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Osteoimmunology and the reconstruction of host immunological status in treponemal infection: Effect of activated immune cells by oral pathogens and *Treponema pallidum* on osteoclastogenesis

By

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Submitted in partial fulfillment of the requirements for Graduation (*summa cum laude*)

University of Louisville

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1. Abstract

Syphilitic infection caused by bacterium *Treponema pallidum pallidum* (Tp) offers an excellent model to study the long-lasting interplay between the immune and skeletal systems and could be used to reconstruct host immunological status. We propose that a hyper-inflammatory phenotype developed during acquired syphilis will have a systemic impact on most bone microenvironments involving inflammatory processes, such as the one developed in periodontal disease (PD). Using osteoimmunological *in vitro* protocols, we explore whether immune activation by Tp antigens and PD pathogens can impact osteoclastogenesis (OCG), ultimately helping to understand the bone alterations caused by systemic inflammatory processes in skeletal material. We used two main protocols, immune cell activation and osteoclastogenesis, that utilized peripheral blood mononuclear cells (PBMC's) from healthy donors. The results showed an increased cytokine expression for IL-6, but not for TNFα, when PBMC's were exposed to Tp antigens. OCG was affected by TNFα expression, but not IL-6 expression. Interestingly, one oral pathogen lysate, *A. actinomycetemcomitans,* showed different results than what was observed for the other three oral pathogen lysates. Chronic syphilis infection, through systemic inflammation, could impact bone microenvironments between seemingly unrelated forms of skeletal inflections such as syphilis and PD, and that association is influenced by the microbiome diversity observed in PD.

2. Lay Summary

Infection with syphilis caused by bacterium *Treponema pallidum pallidum* (Tp) offers an excellent model to study the long-lasting interplay between the immune and skeletal systems and could be used to reconstruct the immune status of skeletal remains. We propose that a shift toward excessive inflammation developed during acquired syphilis will have an impact on the inflammatory processes of bone, which can be observed in the context of periodontal disease (PD). We explore whether immune activation by Tp antigens and PD pathogens can impact bone growth and resorption, ultimately helping to understand the changes to bone structure caused by systemic inflammatory processes. We used two main strategies, activation of immune cells by antigens from *T. pallidum* and oral pathogens present in PD, and using the same cells, the generation of bone growth. The results showed that cells exposed to the *T. pallidum* antigen increased inflammatory responses for some oral pathogens, but not for others. Bone growth was affected by these inflammatory responses, but not consistently. Interestingly, one oral pathogen showed different results than what was observed for the other three oral pathogens. Chronic syphilis infection, through inflammation, could impact bones between seemingly unrelated forms of skeletal inflections such as syphilis and PD, and that association is influenced by the different oral pathogens observed in PD.

3. Introduction

3.1 Osteoimmunology: interplay between the immune and skeletal systems

The human immune system is an intricate complex that can be impacted by a variety of environmental factors. To ward off deleterious pathogens, the immune system can stimulate inflammation, which is one of the most common primary responses to infections or injuries. Inflammation is caused by the secretion of cytokines, which leads to the recruitment of other immune cells and proteins to the site of infection (Abbas & Lichtman, 2005). Inflammation, generally, is a necessary first line of defense against disease. Usually, immune and inflammatory responses are confined to local tissues or organs. However, when inflammation is chronic and strong, a spillover of pro-inflammatory proteins can occur and significantly affect systemic responses, sometimes leading to chronic inflammatory systemic diseases (CIDs). CIDs, like syphilis, rheumatoid arthritis, and type 2 diabetes, can cause inflammation in many organs, even those not near the local site of infection. This is likely due to long-term activation of the immune system (Straub & Schradin, 2016). This chronic systemic inflammatory response can be the result of an over-reactive immune response, or a "hyper-responsive phenotype," (Shaddox et al., 2010). To understand the full impact of CIDs on the immune system, it is important to recognize the interplay between the immune system and the skeletal system.

Systems of the body often work together to perform important functions, such as eliminating pathogens that cause disease. Interestingly, the immune and skeletal systems work closely together to create and distribute immune cells. Chronic infections, such as syphilis, promote long-term inflammatory responses, and wherever inflammatory conditions or diseases occur, systemic impacts on bone develop on some level (Klaus, 2014). Osteoimmunology, the study of the relationship between the skeletal and immune system, is a relatively new and

growing field. It is well known that immune cells arise from bone marrow. However, more recent studies are beginning to show the impact of immune cells on the bones themselves. The bone matrices of skeletal systems are constantly changing; bones are built up by osteoblasts and broken down by osteoclasts, a macrophage immune cell that works to constantly remodel the bone matrix. A common result of CIDs is bone loss, which can be observed in many patient cases. Bone loss occurs when bone resorption happens faster than bone growth. (Arron & Choi, 2000). In other words, a high level of osteoclast activity can lead to bone degradation. Studies have shown that T cells, a common type of immune cell, can also affect bone metabolism by inducing the osteoclasts involved in bone loss. In this case, overactive T cells can lead to bone lesions (Kong et al., 1999). Up to date, there have been no studies conducted using osteoimmunological *in vitro* protocols to apply findings in the reconstruction of chronic immune responses and bone cells in past skeletal remains.

There are several important proteins that can be manipulated *in vitro* to test the level of osteoclast activity in different conditions. One of these proteins is RANKL. RANKL is expressed on activated T cells, binds to RANK on osteoclasts, and thereby induces increased levels of osteoclast activity and bone resorption (Arron & Choi, 2000). In the laboratory, RANKL is used to grow mature osteoclasts from volunteer donor monocytes. Macrophage colony stimulating factor, or M-CSF, is also used in this process for osteoclast differentiation (Takayanagi, 2007). Protein receptors like those for TNF-α, IL-6, and INF-γ are essential for measuring osteoclast activity (Takayanagi, 2007). TNF-α, or Tumor Necrosis Factor-alpha, is a specific cytokine that has been shown to stimulate osteoclast formation in the presence of RANKL and M-CSF. IL-6, or Interleukin-6, mediates bone resorption and regulates the development of mature osteoclasts. INF-γ, or Interferon-gamma, stimulates bone resorption by

enhancing the RANKL and TNF-α production of T cells (Lee et al., 2008). Therefore, the presence of TNF-α, IL-6, and INF-γ in the supernatants of precursors to osteoclasts generated *in vitro* point towards a hyperinflammatory response.

Bone and immune cells share many regulatory molecules, and cytokines play a crucial role in the cross-talk between both systems, where bone remodeling is regulated by the activity and interaction of bone and immune cells. Therefore, systemic inflammatory shifts or systemic inflammation may influence and increase severity in local inflammatory processes detected in bone lesions or alterations (Crespo, 2020).

3.2 Oral pathogens, periodontal disease, and systemic inflammation

A systemic immunological shift produced by CID's could affect other persistent and regular chronic infections such as the ones generated by oral pathogens, namely periodontal disease (PD). One way to study the impact of long-term inflammatory response through the skeleton is through the analysis of PD. The connection between oral pathogens and bone resorption is a novel way to study the relationship between chronic oral diseases and skeletal immune markers (bone lesions). Because oral pathogens stimulate immune cell activity, they can also lead to higher levels of bone resorption. The relationship between oral diseases and systemic inflammation has been proven and shows promise to treat chronic systemic diseases.

The same proteins associated with osteoclastogenesis have been found at increased levels when exposed to oral pathogens known to cause periodontal disease (PD), like *P. gingivalis*. A study conducted in 2010 showed that IL-6 and TNF levels were much higher when exposed to the antigens of *P. gingivalis* (Borch et al., 2010). Another showed that exposure to *P. gingivalis* resulted in an increase of pro-inflammatory cytokines and excessive bone resorption (Shaddox et

al., 2010). While these immune factors enhance the cellular response against the oral pathogens, they also induce excessive osteoclastogenesis and bone destruction (Page, 1991; Birkedal-Hansen, 1993). This chronic inflammatory status leads to bone loss and ultimately possible tooth loss (Irfan et al., 2001). Interestingly, the alveolar bone loss and exposure of underlying trabecular bone associated with PD are frequently identified in skeletal remains (Ogden, 2008; Larsen, 2015). It is important to note that inflammatory response to *P. gingivalis* varied on an individual basis, showing that level of inflammation is based on an individual's immune phenotype.

Several other oral pathogens have been shown in more recent years to cause PD, including *Filifactor alocis* (Fa), *Peptoanaerobacter stomatis* (Ps), and *Aggregatibacter actinomycetemcomitans* (Aa) (Costalonga & Herzberg, 2014). Like *P. gingivalis,* these three pathogens stimulate the secretion of proinflammatory cytokines (Moffatt et al., 2011). *F. alocis*, *P. stomatis*, and *A. actinomycetemcomitans* were originally overlooked as causes of periodontal disease because they were found less frequently than *P. gingivalis*. However, new research on each pathogen suggests that they might play different roles in the stimulation of inflammation than previously thought. *A. actinomycetemcomitans* specifically has been identified as a highly fit oral colonizer (Fine et al., 2019).

We propose that shifts in the host inflammatory phenotypes during acquired syphilis have a systemic consequence, ultimately affecting most bone microenvironments that involve inflammatory processes such as the one developed in other chronic inflammatory processes as observed in PD. The strong association between the expression of pro-inflammatory cytokines and periodontal tissue destruction means that PD in skeletal samples can be used as a marker for the inflammatory status of individuals (Crespo et al., 2017). With more empirical evidence, it

may be possible to reconstruct the inflammatory status of individuals at different stages of acquired syphilis.

3.3 Chronic syphilis as shifter of systemic inflammation

CID's, like syphilis, can cause long-lasting inflammatory immune responses. Syphilis is caused by the bacterium *Treponema pallidum*, and its potential impact on systemic inflammation offers an excellent model to study the lasting interplay between the immune and skeletal systems. While earlier studies suggested that syphilitic infection is correlated with immunosuppression, the current understanding is that *T. pallidum* in fact induces a strong cellular immune response, which is associated with a delayed-type hypersensitivity reaction wherein a delayed (48 hours - one week) cellular response is displayed (Carlson et al., 2011; Stary et al., 2010). This delayed-type hypersensitivity reaction (DTH) recruits antigen-specific T cells, which attracts a system of cytokines and macrophages at the site of infection. DTH is considered to be a beneficial immune response for chronic illnesses. However, hyperinflammation because of DTH can occur and lead to bone resorption and tissue destruction.

The systemic immunological shift produced by a *T. pallidum* chronic infection could affect other persistent and regular chronic infections, such as the ones generated by oral pathogens, namely PD. Therefore, multi-stage infection caused by bacterium *Treponema pallidum pallidum* (Tp) offers an excellent model to study the long-lasting interplay between immune and skeletal systems and to help reconstruct host immunological status using skeletal samples. But first, we need experimental data that will confirm the interplay between activated immune cells by oral pathogens and *T. pallidum* and its potential effects on osteoclastogenesis.

We can propose that shifts in the host inflammatory phenotype (IP) during acquired syphilis will have a systemic consequence ultimately affecting most bone microenvironments that involve inflammatory processes, such as the one developed in PD. Ultimately, the experimental findings of this project could generate the foundation for the integration of osteoimmunology and paleopathology, when reconstructing skeletal inflammatory phenotypes in individuals that faced chronic inflammatory infections, such as syphilis.

4. Objective

The first objective is to test if there is a shift in cytokine expression when cells are exposed to the *T. pallidum* immunomodulatory antigen, then re-exposed to oral bacterial lysates.

The second objective is to study the effect of supernatants from PBMC's activated by the *T. pallidum* immunomodulatory antigen and oral bacterial lysates on osteoclastogenesis and osteoclast differentiation.

Using osteoimmunological *in vitro* protocols, this project will explore whether immune activation by *T. pallidum* antigens and PD pathogens can impact osteoclastogenesis (OCG), which will ultimately explain the bone alterations caused by systemic inflammatory processes in skeletal materials. Therefore, the **long-term objective** is to generate an experimental foundation for the interplay between immune inflammatory responses and osteoclast activity in bone microenvironments. This foundation is integral to the study of host skeletal immune response reconstruction, creating a long-term relationship between osteoimmunology and bioarcheology.

The **preliminary hypothesis** is that immune activation by oral pathogens and/or *T. pallidum* will trigger the expression of inflammatory proteins that will affect osteoclastogenesis, ultimately modulating osteoclast activity and bone resorption.

5. Materials and Methods

To determine if exposing proliferating cells to supernatants collected from activated immune cells will impact osteoclastogenesis and osteoclast activity, we must use multiple, distinct protocols. We believe that there will be increased osteoclastogenesis and osteoclast activity when differentiating cells are exposed to the supernatants collected from the stimulated cells. The sample size was 10 healthy donors (n=10).

5.1 *Immune cell culture*

For the cells that were exposed to infectious antigens and lysates, PBMC's were used from healthy, voluntary donors that were collected at the University of Louisville Nephrology Research Laboratory (IRB# 191.96 and 11.0334). It is important to point out that cells from the same donor were used for all protocols: immune activation and monocyte differentiation into osteoclasts. Two general protocols were used to standardize conditions. See Figure 1 where all experimental protocols are summarized. The monocytes used in this protocol were from the same donor as the cells used in the immune activation. This minimized the confounding variables caused by assorted differences between donors. The differentiation protocol typically lasted between 7 and 21 days. We explored whether or not the shift in pro-inflammatory proteins collected from activated immune cells affected

osteoclastogenesis and/or osteoclast activity. Positive and negative controls were included, as well as conditions treated directly with antigens or lysates. The time during the protocol that the differentiating cells were exposed to the supernatants varied. For quantifying mature osteoclasts, positive TRAP cells with 3 or more nuclei were counted using a TS100F Nikon inverted microscope. For measuring osteoclast activity, a standardized bone resorption protocol was used. After fixation, all cells were removed from the osteoplates using 1M NaOH. In replication, another protocol that determines the optical density in the supernatants collected during the osteoclastogenesis protocol after targeting TRAP expression was also used.

Figure 1

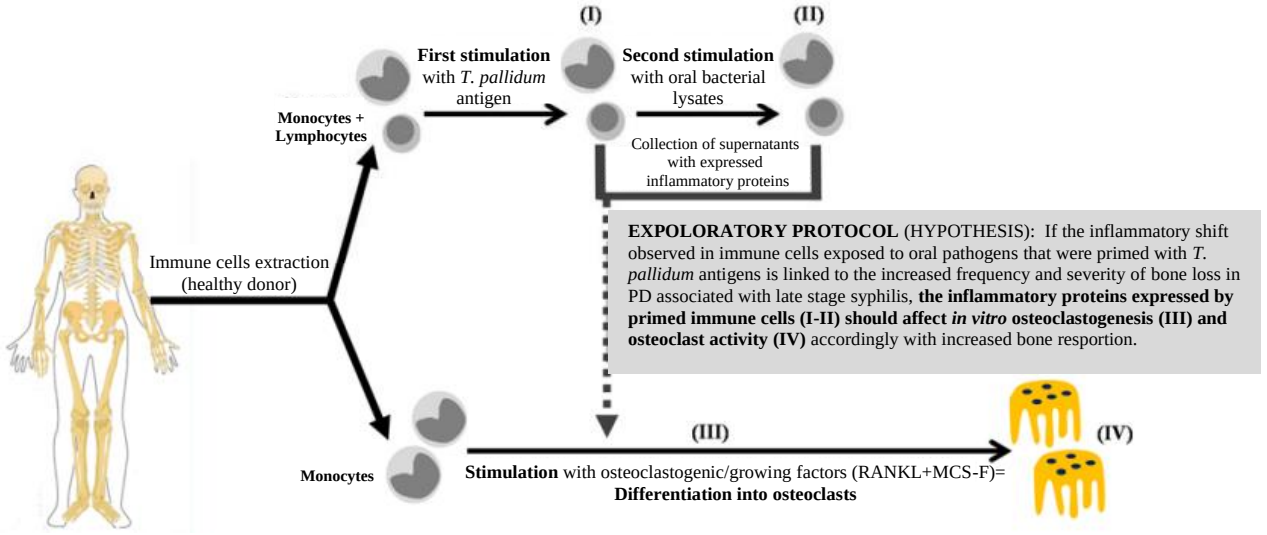

Figure 1: Diagrammatic representation for experimental protocols where initially **(I)** immune cells are activated and pre-stimulated (24 hrs) by *T. pallidum* to generate a "hyper-inflammatory phenotype" (HIP) and re-exposed **(II)** on Day 2 for another 24 hrs to oral bacterial lysates. All final supernatants **(I and II)** are collected and used (interference) during osteoclastogenesis and osteoclast activity protocols **(III and IV)**. (Crespo et al., 2020).

5.2 Immune activation of PBMC's

After receiving the isolated PBMC's from the University of Louisville's Nephrology Research Laboratory, the cells were washed with phosphate buffer saline (PBS), counted, and then resuspended in alpha Minimum Essential Medium (MEM). The cells were plated on a sterile 24-well plate. Each well contained 4,000,000 cells in 1mL of MEM. To complete the first stimulation, reagents were added to each well corresponding to pre-planned conditions (Figure 2). After a 24-hour incubation period at 37℃, the supernatants from the PBMC's were collected from each well, and new media was added along with the corresponding Day 2 conditions. After another 24-hour incubation period at 37℃, the supernatants were collected again from each well.

The stimulation conditions for each well were decided before the experiment based on the interaction between the antigens of different pathogens. The negative control was left untreated throughout the trials. Lipopolysaccharide (LPS) were added to the positive control on both Day 1 and Day 2 of the trial. Antigens from the syphilis pathogen *Treponema pallidum* were added to select wells on Day 1 and select wells on Day 2. Lysates from four different oral pathogens (*Porphyromonas gingivalis-Pg*, *Filifactor alocis-Fa, Peptoanaerobacter stomatis-Ps,* and *Aggregatibacter actinomycetemcomitans-Aa*) were also added to select wells on Day 1 and select wells on Day 2. Figure 2 shows the layout of stimulation for immune activation. 1µL of Tp47, 10µL of LPS, and 100µL of each oral pathogen were added to the corresponding wells.

Figure 2

Day 1 Conditions	**Day 2 Conditions**
Untreated	Untreated
LPS	LPS
Untreated	*Pg*
Untreated	*Fa*
Untreated	*Ps*
Untreated	*Aa*
Tp47	*Pg*
Tp47	*Fa*
Tp47	*Ps*
Tp47	*Aa*
Pg	Untreated
Fa	Untreated
Ps	Untreated
Aa	Untreated
Tp47	Untreated

The number of cytokines present in the supernatants of each condition were determined using an enzyme-linked immunosorbent assay (ELISA). TNFα concentrations were measured for all 10 trials and IL-6 concentrations were measured for 8 trials, beginning at trial 3.

5.3 Osteoclastogenesis

At the same time as the immune activation protocol, PBMC's were plated on a 96-well plate to begin differentiation into osteoclasts. After the cells are washed, counted, and resuspended in MEM, the cells were plated so that each well contained 400,000 cells in 200µL of MEM. 10 total trials were run corresponding to the 10 trials of immune activation. Two different methods of plating were used because of the availability of supplies. In the first method, cells were plated on osteoplates, which have bone matrices for growing osteoclasts. In the second method, a regular plate was used, and bone slices were added to some of the wells to replace the bone matrices. Plates were incubated at 37℃.

During a 10–20-day period, the media was changed every four days. Also on these days, RANK-L and M-CSF were added to induce osteoclast differentiation and colony growth (Figure 3). Inflammatory protein supernatants from the immune activation trial were added to some of the wells near the middle of the incubation period. At the end of the incubation period, the plate was fixed and stained using TRAP, a marker specific to osteoclasts. Mature osteoclasts with multiple nuclei were counted using a TS100F Nikon inverted microscope at 20x magnification.

Figure 3

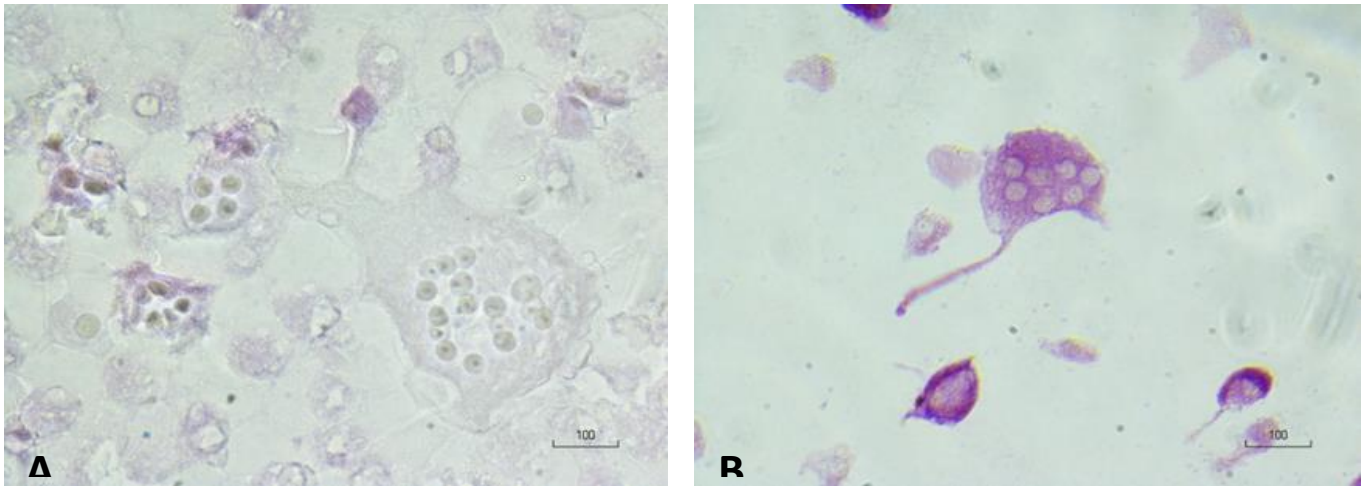

Figure 3: Cell culture of human donor monocytes for osteoclast differentiation. **A:** day 20 (previous fixation and staining); **B**: day 21 after (fixation and staining), see a TRAP-positive osteoclast with 7 nuclei. (40x)

5.4 Statistical Analysis

Results are expressed as mean values ± SD. To evaluate the variability in cytokine expression between donors, the data will be interpreted using a paired Wilcoxson ranked sums test. Statistical significance for all analyses will be determined by a maximum $P<0.05$. Calculations will be completed through R software version 3:2.1.

6. Results

6.1 Sequential immune activation of PBMC's

The goal of the immune activation cell culture protocol was to measure differences in the expression of different cytokines produced by PBMC's exposed to different bacterial lysates or antigens. PBMC's were exposed to a selection of oral pathogen lysates and Tp47 antigens. For this thesis, we prioritized the results for two sets of conditions: untreated (Day 1) and oral pathogen (Day 2), and Tp47 (Day 1) and oral pathogen (Day 2). These experimental conditions

were selected to illustrate the relationship between immune cells to *T. pallidum* immunomodulatory antigen (Tp47) and a secondary exposure to oral bacterial lysates. The experimental rationale for this sequence is to test if there is an "immunological shift" in the expression of inflammatory cytokines when PBMC's had an early exposure to Tp47 that will ultimately affect or change the inflammatory response to oral pathogens. An ELISA test was performed on each condition for 10 experimental protocols (TNFα) and 8 experimental protocols (IL-6) using PBMC's from different healthy donors. This project prioritized the analysis and quantification of 3 inflammatory cytokines: TNFα, IFNγ, and IL-6 in supernatants from activated PBMC's (this thesis presents the preliminary data for TNFα and IL-6).

The results showed differences between TNFα and IL-6 concentrations for each condition after Day 2 (Figures 4 and 5), but the majority of these differences were not statistically significant (Figure 6). For TNFα, cytokine concentration was higher when PBMC's were untreated during Day 1 than when PBMC's were exposed to Tp47 during Day 1 for *P. gingivalis* (Untreated: M = 247.4pg/mL and Tp47: M = 92.0pg/mL), *F. alocis* (Untreated: M = 213.9pg/mL and Tp47: M = 33.6pg/mL), and *P. stomatis* (Untreated: M = 334.0pg/mL and Tp47: M = 106.8pg/mL). The opposite was observed for *A. actinomycetemcomitans* (Untreated: M = 463.9pg/mL and Tp47: M = 527.0pg/mL). The only statistically significant result was for *P. gingivalis* (p-value: 0.02734).

For IL-6, cytokine concentration was higher when PBMC's were exposed to Tp47 during Day 1 than when PBMC's were untreated during Day 1 for (Untreated: M = 275.1pg/mL and Tp47: M = 314pg/mL), *F. alocis* (Untreated: M = 301.3pg/mL and Tp47: M = 323.3pg/mL), *P. stomatis* (Untreated: M = 311.7pg/mL and Tp47: M = 361.3pg/mL), and *A.*

actinomycetemcomitans (Untreated: *M* = 292.8pg/mL and Tp47: *M* = 304.7pg/mL). There were no statistically significant results for IL-6.

Figure 3 and Figure 4 illustrate the supernatant concentration found in each condition for TNFα and IL-6 respectively. Figure 5 shows the p-values for each oral pathogen trial. P-values > 0.05 are not statistically significant.

Figure 4

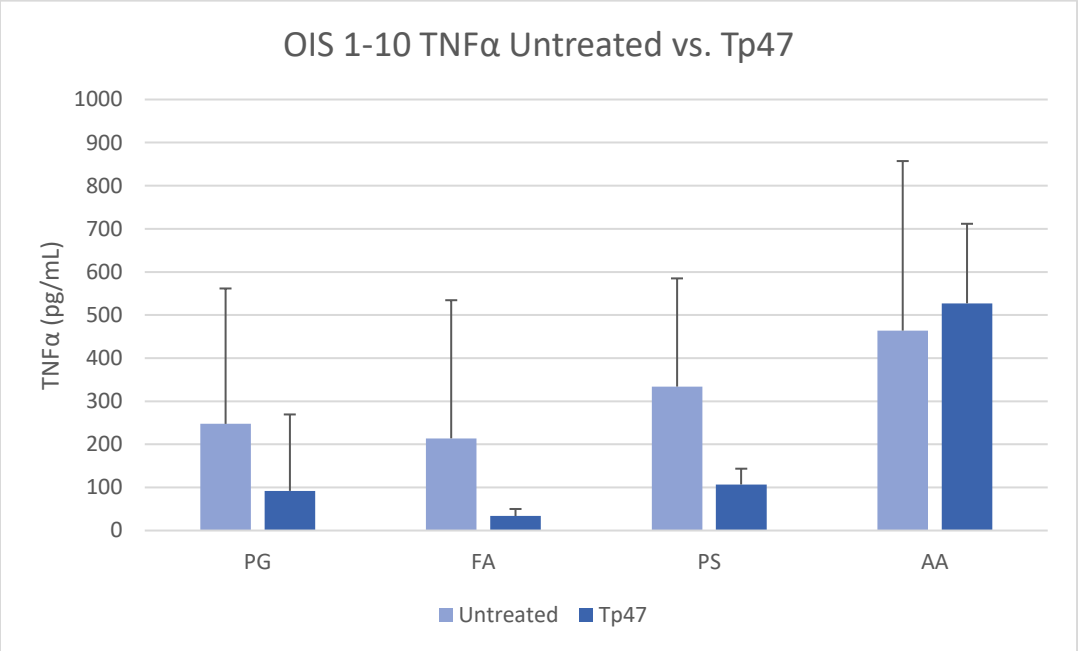

Figure 4: TNFα (pg/mL) produced by PBMC's corresponding to Day 2 of culture. The first column of each oral pathogen lysate represents cytokine expression when Day 1 is untreated, and Day 2 is PBMC's exposed one of the four oral pathogen lysates. The second column represents cytokine expression when Day 1 is PBMC's exposed to Tp47, and Day 2 is PBMC's exposed one of the four oral pathogen lysates.

Figure 5

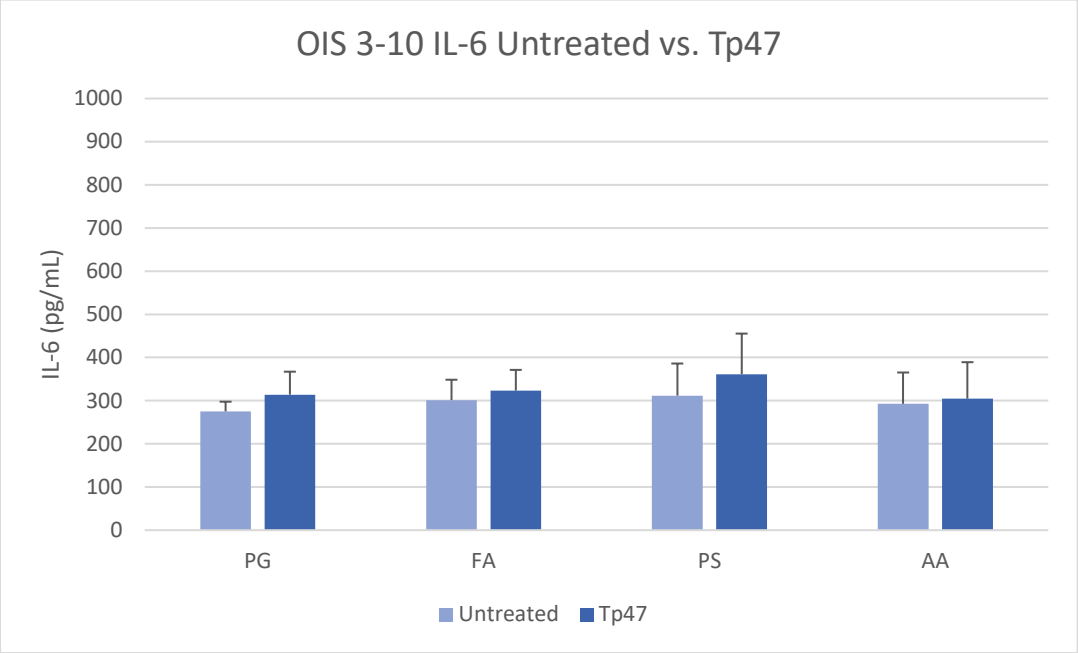

Figure 5: IL-6 (pg/mL) produced by PBMC's corresponding to Day 2 of culture. The first column of each oral pathogen lysate represents cytokine expression when Day 1 is untreated, and Day 2 is PBMC's exposed one of the four oral pathogen lysates. The second column represents cytokine expression when Day 1 is PBMC's exposed to Tp47, and Day 2 is PBMC's exposed one of the four oral pathogen lysates.

Figure 6

Statistical Significance of Untreated vs. Tp47 Treatment:

	TNF	IL-6
Pg	**0.02734**	0.1484
Fa	0.2936	0.1953
Ps	0.08398	0.25
Aa	0.2754	0.7422

Statistical Significance of Oral Pathogens:

TNF

Untreated Day 1

	UNT-PG	UNT-FA	UNT-PS
UNT-FA	0.1415		
UNT-PS	0.1925	0.1415	
UNT-AA	0.03711	0.03711	0.2754

Tp47 Day 1

	Tp47-PG	Tp47-FA	Tp47-PS
Tp47-FA	0.1551		
Tp47-PS	0.1934	0.009152	
Tp47-AA	0.003906	0.003906	0.003906

IL-6

Untreated Day 1

	UNT-PG	UNT-FA	UNT-PS
UNT-FA	0.7422		
UNT-PS	0.25	0.9453	
UNT-AA	0.1953	1	0.674

Tp47 Day 1

	Tp47-PG	Tp47-FA	Tp47-PS
Tp47-FA	0.03906		
Tp47-PS	0.05469	0.07813	
Tp47-AA	0.6726	1	0.03906

Figure 6: Statistical significance of each condition compared to the three other corresponding conditions. Measurements were taken from Day 2 of each condition. Untreated Day 1 means that on Day 1, PBMC's were not exposed to any antigen or lysate. Tp47 Day 1 means that on Day 1, PBMC's were exposed to Tp47. Bolded values represent $P>0.05$, which is not statistically significant.

6.2 Osteoclast Count

During the osteoclastogenesis protocol (as explained in Figure 1), supernatants from the immune activation trials were added to measure the effect of these conditions on osteoclastogenesis and osteoclast differentiation. Five successful trials were recorded for

osteoclastogenesis. Figures 5 and 6 show the number of osteoclasts that differentiated while exposed to each condition. The results were again prioritized for the same two sets of conditions: untreated (Day 1) and oral pathogen (Day 2), and Tp47 (Day 1) and oral pathogen (Day 2).

Generally, as the concentration of TNFα and IL-6 expression increased, the number of osteoclasts decreased. Data from *P. gingivalis*, *F. alocis*, and *P. stomatis* show a trend of lower supernatant concentration and higher osteoclast count (Pg osteoclast count: 26.1 and 15.6, Fa osteoclast count: 12.5 and 17.0, Ps osteoclast count: 12.5 and 15.0), while data from Aa shows a trend of higher supernatant concentration and lower osteoclast count (Aa osteoclast count: 10.0 and 3.5). This is especially visible in Figure 5, the graph depicting data from TNFα.

Figure 7

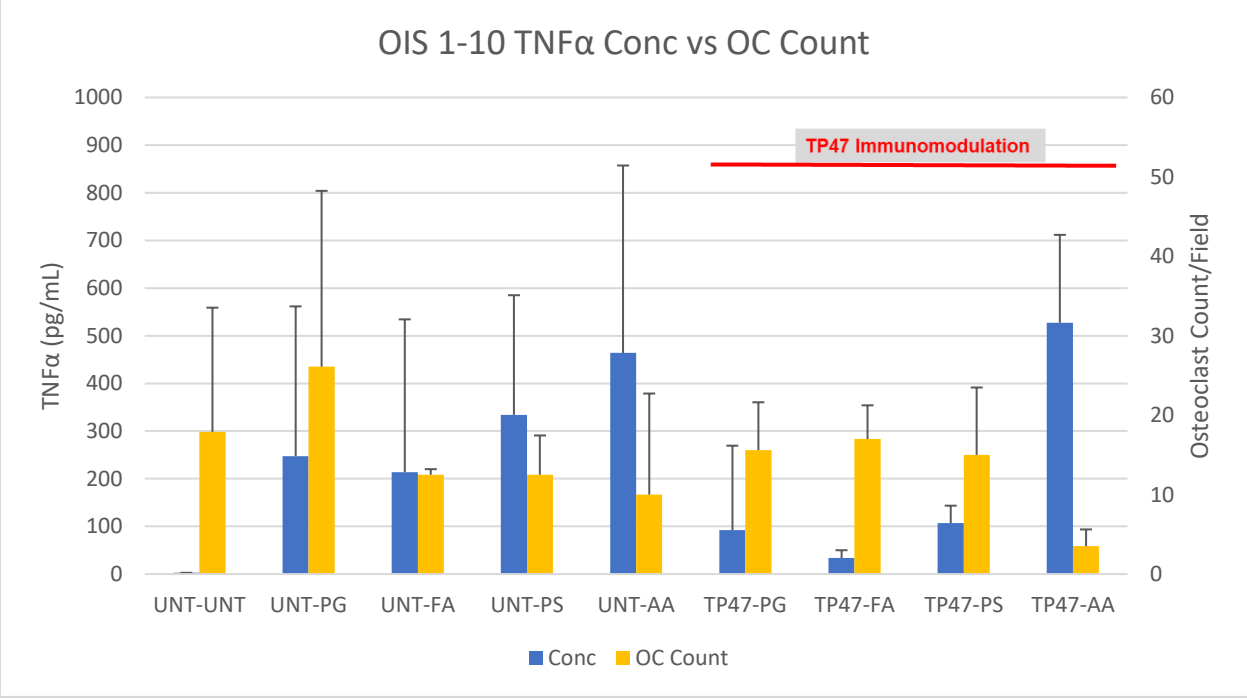

Figure 7: TNFα (pg/mL) produced by PBMC's corresponding to Day 2 of culture correlated with osteoclast count (average field of osteoclast differentiation from monocytes).

Figure 8

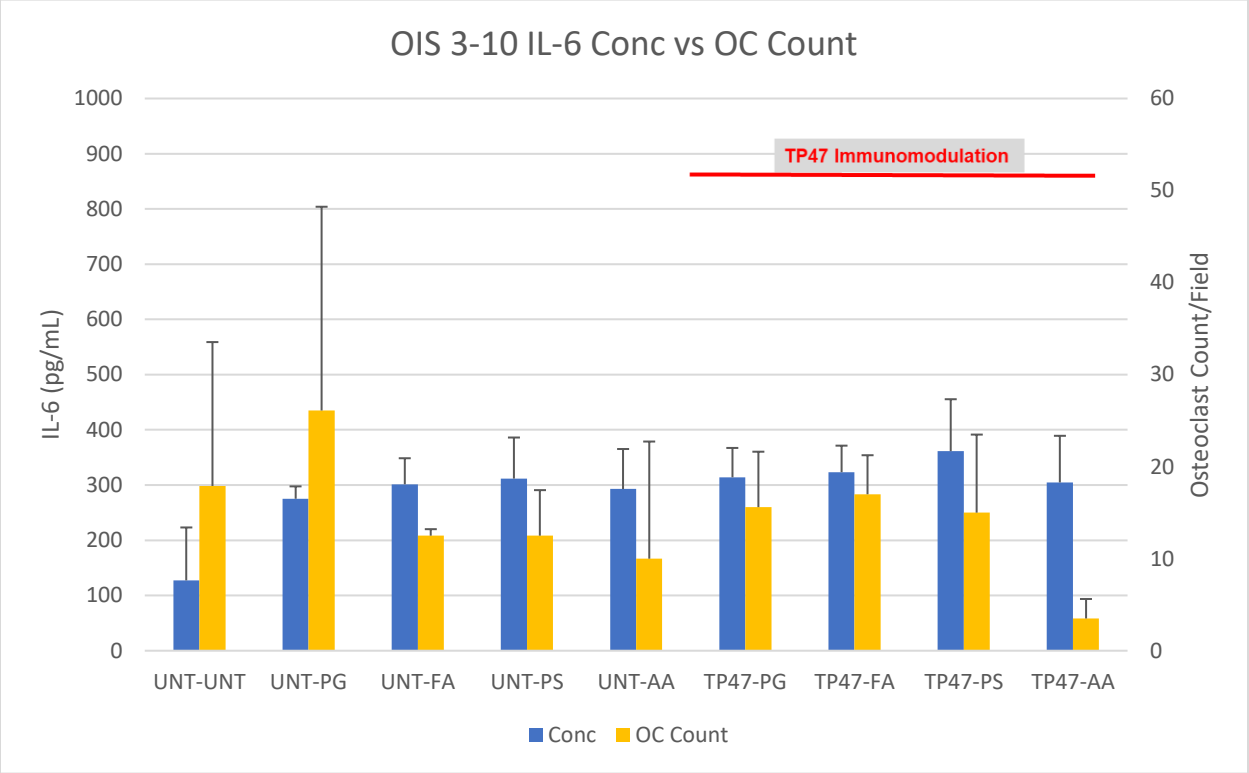

Figure 8: IL-6 (pg/mL) produced by PBMC's corresponding to Day 2 of culture correlated with osteoclast count (average field of osteoclast differentiation from monocytes).

Interestingly, when PBMCs are cultured (Day 1) with the *T. pallidum* antigen (Tp47 immunomodulation) and re-cultured with the secondary stimulation by the oral pathogens (Day 2), lysates show a very similar expression of IL-6 (Figure 8), but the expression of TNFα shows a significant increase when re-exposed to *A. actinomycetemcomitans* lysate, also showing a significant decrease in osteoclast count (Figure 7).

7. Discussion

7.1 Immune activation results

In the immune activation protocol, several controls were used (not shown in graphs). An untreated condition served as the overall negative control, where no antigens or lysates were added. The untreated-oral pathogen conditions served as a negative control to the Tp47-oral pathogen conditions, since only Day 1 was different between the conditions. The LPS-LPS condition served as the positive control for Day 1 and Day 2, showing that PBMC's were viable and active during the whole experiment. LPS is a well-established stimulus of immune cells that causes the cells to produce high levels of both pro-inflammatory cytokines, TNFα and IL-6. The untreated negative control condition produced low or no TNFα and IL-6, while conditions treated with lysates and the *T. pallidum* antigen differentially induced expression of both cytokines. This shows that pathogens, both oral pathogens and Tp47, induce an inflammatory immune response.

There were differences in cytokine expression between each oral pathogen during Day 2, when the PBMC's were untreated in Day 1, as well as treated with Tp47. However, the majority of these differences were not statistically significant. There are several factors that could have contributed to the differences in concentrations observed. *F. alocis* has been shown to have a longer generation time than *P. gingivalis*, by an average of 8 hours. This could have a significant effect on immune activation, which only runs for 1-2 days after the addition of an oral pathogen. Additionally, *F. alocis* tends to grow more when cocultured with other oral pathogens. Because each oral pathogen lysate was added to the monocytes independently of the other lysates, *F. alocis* growth may have been reduced compared to *in vivo* (Wilson Aruni et al., 2011). Our data reflects the trend that *F. alocis* induced lower concentrations of pro-inflammatory cytokines than *P. gingivalis*. Studies have shown that *P. stomatis* induces higher levels of pro-inflammatory

cytokines compared to both *P. gingivalis* and *F. alocis* (Vashita et al., 2019). This is reflected in our results for both TNFα and IL-6. *A. actinomycetemcomitans* has been shown in recent years to be a highly fit oral colonizer that is resistant to most antibiotic inhibition (Fine et al., 2019; Ardila et al., 2020). Additionally, *A. actinomycetemcomitans* has been found more often in aggressive forms of periodontal disease than other oral pathogens, including *P. gingivalis*, which usually results in higher levels of TNFα expression (Casarin et al., 2010). This could explain the increase in TNFα expression for *A. actinomycetemcomitans.*

Contrary to the hypothesis, the data for TNFα showed a higher expression for the untreated conditions compared to the Tp47 treated conditions in all oral pathogens except for Aa, where Pg had the only statistically significant result. Alternatively for IL-6, higher cytokine expression was detected for Tp47 treated conditions compared to untreated conditions for all oral pathogens. However, none of these differences were statistically significant. There are several reasons why Tp47 may not have induced a higher amount of TNFα expression. First, a more advanced level of syphilis infection may be required to induce widespread inflammation (Knudsen et al., 2009). Second, TNFα-blockers may have been present as they are in natural immune systems arising from LPS and components of oral pathogens (Nahman-Tzach et al. 2017; Baer et al., 1998).

7.2 Osteoclastogenesis Results

Like in the immune activation protocol, an untreated condition was used as the negative control and LPS-LPS was used as the positive control. The negative control consistently resulted in 0 osteoclasts, while the positive control averaged 6.7 mature osteoclasts per field. Surprisingly, more osteoclasts were observed in each condition with added pathogen lysates and

antigens compared to the positive control. This could be due to an overpopulation of osteoclasts in the positive control field, leading to cell death. There was only a slight difference detected in osteoclast counts between the untreated and Tp47 conditions. However, Tp47 did have a generally greater number of osteoclasts, with the exception of the untreated-Pg condition and the Tp47-Aa condition. These results support our hypothesis that Tp47 will induce more osteoclast growth.

7.3 Immune Activation and Osteoclastogenesis

When the osteoclast count is compared to the levels of cytokine expression, an inverse relationship can be observed. This is seen most clearly when looking at TNFα expression. Generally, as the concentration of supernatants increased, the number of osteoclasts decreased. Data from *P. gingivalis*, *F. alocis*, and *P. stomatis* show a trend of lower supernatant concentration and higher osteoclast count, while data from *A. actinomycetemcomitans* shows a trend of higher supernatant concentration and lower osteoclast count. This trend does not support our hypothesis that a higher cytokine expression indicates more osteoclast formation equally for all oral pathogens.

8. Conclusions

The results show several important outcomes. First, immune cells exposed to the Tp47 antigen produce more IL-6, but not necessarily more TNFα. Three out of the four oral pathogen results showed a lower expression of TNFα when exposed to Tp47. Second, osteoclastogenesis is affected when healthy donor monocytes are exposed to supernatants from immune cells activated by Tp47 and differentially re-exposed to different oral pathogens. This effect is observed when cells are primarily exposed to Tp47 and secondarily re-exposed to *A. actinomycetemcomitans.*

This differential effect of the immunomodulation by *T. pallidum* antigen on cells re-exposed *A. actinomycetemcomitans* (probably influenced by differential expression of proinflammatory cytokines such as TNFα) suggests the need to study the oral microbiota composition when understanding the severity of periodontal disease in individuals facing chronic inflammatory infections such as the one produced by *T. pallidum*. Ultimately, our results suggest that chronic syphilis infection, through systemic inflammation, could impact bone microenvironments between seemingly unrelated forms of skeletal infections such as syphilis and PD, and our new preliminary findings show that immunological interaction between syphilis and PD is influenced by the microbiome diversity observed in PD.

9. Further Research

9.1 Strengths and Limitations

There were several strengths and limitations of this project that may have impacted the results. First, the same donor was used in each trial for both immune activation and osteoclastogenesis. This reduced the confounding variables that could arise from different immune system makeups of different individuals. The use of the same media type between immune activation and osteoclastogenesis also served to reduce confounding variables. Using the same individual, media, and reagents between each immune activation and osteoclastogenesis trial more closely mimics processes within the human body.

Although using the same donor across one trial was important, it also caused intervariability between trials, since the same donor was not used across all trials. Many factors, including immune system strength, life history, and biological sex could influence the production of inflammatory cytokines. Another significant limitation was a small sample size. Because trials

are expensive and require at least two weeks of stimulation, acquiring a large sample size proved challenging.

9.2 Continuing Research

Because the sample size is a significant limitation, acquiring more samples and running more tests is essential to understanding the relationship between inflammation and the immune system. Reducing donor intervariability would also be useful. This could be done by using the same donor across trials, or by incorporating commercial cell lines that have the same immune characteristics.

One of the most interesting pieces of data obtained from this experiment was the tendency of Aa to behave in opposite ways compared to the other three oral pathogens concerning immune activation and osteoclast growth. Our next area of research should focus on determining a larger trend between Aa and inflammation, as this could have a significant impact on how periodontal disease affects the immune system.

Lastly, expanding the types of cytokines measured after immune activation could provide insight into inflammatory markers. For this experiment, TNFα and IL-6 were used as the only markers for inflammation. Although these cytokines have been widely used to measure inflammation, there are a number of other cytokines, including IFNγ and other interleukins, that could give a larger picture of immune cell reaction to pathogens.

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