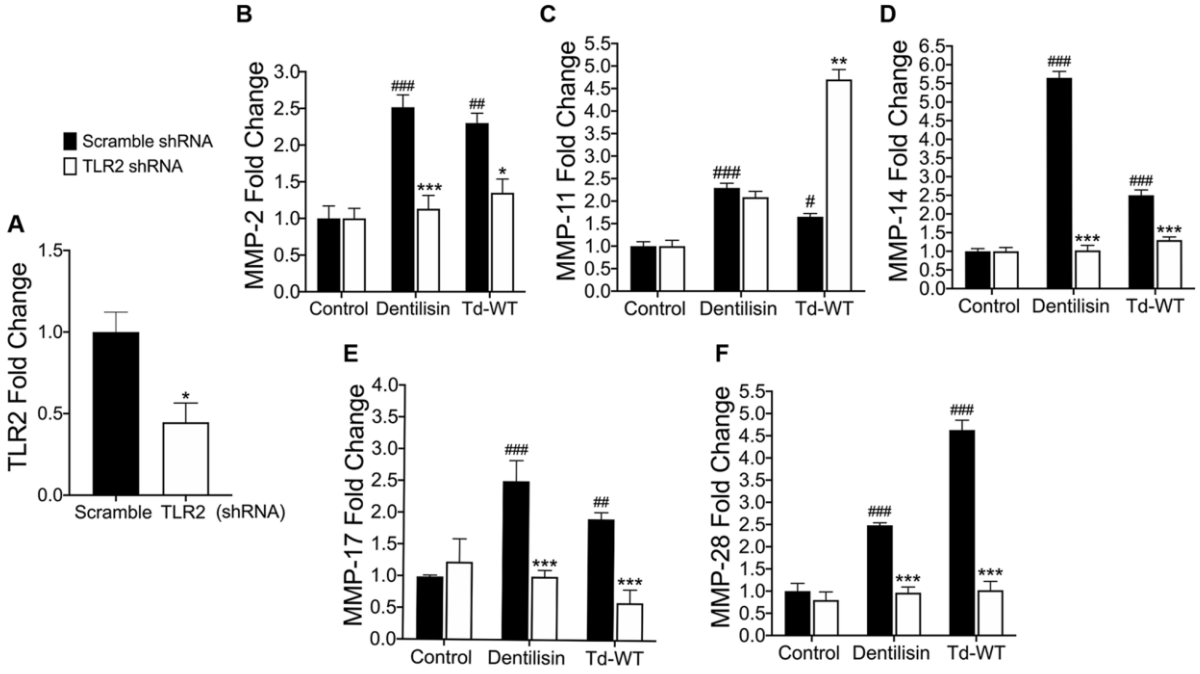


Figure 2.3.4 Suppression of TLR2 inhibits *T. denticola*-stimulated upregulation of MMPs 2, 14, 17 and 28 while exacerbating MMP 11 expression in hPDL cells. A) RT-qPCR validation of stable gene suppression using shRNA vectors targeted against TLR2 in healthy hPDL cells. Cells transduced with scrambled shRNA vectors were used as a control. Statistical significance was determined using an unpaired t-test. Bars represent ± SEM of mean value (n = 3 clones). *p < .05 versus control. B-F) RT-qPCR for MMP-2, MMP-11, MMP-14, MMP-17 and MMP-28 mRNA expression of scrambled shRNA control and TLR2 shRNA hPDL cells challenged or stimulated with Td-CF522, purified dentilisin or Td-WT at an MOI of 50 and concentration of 1 µg/mL for 2-hours in alpha-MEM media with no supplementation followed by a 22-hour incubation in alpha-MEM media supplemented with 10% FBS, 1% Pen/Strep and 1% Amphotericin B. The expression of each gene was normalized to that of GAPDH. Statistical significance was determined using a Two-Way ANOVA followed by post-hoc Tukey's multiple comparisons. Bars represent mean ± SEM (n = 3). #p < .05 versus scramble control group. ##p < .01 versus scramble control group. ###p < .001 versus scramble control group. *p < .05 versus TLR2 shRNA equivalent group. ***p < .001 versus TLR2 equivalent shRNA group.

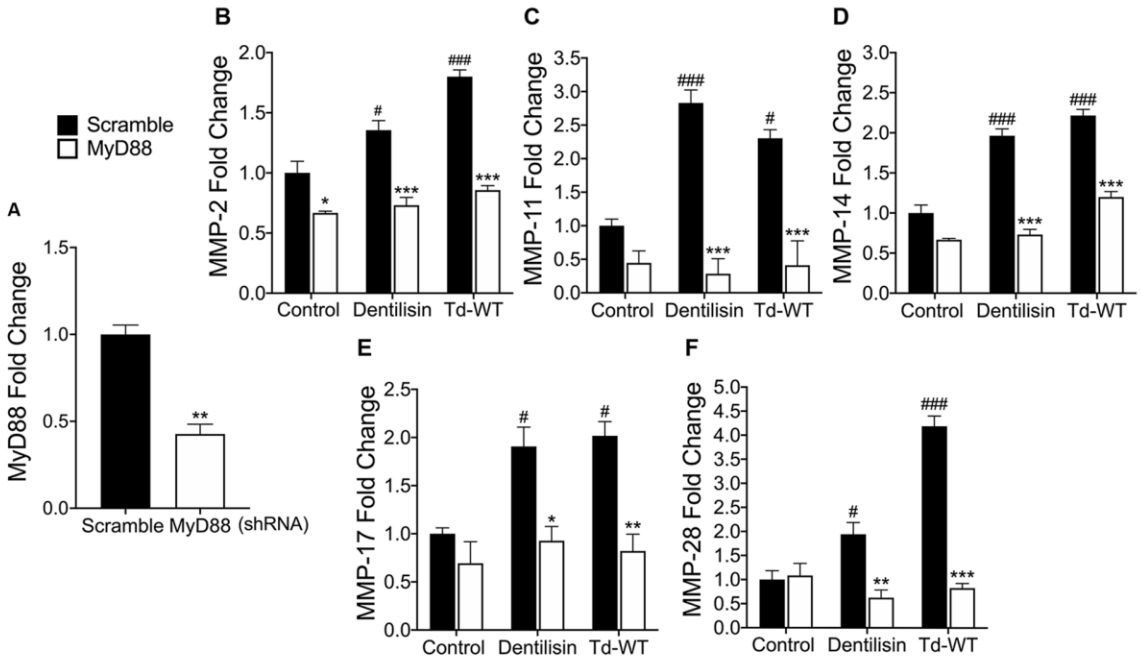


Figure 2.3.5 Suppression of MyD88 inhibits T. denticola-stimulated upregulation of MMP 2, 11, 14, 17 and 28 in hPDL cells. A) RT-qPCR validation of stable gene suppression using shRNA vectors targeted against MyD88 in healthy hPDL cells. Cells transduced with scrambled shRNA vectors were used as a control. Statistical significance was determined using an unpaired t-test. Bars represent ± SEM of mean values (n = 3 clones). *p < .05 versus control. B-F) RT-qPCR for MMP-2, MMP-11, MMP-14, MMP-17 and MMP-28 mRNA expression of scrambled shRNA control and MyD88 shRNA hPDL cells challenged or stimulated with Td-CF522, purified dentilisin or Td-WT at an MOI of 50 and concentration of 1 µg/mL for 2-hours in alpha-MEM media with no supplementation followed by a 22-hour incubation in alpha-MEM media supplemented with 10% FBS, 1% Pen/Strep and 1% Amphotericin B. The expression of each gene was normalized to that of GAPDH. Statistical significance was determined using a Two-Way ANOVA followed by post-hoc Tukey's multiple comparisons. Bars represent mean ± SEM (n = 3). #p < .05 versus scramble control group. ###p < .001 versus scramble control group *p < .05 versus MyD88 shRNA equivalent group. **p < .01 versus MyD88 shRNA equivalent group. ***p < .001 versus MyD88 shRNA equivalent group.

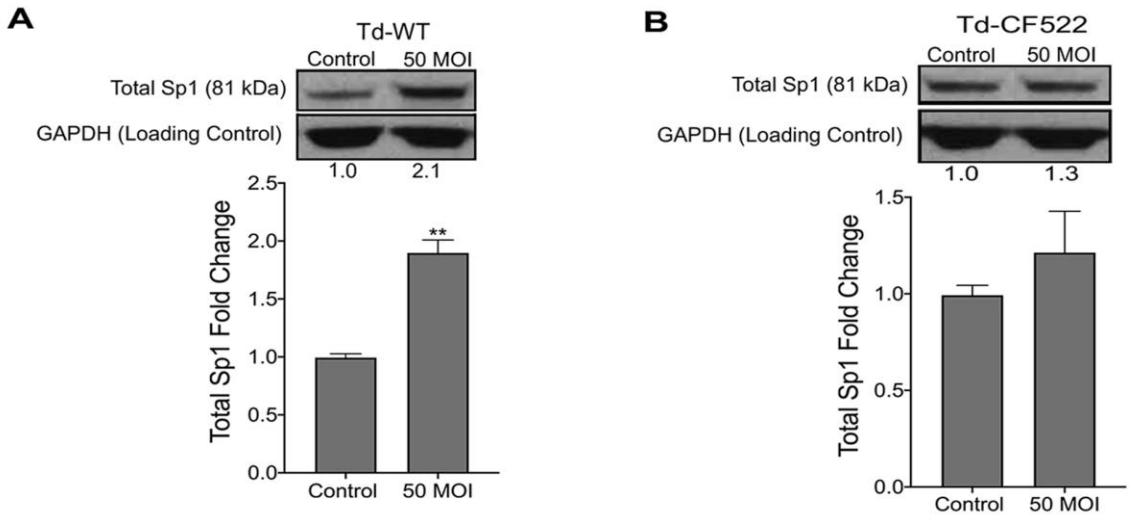


Figure 2.3.6 Wild-type *T. denticola* upregulates Sp1 protein expression while the isogenic dentilisin deificient mutant does not. Healthy hPDL cells were challenged with A) Td-WT and B) isogenic Td-CF522 bacteria at an MOI of 50 as previously described. Whole cell lysates were generated and used for Western Blot analysis utilizing total anti-Sp1 antibodies. Total Sp1 protein expression was normalized against GAPDH protein expression as a loading control. Statistical significance was determined using a paired t-test. Bars represent mean ± SD (n = 3). **p < .01 versus control.

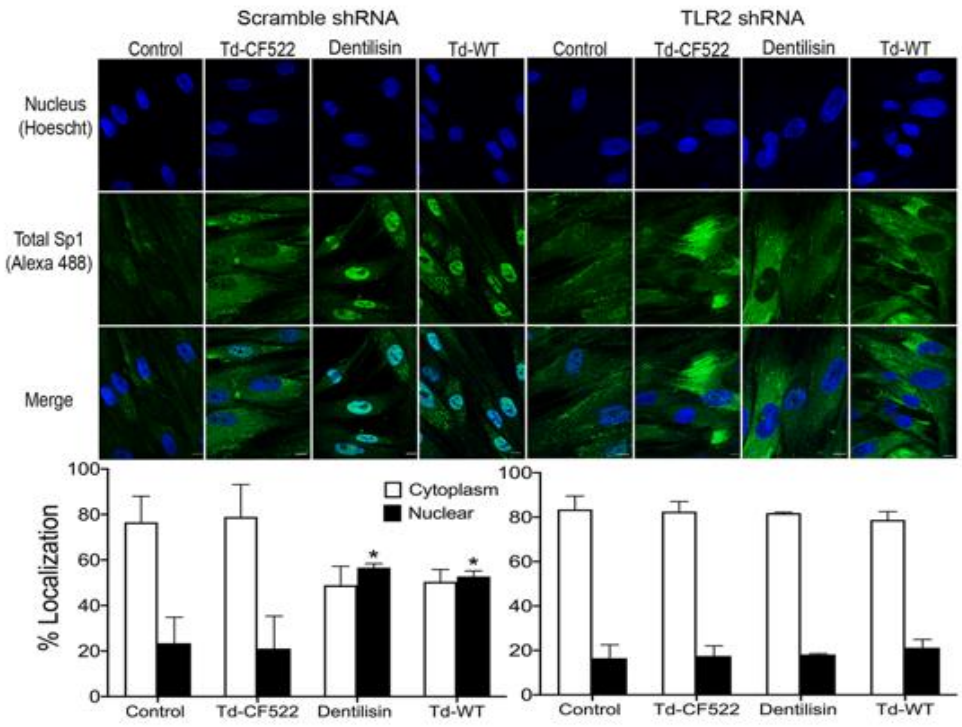


Figure 2.3.7 Knockdown of TLR2-dependent signaling inhibits translocation of transcription factor Sp1 in both *T. denticola* and dentilisin-stimulated hPDL cells. A-B) Healthy hPDL cells were challenged with Td-WT and isogenic Td-CF522 bacteria at an MOI of 50 as previously described. Whole cell lysates were generated and used for Western blot analysis utilizing total Sp1-specific antibodies. Total Sp1 protein expression was normalized using GAPDH as a loading control. Statistical significance was determined using a paired t-test. Bars represent mean ± SD (n = 3). ***p* < .01 versus control. C) Scrambled shRNA control and *TLR2* shRNA hPDL cells were challenged or stimulated with Td-CF522, purified dentilisin or Td-WT at an MOI of 50 and concentration of 1 *μ*g/mL for 2-hours in alpha-MEM media with no supplementation followed by a 22-hour incubation in alpha-MEM media supplemented with 10% FBS, 1% Pen/Strep and 1% Amphotericin B. Cells were stained with Hoescht 33342 (Blue) and total Sp1-specific antibodies (Green), and subjected to confocal microscopy. Scale bar represents 10 *μ*m.

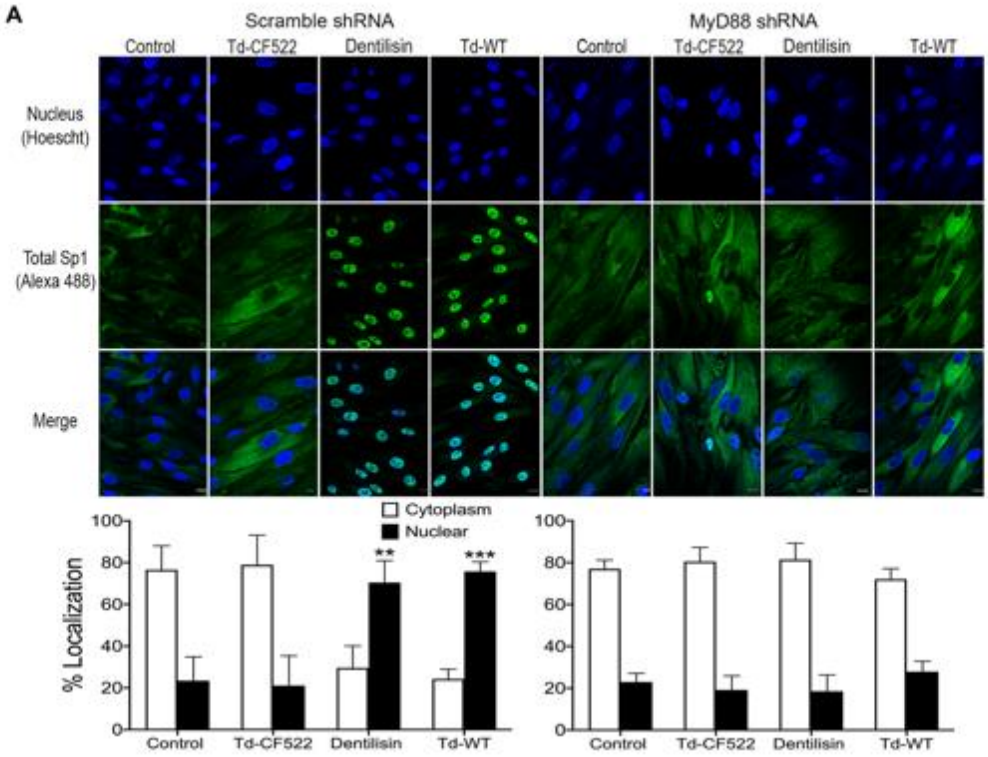


Figure 2.3.8 Knockdown of MyD88-dependent signaling inhibits translocation of transcription factor Sp1 in both *T. denticola*- and dentilisin-stimulated hPDL cells. A) Scrambled shRNA control and MyD88 shRNA hPDL cells were challenged or stimulated with Td-CF522, purified dentilisin or Td-WT at an MOI of 50 and concentration of 1 µg/mL for 2-hours in alpha-MEM media with no supplementation followed by a 22-hour incubation in alpha-MEM media supplemented with 10% FBS, 1% Pen/Strep and 1% Amphotericin B. Cells were stained with Hoescht 33342 (Blue) and total Sp1-specific antibodies (Green) and subjected to confocal microscopy. Scale bar represents 10 µm.

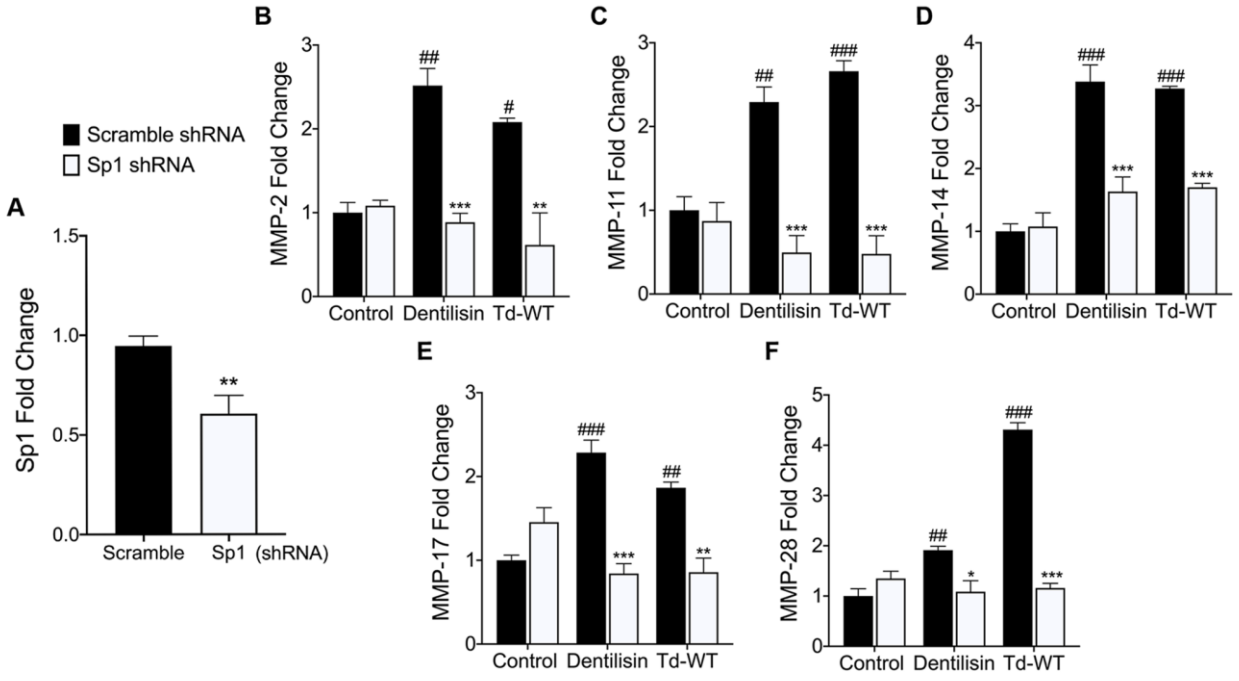


Figure 2.3.9. Stable Suppression of Sp1 inhibits T. denticola-stimulated upregulation of MMP 2, 11, 14, 17 and 28 in hPDL cells. **A)** RT-qPCR validation of stable gene suppression using shRNA vectors targeted against *Sp1* in healthy hPDL cells. Cells transduced with scrambled shRNA vectors were used as a control. Statistical significance was determined using an unpaired t-test. Bars represent ± SEM of mean values (n = 3 clones). ***p* < .01 versus control. **B-F)** RT-qPCR for *MMP-2*, *MMP-11*, *MMP-14*, *MMP-17 and MMP-28* mRNA expression of scrambled shRNA control and *Sp1* shRNA hPDL cells challenged or stimulated with purified dentilisin at a concentration of 1 *μ*g/mL or Td-WT at an MOI of 50 for 2-hours in alpha-MEM media supplemented with 10% FBS and no antibiotics followed by a 22-hour incubation in alpha-MEM media supplemented with 10% FBS, 1% Pen/Strep and 1% Amphotericin B. The expression of each gene was normalized to that of *GAPDH*. Statistical significance was determined using a Two-Way ANOVA followed by post-hoc Tukey's multiple comparisons. Bars represent mean ± SEM (n = 3). #*p* < .05 versus scramble control group. ##*p* < .01 versus scramble control group. ###*p* < .001 versus scramble control group. **p* < .05 versus *Sp1* shRNA equivalent group. ***p* < .01 versus *Sp1* shRNA equivalent group. ****p* < .001 versus *Sp1* shRNA equivalent group.

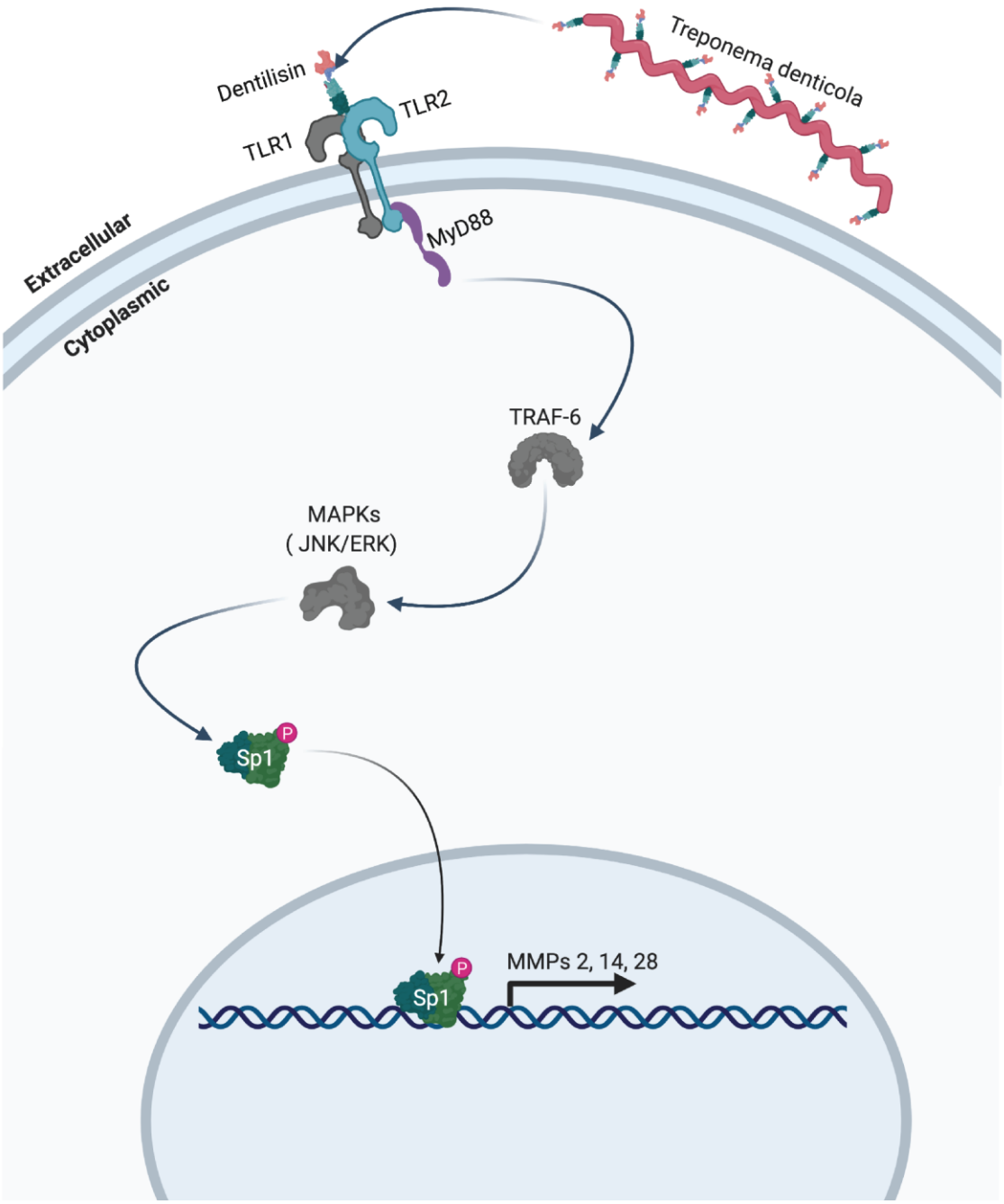
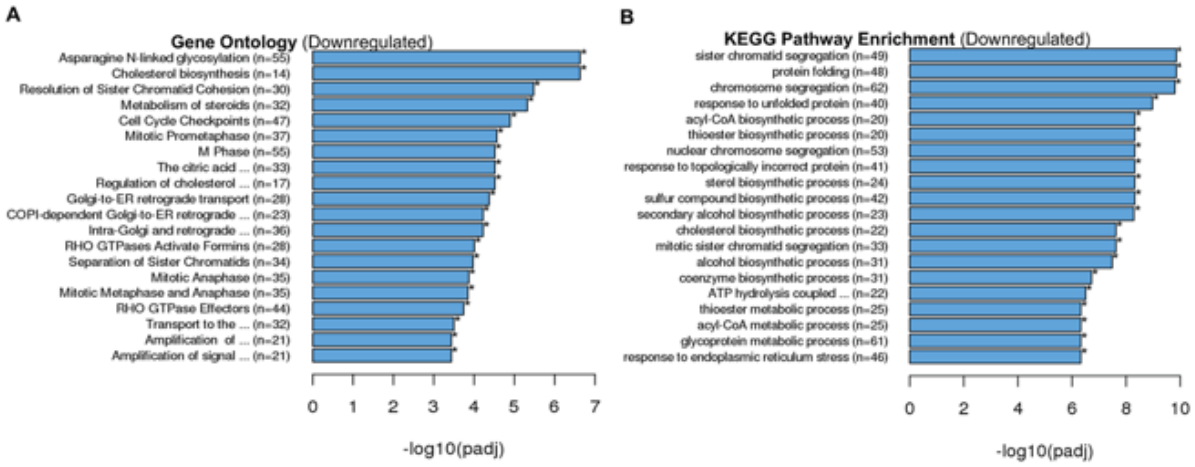
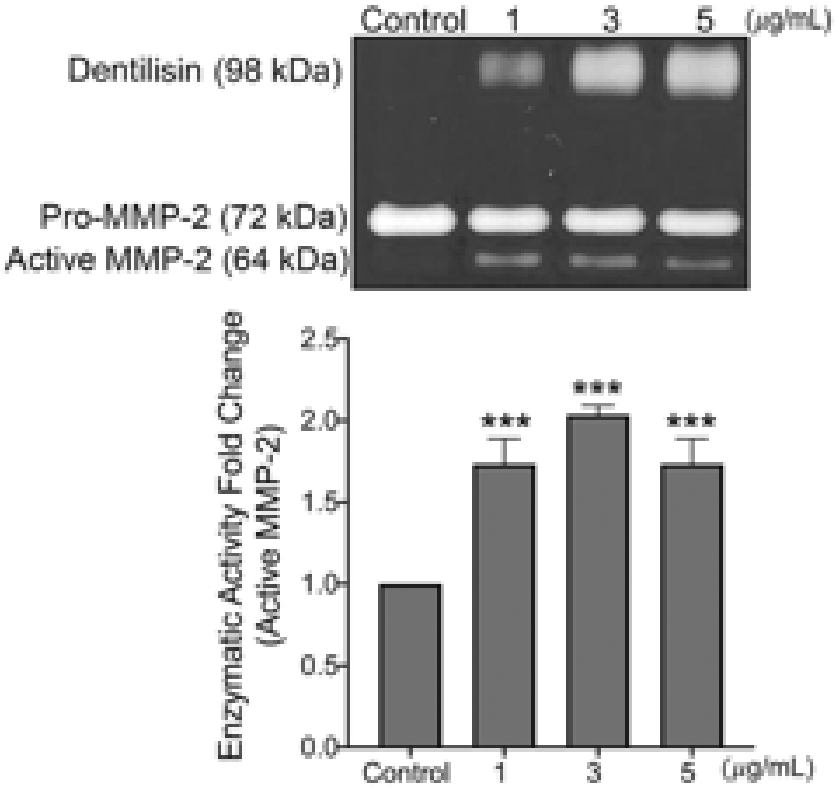


Figure 2.3.10 Model of proposed mechanism. *T. denticola* activates host expressed TLR2 receptors via surface-expressed dentilisin. Subsequent downstream activation of MyD88 and translocation of the transcription factor Sp1 lead to the upregulation of MMPs 2, 11, 14, 17 and 28 in human periodontal ligament cells. Grey colored icons represent genes that were identified through RNA-sequencing and analysis but were not directly validated.

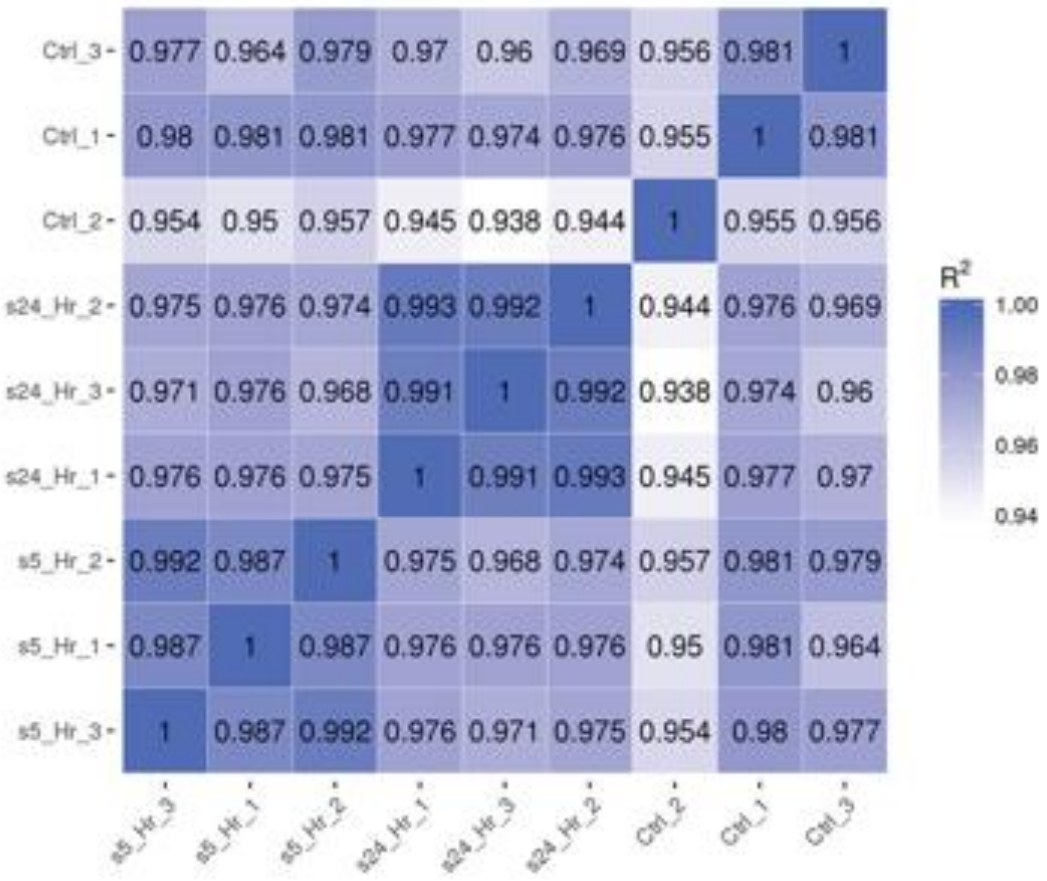
Chapter 2.7: SUPPLEMENTAL FIGURES



Supplemental Figure 2.7.1 Dowregulated GO and KEGG Terms for *T. denticola* stimulated hPDL cells**.** A) Top 20 downregulated Gene Ontology terms of hPDL cells challenged for 2-hours followed by a 22-hour incubation using the Reactome nomenclature. Statistical significance was assessed using a Kolmogorov-Smirnov test followed by Benjamini-Hochberg correction ($p<0.05$). B) Top 20 downregulated signaling pathways of hPDL cells challenged for 2-hours followed by a 22-hour incubation using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database. Statistical significance was assessed using a Kolmogorov-Smirnov test followed Benjamini-Hochberg correction ($p<0.05$).



Supplemental Figure 2.7.2. Assessment of purified dentilisin enzymatic activity after stimulation with purified dentilisin. Healthy hPDL cells were challenged with purified dentilisin at increasing concentrations (1, 3 and 5 *μ*g/mL) for 2-hours followed by a 22-hour incubation in MEM-*α* media free of FBS and supplemented with 1% P/S. Conditioned media from these cells were used to assess the enzymatic activity of Active MMP-2 (64-kDa) and Dentilisin (98 kDa) using gelatin zymography followed by densitometry analysis using Fiji. Statistical significance was determined using a One-Way ANOVA. Bars represent ± SD of mean values (n = 3). ***p < .001 versus control.



Supplemental Figure 2.7.3. Assessing inter- and intragroup gene expression variability across sample replicates for RNA-Seq experiment. The figure displays a Pearson's plot visualizing the correlation between samples. Scale bar represents the range of the correlation coefficients (*R*) displayed.

Chapter 3: *T. denticola* Bacteria and Purified Dentilisin Upregulate RANKL and M-CSF While Suppressing Osteoprotegrin (OPG) mRNA Expression

Chapter 3.1: ABSTRACT

In this study, we investigated the impact of *Treponema denticola* (T. denticola) and its virulence factor dentilisin on the expression of key osteoimmunological markers in human periodontal ligament fibroblasts (hPDLs). hPDLs were subjected to treatments with wild-type *T. denticola* (Td-WT), a dentilisin-functional isogenic mutant (Td-CF522), or purified dentilisin. Each condition included a 22-hour incubation at 37°C with 95% air and 5% CO2. Quantitative real-time PCR (qRT-PCR) was employed to measure mRNA levels of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and macrophage colony-stimulating factor (M-CSF).

Our results demonstrated that both Td-WT and purified dentilisin significantly elevated the expression levels of RANKL and M-CSF compared to the control group ($p < 0.05$). In contrast, the Td-CF522 mutant did not show a significant difference in the expression of these markers, underscoring the crucial role of dentilisin in modulating osteoimmunological responses. Notably, OPG expression remained unchanged across all experimental conditions.

These findings align with existing literature, highlighting the role of *T. denticola* and dentilisin in modulating host immune responses and contributing to periodontal disease pathology. The upregulation of RANKL and M-CSF suggests a mechanism by which dentilisin promotes osteoclast differentiation and activation, contributing to the pathogenesis of periodontal disease. This study enhances our understanding of the molecular mechanisms driving periodontal health disparities and identifies potential targets for therapeutic intervention.

Chapter 3.2: INTRODUCTION

Periodontal disease remains a significant public health concern worldwide, characterized by inflammation and destruction of the supporting structures of the teeth, primarily the periodontium. Central to the pathogenesis of periodontitis are the molecular interactions between receptor activator of nuclear factor kappa-B ligand (RANKL) and Osteoprotegerin (OPG). RANKL, a member of the tumor necrosis factor (TNF) superfamily, plays a pivotal role in regulating osteoclastogenesis, the process by which osteoclasts, the bone-resorbing cells, are formed and activated[204]. In contrast, OPG acts as a decoy receptor for RANKL, competitively inhibiting its binding to RANK receptors on osteoclast precursors, thereby modulating bone resorption[205]. These regulatory mechanisms are crucial in maintaining the delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts, processes orchestrated by a complex interplay involving various signaling pathways and cytokines[206]

Within this intricate network of bone remodeling, the gene encoding macrophage colony stimulating factor (M-CSF), also known as CSF-1, assumes a critical role. M-CSF is indispensable for the survival, proliferation, and differentiation of osteoclast precursors, essential components in the formation and activation of mature osteoclasts[207]. Through its interaction with its receptor, c-Fms, expressed on osteoclast precursor cells, M-CSF initiates signaling cascades that are pivotal for the commitment of these cells to the osteoclastic lineage[208].

Moreover, bone homeostasis is not solely governed by osteoclasts; the reciprocal actions of osteoblasts, responsible for bone formation, and osteocytes, the mechanosensory cells embedded within the mineralized bone matrix, are equally crucial[209]. In mice, when NF-kB activation is blocked in osteoblastic cells but not other cell types, dysbiosis-induced periodontal bone loss is inhibited[210]. This is due to two distinct mechanisms. Blocking NF-kB activation

reduces RANKL expression by osteocytes and other osteoblastic cells to diminish bone resorption. In addition, NF-kB in osteoblast lineage cells inhibits coupled bone formation. NF-kB impairs bone formation by inhibiting differentiation of osteoblast precursors, indirectly stimulating apoptosis of osteoblastic cells or their precursors and reducing production of bone osteoid[211, 212]. The latter occurs because NF-kB inhibits the expression of proteins that make up osteoid[211]. Bacterial dysbiosis significantly increases the number of TNF-α producing cells and increases bone-lining cell death 10-fold. The increased apoptosis is functionally significant since treatment with an apoptosis-specific inhibitor reduces periodontal bone loss through increased coupled bone formation. When the adaptive immune response is stimulated by oral bacteria, coupled bone formation is further inhibited[213]. Thus, activation of NF-kB in osteoblast precursors, osteoblasts and osteoclast precursors plays a key role in periodontitis by promoting bone resorption and limiting coupled bone formation.

Thus, understanding the intricate relationships among RANKL, OPG, M-CSF, osteoclasts, periodontal ligament fibroblast, osteoblasts, and osteocytes is fundamental for unraveling the pathophysiology of periodontal disease and exploring potential therapeutic targets aimed at restoring bone homeostasis and preventing the irreversible consequences of this chronic inflammatory condition.

Chapter 3.3: RESULTS

3.3.1 *T. denticola* Expressed Dentilisin Induces RANKL and M-CSF mRNA Expression While Concomitantly Suppressing Osteoprotegrin (OPG) Expression

Human periodontal ligament fibroblasts (hPDLs) were subjected to various treatments to assess the impact on mRNA expression levels of key osteoimmunological markers. The fibroblasts were challenged with either wild-type *T. denticola* bacteria, a dentilisin-functional isogenic mutant (Td-CF522) at a multiplicity of infection (MOI) of 50, or purified dentilisin at a concentration of 1 μg/mL. Each condition included a 22-hour incubation period at 37°C with 95% air and 5% CO2. Six replicates from healthy patient samples were utilized for each experimental condition to ensure statistical robustness and reproducibility.

Following incubation, mRNA expression levels of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and macrophage colony-stimulating factor (M-CSF) were quantified using quantitative real-time PCR (qRT-PCR). Primer sets specific for these genes were employed to ensure accurate and sensitive detection of transcript levels.

Data analysis was performed using one-way ANOVA, followed by Post-Hoc Tukey's multiple comparisons test to determine statistically significant differences among the groups. The results demonstrated notable differences in the expression of RANKL and M-CSF when comparing the control group to the wild-type *T. denticola* (Td-WT) and purified dentilisin-treated groups. Specifically, both Td-WT and dentilisin groups exhibited significantly elevated RANKL and M-CSF expression levels compared to the control group ($p < 0.05$). However, the dentilisin-functional isogenic mutant group (Td-CF522) did not show a statistically significant difference in the expression of these markers compared to the control, indicating a crucial role for dentilisin in modulating these osteoimmunological responses.

In contrast, no significant changes in OPG expression were observed across any of the experimental conditions. The negative control group, which included cells that were not challenged with either purified dentilisin or bacteria, served as a baseline to confirm that the observed effects were indeed due to the specific treatments applied.

These findings align with previous literature, which has highlighted the role of *T. denticola* and its virulence factor dentilisin in modulating host immune responses and contributing to periodontal disease pathology. The observed increase in RANKL and M-CSF expression is consistent with the role of these factors in osteoclast differentiation and activation, as outlined by Boyle et al. (2003).In summary, our results underscore the critical involvement of *T. denticola* dentilisin in upregulating key osteoimmunological markers, thereby contributing to the pathogenesis of periodontal disease. This study adds to the growing body of evidence elucidating the molecular mechanisms underlying periodontal health disparities and highlights potential targets for therapeutic intervention.

Chapter 3.4: DISCUSSION

In this study, human periodontal ligament fibroblasts (hPDLs) were subjected to various treatments to elucidate the impact of *T. denticola* and its virulence factor, dentilisin, on the mRNA expression levels of key osteoimmunological markers. The fibroblasts were challenged with either wild-type *T. denticola* (Td-WT), a dentilisin-functional isogenic mutant (Td-CF522), or purified dentilisin at a defined concentration. Six replicates from healthy patient samples were utilized for each experimental condition to ensure statistical robustness and reproducibility.

Following incubation, the mRNA expression levels of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and macrophage colony-stimulating factor (M-CSF) were quantified using quantitative real-time PCR (qRT-PCR). Specific primer sets were employed for accurate and sensitive detection of transcript levels. Data analysis using one-way ANOVA, followed by Post-Hoc Tukey's multiple comparisons test, revealed notable differences in the expression of RANKL and M-CSF among the groups. Specifically, both the Td-WT and purified dentilisin-treated groups exhibited significantly elevated RANKL and M-CSF expression levels compared to the control group ($p < 0.05$). However, the dentilisin-functional isogenic mutant group (Td-CF522) did not show a statistically significant difference in the expression of these markers compared to the control, indicating a crucial role for dentilisin in modulating these osteoimmunological responses.

Interestingly, no significant changes in OPG expression were observed across any of the experimental conditions. The negative control group, which included cells that were not challenged with either purified dentilisin or bacteria, served as a baseline to confirm that the observed effects were specifically due to the treatments applied. These findings align with previous literature, which has highlighted the role of *T. denticola* and its virulence factor dentilisin in modulating host immune responses and contributing to periodontal disease

pathology. Td92, an outer membrane protein of *Treponema denticola*, has been shown to induce osteoclastogenesis via prostaglandin E_2-mediated RANKL/osteoprotegerin regulation. However, this is the first study to demonstrate that dentilisin directly upregulates RANKL in hPDL cells, a significant contribution to our understanding of periodontal disease mechanisms. It is also notable that the proteolytic activity of dentilisin appears to be a particular factor in upregulating RANKL and M-CSF. Previous studies, such as those by Takeichi et al. (2004), have shown that other periodontal pathogens, including *Porphyromonas gingivalis*, can induce RANKL expression as a result of the expression of potent gingipains. However, the specific involvement of dentilisin's proteolytic activity in modulating these osteoimmunological markers underscores its unique role in periodontal disease pathogenesis.

The suppression of OPG expression, though not statistically significant in our study, is also worth noting. OPG acts as a decoy receptor for RANKL, inhibiting its interaction with the RANK receptor and thereby preventing osteoclastogenesis. The balance between RANKL and OPG is crucial for bone homeostasis, and perturbations in this balance can lead to enhanced bone resorption, a hallmark of periodontal disease.

In summary, our results underscore the critical involvement of *T. denticola* dentilisin in upregulating key osteoimmunological markers, thereby contributing to the pathogenesis of periodontal disease. This study adds to the growing body of evidence elucidating the molecular mechanisms underlying periodontal health disparities and highlights potential targets for therapeutic intervention. The unique contribution of dentilisin's proteolytic activity in modulating RANKL and M-CSF expression opens new avenues for exploring targeted treatments aimed at mitigating periodontal disease progression and its associated bone loss.

Chapter 3.5: MATERIALS AND METHODS

Human PDL Cell Cultures: As described previously, the primary culture of PDL cells was conducted via the direct cell outgrowth method by isolating cells from the PDL tissue around the middle third of extracted healthy human teeth[115, 116]. Cells were maintained in Minimum Eagle Medium-α (MEM-α) (Gibco, USA) augmented with 10% fetal bovine serum (FBS) (Gibco, USA), 1% penicillin/streptomycin (P/S), and 1% amphotericin B (Gibco, USA) in a Steri Cycle 370 incubator (Thermo-Fisher Scientific, USA) with a humid atmosphere containing 95% air and 5% CO_2 at 37 °C. Cell outgrowths were passaged before reaching confluency. Only cells passage three to seven were used for experimentation. Cells were validated by morphological assessment and gene expression of confident biomarkers such as *Periostin*.

Anaerobic Bacterial Cultures: *T. denticola* ATCC 35405 (Td-WT) and its isogenic Td-CF522 dentilisin-deficient mutant Td-CF522 were grow in in Oral Treponeme Enrichment Broth (OTEB; Anaerobe Systems, USA) at 37 °C) grown in tanks filled with an anaerobic gas mixture [198]. *Veilonella parvula* ATCC 10790 was grown under the same anaerobic conditions as *T. denticola* in brain heart infusion (BHI) media supplemented with 0.075% sodium thioglycolate, 0.1% Tween 80 and 1% of 85% lactic acid and pH adjusted to 6.5-6.6 using 1 M NaOH.

Purification of *T. denticola* dentilisin: The dentilisin protease complex was purified from the detergent phase of Triton X-114 extracts of an isogenic *T. denticola* mutant that lacks Msp ,the dominant outer membrane protein, as described previously. Briefly, following overnight Triton X-114 treatment, cells were pelleted, the supernatants were phase-separated, the detergent phase was re-extracted twice, precipitated in acetone, then subjected to preparative SDS-PAGE electrophoresis. Fractions of interest, eluted in detergent-free buffer, were concentrated by ultrafiltration, subjected to buffer exchange into PBS containing 0.1% CHAPS {3-[(-[(3-

cholamidopropyl)-dimethylammonio]-1-propanesulfonate} and concentrated again. Purified [59]. Aliquots of purified dentilisin were stored at -80˚C. Activity of purified dentilisin samples was monitored periodically, using the chromogenic substrate succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine-*p*-nitroanilide (SAAPFNA) as described previously[128].

Bacterial Challenging of hPDL Cells: ~9x10^5 healthy hPDL cells were seeded on 6 cm plates. On the next day, these cells were challenged with either *T. denticola,* Td-CF522 or *V. parvula*, as previously described by Ateia et al.[56]. Briefly, the bacteria were centrifuged at 4000 RPM for 15 minutes and the supernatant was removed. The pelleted bacterial cells were then resuspended in antibiotic-free MEM-α (without phenol red) at an optical density (OD) of 0.1 at 600 nm using a Spectramax M2 microplate spectrophotometer (Molecular Devices, USA). It has been previously established that an OD of 0.1 at 600nm is equivalent to 2.4x10^8 CFU/ml for *T. denticola[198]* and 2x10^8 CFU/mL for *V. parvula* strains[199]. Next, hPDL cells were challenged with the bacteria at 50 multiplicity of infection (MOI), whereas the control group was challenged with antibiotic-free MEM-α. Next, the cells were incubated for 2h at 37 °C and 5% CO_2, washed twice with Phosphate Buffer Saline (PBS) (Gibco, USA) and incubated again at 37 °C and 5% CO_2 overnight in antibiotic-free MEM-α. On the next day, cells were either harvested for RNA isolation, generation of cell lysates and stored at -80 °C for further investigations.

Stimulation of hPDL Cells Using Purified Dentilisin: A BCA protein assay kit (CAT # 23227 Thermo Scientific, USA) was used to determine purified dentilisin sample concentrations as described above. Assessment of enzymatic activity was determined using gelatin zymography as described below. ~9.5x10^5 cells were seeded on 6 cm plates. Purified dentilisin was added to

MEM-α media with no serum and 1% P/S at a final concentration of 1 μg/mL and added to healthy hPDL cell cultures for 2-hours. The treated cells were washed with PBS and incubated overnight in MEM-α media free of FBS and supplemented with 1% P/S. Next, the Supernatant (conditioned media) was collected and total RNA was extracted using an RNeasy Mini kit (Cat# 74134, Qiagen, Germany). The resulting samples were stored at −80°C until analyzed further in downstream applications. This protocol has been formally published[214].

Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR): Total hPDL RNA was extracted and purified using the RNeasy mini kit (Cat# 74134, Qiagen, Germany) according to the manufacturer's instructions and quantified using a NanoDrop UV-Vis Spectrophotometer (Thermo Scientific, USA). Reverse transcription of the RNA into cDNA was then performed using the SuperScript III cDNA synthesis kit (Cat #11754050, Invitrogen, USA). Samples were ran on a Bio-Rad MyCycler Thermal Cycler according to manufacturers protocol. All cDNA samples were diluted to 5 ng/mL working concentrations and stored at -20°C. cDNA samples were probed for target gene expression via RT-qPCR using PowerUp SYBR Green Master Mix (Applied Biosystems, USA) and primer sequences (Table 1) on a QuantStudio 3 platform (Applied Biosystems, USA). The relative expression levels of target genes were plotted as fold change compared with untreated or negative controls. The $2^{-\Delta\Delta}$CT method was used to determine relative change in target gene expression, and gene expression was normalized against GAPDH expression.

Chapter 3.6: FIGURES

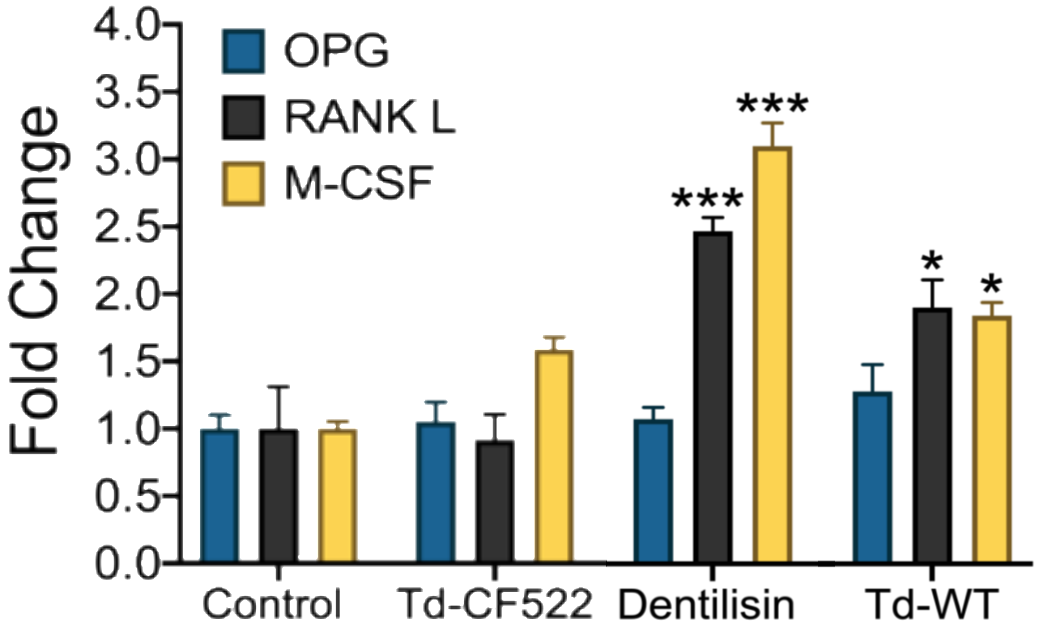


Figure 3.3.1. *T. denticola* Expressed Dentilisin Induces RANKL and M-CSF mRNA Expression While Concomitantly Suppressing Osteoprotegrin (OPG) Expression. mRNA expression levels of osteoprotegerin (OPG), receptor activator of nuclear factor kappa-B ligand (RANKL), and macrophage colony-stimulating factor (M-CSF) in human periodontal ligament fibroblasts (hPDL) challenged with either wild-type *T. denticola* bacteria, a dentilisin functional isogenic mutant (Td-CF522) at a multiplicity of infection (MOI) of 50, or purified dentilisin at 1 µg/mL. The data were obtained from six healthy patient sample replicates using quantitative real-time PCR (qRT-PCR) with specific primer sets for each target gene. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons post-hoc test. Significant differences in RANKL and M-CSF expression were observed in the groups challenged with wild-type *T. denticola* and purified dentilisin compared to the control group. However, no significant differences were found in the Td-CF522 group. This indicates that the presence of functional dentilisin is crucial for the upregulation of RANKL and M-CSF expression in hPDL cells.

Chapter 4: Conclusion

The present study delves into the multifaceted role of *Treponema denticola* (*T. denticola*) and its virulence factor dentilisin in modulating host immune responses and contributing to the pathogenesis of periodontal disease. The dysbiotic periodontal community faces a challenging paradox: while these bacteria must evade immune-mediated destruction, they also rely on inflammation to obtain nutrients from tissue degradation, including collagen peptides and hem-containing compounds. This dynamic leads to a dysbiotic subgingival biofilm that alters the overall biomass, virulence factor output, synergistic effects, and the ability to subvert the normal immune host-response. Our findings illuminate the sophisticated mechanisms by which *T. denticola* interacts with human periodontal ligament fibroblasts (hPDLs), influencing the expression of key osteoimmunological markers and contributing to the inflammatory milieu associated with periodontal infections.

T. denticola's virulence is characterized by its ability to manipulate host immune responses rather than inducing overt inflammation. By subverting innate immune signaling, including the crosstalk between complement and Toll-like receptors (TLR), *T. denticola* effectively evades immune surveillance[61, 141]. Notably, key genes involved in innate immune responses, such as those encoding pro-inflammatory cytokines (IL-8, IL-6) and chemokines (ICAM-1, HβD-2), are not induced by *T. denticola*, although TLR2 signaling remains active[62]. This indicates a nuanced regulatory mechanism wherein *T. denticola* maintains a low inflammatory profile to avoid detection while still manipulating host defenses to its advantage. This thereby allows for other keystone pathogen to proliferate and begin shifting the subgingival biofilm composition.

Dentilisin, a surface-expressed protease complex of *T. denticola*, emerges as a pivotal factor in immune evasion, tissue degradation, and modulation of host cell responses. This protease complex plays a crucial role in several pathogenic processes. It exhibits significant proteolytic

activity that enables the bacterium to dismantle various components of the host immune system. A notable target of dentilisin is Factor H, a critical regulator of the alternative complement pathway. By degrading Factor H, dentilisin impairs the host's ability to regulate complement activation, leading to diminished complement-mediated bacterial killing[61]. Furthermore, dentilisin can degrade other complement components such as C3 and C4, further weakening the host's immune defense mechanisms.

Beyond its effects on the complement system, dentilisin also degrades key pro-inflammatory cytokines, including IL-6 and IL-1β, thereby attenuating the inflammatory response at the protein level[65]. This modulation of the host's immune response is complemented by dentilisin's ability to activate matrix metalloproteinases (MMPs), particularly MMP-2 and MMP-9[59, 215]. These MMPs are crucial for the degradation of integral extracellular matrix (ECM) components, cytokines, chemokines and recently discovered intracellular substrates which regulate the inflammatory response. Additionally, MMP-11 and MMP-17 have not been described in the context of periodontal disease and lack clearly defined functions in development and homeostasis.

Matrix metalloproteinase-28 (MMP-28) exhibited the highest response among MMP genes following *T. denticola* infection and purified dentilisin stimulation, underscoring its pivotal role in periodontal disease pathogenesis. Illman and colleagues demonstrated that overexpression of MMP-28 leads to the upregulation of MMP-2 mRNA expression and activity, indicating a regulatory cascade that may exacerbate tissue degradation[171, 216]. Additionally, E-cadherin, a critical cell adhesion molecule, has been identified as a substrate of MMP-28, suggesting that MMP-28 activity could disrupt cell-cell junctions and compromise tissue integrity[171]. The heightened expression of MMP-28 in response to *T. denticola* and its virulence factor dentilisin highlights its significant role in the host's osteoimmunological response. These findings provide valuable insights into the molecular mechanisms driving periodontal pathology and underscore

the importance of better understanding the roles of MMPs and all of their substrates to better target them in therapeutic strategies. Given the increasing concern over bacterial resistance to antibiotics, there is an urgent need to develop alternative treatment approaches. Inhibiting dentilisin and other potent host modulators represents a promising avenue for therapeutic intervention. By focusing on these virulence factors, it may be possible to reduce bacterial pathogenicity and enhance host resilience without relying solely on traditional antibiotics.

By better understanding the pathways and molecular mechanisms utilized by periodontal pathogens such as *T. denticola*, we have gained valuable insights into the downstream effects on host tissues. This knowledge underscores the importance of restoring the host tissue milieu to its normal state as the ultimate goal in treating patients with periodontal disease. Recently, BET bromodomain inhibitors or epigenetic drugs like JQ1 have emerged as promising therapeutic agents. Studies have shown that JQ1 reduces inflammatory responses both *in vitro* and *in vivo*. While it effectively suppresses most pro-inflammatory cytokines, JQ1 also inhibits IL-10 production, an anti-inflammatory cytokine, in treated cells and mice[42]. The downregulation of MMP-9 and RANKL in gingival tissue treated with JQ1 suggests a protective effect against connective tissue degradation and bone destruction, which are critical aspects of periodontal disease pathology[41]. These findings indicate that JQ1 not only mitigates inflammation but also contributes to preserving the structural integrity of periodontal tissues.

Moreover, the ability of JQ1 to modulate the immune response highlights its potential as a multifaceted therapeutic option. However, despite these promising results, the complexities of periodontal disease necessitate further research. It is crucial to continue investigating how these oral pathogens interact with host systems and the broader implications for systemic health. Understanding these interactions will be pivotal in developing comprehensive treatment strategies that not only address local periodontal issues but also mitigate any potential systemic effects. While JQ1 serves as a beacon of progress in the field of periodontology, there remains

a significant need to delve deeper into the molecular crosstalk between periodontal pathogens and host responses to fully harness the potential of such therapeutic approaches.

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