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Experimental immunology and the potential for osteopathological reconstructions : pursuing an experimental foundation for the skeletal inflammatory index.

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EXPERIMENTAL IMMUNOLOGY AND THE POTENTIAL FOR OSTEOPATHOLOGICAL RECONSTRUCTIONS: PURSUING AN EXPERIMENTAL FOUNDATION FOR THE SKELETAL INFLAMMATORY INDEX

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ABSTRACT

EXPERIMENTAL IMMUNOLOGY AND THE POTENTIAL FOR OSTEOPATHOLOGICAL RECONSTRUCTIONS:

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Megan E. Duncanson

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Individuals who mount a strong inflammatory response may produce a shift in the systemic levels of inflammatory mediators, which may lead to a potential hyper-inflammatory phenotype (HIP). Systemic inflammation can increase severity in local inflammatory processes detected in bone lesions. This thesis investigated *in vitro* if human immune cells pre-treated with inflammatory inducers would affect the inflammatory response against *Porphyromonas gingivalis* or *Staphylococcus aureus* (both associated with osteological lesions). We exposed human peripheral blood mononuclear cells to bacterial lysates, or pro-inflammatory cytokines. Sequentially, we exposed the same culture to either *P. gingivalis* or *S. aureus*. The final expression of TNFα and IFNγ was measured by ELISA. Our results showed that early exposure to the inflammatory inducers increased the expression of inflammatory cytokines. These findings could be useful in osteological analyses when considering how systemic inflammation may affect local inflammatory responses, and how this could be influenced by a HIP.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

The human skeleton provides a unique window into the past offering a glimpse of an individual long gone, even if no written records or other information remain. As such, understandings of health and disease in the past are often reliant on the osteological record as a primary source of evidence. Human skeletonized remains provide a wealth of information about that individual, their lifestyle, and in some instances, the diseases they carried (Larsen 2015). Still, despite the wealth of information that a skeleton can provide, there are limits. Physical abnormalities and lesions on bone do not necessarily reflect poor health in life, and recognition of this paradox exemplifies the necessity for careful consideration of what information the bones do provide (Wood et al. 1992). The very notions of what health and disease constitute are complex concepts, reliant on both specific biological and social contexts, as well as a broader evolutionary understanding of human history (Cohen 1989). However, utilizing different multidisciplinary perspectives can add necessary depth and nuance to what information can be taken away from the skeleton. These multidisciplinary perspectives allow for the potential synergistic interactions of multiple factors, such as environment or culture, to be taken into consideration in analyses of health in the past; a recognition of the complexities of health both past and present (Reitsema and McIlvaine 2014).

There are multiple etiologies of the diseases that present in the osteological record, and pathogens are not always the sole causative agents of lesions and

abnormalities detected on the bone. Stress, physical trauma, or even mechanisms of the human body itself may create or contribute to what is observed in the osteological record (DeWitte and Stojanowski 2015). Inflammation, one of the fundamental processes of the immune response, is not only capable of causing tissue damage, but may also exacerbate other underlying causative processes of tissue damage, including that of bone alteration (Abbas et al. 2015). Therefore, in order to contextualize and better understand what information the osteological record does provide, it is necessary to understand the context of the bone during life, that is, the context in which those lesions and abnormalities formed.

The immune system is both a defense and a maintenance mechanism. Its complexity allows for a flexible and varied response to disease, yet these same protective mechanisms can also contribute to tissue damage and pathological consequences. The entire system is a balancing act: deficient immune responses allow for increased susceptibility to further encroachments from pathogens, while excessive responses may result in harm to the individual and compromise subsequent immune responses. The skeletal record represents those deceased, yet understanding the mechanisms of immune function, how inflammation manifests, and the consequences of such in life are still relevant to further osteological analyses (Crespo et al. 2017). Highlighting the myriad complexities of health, considerations of immune function are increasingly pertinent for broader analysis of bone.

Given the complexity of the biological context of bone, application of a combination of assessments afforded by different disciplines creates a more nuanced end perspective. Related, yet diverse fields of study may provide new insights regarding the

paradoxical evidence of health observed in the skeletal record. Here, in addition to an overview of the immune system and inflammation, the potential insights of experimental immunology, osteoimmunology, archaeoproteomic analysis, and paleopathology will be briefly considered. However, the potential perspective that experimental immunology specifically may add to bioarchaeological considerations regarding the role of inflammation, as part of larger multidisciplinary reconstructions of health and stress from the osteological record, will be considered as the primary, and necessary, starting point for this line of inquiry.

Immunology: A starting point for inquiry

In order to consider what evidence experimental immunology may contribute to bioarchaeological considerations, a review of what experimental immunology is, and more important foundationally some of the basics of immunology, is warranted to provide necessary context for understanding inflammation. Immunology is the study of the immune system, a complex network of organs and cellular components that work together to maintain the homeostatic balance while protecting an individual from insults, mainly pathogens. A pathogen is defined as "any agent capable of causing infection or disease in a cell or organism" (Karp 2009). It is important to note that pathogens are not exclusively bacteria. Historically, immunity referred to the protection from disease, specifically infectious disease, but noninfectious foreign substances, and even products of the body itself can elicit an immune response (Abbas et al. 2015). The immune system itself is not a singular entity: it is a complex network of systems (including organs, tissues, and cellular components) that work in concert to elicit a response. This inherent complexity allows for an overall flexible response depending on the insulting stimulus.

This system functions as a whole but can be understood as two large complementary and overlapping systems; innate immunity and adaptive, or acquired, immunity.

Innate immunity can be thought of as the first line of defense for an individual. The adaptive immune response requires time, so the effector mechanisms of innate immunity clear out, or at least hold off the infection until the acquired response is ready (Abbas et al. 2015). Detection of a pathogen elicits a rapid response in which these effector mechanisms recognize foreign molecular structures that set apart bacteria and other pathogens from human cells. The innate system recognizes broad groups of conserved structures (pathogen-associated molecular patterns), rather than with the specificity that characterizes the adaptive response (Medzhitov and Janeway 2000). Players on the innate side of immunity include the phagocytic cells (macrophages and neutrophils), as well as a system of blood proteins and chemical mediators that comprise the complement system (Abbas et al. 2015). These innate reactions are generally not specific, and the same responses are elicited regardless of the stimulus. The innate response works quickly to neutralize and eliminate the insult, preventing further survival or encroachment of the pathogen. In addition to the traditional understandings of innate immunity as the initial pathogen response, the innate system also works to help control the adaptive response (Medzhitov and Janeway 2000). These two systems do not function in isolation from one another, and mechanisms of one system, such as complement, are increasingly recognized as bridging the innate and adaptive immune responses (Markiewski and Lambris 2007).

Although innate immunity evolved first, it has its limitations. Not every pathogen is eliminated by this initial response. The scope of recognition of innate immunity is

limited, and the ability of pathogens to mutate or otherwise avoid detection spurred the development of the adaptive side of immunity (Bonilla and Oettgen 2010). Adaptive immunity generates a specific response to each pathogenic insult via highly variable recognition receptors on adaptive cells. This system can recognize a wide variety of insults and even has the ability to recognize specific pathogens encountered previously, creating an immunological memory that allows for a much faster response time upon reinfection (Bonilla and Oettgen 2010). However, this process is not instantaneous, and it can take from several days up to a full week for this system to effectively mount a response (Abbas et al. 2015). The adaptive response can be broken down into two major categories: humoral immunity which utilizes B lymphocytes and antibody production, and cell-mediated immunity mediated by T lymphocytes, which generally target infected cells. These lymphocytes are mobile and traffic to infected areas upon recruitment by innate immune signals.

The two arms of the immune system must be able to communicate with one another and do so via chemical mediators, cytokines. Cytokines are proteins produced and secreted by a variety of cells, and are the primary mediators of the immune system (Abbas et al. 2015). There is tremendous diversity in cytokines and their functions, and many effector functions of such depend on context. Individual cytokine function can vary depending on the type of cell producing it and its microenvironment (Turner et al. 2014). For example, the role of localized cytokines in a microenvironment which are indicative of either a type I or type II response, are a reflection of which effector T cells are operating within that specific context. In addition, the production of cytokines in and of themselves can alter the functional states of the targeted tissues and organ systems

(Abbas et al. 2015). Cytokines are of particular interest when considering inflammation, as these ubiquitous proteins are a fundamental part of the overall immune response, with effector and regulatory functions across both innate and adaptive responses (Abbas et al. 2015; Turner et al. 2014). Specific cytokines IL-1β, TNFα, and IFNγ are of particular interest for their proinflammatory roles and implications for the human skeleton.

As a complex whole, diverse mechanisms from both innate and adaptive immunity can influence one another and even subsequent immune responses. However, a full review of the intricacies of the immune system is well beyond the scope of this project. Instead, focus will be given to one of the most important aspects of the immune response, inflammation, specifically because the consequences of elevated or pathological inflammation may contribute to abnormalities observed in the osteological record. Inflammation does not exist in a vacuum, and increasingly the consequences of the inflammatory response must be addressed in the context of osteological interpretations, as inflammation has a direct impact on bone physiology (Crespo et al. 2017; Hardy and Cooper 2009; Klaus 2014; Redlich and Smolen 2012).

Inflammation

A familiar process accompanying injury and sickness, the first to describe the hallmark clinical symptoms of inflammation (redness, swelling, heat, and pain) was Cornelius Celsus in the 1st century CE (Medzhitov 2010). Specifically, it is the recruitment and accumulation of leukocytes and plasma proteins at a specific site of infection or tissue injury for purposes of containment and control (Soehnlein and Lindbom 2010). The extravasation of leukocytes out of circulation and towards the site of the infection or injury, and their subsequent work there to neutralize and eliminate the

problem, produces these standard clinical symptoms (Ashley et al. 2012). Both the arms of the immune system utilize inflammation, but the local, acute inflammatory response is the hallmark of innate immunity, developing in as little as a few minutes and capable of persisting for days (Abbas et al. 2015). Organ systems are linked via intricate cellular pathways trafficked by the specialized immune cells and chemical mediators (cytokines and chemokines) at both the local and systemic level, and inflammation as a whole can be thought of broadly as "…a system of information flow in response to injury and infection" (Nathan 2002).

The inflammatory response can be conceptualized as moving through a series of checkpoints, with multiple factors built in to prevent inappropriate escalations of the inflammatory response (Ashley et al. 2012; Nathan 2002). This response can be understood in terms of inducers and mediators of inflammation: inducers are signals that initiate the response, such as a pathogen, while mediators are the effectors of the response (cytokines). The production of these mediators alters the functional state of the targeted tissue and organs systems and while it remains unclear whether the nature of the inducer influences the production of a specific mediator, in general, inflammatory mediators often induce the production of additional mediators, escalating the inflammatory response (Abbas et al. 2015; Medzhitov 2008). Initially local, inflammation is triggered by some noxious stimulus that includes a "tissue-based startle reaction to trauma", which sets into motion recruitment of cells to deal with the stimulus (Nathan 2002). However, the very cytokines that orchestrate an inflammatory event may contribute to inappropriate inflammation later if concentrations are increased, regulatory mechanisms fail, or even the population of cells transitions away from neutrophils to macrophages (Ashley et al.

2012; Medzhitov 2008; Turner et al. 2014). The extent of influence inflammatory mediators have extends beyond the immediate site of inflammation: when these mediators induce the formation of an exudate there are additional effects on the associated neuroendocrine and metabolic functions of the targeted tissue, as well as maintenance of tissue homeostasis in general (Ashley et al. 2012; Medzhitov 2008). Every step of the inflammatory process should be tightly regulated by multiple checks and balances on the system. However, the lack of inflammation is not a passive absence of inflammatory stimuli; rather, the maintenance of health requires checkpoints that suppress inappropriate inflammatory reactions (Nathan 2002).

Inflammation is a dynamic process and can be understood in the context of both duration and location. Failure to resolve the initial crises, or failure of the immune system to resolve the initial inflammation, shifts the acute response to a chronic one (Ahmed 2011; Ashley et al. 2012). Furthermore, just as acute can become chronic it is possible for local events to become systemic. When leukocytes secrete cytokines in response to bacterial products this also stimulates the production of acute-phase proteins, leading to systemic manifestations of the local event (Abbas et al. 2015). The process of spillover is the overflow of excess proinflammatory cytokines (including TNFα and IL-1β) and inflammatory mediators (activated immune cells and proteins) from the initial local site and out into circulation (Ashley et al. 2012). The subsequent activation of sensory nerves from this spillover report inflammation to the entire body (Straub 2011). This type of spillover process is often concomitant with cytokine storms, the abnormal and often pathological increase in cytokine signaling and production (Tisoncik et al. 2012). Cytokine storms can increase the severity of the inflammation as well as the duration,

often more so than the spillover observed in peripheral blood (Tisoncik et al. 2012). However both cytokine storms and spillovers have systemic consequences in terms of the severity of the continuing inflammatory response, as well subsequent immune reactions (Crespo et al. 2017).

While systemic inflammation does not necessarily have to be chronic it often is, and highlights the point that inflammation is not one singular uniform process. Conceptualizing inflammation as "a diverse range of inflammations", or a diverse range of the inflammatory response, may be more useful than considering inflammation as a single entity (Ahmed 2011; Ashley et al. 2012). Initiated by numerous stimuli and regulated by differing mechanisms inflammation is highly variable: indeed the characteristics of chronic inflammation even differ simply depending on which effector class of T cell is present (Medzhitov 2008). Likewise, local microenvironments exert influence on the inflammatory response when immune cells interact with local cells comprising the tissues of the organs experiencing the inflammatory infiltrate (Libby 2007). Aberrant expression of adhesion molecules in these microenvironments subsequently leads to inappropriate leukocyte retention and survival in tissues. For example, fibroblasts, once thought to provide structural support to tissues, are increasingly implemented in active contribution to the retention and persistence of the inflammatory response (Buckley 2011). In addition, fibroblasts themselves appear to be modified by local inflammation highlighting the inherent complex give and take of any immune response, and consequently how pathological responses may quickly compound into chronic situations (Buckley 2011).

Even processes that have not been traditionally considered inflammatory are increasingly being recognized as playing a role in inflammation, such as with the activation of the complement system. Here the formation of an enzyme complex with proteolytic activity leads to the lysing of the targeted cell. This serves to highlight that different parts of an enormously complex system can contribute to inflammation (Markiewski and Lambris 2007). While classical infection-inflammatory pathways are well elucidated, other inflammatory processes are not as much, and with such inherent variability it is becoming apparent that the mechanisms of much of sustained inflammation, especially states of systemic chronic inflammation, are poorly understood (Ahmed 2011; Ashley et al. 2012; Medzhitov 2008). It is important to remember the complexities inherent to the immune response in subsequent considerations of inflammation. As the pathogenesis of diseases are increasingly recognized as heterogeneous, so too is inflammation (Straub 2011).

Inflammation as a double-edged sword: Consequences of hyperinflammation

It is a paradox then that one of the fundamental processes of the immune response meant to protect an individual can in turn become detrimental, and inflammation exemplifies a double-edged sword. The shift from the acute local response to the chronic systemic one is of particular interest as in general it is chronic systemic inflammation that is implicated in states of chronic disease and subsequent pathological immune responses (Ahmed 2011; Medzhitov 2008). Indeed, “the pathological potential of inflammation is unprecedented for a physiological process” (Medzhitov 2008). One function of inflammation is to clear damaged cells and initiate tissue repair in response to injury (Soehnlein and Lindbom 2010). Characterized by the influx of cells to the local site of

injury, this process becomes pathological with dysregulation of the normal resolution mechanisms (Libby 2007; Medzhitov 2010). The mechanisms of efficient removal of pathogens and apoptotic cells, that is, the utilization of reactive oxygen species, cytokines and other inflammatory mediators, in turn damage host tissues with prolonged exposure (Libby 2007). Individuals are not immune to their own inflammation, and failure to resolve the response, or prolonged exposure to the potent molecular components of the inflammatory response, can be quite harmful to the individual (Ashley et al. 2012).

Dysregulation of control mechanisms leads to pathology, and so too do inappropriate responses to pathogens or other insults. Cytokine spillover opens the door to systemic consequences of initial inflammatory responses: indeed, chronic inflammatory diseases are systemic in their very nature (Straub 2011). Regulation of inflammation is not passive, it takes active regulation to avoid an overreaction to minimal threats (Nathan 2002). Signaling pathways in essence program for normal self-limitation and termination in the very initial phase of the activation of the acute inflammatory response (Serhan and Savill 2005). Even specific cells play a part in resolution: leukocytes have the ability to trigger a self-limiting response in acute inflammation, and neutrophils undergo increased rates of apoptosis after certain time points (Serhan and Savill 2005; Soehnlein and Lindbom 2010). These self-limiting responses help to facilitate resolution as clearance of the initial inflammatory cells helps to terminate the inflammatory response.

Active events in early signaling pathways as well as continuous checkpoints throughout the development of the entire inflammatory response are crucial for normal functioning, and any dysregulation at any point along these pathways has the potential to

influence downstream events (Nathan 2002; Serhan and Savill 2005). Indeed, any dysregulation in the balance of pro-and-anti-inflammatory mechanisms may contribute to the development of cytokine storms (Tisoncik et al. 2012). Furthermore, the establishment of positive feedback loops quickly escalates the inflammatory response. Increasingly chronic inflammatory conditions are described where the initiating trigger (injury, pathogen) is undefined, yet the escalation and positive feedback loops are present (Medzhitov 2010). Dysregulation in pathways necessary for control of inflammation may be responsible for some of these chronic situations where the stimulating trigger is unknown (Nathan 2002). Dysregulation is increasingly recognized more in the underlying etiology of chronic inflammatory conditions, and while multiple layers of regulatory safeguards exist to check against pathological consequences, these are not always failsafe (Barton 2008; Medzhitov 2010). Indeed, with signaling pathways so responsive that distinct inflammatory outcomes may depend in part on which specific type of ligand is recognized, let alone the context in which that ligand is recognized, it is important to keep in mind the myriad complexities of inflammation as a whole (Barton 2008).

Keeping these myriad complexities of inflammation as a whole in mind is also necessary to provide bioarchaeological considerations of inflammation proper context. With the pathological consequences of inflammation regardless of the etiology that spurred an inflammatory response, it is important to keep in mind the potential effects on bone that sustained inflammation may exert (Redlich and Smolen 2012). The consequences of inflammation are important to osteological questions, as chronic inflammatory disorders are often associated with bone loss and remodeling (Hardy and

Cooper 2009). Considering these pathways and their cellular components provides context to better frame questions that relate to both immunology and bone.

Homeostasis, energy, and sustained inflammation

The mechanisms of inflammation and the function of the immune system as a whole should also be considered within the larger context of the entire body. How inflammation is sustained, and how it relates to the body functioning as a whole is necessary background to understand how in turn inflammation will relate to and influence bone. The immune response is implicated in more than just pathogen responses: it also plays an important role in maintaining homeostasis (as well as extending and complementing homeostatic capacity) and overall stress responses of the body (Chovatiya and Medzhitov 2014; Medzhitov 2008). Broadly, when homeostasis is disrupted a stress response is mounted, and if this is insufficient to restore homeostasis, then an inflammatory response is induced (Chovatiya and Medzhitov 2014). Immunological homeostasis as evident in the adaptive arm of the immune system, contributes to homeostasis by controlling the expansion and contraction of the immune response, especially in regards to the resolution of inflammation (Abbas et al. 2015; Andersson and Tracey 2012).

The maintenance of homeostasis occurs at multiple levels (systemic; tissue; cellular) and helps to maintain the organism at an acceptable and stable range, tightly regulated by the endocrine and autonomic nervous systems, although immunity and the immune response has long been considered an autonomous response to stimuli, separate from the regulations of the rest of the body (Andersson and Tracey 2012; Chovatiya and Medzhitov 2014). Yet increasing evidence shows that the immune system is functionally

and anatomically connected to the nervous system, with systemic implications for the regulation of the immune response. Nerve endings have been found nanometers away from immune cells that happen to express receptors for primary neurotransmitters: anatomically pointing to the communication possible between the two systems (Andersson and Tracey 2012). The connectedness of these systems is of interest as neuroendocrine regulation may help to explain both the control and the maintenance of inappropriate inflammation, such as in the absence of injury or disease. With the recognition that behavior, mental illnesses, and even chronic psychological stress are often accompanied by chronic smoldering inflammation, it is necessary to consider the role of the neuroendocrine system in factors of chronic inflammation (Padro and Sanders 2014; Straub 2011). The vagus nerve serves as a highway for hormones and molecular products of the immune system to communicate with the brain, and lesions (anatomical, functional, or molecular) in such are associated with enhanced cytokine production observed in non-resolving inflammation (Andersson and Tracey 2012). In addition, close interactions between the cytokine system as a whole and the endocrine system exemplify the reciprocal relationships between mediators that may come into play, influencing disease progression and prognosis (van der Poll et al. 1994). The importance of the communication between these systems becomes apparent with pathological consequences of disruptions in these signaling pathways, and even though a disruption may be short term, the health consequences can become chronic, and due to the bidirectional nature of this relationship, one system can influence the other (Padro and Sanders 2014). This is one such context in which inappropriate inflammation may manifest, leading to systemic

consequences (such as observed in the osteological record), without pathogens or injury present.

One of the most important aspects of homeostatic control is energy regulation, especially considering that the immune system is one of the top three consumers of energy, including the brain and muscular system (Straub 2014; Straub et al. 2010). Much of inappropriate systemic inflammation can be considered within the context of energy regulation. An increase of inflammatory cytokines at even low serum levels can induce an energy reallocation program, funneling fuel to an activated immune system (Straub 2011). This is an important consideration as the activated immune system is costly, consisting of up to 25% of the basal metabolic rate (Straub et al. 2010). Yet the utilization of these energy stores can come at a cost, especially when bone is used to support immune function. Higher incidences of multiple bone fractures are correlated with an increase in the metabolic rate by 15-30% (Straub et al. 2010). Bone is a repository of calcium and phosphorous, and retrieval of this energy comes at a cost to bone: even low grade chronic inflammation is associated with a decrease in bone quality (van Niekerk et al. 2018). The utilization of bone initially makes sense, as it is a quick source of phosphate (necessary for effector cell function) and calcium (necessary for cell survival) (van Niekerk et al. 2018). In cases of severe disease, calcium is only provided from boney sources, not from the intestine (Straub et al. 2010). Vitamin D is a major hormone for the storage of calcium and phosphorous in bone, and bone remodeling is a direct function of vitamin D, highlighting one pathway leading to observable lesions on bone (Straub 2014).

Like most bodily systems homeostasis is not discrete and "inflammation can be viewed as the end of the spectrum of mechanisms that maintain and defend homeostasis" (Chovatiya and Medzhitov 2014). However, existing on a spectrum, it is possible for a shift to occur in the relevant set points of homeostasis so that they become reset, in that the abnormal becomes the new normal (Medzhitov 2008). For whatever underlying reason, in this context chronic inflammation becomes the new set point, and while this inflammation is of a lesser magnitude than the stereotypical acute inflammatory response, it still occurs at the expense of other physiological processes, and a decline in function in affected areas (Medzhitov 2008). That is, for any disruption in homeostasis, there should be a corresponding stress response, and if this fails to restore the system, inflammation is induced (Chovatiya and Medzhitov 2014). Stress responses are thorny, as the implementation of inflammation can be both in response to stress, as well as a defense mechanism of the system. That is not to say stress is always bad, rather it is an evolutionary response, and under certain conditions stress may prep and even enhance the immune system for upcoming stressors (Dhabhar 2014). It is the dysregulation of the response to stress that helps to create the physiological environment of inappropriate and sustained inflammation. A more nuanced approach may be to consider stress and subsequent responses in terms of allostatic loads, with high loads resulting in dysregulation or suppression of immune function (Dhabhar 2014).

The evolutionary context: Why does this happen?

Placing inflammation in a larger evolutionary context helps to clarify why pathological inflammation exists, as well as how to recognize when and why this might happen. The immune system is a powerful system, but one influenced by the brain and

constrained by limits on available energy. The evolutionary perspective seeks to contextualize observed phenomena, such as sustained inflammation, within the long-term perspective of our species. This broad view elucidates the why for some responses that are not immediately apparent. It is likely that inflammation evolved as an adaptive response for restoring homeostasis, and the immunoenhancement observed during acute stress is part of this adaptive response (Ashley et al. 2012; Dhabhar 2014; Medzhitov 2008). This does not appear unique to mammals either, as evidence that neural circuits have coevolved with, and influenced the evolution of such, at least as far back as the invertebrate period (Andersson and Tracey 2012). Yet sustained inflammation exerts a costly and overall negative effect on reproductive fitness.

Inflammation triggered by noxious stimuli is generally a short-term response, and the homeostatic networks between the neuroendocrine system and the immune system came under positive selection for the benefits related to survival and increased fitness from short term, yet strong, inflammatory responses (Straub and Schradin 2016). This response is mounted at the expense of other physiological processes, so therefore it cannot be sustained for an extended period without detrimental consequences to the host (Medzhitov 2008). The acute inflammatory response has come under selection for the rapid resolution of infection, with a general time frame that corresponds with that of germinal center B cell expansion and contraction of approximately 21-28 days (Straub and Schradin 2016). Longer responses take a toll on fitness, so there exists a dilemma between the needs of the active immune system and the rest of the body, yet short-lived inflammation increases fitness (Straub and Schradin 2016). Beyond set time limits of acute inflammation (lasting anywhere from 21-60 days broadly), regulation and

resolution of inflammation fails after this time, as it falls beyond the limits of adaptedness (Straub et al. 2015). Consuming infectious diseases may run through available energy stores (capped around 43 days), so limits on energy availability can help explain the relatively constant course of adaptive immune responses and define the point of transition to chronic disease at the time point of complete energy consumption (Straub 2012).

The pathological potential of inflammation is unprecedented for a physiological process (Medzhitov 2008). It is a paradox that a beneficial response can also be so damaging. Evidence of such damage, such as observed in bone, is contextualized by the evolutionary perspective. The use of phosphate from bone resorption is evolutionarily conserved to sustain the biosynthetic activities of activated immune cells, a phenomenon also observed in multiple species, indicative of an evolutionary conserved immune response (van Niekerk et al. 2018). The use of bone for energy is not a coincidence; use of bone has been selected for in acute responses and it is only in chronic situations that the use of bone becomes pathological (Straub et al. 2015). In addition, as part of sickness behavior, the decrease in mobility also stimulates bone loss, feeding energy to the system (Straub et al. 2015).

The neural circuits that regulate immunity evolved in response to products of the immune system formed in response to infection and injury (Andersson and Tracey 2012). Yet these same neural pathways selected for in regard to acute inflammation are the same implicated in chronic inflammatory diseases (Straub 2014; Straub and Schradin 2016). “The potential of chronic development increases with the number of erroneous adaptational reactions…” or, the accumulation of inappropriate uses of those conserved pathways contribute to pathological inflammation (Straub and Besedovsky 2003). In

addition, long standing use of neuroendocrine pathways is in of itself a disease aggravating etiologic factor (Straub 2014). This makes sense, as the evolution of the immune response is heavily tied to energy regulation and homeostatic regulation.

Energy concerns are not the only selective pressure in play, and it should be briefly mentioned that the immune response also reflects the inherent reactivity of the system as a whole, one shaped by evolutionary forces to keep pace with pathogens and their immunoevasive and immunosuppressive maneuvers (Graham et al. 2005). Indeed, pathogens may be a contributing and even causal factor in chronic inflammatory diseases. Mycobacteria species especially are well supported candidates for pathogen-mediated selection altering inflammatory pathways, and increasing the frequency of chronic disease alleles in humans (Brinkworth and Barreiro 2014). Pathogen interactions may modulate the host immune response towards immunopathological responses, all in an attempt to eliminate the pathogen (Graham et al. 2005). Increasingly, progress of an infection is a function of pathogen load and the virulence of a particular strain (Gluckman et al. 2009). The role of pathogen survival contextualized by the mechanisms of the immune response may help to better understand some of the pathological responses of the host immune system. The long-term arms race between pathogen and host has no end in sight, and this may help to frame considerations of inappropriate inflammation and subsequent consequences moving forward.

Searching for an inflammatory phenotype

Bone is biologically complex, and investigations of such from the bioarchaeological record would do well to consider the inflammatory response. The shift from local to systemic, from short term to chronic, and the implications in terms of

impact on the type of, and severity of the inflammatory response, and in turn the potential impact on bone, must be addressed in these bioarchaeological interpretations (Crespo et al. 2017; Klaus 2014). Given the inherent complexity of the immune system as briefly reviewed above it is no surprise that there exists significant heterogeneity and variability of the immune response to disease in any given population. Yet it is not always possible to study complete populations in the past. Nonetheless, individuals can provide a great source of information. How well an individual can response to various insults and the ability of the immune system to generate an appropriate response to an insult comprises an individual's immune competence (IC) (Crespo and Lawrenz 2014).

Immune competence is an important concept: it is a reflection of the immune system's responsiveness, including both innate and adaptive functions. It reflects the balancing act of maintaining homeostasis, as excessive or deficient responses are pathological. While competence is maintained by the cellular and chemical mediators of the overall system, the competence of an individual is more than just a tally of cell count and type: immune competence is a reflection of an individual's evolutionary history, as well as their environment, both physical and social. The complex interplay of these factors shapes the reactiveness of the system as a whole, with moving pieces comprising an individual's immune competency. This competence is a reflection of the individual and is not merely the ability of the body to mount an immune response. Competence here is a reflection of function and individual life history and context.

As neither populations nor individuals are fixed entities, immune competence can shift over an individual's lifetime as their environment, biological or social, changes (Crespo and Lawrenz 2014). Heterogenous environments are comprised of varied

challenges and stresses that stimulate the immune system. The presence of comorbidities or even subsequent exposures to additional pathogens may elicit an inappropriate response depending on an individual's environment. Indeed, this type of immunomodulation is grounded in the environments encountered in infancy and early childhood; these early environments shape an individual's immunophenotype, one that is modified over the course of the individual's lifespan (McDade 2012). Given the interconnectedness of systemic responses factors such as diet or even social and psychological stress can influence the immune response (Dantzer et al. 2008; Dhabhar 2014; McDade 2012). Stress is one such factor that has been implicated in systemic inflammation, associated with the dysregulation of the stress response and high allostatic loads (Dhabhar 2014). Moreover, the examination and redefinition of the role of stress in analyses of health needs to be incorporated into analyses of the immune response; relevant here for contextualizing both the individual inflammatory response (competence), as well as for osteological analyses (Dhabhar 2014; Temple and Goodman 2014).

Yet it is important to emphasize that immune competence is not a purely biological concept (Crespo and Lawrenz 2014). Humans are complex biosocial creatures, that is to say, influenced by both our genes and culture, and as such cannot be removed from environments framed and shaped by culture and society. In addition, human biology and human beings cannot be reduced to only biological processes: people are the product of complex dialectical relations between biology and culture, and there is an inextricable connection between the two (Goodman 2013; Larsen 2015). Taking our biosocial environment into consideration is necessary to contextualize the how and the why of the

potential immune system reaction to certain or even perceived threats. Moreover, while all people share the same fundamental components of the immune system, the same cellular players and pathways, the responses of such that comprise competence are highly variable and dependent on individual context: that is, individual life histories and environments (McDade 2012). Furthermore, as the immune system is so plastic, it stands to reason that events early in life, if not *in utero*, can shape later immune responses as well as the long-term inflammatory phenotype (McDade 2012; McDade et al. 2010). This idea of "immunological plasticity", that is the capacity to adjust or modify responses depending on intra-or-extra-cellular conditions, also helps to frame the idea of the variability and flexibility of the immune system over time (Crespo and Lawrenz 2014).

Inflammation is the hallmark response of the immune system and as such is an important part of immune competence. Due to its potent capabilities and systemic reach, it is also the one immune response most likely to inadvertently cause damage to the host. Its dual function of defense and tissue repair can quickly become pathological with dysregulation and failure of systemic checks to control the response. An individual's inflammatory response, as a component of competence, comprises the inflammatory phenotype (IP), shaped extensively by individual biosocial contexts and life history (McDade 2012). Thus, the inflammatory phenotype provides clues to how the individual may react to stressors, informed by specific environmental contexts. Within this biosocial context then, those individuals who mount a stronger initial inflammatory response could experience a shift from the initial local response to a systemic one, a hyper-inflammatory response (Crespo et al. 2017). This hyper-inflammatory response is the combination of the inflammatory reaction to a persistent insult, the environment, and

even the pathogens' own reactions to its host, creating a hyper-inflammatory phenotype (HIP), especially within chronic situations (Crespo et al. 2017). However, due to the specificity of the individual response, not all individuals will experience a shift to a HIP when encountering a pathogen.

Understanding why this shift to a HIP happens, how it is maintained and the health consequences of such, all within the biosocial context of the individual are compelling reasons for further investigation. Like the individual, the immune system and its inflammatory response do not exist in a vacuum, and neither should the multiple disciplines that consider health, especially any investigation of health in the past (Reitsema and McIlvaine 2014). The challenge then becomes, how would it be possible to study this phenomenon in past populations, and what sort of information could it provide? With the obvious ethical and logistical problems of studying these questions in living populations, evidence about both contemporary and past populations must be derived from other sources. While the work of many biological anthropologists utilize modern populations and synthesize human biology with anthropological frameworks, even here there are limitations, including how much can be extrapolated to the past. One of the draws of bioarchaeology is its interdisciplinary nature, and it is this dynamic approach to analyses that even allows for collaborative work, such as proposed here with connections made between immunological and bioarchaeological perspectives, with potential for understanding immune competence in the past (Crespo et al. 2017; Larsen 2015; Larsen 2018). Indeed, a multidisciplinary approach allows for different methodologies and perspectives to be taken together and is therefore necessary to begin

to reconstruct understandings of health, disease, and stress in past populations (Klaus 2014; Reitsema and McIlvaine 2014).

The IP in the skeletal record: Are reconstructions possible?

The inflammatory phenotype provides valuable information that can help to better contextualize analyses of health and responses to stress. The problem becomes then, is it even possible to reconstruct an individual's inflammatory phenotype? If so, it must be based upon osteological evidence, given the importance of the skeletal system for immune function and the interplay between these systems in life. This undertaking, to even start to reconstruct something as complex and varied as immune competence and an inflammatory phenotype from a skeleton, cannot be pursued via a single lens. Rather multiple perspectives must be engaged to help create a more nuanced perspective of what is observed in the bioarchaeological record. Here, the capacity of the field of bioarchaeology as a whole to embrace other disciplines, to integrate, contextualize, and synthesize data from outside the discipline, is crucial for a more holistic end assessment (Klaus 2014; Reitsema and McIlvaine 2014; Temple and Goodman 2014).

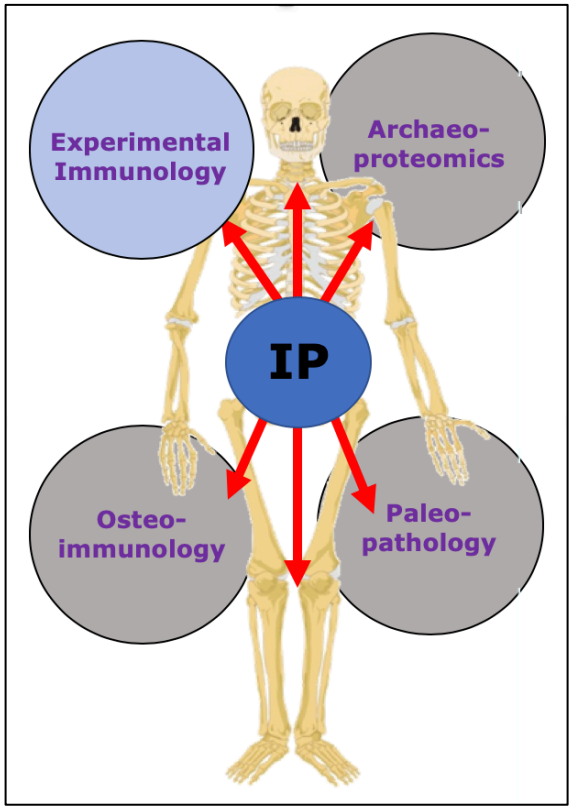

Figure 1 The Multidisciplinary Model

The multidisciplinary approach (model)

There are four prominent fields of study that lend themselves well to this task of reconstruction of an inflammatory phenotype:

experimental immunology, osteoimmunology, archaeoproteomic analysis, and paleopathology. These fields were brought together in a recently proposed framework which established a trajectory for how to pursue this type of analysis (Figure 1) (Crespo 2018).

Experimental immunology

Experiments performed *in vitro* consider the mechanisms of inflammation itself, the cellular and chemical mediators of the inflammatory response. Teasing apart how specific inflammatory factors respond to insults via *in vitro* models may provide insights, i.e., how activated immune cells can affect inflammatory responses to a variety of secondary insults, which may then be extrapolated to other levels of analysis.

In the context of chronic infections, and the inflammatory response on bone in that environment, experimental immunology provides tools to investigate a potential inflammatory shift at the level of the immune cells themselves (Crespo et al. 2017). Observation of lesions on the bone provides a wealth of information. Yet lesions and abnormalities may be nonspecific, and without context the true etiology of those abnormalities may not be known. Understanding the cellular players behind what is observed on bone, especially in the context of the HIP and chronic systemic inflammation, may not provide specific context to an individual, but it does provide another perspective of how what is observed came to be. Incorporating what information immunology and inflammation provide may help to elucidate more clearly what is left behind in the osteological record.

This study into the dynamic interactions between the immune system and the rest of the body, and the immune system in response to actual or perceived threats, justify the

foundations of this model upon experimental immunology. These interactions between host and pathogen, and of the host with itself, create these sustained inflammatory environments in which change may happen to the bones, and teasing out the inflammatory pathways provides a starting point to work up from.

Osteoimmunology

The logical follow up to and extension of experimental immunology lies in the emerging discipline of osteoimmunology. Osteoimmunology investigates how the immune and skeletal systems interact (Caetano-Lopes et al. 2009). Experimental osteoimmunology performed *in vitro* provides opportunity to investigate the underlying relationship between the bone at its fundamental cellular level and the immune system, i.e., how activated immune cells can affect bone cell differentiation and physiology, and how the cellular players from both systems interact and influence one another (Nakashima and Takayanagi 2009). While experimental immunology looks at how the immune system interacts with itself, and with outside players (pathogens), osteoimmunology examines the more specific relationship of how the immune system interacts with bone. Both perspectives are necessary, and to understand lesion formation bone physiology and biology within the context of immune interaction must be considered.

There is plenty of overlap within these two experimental fields. Osteoclasts and immune cells share many of the same regulatory molecules, such as cytokines. The cytokines that are implemented in proinflammatory activity in turn exert influence on bone cells as well (Nakashima and Takayanagi 2009). It stands to reason that in chronic inflammatory environments there will be unintended consequences for bone at the very

least because these molecules can influence multiple types of cells (Caetano-Lopes et al. 2009). There are already many cases of bone damage and loss associated with inflammatory disorders (Takayanagi 2005). In addition, overlap with endocrine and homeostatic mechanisms provides another pathway leading to potential bone damage outside of traditional pathogens, especially since many of the molecular players in energy storage and retrieval (including vitamin D, osteocalcin, and cortisol) exert influence on osteoblasts and osteoclasts (Caetano-Lopes et al. 2009). Osteoimmunology provides a framework to start to investigate how interactions between the inflammatory response and bone cell physiology may be translated into the lesions or abnormalities observed in the osteological record.

Archaeoproteomic analysis

The structure of bone is one of the most important aspects of the tissue, and the proteins that comprise bone are just as important for analysis as the cells and mediators that interact in the living system. Archaeoproteomic analysis provides the potential for the inquiry of bone at the level of its proteins. As bone is a dynamic living tissue, not all of its protein composition will be structural. After death, the chemical analysis of bone provides a wealth of information. Isotopic analysis provides insight into diet, with the potential for behavioral and ecological inferences from such, laying a groundwork for this type of analysis (Larsen 2015).

One of the major setbacks to chemical analysis has been the changes that bone undergoes postmortem. Bone is comprised mainly of type I collagen, and non-collagenous proteins are only a small fraction of bone proteins, yet these non-collagenous proteins are the ones of interest. After burial, these organic proteins undergo deterioration

and other chemical changes, making the analysis of ancient bone problematic. However, recent work on refined protocols and analyses are bringing the potential of archaeoproteomic analysis on bones forward (Cersoy et al. 2019; Sawafuji et al. 2017). Indeed, a recent study was able to use protein analysis to identify the presence of pathology biomarkers in the bone (Pérez-Martínez et al. 2016).

The great potential of this emerging field is detecting proteins that can be indicative of the processes that occurred in life. Analyzing protein from a miniscule sample of powdered bone could potentially capture the presence of inflammatory (or other stress) markers in the bone postmortem, something previously undetectable. The presence of these proteins may be more indicative of the inflammatory processes that occurred in life, providing a more nuanced understanding of that individual's health, regardless of whether there was observable lesion formation. A potential correlation in detectable immune proteins (or signals thereof) and lesions provides another new avenue to expand understandings of health from the bioarchaeological record. This type of proteomic analysis may potentially provide a much more powerful understanding of health in life than previously possible from skeletonized remains alone (Sawafuji et al. 2017). For example, detection of osteocalcin (important in life in part for its role in energy regulation) from bone not only sets up the potential for further analysis of health and stress during life from bone, but the potential to identify stress in an individual before any physical lesions had a chance to manifest (Scott et al. 2016).

Paleopathology

No analysis of health, stress, or disease in the past is possible without utilizing the basics of paleopathological research. Paleopathology relies on both the biological and

social sciences to describe and classify the diversity of abnormalities of the human skeleton (Ortner 2011). Yet this discipline is more than just route classification. Paleopathology at its core is an interdisciplinary pursuit, utilizing methodologies and ideas from other disciplines to substantiate its own foundations. It is a complementary field to bioarchaeology, drawing on much of the context that bioarchaeology provides, and vice versa. Fundamentally, paleopathology is rooted in the anatomical analysis of bone abnormalities, but collaborative work with a variety of other disciplines allows expansion of the application of paleopathology to multiple questions, not least of which is the role of disease in human societies and reconstructions of pathogen-human interactions (Ortner 2011).

The analysis of observable lesions and abnormalities is the final piece of this model to begin to reconstruct the inflammatory phenotype. All of these fields provide valuable information about some specifics of health, but paleopathology grounds these disciplines in the observable anatomical record. Archaeoproteomics may analyze the proteins from bone, but paleopathology sets the foundation for observing and classifying the abnormal. While cellular and molecular studies work out the myriad intricate pathways that eventually lead to lesions, paleopathology has the tools for the immediate observation. The actual study of bone, and the continued observations of such are necessary to ground and orientate the research questions and goals of the other fields. Paleopathology must be consulted as it provides this foundation for interpreting the diseased skeleton within the context of these other analyses.

That is not to argue that any one field is of more importance than another within this model. Rather, this model provides a starting point to frame questions about the

potential of reconstructing past inflammatory processes, with each part contributing a piece to the puzzle. These four disciplines each provide unique perspectives that work well in concert with one another, building off of, and expanding the analysis. It is not possible to immediately compare or extrapolate the results of an *in vitro* experiment to an observable lesion in bone. However, by taking the information from experimental immunology and expanding that to questions of osteoimmunology, it is possible to start to build a context in which questions of inflammation are relevant. Archaeoproteomics moves that context of inflammation and bone from *in vitro*, to proteins sampled directly from bone. Paleopathology provides the examples of what the osteological record can provide, that may help to orientate and contextualize what the other three fields have built up to. Inflammation cannot be understood in the context of the skeleton without understanding the mechanisms that occur in life. This model provides a starting point, building off of each disciplines' relevant contributions to start to piece together a more nuanced picture of the whole: a reconstruction and understanding of the inflammatory phenotype from the bioarchaeological record.

The IP in skeletal samples: Searching for experimental evidence

It is not enough to make inferences about inflammatory processes based solely on osteological observations. If it is possible to reconstruct an inflammatory phenotype using the above model, then the start of any such analysis must be grounded in the experimental component. Understanding inflammation and inflammatory environments is the necessary first step to provide the context to test subsequent hypotheses. The how and the why of inflammation provide the background to better understand events that may push an individual into a HIP. Findings from the experimental component are necessary for

further considerations of how inflammation, and the HIP, may affect subsequent pathological developments, and what may be observed in the osteological record. The starting point for reconstructing an inflammatory phenotype must be with experimental immunology, to provide the basis for understanding the how and the why of the inflammatory process. These *in vitro* models provide a chance to start to look at the underlying roles of, or at least some specific responses of, inflammatory mediators not possible with the other disciplines of this model. Questions that aim to tease out the basics of immune and altered immune responses start to lay this groundwork.

Would the shift into a HIP be an isolated occurrence, or could typical host-pathogen interactions spur a HIP within the right context? In consideration of the HIP, it becomes prudent to consider a situation in which a familiar pathogen that elicits a known inflammatory response, may end up in turn modifying subsequent inflammatory responses, albeit indirectly. Based on how interconnected the immune system is this makes sense, and this indirect modification of subsequent responses is of particular interest, as this may play into the shift to a HIP. For example, does an infection with *Mycobacterium tuberculosis* indirectly modify the host's subsequent response to later secondary infections, such as those produced by *Porphyromonas gingivalis* or *Staphylococcus aureus* (pathogens commonly associated with nonspecific osteological lesions)? That is, does the initial insult (tuberculosis) influence the host's inflammatory response to the second insult (staph infection), eliciting an inappropriate response to that second insult, which would not have occurred without the initial insult? Is this enough to potentially push the individual into a HIP? An *in vitro* model that compares basic

immune cell reactions to one or more threats provides some insight into whether subsequent insults would be enough to shift the inflammatory response (Figure 2).

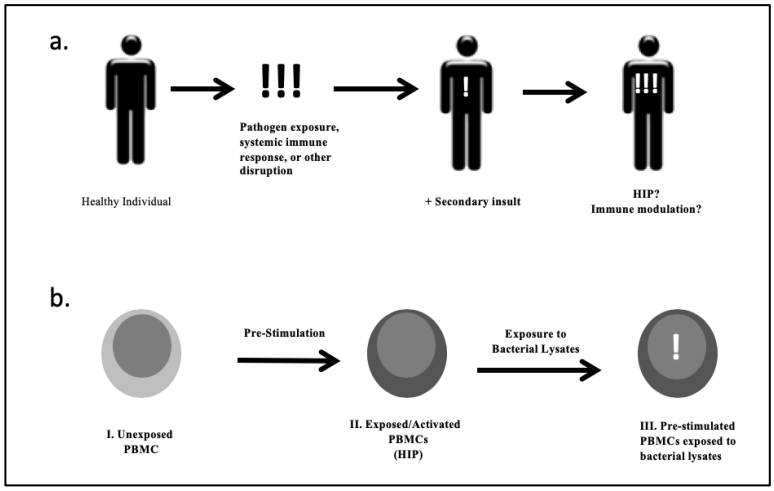

Figure 2 Shifting into a HIP

How might the shift into a HIP occur? a.) The sequence of events in life that may promote a HIP, from an initial insult, followed up with a secondary exposure in the context of an activated immune response, and the potential end result of a sustained inappropriate immune response or HIP. While not directly comparable, b.) presents a basic *in vitro* model that attempts to follow the same trajectory, that compares basic immune cell reactions to initial and secondary exposure and measures the end response. Is this series enough to potentially push an individual into a HIP?

The immune response is more intricate than just a series of set responses. Pathways other than direct pathogen interaction may modulate the inflammatory response. Cytokines are a basic part of the proinflammatory response, and while generally produced by cells at the site of infection, they also play a part in systemic inflammation. Exposure to proinflammatory cytokines can act as primers of immune cells. The next experimental question then becomes, do those immune cells exposed to other proinflammatory mediators subsequently adjust their own inflammatory response? Does, in a sense, priming these immune cells (via contact with proinflammatory mediators) influence their subsequent responses to other insults? An altered immune response based on subsequent exposures, or priming, may be part of the process that contributes to altered or inappropriate inflammatory responses.

The questions raised by experimental immunology reflect back on the broader question of what are the potential consequences of the shift to a hyper-inflammatory phenotype? If it is possible to tease out some of the consequences of such a shift, could

that be used in turn to better understand and reconstruct the inflammatory phenotype, or even immune competence in past populations via the osteological record? While reducing the inflammatory process to basic *in vitro* models leaves out the nuances and confounding factors of real life, it does allow for a starting place to understand the fundamental underlying mechanisms of inflammation, providing a clearer look at the potential ways that this process can be initiated.

Utilizing this four-field framework, and the context and grounding that the experimental components add. as well as understanding the immunological shifts that create inflammatory environments, can help contextualize the end result of pathological processes. Adding in the context of the biosocial environment, reconstructions of the (hyper) inflammatory phenotype have the potential to provide a more nuanced look at health and disease in the past. Specifically, the great potential of the HIP placed within these contexts (immunological; inflammatory; osteological; biosocial) is its integration into analysis of skeletal lesions. The identification of lesions on the bone where inflammation and inflammatory processes were present, or likely present, adds more physical grounding to these questions investigating reconstructing the IP. The pursuit of experimental evidence at all levels lends itself to the creation of a 'skeletal inflammatory index' (SINDEX), a framework which further integrates the cellular and immunological processes in conjunction with mechanisms of lesion formation (Crespo 2018). This index could be used to better understand inflammation and the inherent issues of such, with what is observed in the osteological record. While the IP and immune competence vary from person to person, this index could help to contextualize individuals within a population and a specific environment. The use of this index, utilizing the

multidisciplinary model that provides the backbone for analysis, allows for the potential of creating more comprehensive bioarchaeological reconstructions of health, disease, and stress in these past populations, one piece of the puzzle at a time, all starting with an experimental foundation.

OBJECTIVE AND HYPOTHESIS

Objective

The general objective of this thesis is to provide experimental evidence to contribute to the multidisciplinary reconstruction of IP in skeletal samples (presented in Figure 1).

The specific objective of this thesis is to determine if immune cells activated, or primed, *in vitro*, can generate an inflammatory shift altering their immune responses to pathogens (*P. gingivalis* and *S. aureus*) commonly associated with persistent infections (such as those associated with periodontal disease [PD] or periosteal lesions [PL]).

Hypothesis

We predict that after the first day, pre-stimulated cells (mimicking a hyperinflammatory phenotype) will show increased expression of pro-inflammatory cytokines when these same cells are exposed to oral pathogen *P. gingivalis* or periosteal associated pathogen *S. aureus*. After the second day, we predict that those cells exposed to *P. gingivalis* or *S. aureus* will also show higher expression of inflammatory cytokines (TNFα; IFNγ; IL-1β), when compared to corresponding controls (i.e., non-pre-stimulated cells and *P. gingivalis*, or non-pre-stimulated cells and *S. aureus*).

Rationale

Repeated experiences of acute or chronic stress can induce chronic inflammation, and this can potentially lead to dysregulation of the systemic and local expression of inflammatory proteins, consequently impacting inflammatory processes against local pathogens; ultimately leading to a hyper-inflammatory phenotype (HIP) (Black 2003; Stelekati and Wherry 2012). To "mimic" an inflammatory shift (i.e., to generate cells with a "hyperinflammatory phenotype"), we propose to pre-expose human immune cells to inflammatory inducers such as: lipopolysaccharide (LPS), and pro-inflammatory cytokines interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), and later expose those "pre-stimulated" cells to the pathogens often linked to the osteological markers commonly associated with inflammatory processes (Chen et al. 2015; Kraaij et al. 2014; Li and Lin 2008). Our predictions lend support to the preliminary hypothesis where preliminary exposure to inflammatory inducers will generate an inflammatory shift that will persist and impact a secondary infection by oral or periosteal pathogens. This line of evidence will help in the reconstruction of increased severity (inflammation) in PD and PL in individuals with systemic stress and potentially with systemic inflammation. Recently published evidence on how chronic infections (such as tuberculosis and leprosy) can impose an inflammatory shift on the immune response to oral pathogens may help to partially support these predictions (Crespo et al. 2017).

MATERIALS AND METHODS

Ethics approval

For the *in vitro* analysis peripheral blood mononuclear cells (PBMCs) from healthy voluntary donors were collected at the University of Louisville Nephrology Research Laboratory. The blood sample collection, including the corresponding subject informed consent document, was approved by the Institutional Review Board (IRB) of the University of Louisville; the assigned number is 191.96. The collection of PBMCs and experimental protocols for the current study was marked as Exempt within the same IRB (.96), under tracking number 11.0334.

Cell culture

The PBMCs were collected and subsequently isolated from venous blood at the University of Louisville Nephrology Research Laboratory by dextran sedimentation and density centrifugation in a Percoll gradient as described previously (Haslett et al. 1985). PBMCs were then transported on ice to the laboratory where the experiments were carried out in full. The cells were centrifuged for 10 minutes at 1500rpm at 4 degrees Celsius. The original media was removed, and the pellet was resuspended in 40ml of PBS (Phosphate buffered saline, ph7.4 (*Gibco Life technologies*, ref#10010-02)) in order to wash the PBMCs. A sample (125µL) of the resuspended cells was taken and placed into a 2ml tube on ice. An additional 125µL of Trypan blue stain (*Gibco Life Technologies* .4%

lot #1311086) was added to stain the cells. 10µL were taken from this sample and counted on a hemocytometer. Using a standard microscope, cells were counted according to what fell in the hemocytometers grid. An average of cells per grid was taken. The cell count was determined using the following formula: **N = cell count in grid x 10,000 x 2(dilution factor)**. The final cell count is N x the final volume (40ml). After counting, the cells were centrifuged again at the same conditions. The PBMCs were then re-suspended in complete medium RPMI 1640 containing 10% FCS, penicillin (100 U/ml), streptomycin (100 g/ml), sodium pyruvate (1 Mm), and nonessential amino acids (0.1 Mm).

These protocols are based on a cell density of 4 x 10^6 in 1ml of media per well. The volume of media required for resuspension to achieve the final cell density per well, was determined by the number of PBMCs available from the donor on that day. The total number of available PBMCs varied with each donor, but all wells and conditions had the same cell density per well. Immediately after resuspension in the appropriate volume of media, the cells were seeded into 24 well plastic cell culture plates at a density of 4 x 10^6 in 1ml of media per well and subsequently followed the corresponding experimental protocols. This collection protocol has been successfully utilized in this laboratory previously (Crespo et al. 2017).

Reagents for experimental protocols

PBMCs were pre-stimulated/primed *in vitro* with lipopolysaccharide (LPS) (from *E. coli* 0111: B4; a general proxy for bacterial infection) (*Sigma*, St. Louis, MO) at a working concentration of 10µL per the final volume of 1ml for each well. LPS was used to act as a general T-cell receptor stimulus agonist to induce an inflammatory response

and used primarily as a positive control. Recombinant TNFα and recombinant IFNγ (*Affymetrix-eBioscience*, San Diego, CA) were used for the priming of PBMCs, as well as to induce a hyperinflammatory response. TNFα was used at a working concentration 10µl/well, and IFNγ was used at a working concentration of 5.25µl/well. For mimicking the bacterial infection several different whole sonicated bacterial lysates were used. *Mycobacterium tuberculosis* (Seeley et al. 2008) (BEIResources, NR-14822) was added at a working concentration of 1µL/well. *Mycobacterium leprae* (LP) (BEIResources, NR-19329) was added at a working concentration of 10µL/well. *Porphyromonas gingivalis* (Pg) (obtained from Dr. Richard Lamont's lab at the Department. of Oral Immunology and Infectious Diseases; University of Louisville) was used at a working concentration of 100µL/well. Finally, *Staphylococcus aureus* (Sa) (Pansorbin cells-Calbiochem # 507861, EMD Millipore, Billerica, MA), was used at a working concentration of 4.4µL/well. *S. aureus* was heat killed and formalin fixed unlike the other three whole sonicated lysates.

Experimental protocols

A 48-hour experiment was set up (Figure 3) to investigate how pre-stimulated/primed PBMCs *in vitro* can generate an inflammatory shift, that is, alter their inflammatory response to sequential exposure to pathogens. After re-

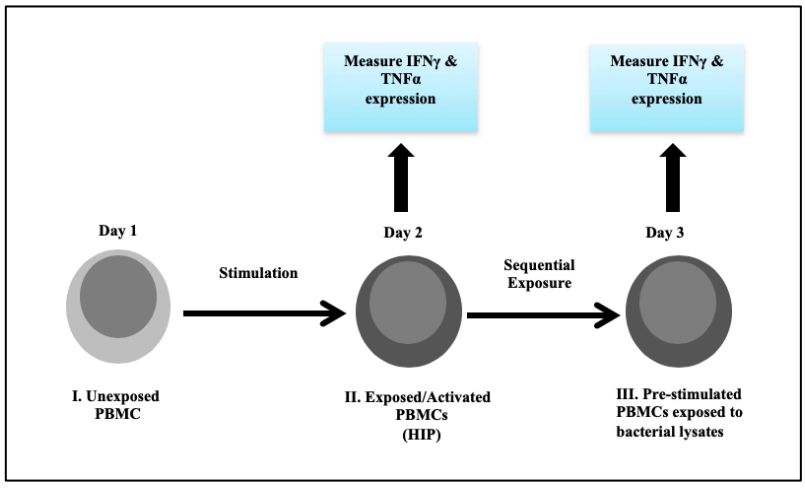

Figure 3 The General Experimental Model
The multiday in vitro experimental model to investigate PBMCs response to stimuli, that is, how the inflammatory response may be altered.

suspension and seeding onto a 24 well plate, PBMCs were exposed to either LPS, TNFα, or IFNγ (as well as bacterial lysates). The cells were left for a period of 24 hours, constituting the first day. On the second day, the PBMCs were collected, and pipetted into 1.5ml tubes. The cells were then centrifuged at room temperature for 2 minutes at 6000rpm. The supernatants were collected and aliquoted into 300µl portions and stored at -20 degrees Celsius. The cells were then re-suspended in 1ml of fresh RPMI and reseeded onto the plate, in previously unused wells. The cells were then exposed to bacterial lysates or other controls and left for an additional 24-hour period (constituting day 2). This process was repeated at the end of day 3 (the end of the 48-hour period), with 300µl aliquots of supernatant placed into storage at -20 degrees Celsius.

The aliquots of the supernatants were used primarily for cytokine analysis of TNFα; IFNγ; or IL-1β by an enzyme-linked immunosorbent assay (ELISA) (*Affymetrix-eBioscience*, San Diego, CA). Extensive controls were used on both days of the experimental protocol (see appendix 1 for example of an in-lab experimental protocol form). To avoid disruption in cytokine expression, the experimental protocols were not run longer than a total of 48 hours. While experimental time length varies depending on the clinical or research question, this time window (48 hours) was the most conservative and allowed for the comparison of the results with most cytokine *in vitro* profiles in healthy donors (for a comprehensive review see (Warle et al. 2003)). Three variations on this basic experiment were run.

Protocol 1: Pre-activation with bacterial lysates and sequential immune responses to S. aureus and P. gingivalis

The objective of this protocol was to determine if *in vitro* exposure of PBMCs to *Mycobacterium tuberculosis* or *Mycobacterium leprae* (LP) lysates (mimicking a HIP) impacted subsequent immune responses to persistent, or local, pathogens, either *S. aureus* or *P. gingivalis.* That is, does a higher expression of inflammatory cytokines (in circulation due to pathogen exposure) generate a systemic shift, and if so, does this systemic hyperinflammation in turn affect inflammatory responses against other persistent infections, such as the one often present in periosteal lesions (commonly associated with *S. aureus*) or periodontal disease (commonly associated with *P. gingivalis*)? To measure whether cytokine expression changed with early exposure to TB or LP lysates *in vitro* human PBMCs were exposed to Sa, TB, or LP lysates on day 1, and subsequently on day 2, cells were collected, washed, and resuspended in fresh medium, and exposed for another 24-hour duration to the same, or a different pathogenic insult (Figure 4). All supernatants were collected on day 2 and day 3.

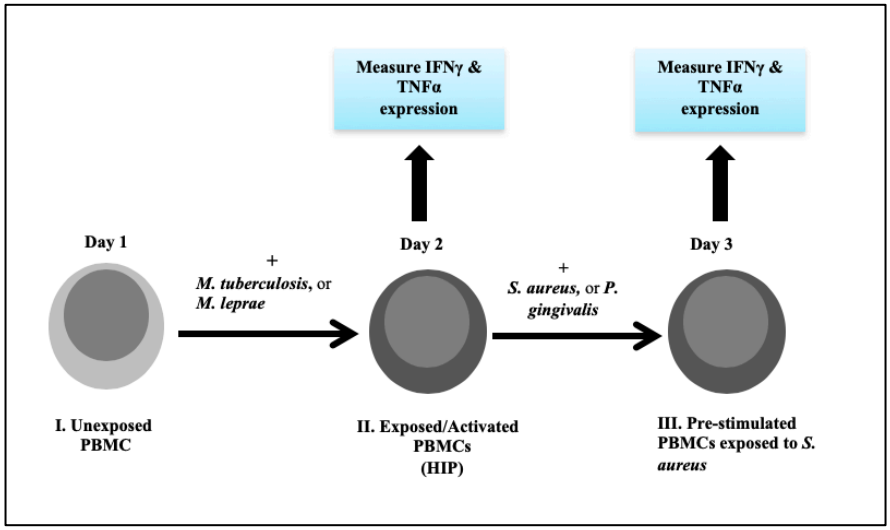

Figure 4 Protocol 1: Pre-activation with bacterial lysates
In this protocol, naïve PBMCs from healthy donors were first exposed to bacterial lysates, and then sequentially exposed to a second bacterial lysate, either Pg or Sa. The initial exposure to TB or LP mimicked a systemic HIP, and the subsequent responses to further pathogen exposure were measured.

Protocol 1 contributes to the objective of the thesis by exploring what might generate an inflammatory shift, altering subsequent immune responses to pathogens that are commonly connected with persistent infections associated with periosteal lesions and periodontal disease, observable in the osteological record. That is, can other infections be a driving factor in generating a systemic inflammatory shift?

Protocol 2: Pre-activation with proinflammatory mediators and sequential immune responses to S. aureus and P. gingivalis

The objective of this protocol was to determine if pre-stimulated PBMCs would shift the inflammatory response when those same cells were exposed to subsequent pathogenic insults. In this approach (Figure 5) naïve PBMCs were initially exposed to LPS, or proinflammatory cytokines TNFα, or IFNγ, to prime the cells, but not bacterial lysates. After 24 hours, the cells were collected, washed, and resuspended in fresh medium (same as previous protocols) and then exposed to either Pg or Sa. Supernatants were collected at the end of day 2 and day 3. This approach allowed for investigation of whether the pre-stimulation of naïve PBMCs (mimicking the hyperinflammatory phenotype) could induce an immunological shift, that

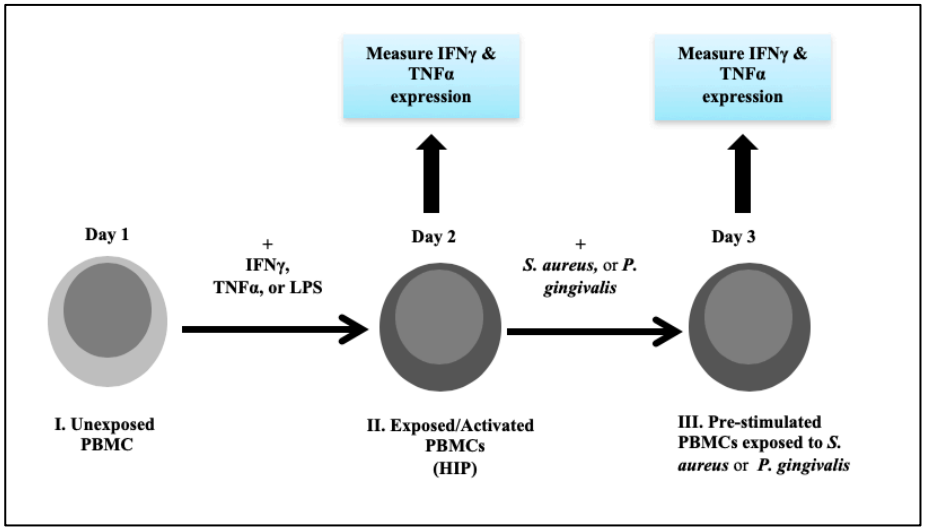

Figure 5 Protocol 2: Pre-activation with proinflammatory mediators
In this protocol, naïve PBMCs were primed via exposure to a proinflammatory mediator (cytokine or LPS), and then sequentially exposed to a bacterial lysate, either Pg or Sa. The priming of the PBMCs was in order to determine if the stimulated cells would then alter the inflammatory response with subsequent pathogen exposure.

is, change the inflammatory response with sequential exposure to oral or periosteal pathogens (*P. gingivalis* or *S. aureus*). Those pathogens are commonly associated with persistent infections (such as those associated with periodontal disease [PD] or periosteal lesions [PL]), and often linked to the osteological markers commonly associated with inflammatory processes.

Protocol 2 directly reflects the specific objective of this thesis: to determine if immune cells activated/primed *in vitro*, can generate an inflammatory shift altering their immune responses to pathogens (*P. gingivalis* and *S. aureus*) commonly associated with persistent infections and osteological markers associated with inflammation, even if the stimulating factor was not direct exposure to a pathogen.

Protocol 3: Indirect immune responses

The objective of this protocol was to investigate if immune factors released by PBMCs exposed to a pathogenic insult would affect subsequent cytokine expression by naïve PBMCs, or PBMCs previously exposed to *S. aureus*. In this approach, (Figure 6) supernatant from PBMCs that had been

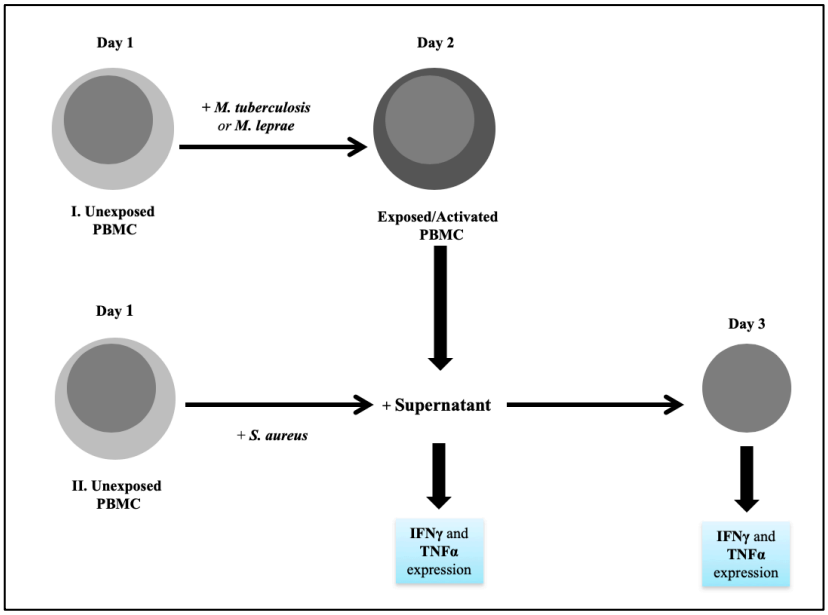

Figure 6 Protocol 3: Indirect Immune Responses

In this protocol, the supernatant of previously exposed PBMCs was used to determine if an indirect immune response might elicit an inflammatory response, for either naïve cells, or PBMCs previously exposed to Sa. Only the products from PBMCs previously exposed were used in the sequential exposure, no new lysates or proinflammatory mediators were used to elicit a response.

exposed to TB or LP and collected on day 1 was subsequently used on naïve PBMCs (or primed by Sa) for the day 2 exposure. All supernatants were collected on day 2 and day 3, following the same procedure in protocol 1. This allowed for an investigation of whether there would be a subsequent shift in the expression of immune proteins, as well as tested whether immune factors (cytokines TNFα; IFNγ) released by activated PBMCs could affect the cytokine expression of PBMCs (naïve or exposed to Sa) mimicking a systemic impact on distant tissues.

Protocol 3 contributes to the objective of the thesis by investigating one route that immune cells may become activated/primed, even indirectly via exposure to other circulating cytokines and not through direct pathogen contact. Indirect stimulation of the immune system can have systemic consequences, of importance to inflammatory processes associated with osteological lesion formation. Protocol 1 measures cytokine expression from PBMCs directly exposed to lysates, while this protocol measures cytokine expression from naïve cells with indirect exposure.

Cytokine assays

Cytokine expression (TNFα; IFNγ; IL-1β) was determined by an enzyme linked immunosorbent assay-ELISA (*Affymetrix-eBioscience*, San Diego, CA). This technique allows for the quantitation of soluble proteins in the samples; that is, which immune proteins were present in the collected supernatants from the experiments. The established protocol outlined by the manufacturer of the assay kits was followed, a brief summary follows: the provided 96 well plates were coated (diluted coating buffer and deionized water) and plated with the capture antibody. The plate was left at 4 degrees Celsius overnight. On the second day, the plate was washed three times with washing buffer

(450µl deionized water, 50µl PBS, and 250µl Tween (Sigma Life Sciences, #P2287)). The supernatants to be used were thawed on ice, while the plate was blocked with diluent for an hour. 200µl of each supernatant was pipetted into the appropriate wells, and a set of standards was also added (see appendix 2 for an example of an ELISA plate layout; one was filled out for each plate run). The plate was left overnight at 4 degrees Celsius. On the third day the plate was washed thoroughly with washing buffer, the detection antibody was added and left at room temperature for one hour. After another round of washing, the enzyme was added. After 30 minutes the substrate was added. At this point the color of the plate changed from clear to blue if the plate had been set up correctly, visible at least in the 6-8 standards and controls added to every plate. After 10 minutes a stop solution was added (H_2SO_4 and deionized water). The color changed from blue to yellow and the plate was read on a microplate reader (*Biorad*, iMark reader). Concentrations were calculated by comparison with recombinant cytokine standards. Within each sample (healthy donor) all experimental conditions were run by duplicate, and the results were compared to stimulus-free controls and standard positive controls.

Statistical analysis

The statistical analyses were run on data collected over the course of several years. Data collected specifically for this thesis is based on 26 individual healthy donors. Previous relevant data and conditions from the laboratory consisting of an additional 27 healthy individual donors are included where relevant to help substantiate experimental conditions. As previously conducted for similar experimental protocols, within each sample (one healthy donor) all experimental conditions were run by duplicate, and the

results were compared to both stimulus-free controls (untreated conditions) and positive controls (Crespo et al. 2017).

Some conditions expressed low enough cytokine expression that it was below the limit of detection of the plate reader and is represented by a (-) sign in the raw data. While the exact value is unknown, for purposes of statistical analysis all zero values are replaced with .005, the plate readers mechanical limit of detection. Using the limit of detection as a place holder allows for analysis without assuming that those values are a true zero. Some conditions expressed a high enough cytokine reading that it was also beyond the limits of the plate reader. The plate readers photometric optical density limit is 3.5. Values higher are represented by a (+) sign in the raw data. For the purposes of statistical analysis, all of these values were interpolated based on a line of best fit ($y=mx + b$), based on the particular slope and intercept for the specific plate that the high readings were run on.

For the statistical analysis outliers were identified and removed. While outliers from biological data do not necessarily represent mistakes or errors, they were removed here for the purposes of subsequent between group comparisons (Motulsky 2014). Including outliers allows for the possibility that the interpretations will be skewed. To balance between removing outliers, but not removing important data, a conservative approach was taken for identification of outliers. Outliers were determined as falling outside a range of three times the standard deviation plus the mean, or the mean minus three times the standard deviation. Using three times the standard deviation (as opposed to two times or less) helps to reduce the absolute size of bias, as by extending the range fewer observations are removed (Miller 1991). The extended range captures as much of

the inherent variation as possible, but also removes extraneous values well beyond the distribution of the group. Every condition was placed into a scatterplot to visually check the distribution for outliers as well.

Outliers were removed from conditions as opposed to from donors. Every experimental condition was tested for outliers, for example the condition LP/LP for TNFα with a sample size of 38, had one outlier. Each condition has a different sample size, as not all conditions were run for every donor. Individual donors are not compared to one another, rather it is the group of donors as a whole that contributes to each condition, and these groupings are compared to one another. This more conservative approach to outliers allows each condition regardless of sample size to keep as many donors as possible to preserve inherent variability, but also removes data that is likely to skew the end results. Removing an entire donor could impact the distribution of all other conditions run with that donor. However, if a donor presents with multiple outliers over the experimental conditions in which they were run, then by removing those from each experimental condition where they present amounts to the same as removing the entire donor.

The interpolation of values and the removal of outliers prepared the data for analysis. The initial statistical analysis was run on GraphPad Prism. Before comparing any of the groups, F-tests were run, which tested the hypothesis that two groups being compared will have identical standard deviations and thus equal variances (Motulsky 2014). The F-test was used as a test against the normality assumption, that is, the distribution of each group is identical. Distribution matters, as comparing groups

assumed to be in Gaussian distribution with groups that are not would not produce valid results; Gaussian distribution was not assumed.

The F-test determined distribution of the group, indicating whether a parametric test or nonparametric test was relevant. Based on the results of the F-test, which indicated that the groups were not equal in variance, a nonparametric test was chosen: the Kruskal-Wallis one-way ANOVA. This compared the means of three or more groups (Motulsky 2014). The "one-way ANOVA computes a single P value that tests the null hypothesis that all groups were sampled from populations with identical means" (Motulsky 2014). Groups were limited to four to five conditions, generally the 24-hour condition and subsequent 48-hour exposures. The one-way ANOVA indicated whether within this group if there was any significant difference between the means compared. However, this does not indicate which conditions are different from one another within the group. To determine which conditions are significant from the Kruskal-Wallis test, a post hoc nonparametric test is required.

Dunn's multiple comparison is a post hoc nonparametric test, which tests the difference between groups, and tends to produce very conservative results (Motulsky 2014). For our purposes, the groups Dunn's multiple comparison test is testing are the individual conditions within the subset group the one-way ANOVA was run on. Most importantly, groups can be unequal in size, as this is a nonparametric test. From this, an adjusted P value of less than .05 was used to determine significance, with an adjusted P value of less than .1 to be considered a trending value and notable for discussion.

For the case of the cytokine IL-1β, where limited samples were available, the number of comparisons of groups available was often only two at a time, less than the

required number for the previous tests. A one-way ANOVA was run for all of the IL-1β experimental conditions and groups when available, such as broad comparisons of Pg or Sa. For further comparisons, the Mann-Whitney test was used. This nonparametric test is used to compared two unpaired groups to compute the P value, that in larger groups the Dunn's multiple comparisons was used for (Motulsky 2014).

While nonparametric tests do not have the same power as parametric tests, for the purposes here nonparametric tests best fit the data. The nonparametric tests which focus on the median, are a better description of this data. Even with the removal of outliers, high data points within a group can skew the mean. The nonparametric tests observe the ranking of the data instead, and while these tests tend to be quite conservative, these are more appropriate for the data.

RESULTS

The specific objective of this thesis is to determine if immune cells activated/ primed *in vitro*, can generate an inflammatory shift altering their immune responses to pathogens (*P. gingivalis* and *S. aureus*) commonly associated with persistent infections (such as those associated with periodontal disease [PD] or periosteal lesions [PL]).

Protocol 1 Pre-activation with bacterial lysates

The objective of this protocol was to determine if *in vitro* exposure of PBMCs to *Mycobacterium tuberculosis* or *Mycobacterium leprae* lysates (mimicking a HIP) impacted subsequent immune responses to persistent/local pathogen *S. aureus* commonly associated with periosteal lesion formation (Figure 7).

Of particular interest in these experiments are the comparisons of conditions that are followed up by exposure to Sa. Overall, exposure to Sa appears to elicit a response as measured by

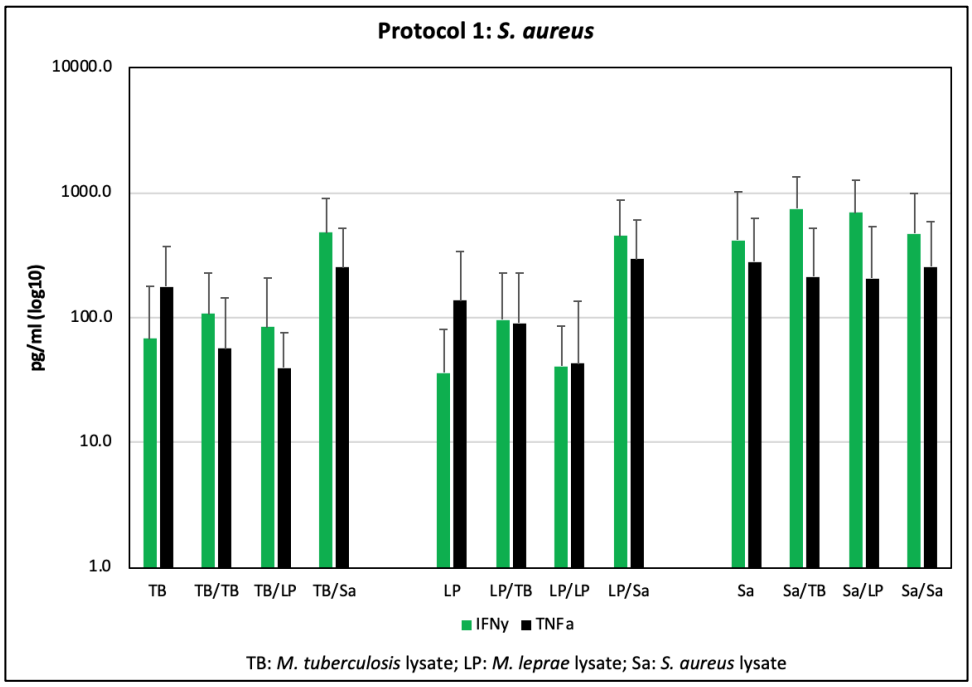

Figure 7 IFNγ and TNFα expression from PBMCs exposed to bacterial lysates TB, LP, or Sa, followed with sequential exposure to TB, LP, or Sa.

cytokine expression of both TNFα and IFNγ. TNFα expression for conditions TB/TB compared to TB/Sa is trending with an adjusted P value of .0654, showing an increase in TNFα for TB/Sa. Initial exposure to LP also appears to promote an increase in TNFα, with comparison of TNFα expression for LP/LP to LP/Sa, with a significant adjusted P value of .0009, where expression is increased with exposure to Sa. Even with the same background (early exposure to LP), follow up with Sa produced a statistically significant increase in TNFα expression. The initial response to Sa, and subsequent exposure to Sa produces a uniform response for TNFα expression. Variations on TNFα expression are most apparent with initial exposure to either TB or LP.

IFNγ expression is in general higher than TNFα. Similar significant differences are observed with an initial exposure to TB followed by a second exposure. IFNγ expression for TB/TB as compared to TB/Sa is significant with an adjusted P value of .0135, and TB/LP as compared to TB/Sa, is significant with an adjusted P value of .0007. In both cases, subsequent exposure to Sa increases cytokine expression. In addition, LP/LP as compared to LP/Sa is also significant, with an adjusted P value of .0003. Even in comparing UNT/LP to UNT/Sa, there is a difference in how these cells respond to the different lysates, with Sa inducing greater cytokine expression, with a trending adjusted P value of .0521 (see appendix for expanded results and adjusted P values).

This question can also be extended to *Porphyromonas gingivalis*: does *in vitro* exposure of PBMCs to TB or LP lysates (mimicking a HIP) impacted subsequent immune responses to persistent/local pathogen Pg commonly associated with periodontal lesion formation (Figure 8)? Initial exposure to LP appears to promote an increase in TNFα expression, as observed in comparison of LP/LP to LP/Pg, with a significant

adjusted P value of .0167. For exposure to TB, a comparison of TB/Pg to Pg/Pg TNFα expression was also significant, with an adjusted P value of .0414. In both cases, subsequent Pg exposure exhibited increased TNFα expression. While cytokine expression was uniform following exposure to Sa and subsequent lysates, here exposure to Pg and subsequent exposures generally decreased both TNFα and IFNγ expression (see appendix for expanded results and adjusted P values for Pg).

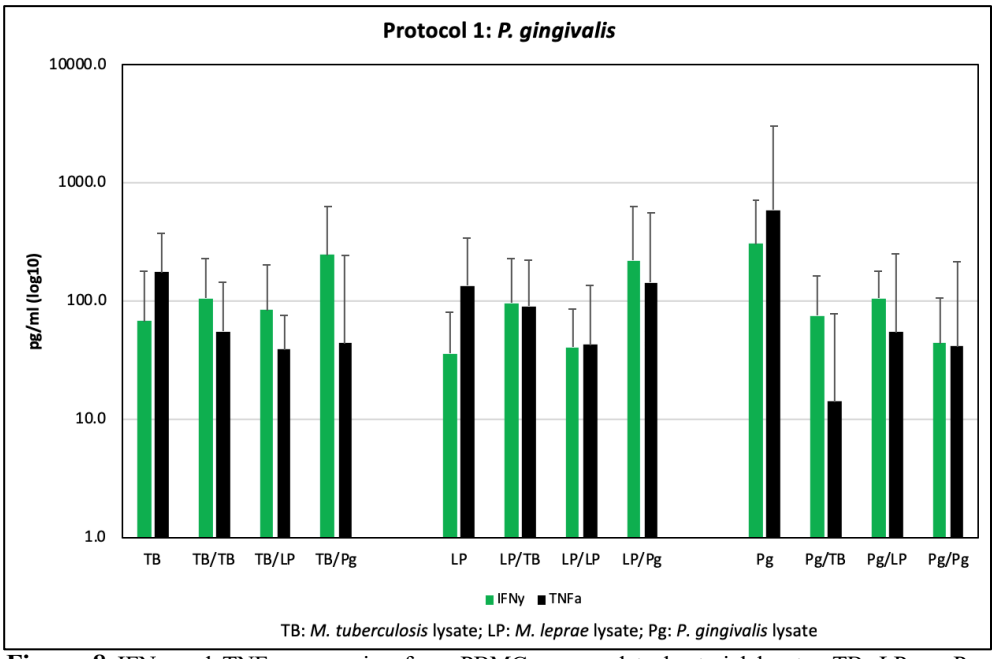

Figure 8 IFNγ and TNFα expression from PBMCs exposed to bacterial lysates TB, LP, or Pg, followed with sequential exposure to TB, LP, or Pg

Protocol 2 Pre-activation with proinflammatory mediators

The objective of this protocol was to determine if pre-stimulated or primed PBMCs can generate an inflammatory shift altering their immune responses to pathogens commonly associated with persistent infections. Here, PBMCs were primed with proinflammatory cytokines and then exposed to either PG or Sa lysates (Figure 9; Figure 10). As was expected, there were significant differences in cytokine expression between the untreated controls followed up with exposure to one of the bacterial lysates (see appendix for full results and adjusted P values).

Changes in TNFα expression were of particular interest with secondary exposure to Pg. LPS/Pg as compared to IFNγ/Pg showed an increase in expression with an adjusted P value of .0163, an interesting difference given the different proinflammatory conditions. However, TNFα expression was decreased as compared between TNFα/Pg compared to Pg/Pg with an adjusted P value of .0011, and between IFNγ/Pg compared to Pg/Pg, with an adjusted P value of <.0001. It appears here that early exposure to a proinflammatory cytokine elevated TNFα expression, as opposed to only exposure to the bacterial lysate, and for initial priming with IFNγ, significant increases in TNFα are observed following

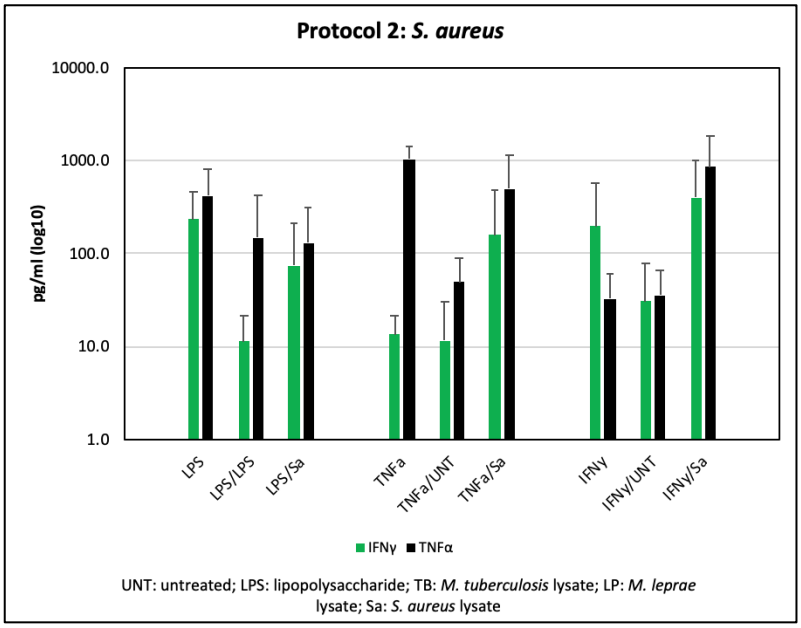

Figure 10 IFNγ and TNFα expression from PBMCs primed with proinflammatory mediators, followed with sequential exposure to Sa.

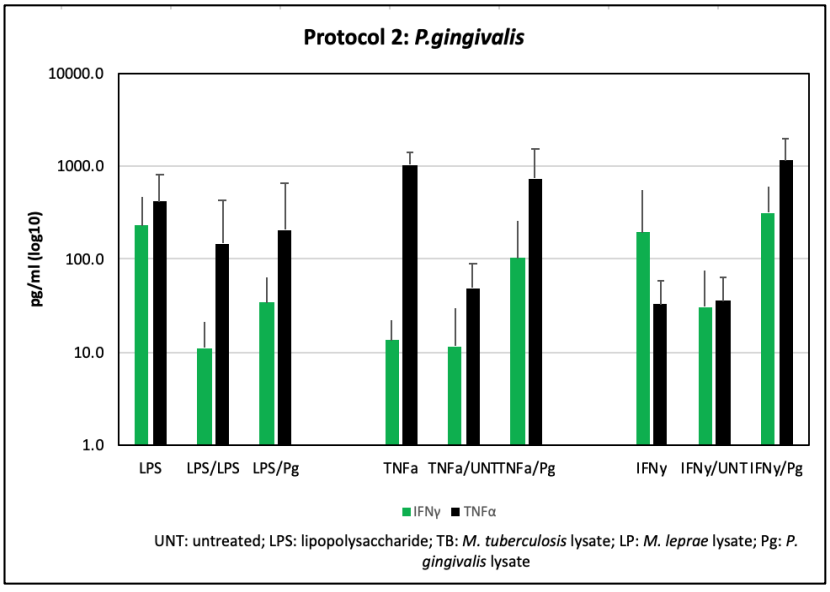

Figure 9 IFNγ and TNFα expression from PBMCs primed with proinflammatory mediators, followed with sequential exposure to Pg.

with either Pg or Sa compared to UNT. Early priming with either TNFα or IFNγ produced significant increases in TNFα expression when followed by Pg as compared to Pg/Pg only. For early exposure to LPS, a component of gram-negative bacteria and used as a proxy for pre-existing bacterial infection, there was little difference in follow up exposures for TNFα expression. Early exposure to LPS did produce elevated TNFα expression, but less so as when compared to the same conditions for IFNγ (LPS/Pg compared to IFNγ/Pg, and LPS/Sa compared to IFNγ/Sa). While most comparisons regarding Sa were not significant, LPS/Sa as compared to IFNγ/Sa was borderline trending with an adjusted P value of .1242 for TNFα expression. While not significant, it is more interesting than the other conditions that generated adjusted P values of >.9999.

IFNγ expression also displayed significant differences in the untreated controls with follow up exposure to a bacterial lysate (see appendix). IFNγ/Pg compared to Pg/Pg was statistically significant with an adjusted P value of .0268, with a decrease in IFNγ expression. LPS/Pg as compared to IFNγ/Pg was trending with an adjusted P value of .0991, with an increase of cytokine expression following priming by IFNγ, even though LPS is proinflammatory. IFNγ/Pg as compared to IFNγ/UNT presented a trending value for an increase in expression with an adjusted P value of .0531. Interestingly, LPS/Sa as compared to Sa/Sa, with a trending adjusted P value of .0875 and TNFα/Sa as compared to Sa/Sa, with a trending adjusted P value of .0875, displayed decreased cytokine expression as compared to just exposure to Sa, the opposite of what was observed with Pg and TNFα expression. LPS as compared to LPS/LPS actually exhibited a decrease in IFNγ expression, and LPS in general did not elicit a strong response regardless of follow

up. Interestingly there were not significant differences observed with initial exposure to IFNγ for IFNγ expression, unlike the dramatic changes observed with TNFα.

Due to the limits of how many analyses could be run on the supernatant from one well (condition) (each ELISA used 200µl of supernatant) limited conditions were run for IL-1β. IL-1β expression measured in the same range at IFNγ and TNFα, with generally homogenous expression regardless of the priming factor or subsequent exposure (Figure 11). Of interest for IL-1β, were the proinflammatory mediators. A comparison of LPS/Sa to IFNγ/Sa was significant with an adjusted P value of .0112, with LPS/Sa exhibiting a higher IL-1β expression. The same comparison for TNFα expression was slightly trending with an adjusted P value of .1242, where IFNγ/Sa exhibited the higher expression. IFNγ/Sa as compared to IFNγ/Pg was significant with an adjusted P value of .0317, with IFNγ/Sa exhibiting a reduced IL-1β expression as compared to IFNγ/Pg. Sa/Sa as compared to LPS/Sa was not significant, but with a trending adjusted P value of .0986 is of interest, with LPS/Sa exhibiting a higher IL-1β expression. Interestingly, LPS/Sa as compared to Sa/Sa for IFNγ was

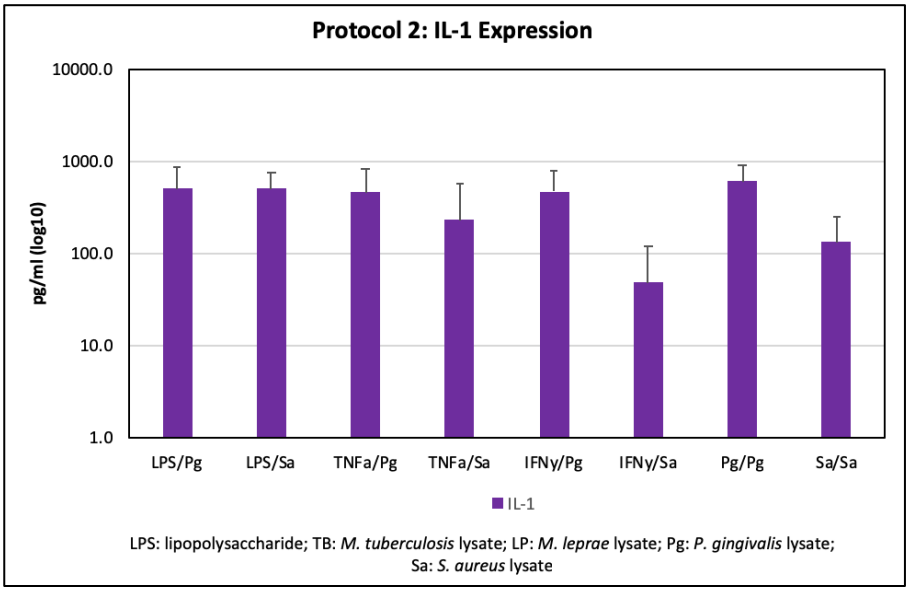

Figure 11 IL-1β expression from PBMCs primed with proinflammatory mediators with subsequent exposure to either Pg or Sa.

trending with an adjusted P value of .0807, with Sa/Sa exhibiting a higher expression, the opposite of what was observed with IL-1β.

For this protocol, a comparison of all cytokine expression was also made. Expanding the comparisons to the expression between the cytokines themselves for this protocol showed some significant differences in cytokine expression to different experimental conditions. Comparisons were also run between each of the three cytokines for the available conditions, results as follows. IL-1β expression was elevated significantly compared to IFNγ (adjusted P value .0042), but not TNFα for the LPS/Pg condition. IL-1β exhibited increased cytokine expression as compared to both TNFα (adjusted P value .0339) and IFNγ (adjusted P value .0009) for the LPS/Sa condition. Both IL-1β and TNFα were significantly elevated as compared to IFNγ (adjusted P value .0361 for IL-1β and .0046 for TNFα) for TNFα/Pg, but IL-1β and TNFα again showed no significant difference between the two. No significant difference for TNFα/Sa, but a trending difference between IFNγ and TNFα expression, with elevated TNFα expression. TNFα was elevated compared to IFNγ (adjusted P value .0225) for the condition IFNγ/Pg, but no difference was detected between TNFα and IL-1β expression. IL-1β was significantly reduced compared to TNFα for IFNγ/Sa (adjusted P value .022). For the Pg/Pg response, IL-1β expression was significantly elevated as compared to the IFNγ (adjusted P value .0002) and TNFα (adjusted P value .0037) response for Pg/Pg. There was no detectable difference in response to Sa/Sa.

IL-1β expression in general was elevated as compared to the other two cytokine responses (see appendix). While no significant difference in response was observed in response to Sa/Sa between the cytokines, it is clear however, that there is immune cell

activation, as these PBMCs are still continuing to produce cytokines. Visually examining the experimental groups in addition to the statistical analysis provides additional information. Each dot on these column scatterplots (Figures 12,13) represents the cellular response for one individual donor. In certain instances, there is a clear division within the group of responders and non-responders. Responders are those PBMCs that exbibit cytokine production in response to the experimental conditions, regardless of whether it is significant or not. The non-responders are those PBMCs that exhibit little to no cytokine activity regardless of the exposure. Here it does appear that there is variation in how each sample is reacting within the different cytokine groupings, a difference that would be worth further consideration in future experiments, as IFNγ and TNFα show variation in responders while lL-1β does not (Figure 12).

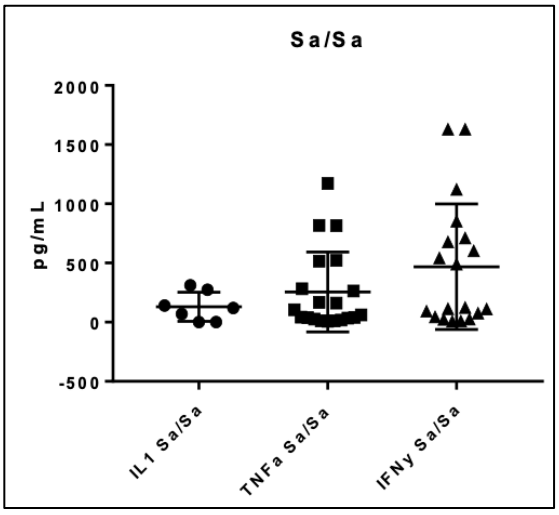

Figure 12 Variation in cytokine expression for Sa/Sa conditions.

The same observation can be made for the TNFα/Sa condition as well, with no significant differences observed, but a trending difference noted between IFNγ and TNFα expression, with elevated TNFα expression. Here, there is notable distribution variation (Figure 13). Responders in the TNFα grouping are

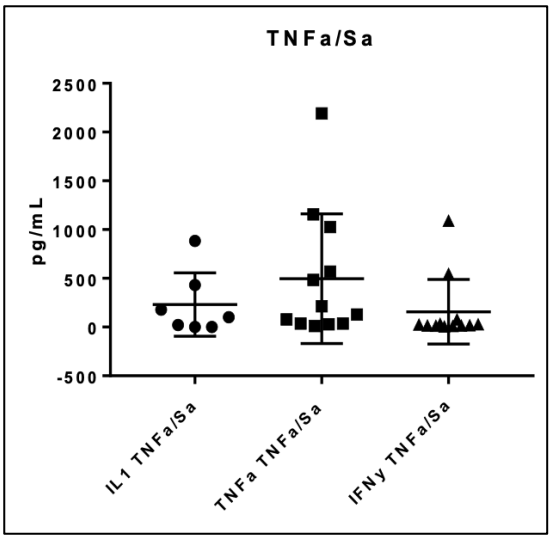

Figure 13 Variation in cytokine expression for the TNFα/Sa condition.

varied for both this condition as well as for Sa/Sa. This response may also suggest that those individuals whose cells are more likely to respond aggressively to stimuli, might also be those more likely to enter into a HIP.

Protocol 3 Indirect immune responses

The objective of this protocol was to investigate if immune factors released by PBMCs exposed to a pathogenic insult would affect subsequent cytokine expression by naïve PBMCs, or PBMCs previously exposed to Sa. For this particular protocol we chose to focus only on the stimulation of PBMCs that had been exposed to, or subsequently exposed to Sa, or Sa supernatant. Pg and Pg supernatant was not available for this round of experiments. One of the limitations of this experiment was that there were limited samples available, and the sample size for these experimental conditions tended to be smaller than the conditions for the other two protocols. Overall, previously unexposed PBMCs did exhibit activity based on exposure to supernatants alone on the second day

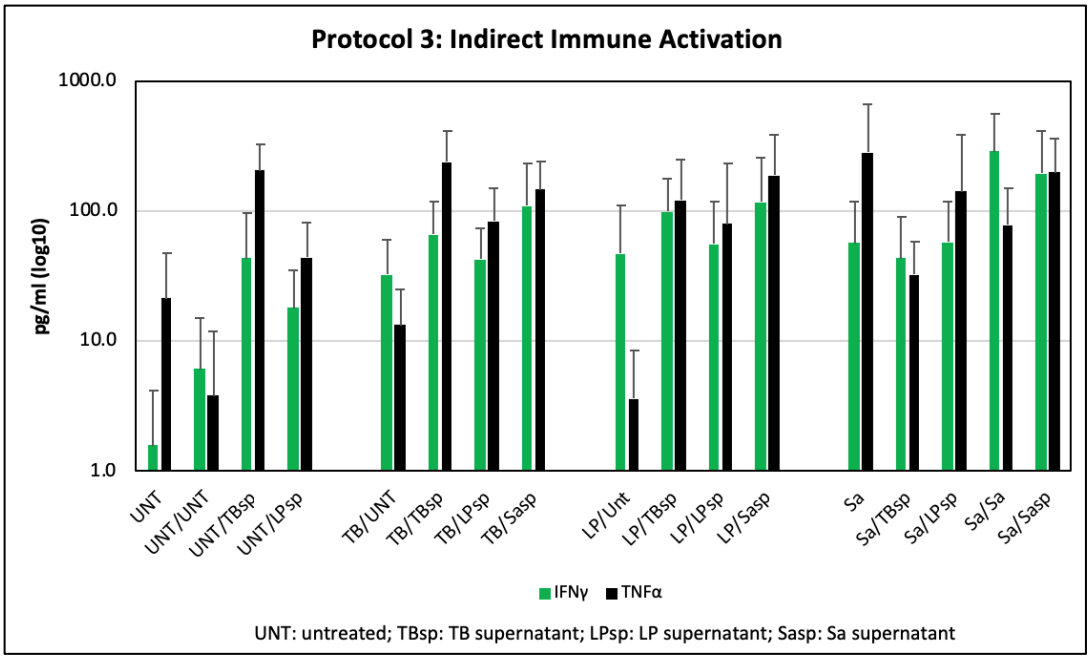

Figure 14 IFNγ and TNFα expression from PBMCs sequentially exposed to supernatant from activated PBMCs

and cytokine expression does not appear to decrease with exposure to the supernatants (Figure 14).

For TNFα expression, there was a significant difference between the UNT/UNT and the UNT/TBsp condition, with TBsp eliciting an increase in cytokine expression, with an adjusted P value of .0017. This elevated response for TNFα was also observed in comparison of TB/UNT to TB/TBsp with an adjusted P value of .0367. Here the early exposure to TB followed by exposure to the supernatant of PBMCs previously exposed to TB maintained an elevated cytokine response, one not observed when TB was followed up by the UNT condition.

Early exposure to LP provided significant increases of TNFα expression followed up with Sasp as compared to LP/UNT (adjusted P value of .0299), or followed up with TBsp as compared to LP/UNT (adjusted P value of .0419), highlighting again the increase in cytokine production that would likely not occur without the supernatant stimulus. For IFNγ expression, a significant increase was observed in comparing the UNT condition with UNT/TBsp, with an adjusted P value of .008. IFNγ expression for UNT as compared to UNT/Lpsp, was trending with an adjusted P value of .0776 (see appendix for full additional adjusted P values).

For early exposure to TB, LP, or Sa, there were no significant differences to report within or between any of the groups with subsequent exposures to supernatants. It is clear however, that there is immune cell activation, as these PBMCs are still continuing to produce cytokines. One of the limits of the tests used to determine significance here is that nonparametric tests tend to have less power with smaller sample sizes, and will not pick up on nuances in the data (Motulsky 2014). It would be inappropriate to use another

test for Protocol 2 data alone, and the distribution and methodology of this experiment indicate that this nonparametric test is still the best test for this data despite the small size limitation. Visually examining the experimental groups in addition to the statistical analysis provides additional information. By looking at the conditions grouped together by exposure in a column scatterplot, even with small samples sizes, this provides information about the distribution of the samples taken.

In certain instances, there is a clear division within the group of responders and non-responders. Responders are those PBMCs that exbibit cytokine production in response to the experimental conditions, regardless of whether it is significant or not. The non-responders are those PBMCs that exhibit little to no cytokine activity regardless of the exposure. For example, in comparing conditions that are exposed to LP first and then followed up with supernatants, LP/TBsp is not significantly different from any other group except for the LP/UNT. The distribution of the samples shows that for LP/TB, half exhibit limited cytokine expression, but several others have elevated expression (Figure 15). Non-response may occur for a variety of reasons, and for future experiments an increased sample size would allow for the removal of non-responders (not just outliers). Analysis of only the responders to the experimental prompt may provide more information. The non-responders are not the same as an outlier, and do not meet the

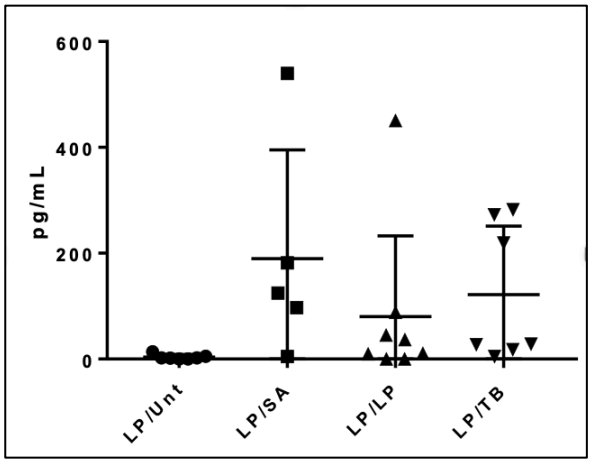

Figure 15 Variation in cytokine expression for PBMCs exposed to LP and sequentially exposed to various supernatants in protocol 3

criteria for exclusion based on three times the standard deviation subtracted from the mean.

DISCUSSION

Pre-activation with bacterial lysates

The first experimental protocol investigated what might generate an inflammatory shift by determining if exposure to bacterial lysates would impact subsequent immune responses to another pathogen, in this case lysates of either *S. aureus* or *P. gingivalis*. TB and LP lysates served to create and mimic an environment in which there would be a higher expression of inflammatory cytokines already in circulation due to the previous exposure. In general, for both IFNγ and TNFα, the PBMCs exhibited increased cytokine expression between the untreated controls and subsequent exposures. This suggests that subsequent exposure to these lysates (Sa and Pg) elicits a greater cytokine response, which may be the type of response needed to push an individual into or maintain a HIP. That there are differences between the PBMCs' response to different lysates with no prior experimental exposure (UNT), highlights an interesting response, and one that appears amplified with prior exposure to other lysates as observed in other conditions. These differences in subsequent exposure suggest that some immune alteration is occurring.

Subtle variations in specific cytokine expression are both a reflection of individual variability as well as the physiological role of that cytokine. While the *in vitro* lab experiments do not capture the inherent complexity of these infections, some of the basic cytokine responses of such are still relevant. One of the limiting factors of these experimental models is the focus on a selected few cytokines, while in reality there are a multitude of cytokines that act in concert with one another and exert profound influences

on production and maintenance of cytokines, which in turn influences control of bacterial growth (Cooper et al. 2011). Cytokines play an array of roles depending on context, and for mycobacterial species they can act as both effectors and regulators of immunity against these species, as well as part of the innate immune defense against the initial infection (Cooper et al. 2011). IFNγ and TNFα have specific physiological roles, but act synergistically together in the context of mycobacterial infections (Lin and Flynn 2010). Together these proinflammatory cytokines activate microbial effector mechanisms in macrophages, and an observable increase in IFNγ often reflects an increased bacterial burden (Lin and Flynn 2010; Ottenhoff 2012). To aggravate matters there is evidence that mycobacterial infections delay the onset of acquired immunity, as opposed to the course of immune activation observed in other infections (Ottenhoff 2012). This helps to contextualize the increases in cytokine production observed in the *in vitro* plates, where the PBMCs are displaying increased production of these two cytokines that work in concert with one another.

Cytokine expression however is a double-edged sword, with consequences following a shift too far in either direction. Repeated exposure of *M. tuberculosis* antigen in infected individuals can lead to the development of purulent inflammation, which is quite detrimental to the host (Cooper et al. 2011). Small shifts in cytokine expression clearly can have detrimental systemic impacts, and in regards to specific types of interferon production in specific contexts can promote, not hinder, bacterial growth (Cooper et al. 2011). Sensitization to cytokines and their effects must also be considered as well. In an animal model repeated injections of TNFα led to differing immune results dependent on dose and timing, but with the potential of decreased response to the

stimulating bacterium (Dantzer 2001). Even interactions of host receptors (in the case of mycobacterial species: TLR2) with LPS during the act of phagocytosis, can lead to the impediment of continued cytokine synthesis and decreased cytokine responsiveness (Huynh et al. 2011). Cross-sensitization between cytokines and nonimmune stressors has been reported, and further complicates the balance of cytokine production and effectiveness of immune response (Dantzer 2001). Perhaps such sensitization may explain the lack of significant increases in TNFα expression of the PBMCs exposed to TB, with subsequent exposure to the same or similar pathogen.

This dynamic back and forth with the mycobacterial infections set up a busy environment in which unrelated additional pathogens enter into. Adding into this mix an infection with *S. aureus* is of interest, as this is a bacterium already renowned for immune system avoidance (Fedtke et al. 2004). *S. aureus* may be adept at perseverance in the host, but it does not completely avoid detection. Detection of *S. aureus* is enough to promote a systemic cytokine response, especially in regards to TNFα production (Fournier and Philpott 2005). However, in cases of gram-positive infections, such as *S. aureus*, the initial cytokine response is considerably delayed as compared to gram-negative infections (Fournier and Philpott 2005). This delay is problematic, as the continued increase in cytokine production to deal with an evasive pathogen is also associated with the development of septic shock and multiorgan dysfunction (Fournier and Philpott 2005). In this context of an immune system fighting two different types of infection, the substantial increase in proinflammatory cytokine production would also be enough to spur the development of a cytokine storm, and the major proinflammatory cytokines (IFNγ and TNFα) considered in these infections are also major systemic

players in such storms (Tisoncik et al. 2012). All of this makes sense why increases in cytokine expression following subsequent exposure to Sa are observed in this protocol. It also suggests that this secondary unrelated infection would be enough to trigger immune alteration, abnormal cytokine cascades, and induce both cytokine storms and push an individual into a HIP.

This protocol used *M. tuberculosis* and *M. leprae* for the initial exposure not only because these lysates represent two closely related prevalent chronic infections, but also because these infections are associated with similar biological and social factors in humans (Crespo et al. 2017; Roberts 2011). *S. aureus* was chosen for this experiment in part because of its association with periosteal lesions, and the potential compounding interaction it could have in the face of prior infection with mycobacterial species. The experimental question also applies to *P. gingivalis*, and this particular pathogen was chosen in part for its association with periodontal lesions. Similar observations are made for cytokine expression for follow up with Pg following TB or LP as were made with Sa. Again, there are also observed differences between the response to the untreated controls followed by different pathogens.

P. gingivalis elicits a strong host reaction provoking different immune responses depending on whether it initially stimulates endothelial or epithelial cells, resulting in differing cytokine expressions (Kocgozlu et al. 2009). Interestingly studies have shown that only epithelial cells (not endothelial) will secrete IFNγ and TNFα in response to *P. gingivalis*, and this stimulation was associated with periodontal sites of infection (Kocgozlu et al. 2009). The variable responses that *P. gingivalis* exerts from the host may also help to explain why as a local, yet chronic, infection it might exert systemic

influences (Kocgozlu et al. 2009). Exposure to Pg stimulated cytokine expression in our experimental model; exposure to Pg initially, or subsequently, produced increases in cytokine expression as compared to the untreated control. However, the patterns of expression are quite different from that of Sa, where exposure to Sa tended to elevate cytokine expression, and initial exposure to Sa increased cytokine expression across the board. Early exposure to Pg did not dampen cytokine expression but did not significantly elevate it either. However, with early exposure to LP or TB, then Pg elevated the response as compared to similar controls. Clearly *P. gingivalis* does not operate within the host immune response the same way that *S. aureus* does, and *P. gingivalis* while inflammatory, is not so much to the extent that *S. aureus* is. However, these preliminary results show that there is likely immune modulation occurring with subsequent exposure, and *P. gingivalis* should be considered seriously as a potential candidate for helping to push an individual into a HIP.

The initial results of this first experimental protocol suggest that there is some immune alteration that occurs in response to secondary exposure to Pg or to Sa, following preliminary exposure of PBMCs to TB and LP. This suggests that chronic infections, followed by a secondary infection or insult, may create an environment in which it is likely that this second insult could push the individual into a HIP.

Pre-activation with proinflammatory mediators

The goal of the second experimental protocol was to determine whether PBMCs could generate an inflammatory shift and alter their immune responses to pathogens if they had been primed with proinflammatory factors. This experiment highlighted broad

differences in cytokine expression based on the experimental conditions, specifically the influence of the proinflammatory primers.

Priming with IFNγ increased cytokine expression, but follow up with a pathogen significantly increased it, suggesting that an initial inflammatory environment may exacerbate subsequent responses, and may help to push an individual into a HIP, or maintain a HIP. Early exposure to TNFα prompted a large increase in TNFα expression unsurprisingly, as observed in the initial condition as well as in elevated expression of both the Pg and Sa conditions. The robust expression of TNFα may be explained in part due to its synergistic relationship with IFNγ, where IFNγ can stimulate and promote macrophage production of TNFα, especially in contexts with mycobacterial species (Van Crevel et al. 2002). The combinatorial effects of these two cytokines are most noticeable together in the context of pathogen exposure. The interplay between cytokines themselves provides some context to how an infection or insult could shift an individual into a HIP. TNFα is a potent inflammatory cytokine on its own, capable of inducing additional cytokine production and activating the innate immune system (Turner et al. 2014). IFNγ, also proinflammatory on its own, functions to stimulate macrophage activation as well as enhance neutrophil and monocyte functions in the innate response (Turner et al. 2014). Together these potent cytokines enter a feedback loop, magnifying their impact, and creating the setup for potential spillover allowing an initially local event to exert a systemic impact. Yet even acting synergistically these cytokines are also functioning as part of a diverse array of complex signaling pathways, the regulation of which triggers either pro or anti-inflammatory actions. Dysfunction in basic cytokine signaling pathways can contribute to inappropriate sustained inflammation, and the

creation or maintenance of a HIP may also be a reflection of such dysfunction (Hanada and Yoshimura 2002). It is not only the pathogen that exerts a response, even the messengers and mediators of the immune system itself can exert effects on the host immune response.

Of note, exposure to Pg elicited the strongest response in regard to both IFNγ and TNFα. One potential explanation is that periodontal diseases tend to elicit a strong inflammatory response, recruiting multiple cytokines including IFNγ and TNFα to the site of infection. This proinflammatory response not only has consequences in terms of potential spillover, but the potential for the development of alveolar resorption, something readily identifiable in the osteological record (Cochran 2008). It is also interesting to note that while LPS was used as a positive control to definitively prompt an inflammatory response in the first two protocols, here it was used as its own condition, and did not elicit as strong of reactions as observed from the two cytokines. The response that LPS elicits is an alarm to the host and not necessarily in the bacterium's best interest, perhaps explaining in part why the response elicited is not to the same extent as was observed with the proinflammatory cytokines, where the job is to mount and sustain an effective response to a persistent infection.

For this protocol, a third cytokine was investigated: interleukin 1 (IL-1). IL-1 is now recognized as an entire family of proteins, and this protocol used IL-1β specifically. IL-1β is another potent proinflammatory cytokine, expressed by numerous cell types, including macrophages, and is also synthesized by multiple immune cells (Turner et al. 2014). Of particular note here was the general robustness of IL-1β expression observed in these experiments, especially for conditions that featured Pg. Manifestations of

periodontal disease are heterogenous, just as the various immune responses such disease triggers. This heterogeneity explains in part differing periodontal outcomes, ranging between stable long term infections that do not present with tissue damage, and highly inflammatory conditions with rapid degradation of tissue (Seymour and Gemmell 2001). IL-1β is one of the major mediators of tissue destruction associated with bacterial periodontal disease. *P. gingivalis* stimulates macrophages, which in turn are major producers of IL-1β. Yet in cases of advanced periodontitis macrophage numbers were not observed to be significantly elevated, and *P. gingivalis* appeared to be eliciting an IL-1β response from B cells rather than the expected macrophages (Seymour and Gemmell 2001). Add in considerations of Th1 and Th2 responses, which if are insufficient, and where for example IFNγ production does not adequately address plaque biofilm production, the burden of infection control falls again to B cell production, with an increasing amount of IL-1β expression and subsequent tissue consequences (Seymour and Gemmell 2001). Interestingly, a study conducted using PBMCs stimulated with mitogens from periodontitis patients showed reduced IFNγ, which was observed as well for the Pg/Pg condition in this protocol, along with significantly elevated IL-1β expression over both other cytokines (Sigusch et al. 1998).

Both *P. gingivalis* and *S. aureus* are of interest here not only for their dynamic interactions with the host immune system, but for the potential each presents in terms of consequences for bone loss. *P. gingivalis* and periodontal disease encapsulate the combination of inflammation and bone physiology so well that it has been noted that it is now appropriate to consider these diseases under the purview of the field of osteoimmunology, a particularly useful interdisciplinary field that aligns with, and is

included in, the four-field model proposed above (Cochran 2008; Crespo 2018). Bone resorption does not happen instantaneously with infection; rather resorption depends in part on the expression of proinflammatory cytokines, including IL-1β and TNFα. TNFα in the context of periodontal disease plays a role in ramping up the inflammatory response, as well as upregulating IL-1β, and animals models have shown that those with a deficient TNFα receptor exhibited reduced alveolar bone loss (Garlet 2010). The role of IFNγ is complicated in the context of periodontal disease. Studies have shown that the down regulation of IFNγ acts to inhibit bone resorption (Cochran 2008). *In vitro* studies demonstrate that IFNγ plays a role in inhibiting pathological osteoclastogenesis (the bone cells responsible for bone resorption), yet *in vivo* studies demonstrate that IFNγ upregulates TNFα and IL-1β, promoting inflammation and bone resorption (Garlet 2010). The contradictory information just highlights the numerous complex potential pathways that are balanced on the presence or absence of just a few factors. The destructive effects of proinflammatory cytokines, such as the case with TNFα, may reflect the necessity of balancing those detrimental inflammatory effects with its role in fighting the bacterial infection behind the disease.

The initial results of this second experimental protocol suggest that there is some immune alteration that occurs in response to the pre-activation, or priming, of PBMCs prior to secondary exposure to Pg or to Sa. This suggests that chronic infections, or other contexts of sustained elevated inflammation, followed by a secondary infection or insult, may create an environment in which it is likely that this second insult could push the individual into a HIP, or maintain the presence of such.

Indirect immune responses

The third experimental protocol focused on the question of whether immune factors released by PBMCs exposed to a pathogenic insult would affect subsequent cytokine expression by naïve PBMCs, or PBMCs previously exposed to Sa. That is, would those cells that did not have direct access to the pathogen still alter their cytokine production? The use of supernatants from conditions with pathogen exposure were used to model this secondary influence. In addition, for this protocol we were only able to focus on the subsequent reactions to Sa and Sa supernatant. One additional limitation of this experiment was limited sample availability, thus the sample size for these experimental conditions tended to be smaller than the conditions for the other two protocols.

Clearly, there is some immune alteration occurring based on some of the specific responses from exposure to supernatants. Overall, previously unexposed PBMCs did exhibit activity based on exposure to supernatants alone on the second day and cytokine expression does not appear to decrease with exposure to the supernatants. This indicates that it is possible that immune factors can induce expression changes in these naïve cells without direct exposure to the provoking insult. Based on visual examination of the data (see Figure 15) it is also possible to infer that there are divisions within the conditions themselves, with clear divisions between PBMCs that are responders versus those cells that are not responsive to the stimuli.

The triggering of an immune response without direct contact with the insult makes sense, as alerting the rest of the immune system helps the body as a whole deal with the potential crisis. Cells of the innate immune system traffic to the local lymph

nodes where they encounter the cells and mechanisms needed to help ramp up the initial immune response as well begin activation of acquired immunity. Cytokines and chemokines serve as chemical messengers, and in situations where a strong cytokine response results in spillover, this creates a state in which these proinflammatory messengers are now no longer contained to the initial site of infection. They can now exert a proinflammatory influence on other cells outside the pathway of the lymph nodes and traditional activation. There are many different ways in which this might happen, ranging from the interaction with byproducts of complement activation to the influence of a pre-existing HIP. Further, if such immune activation is possible, and this experimental model suggests that it is, logically this effect and the consequences of such could be increasingly exaggerated when preconditions, such as chronic infections, are in play. The presence of non-responders in the data does not rule out this possibility. Rather it highlights the variability inherent in humans. Non-response to an experimental condition, or even limited response of one cytokine does not rule out the potential of other cytokines, other signaling pathways, or other stimuli to prompt these systemic reactions indirectly *in vivo*.

This is a good reminder that experimental models have their limits, and while *in vitro* experiments can tease out the nuances of cell behavior, it cannot be conducted in isolation. Other fields, and other studies must complement it, as the *in vivo* reality may differ. This is not to argue against these experimental models; they serve as the foundation for working out the intricacies of cellular behavior and have elucidated the immensely complicated pathways that the immune system utilizes. These experiments cannot be an end in of themselves, rather, incorporation of multiple disciplines and

perspectives add nuance to interpretation of results, and also highlight areas that need a closer look, such as with the discrepancies of IFNγ and periodontal disease. The context in which these microscopic pathways play out matters. It is not only the local immediate environment at the site of infection or injury that matters. Humans are biosocial creatures, and broader context is necessary to better frame what is happening at the cellular level. That is, these molecular players can be influenced by situations other than pathogen contact or direct bodily injury. The various potential ways in which the immune system reacts to pathogens, as well as to its own products, are the starting points and foundation for considering ways in which subsequent immune reactions influence the creation or a maintenance of HIP.

Expanding on an experimental foundation

While the data presented above are strictly experimental, they can be further contextualized by both previous work as well as the future potential that this line of research may take. The experimental design and protocols here follow that previously published by this lab; experimental protocols developed to investigate the potential of how exposure to pathogens might generate an inflammatory shift, further affecting the immune response against other persistent pathogens (including the Pg investigated here) (Crespo et al. 2017). Crespo et al. (2017) found that direct exposure to bacterial lysates (TB and LP) induced an inflammatory shift when PBMCs were subsequently exposed to Pg. The experiments presented above add on to this initial question, with the addition of the *S. aureus* lysates in addition to Pg, as well as extend it, to question whether early exposure to pro-inflammatory mediators might also have an impact as well.

The results of this work highlight similar conclusions to that of Crespo et al. (2017) in regard to exposure to bacterial lysates followed by subsequent exposure to a lysate representing a persistent infection, in that there is a shift in immune response as demonstrated by the PBMCs' cytokine expression. The second question of how this might look with early exposure to the proinflammatory mediators themselves (cytokines and LPS) adds on to this, showing another route immune modulation may take. In addition, the work here suggests that there are nuances in the inflammatory response to different pathogens. This is especially relevant as the different model pathogens used, *S. aureus* and *P. gingivalis*, generally present at different locations, but are both associated with generalized bone lesions. Nuanced and tailored responses by the immune system to pathogens are not a novel idea, nor is the general underlying framework of these experiments novel. The considerations of these cellular reactions within the framework of an inflammatory response are of interest for the potential pathological consequences to the host, absolutely relying on the groundwork from the field of experimental immunology. The questions asked, and the protocols run here, only serve to provide further experimental grounding with which to cement how this experimental work is relevant to broader systemic questions of inflammation and bone.

In sickness and in health the immune system functions with great complexity, and context matters for any understanding of immune function, especially inflammation. The microenvironments of the initial inflammatory response are as important to understanding hyperinflammation as much as the external environment and the social and psychological well-being of an individual. It is increasingly recognized that other stimuli can trigger inflammatory events beyond pathogen exposure and injury. The inflammatory phenotype

and immune competence vary from person to person, hence the necessity to consider an expanded analysis beyond the immediate inflammatory triggers, especially one that takes into account the biosocial context. While the data presented here are strictly experimental, the future potential of this line of research must take into consideration a comprehensive, more holistic context to frame any broader conclusions.

The application and extension of questions regarding the role of inflammation and consequences of inappropriate manifestations of such into other lines of inquiry, such as biosocial and bioarchaeological realms, requires nuance in analysis and context to both ground and frame these queries. In trying to understand hyperinflammation, and the potential of reconstructing it from bones, it is important to recognize there are multiple pathways available to trigger the inflammatory response, some of which will not be as immediately obvious as the characteristic lesions made by specific pathogens, or the marks of blunt force trauma. Understanding the broader biosocial context of an individual may help to frame the observations made upon their skeleton, as well as guide what questions can be raised. The experimental protocols outlined above are not a means to an end in of themselves. Rather, these experiments are the start of a foundation for a line of inquiry that stretches from the experimental to the observational. The development of the four-field model (see Figure 1) requires an experimental foundation. However, this experimental foundation must be augmented with an expanded biosocial perspective in order to be feasible for further applications, such as contributing to the development of a skeletal inflammatory index.

Stress is an ubiquitous term, but its usage in bioarchaeological models is useful here as an understanding of synergistic interactions between the environment, culture,

and psychological disruption, which provides a model for understanding alternative pathways that might trigger inflammation (Reitsema and McIlvaine 2014). Over the course of the 20th century notions of stress have evolved from a pure physiological response to stimuli, to a complex and nuanced biobehavioral response which occurs in reaction to both biological as well as nonbiological (i.e., socioeconomic; political) stimuli (Goodman et al. 1988). Contextual biosocial information is not always available such as nutritional status or community support, but these and other factors do play an important role in experiences of stress (Reitsema and McIlvaine 2014). It is important not to box in the use of stress as a purely typological counterpart to notions of health, but rather to utilize it in a more comprehensive manner which further amplifies its potential applicability to interdisciplinary studies (Temple and Goodman 2014).

In regards to inflammation, the immune system responds to both psychological as well as psychosocial stressors as evidenced by circulating markers such as proinflammatory cytokines (Steptoe et al. 2007). Physiological responses such as increased blood pressure in turn have cascading effects, as the physical changes including increased hydrostatic pressure force plasma into interstitial spaces, creating new opportunities for mediators to interact (Steptoe et al. 2007). This makes sense, as many proinflammatory mediators act in concert when in contact with one another. Stress hormones generally exert an anti-inflammatory response, but for local responses the opposite occurs with an upregulation of pro-inflammatory cytokines (Elenkov and Chrousos 2002). Indeed, it is also the case for more typical stressors that if a generalized stress response is unable to return the system back to homeostatic normal then an inflammatory response can be induced (Chovatiya and Medzhitov 2014). Cytokines

themselves have also been implicated in the onset of sickness behavior (i.e., weakness, malaise, suppression of appetite), and it stands to reason that in cases of increased cytokine activity prompted by another stimulus could also implement sickness behavior (Dantzer 2001). That is, when cytokines are interpreted by the brain to implement an adaptive program of sickness behavior to preserve resources to fight an infection, it triggers certain behavioral responses. All of this to say that stress from nonpathogenic stimuli clearly can exert an effect of the body, and inflammation is not restricted to tissue injury or control of infection. Stress is one mechanism that accounts for the biological as well as the social impact of environment, and the potential mechanism which may help to explain the creation or maintenance of a HIP, especially when considering stress as an additional factor layered onto underlying injury or infection.

Stress is a prime example of how to further contextualize and frame experimental questions moving towards bioarchaeological and biosocial considerations. Of course the challenges of stress are amplified when trying to reconstruct health status in a past population, and stress cannot be considered merely the lack of health, reducing both constructs to mere typological categories (Temple and Goodman 2014). While the field of bioarchaeology has been grappling with stress as well as how to reconstruct and better understand health in the past since at least the early 1980s, recent calls have been put forth to reevaluate how to better utilize stress (Goodman et al. 1988; Reitsema and McIlvaine 2014). However, even before this most recent renewed interest in the conceptual problems of stress, researchers had begun to wrangle with the problems associated with reconstructing health in the past. Conceptually, researchers took the next step and developed a health index; a tool that could account for multiple data points and

standardize skeletal measurements to better discuss and interpret results (Steckel et al. 2002). While this health index was not without some critiques it did account for the importance of distinguishing between material and health aspects, as well as the underlying importance of context. It is the development of a tool such as the health index that allows for some degree of standardization, which ultimately allows analyses to be built upon and expanded upon.

More holistic and encompassing analyses of data that can incorporate local histories and archaeological context as well as biology and physiology produce data with more depth than just answers of presence or absence for typological categorization (Klaus and Tam 2009). The development of indices as tools to better grapple with complex and nuanced data also aligns with a more holistic approach to studying stress in skeletal remains; one that accounts for both biological responses within physiological as well as local biocultural contexts. The development of a skeletal frailty index for example, attempts to account for relationships between health and stress from standardized indicators of stress already utilized in bioarcheological research to tease out and evaluate frailty in past human populations (Marklein et al. 2016). This index which builds upon a solid foundation of work in the field allows for a more nuanced interpretation of the same skeletal data and allows for a shift in perspective to occur. The frailty index is compelling because it addresses a very abstract concept yet is grounded in concrete analyses. Immune competence and hyperinflammation are similar, abstract in concept yet also material in that the physical cells and mediators exert physical changes.

It is in this context that the importance of developing and building an experimental foundation within a model is especially relevant, as this foundation may

help to better develop additional tools, such as the development of a skeletal inflammatory index (SINDEX). To get to the point where it possible to utilize something like a SINDEX, a solid foundation of experimental and observational data and theory is necessary. The experiments laid out here are the start of how such experimental data might be generated. These experiments look at specific cells and specific reactions, but the potential to consider these immune reactions in broader biosocial context, such as placed within analyses of stress, combines multiple perspectives to develop a more nuanced conclusion. The great potential of a tool such as SINDEX can only be realized because it will be built upon, and it will rely upon, an experimental foundation strengthened by multiple contributors (experimental immunology, osteoimmunology, archaeoproteomic analysis, and paleopathology), all to be synthesized into a framework that provides the structure, and this experimental grounding, to begin reconstructing the inflammatory phenotype in the past.

The broad development of an experimental base upon which to build a model, or to begin to construct an index, reflects trends in the development of the field of bioarchaeology as well. These trends are calls for critical evaluations of concepts and usages that have become standard practice (i.e., health and stress). Reevaluation of what has become accepted practice provides opportunity to critically evaluate both the results of a study, moving the field beyond set paradigms of what information the osteological record does and does not provide. Careful considerations utilizing rubrics and indices are necessary for observation and standardized recordings of data, yet a balance must be struck between rigor in use of terminology and data collection, and the flexibility to adjust these tools when reevaluation is necessary (Klaus 2017; Temple and Goodman

2014). These trends in bioarcheology are also calls to advance the field forward, not just reevaluations of the past. Indeed, the development of these experimental models are in of themselves a response to a call for cross-disciplinary research in order to promote more complete understandings of health within the bioarchaeological context (Klaus 2014).

Factoring in the biocultural perspective is necessary to frame interpretations from the bioarchaeological record, as humans are biocultural creatures and our biology and physiology should not be investigated in isolation. In the context of disease interaction, of relevance here for the potential inflammatory impact of multiple or comorbidities, it is becoming apparent that this expanded perspective is necessary to better understand experiences of health. This is especially relevant for understanding the varied and dynamic factors that contribute to normal inflammation, yet alone the myriad complexities that allow for systemic, chronic, and, severely pathological manifestations of such. Inflammation is not only the actions of the cytokines and the chemical responses to stimuli, but it is also a product of that individual's own history, their evolutionary context, their social, political, and economic standing, and more. The syndemic approach should be briefly mentioned here, as it factors in consequences of disease in the context of social, economic, and environmental factors, expanding beyond comorbidities as simple additive layers of disease, and investigates these disease interactions along the lines of multifactorial interactions (Singer et al. 2017). The syndemic approach considers the population level clustering of diseases, both biological and social. The complexities of disease interactions are better explained in context of the socioeconomic and cultural environments. The syndemic approach in conjunction with the reconstructions of inflammatory processes in the past creates the potential for a new perspective on health in

past populations, by combining the individual inflammatory experiences with considerations of population level trends in regard to health and disease.

Recognizing the dynamic nature of human biology and that humans are biocultural creatures, sets the stage for increasingly synthesized analyses. The potential of reconstructing inflammatory phenotypes, of understanding immune competence, is the extrapolation of this information, via tools and indices such as the proposed SINDEX, to larger frameworks (including the syndemic approach). It is at this intersection where these disparate fields come together allowing for the creation of multidisciplinary models, whose utilization allows for these advancements in theory and knowledge to take place. It is the product of these sorts of intersections that allows for the expansion of the field of bioarchaeology and allows for this better integration of fields of study once thought removed from the purview of one another. The combinatorial analyses provided by disparate fields creates the context in which a more holistic understanding is possible, one that captures the nuances of the dynamic interplay between multiple factors.

This nuanced approach that incorporates both biology and culture, falls completely under the purview of one discipline: anthropology. The anthropological lens, and the biocultural synthesis, allows for this approach to immunological and osteological perspectives. It is within the anthropological lens that biology and culture can be considered together, and not as mere dialectical opponents. It is the extrapolation and expansion of the realm of analysis, and the additive totals that allow for the development of something such as analysis of health in the past, which attempt to provide additional perspective to old bones. Moving beyond a biocultural synthesis, incorporating culture into human biology, and both again back into anthropology, creates these advancements

in what is possible and what potential information may be gleaned from such combinatorial efforts (Goodman 2013).

This synthesis is not an abandonment of what each individual field has to offer, or the core tenants of each. Day to day osteological analysis must still rely on anatomical and terminological rigor. The field of bioarcheology must rely on the establishing principles and guidance of researchers past, just as immunologists in the lab must still follow the established protocols that allow cells to live in a petri dish long enough to survive for chemical analysis. The anthropological lens permits the combination of the insights from multidisciplinary collaboration into a new synthesis of results and analyses, while retaining (and refining) the underlying methodologies and tools of data collection. Pursuing inflammation in the past, via reconstructions of potential immune competence and the search for a hyper-inflammatory phenotype embody the anthropological method well. Here then lies the potential to better understand the available evidence from the human biological past, a starting point for the potential to investigate mechanisms of health in life (immune competence; inflammatory phenotypes) in those long since passed. Yet the potential of these broad analyses will always fall back on the foundation holding them up. For investigations of health in the past experimental work will continue to provide this foundation. For considerations of inflammation in the past, only hinted at in the bone, experimental methods building one on top of the other (osteoimmunology on top of experimental immunology, and archaeoproteomics on top of both and so on), will create the platform for this analysis, and the potential reconstructions, one piece of the puzzle at a time.

Limitations & Pitfalls

As with any study there will be limitations and potential pitfalls. No experimental design is perfect, and in regard to experimental immunology certain inherent dilemmas must be dealt with. For the experimental models used here, these are understood to be limited models based on specific cells reactions *in vitro.* These models do not reflect the complexity of the immune system functioning *in vivo*, and all interpretations of such are framed and limited by the applicability of these isolated results to the whole.

In regard to the cells utilized, peripheral whole blood mononuclear cells (PBMCs) are not truly naïve immune cells, even though they are treated as such for the experimental conditions. Truly naïve immune cells will not be found in circulation and must be taken directly from germinal centers such as the spleen, something possible only in animal models. Our PBMCs came from healthy donors' bloodstreams, so while in theory these were not actively fighting infection, they are not naïve. However, it is accepted that one of the better ways of assessing peripheral cytokine function is to study the ability of PBMCs to produce cytokines when placed in culture and stimulated, so there is some debate still about this practice (Dantzer 2001).

For the statistical analyses, interpolated values were used for values that were above the plate readers limit of upper detection. Ideally those samples would have been rerun at dilution and then the results multiplied by the dilution factor in order to capture those larger values. Constraints on laboratory funding, sample availability, and time did not allow for dilutions to be run. One well from a plate provided a little under 1ml of supernatant, to be divided into two, sometimes three aliquots for analysis, and in some cases not enough supernatant was available for reruns or dilutions.

As discussed, the immune system is complex and varied. Inflammatory responses may be triggered by a multitude of factors. One such factor is seasonality, in which inflammation varies by season (Ashley et al. 2012). Louisville, Kentucky does experience all four seasons, and the timing of when future experiments are run to account for potential seasonal variability might be taken into consideration, but the realities of when funding for said experiments is available and when there is time to run experiments cannot be dismissed either.

In broader discussions of placing inflammation in context, multiple perspectives lie well beyond the scope of this thesis. Neuroendocrine regulation and related pathways are one such area that warrants a closer look. For the goal of a broad baseline of basic context to inflammation, this thesis focused briefly on homeostasis in regard to contextualizing mechanisms of inflammation and placing inflammation in an evolutionary context. However, it is increasingly recognized that homeostasis may not be the most appropriate phenomenon to understand stress and inflammation, and not taking into further consideration the role of allostasis and disruption of allostatic loads is one potential misstep of this thesis (Klaus 2014).

CONCLUSIONS

Pursuing experimental work of any type is an undertaking requiring multiple steps and considerations. The objective of this thesis was to provide some experimental evidence to contribute to the proposed multidisciplinary reconstruction of inflammatory phenotypes in skeletal samples. More specifically this thesis questioned whether if *in vitro* priming of immune cells could generate an inflammatory shift, altering the immune response to pathogens (exposure to bacterial lysates) commonly associated with persistent infections such as those associated with periodontal disease and periosteal lesions.

Broadly, these three experimental protocols do show that there is some immune modulation occurring in response to exposure to bacterial lysates or other proinflammatory stimuli. Even with the limitations of *in vitro* extrapolation, these results are of interest as they are the first step hinting at potential pathways and mechanisms that would create these inflammatory environments in which exacerbation of lesion formation may occur. The results of the first experimental protocol indicate that some immune modulation does occur in PBMCs initially exposed to bacterial lysates (TB; LP) and sequentially exposed to additional lysates (Pg; Sa), that represent common, persistent infections. The second protocol also showed that priming of naïve PBMCs with proinflammatory mediators also produces a change in PBMCs' cytokine expression following subsequent exposure to bacterial lysates (Pg; Sa). Finally, the third protocol showed that immune modulation is also likely occurring in response to indirect

stimulation from the byproducts of other PBMCs, which had previously been exposed to bacterial lysates.

What these experimental protocols together suggest is that stimulation, direct or indirect, and compounded with sequential exposure, does produce a shift in the inflammatory response of these cells. This suggests several pathways in which an inflammatory environment might be created or maintained, with this implication that these inflammatory environments may also play a role in the creation or maintenance of an individual's hyper-inflammatory phenotype. Of particular interest are the differences in cellular response to the varied lysates, particular Pg and Sa. The dynamic nature of the pathogens represented in these experiments and the preliminary experimental results are of particular interest given their association with certain (non)specific osteological lesions. There is great potential for future osteopathological reconstructions to consider the role of the pathogen at play, as well as considerations for the overall role of inflammation and an individual's inflammatory phenotype.

Finally, this inflammatory context is of great importance for teasing out nuances in health, past and present, and the causes and mechanisms of inflammation are an important background for these considerations. The foundation for all analysis here, and for future related studies, must be grounded in experimental work: immunological, bioarchaeological, and anthropological

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APPENDIX

1. Cell Culture Template

SINDEX (Systemic **In**flammatory In**DEX)**

Sample or Experiment #: **Date:**

Cells:

---

1) Cell collection following Protocol # 1 for Day 1 and Day 2 (in binder)

2) Experimental conditions for Day 1 and Day 2:

Final volume/well: Cells (total count/initial):

Cells/ml:

LPS [stock]: - Added to well:

IFNγ [stock]: - Added to well:

TNFα [stock]: - Added to well:

P. gingivalis [stock]: - Added to well:

S. aureus [stock]: - Added to well:

Well #	24 hrs	48hrs
1	UNT	UNT
2	Pg	Pg
3	LPS	Pg
4	IFNγ	Pg
5	TNFα	Pg
6	Sa	Sa
7	LPS	Sa
8	IFNγ	Sa
9	TNFα	Sa

3) Observations:

2. ELISA Template

Example of ELISA notetaking form to help organize ELISA plates

Project:

Date:

Personnel:

Cytokine/Protein:

Kit / Reagents: eBioscience Ready Set Go

Plate:

	1	2	3	4	5	6	7	8	9	10	11	12
A	NC	NC	ST7	ST7								
B	BLK	BLK	ST8	ST8								
C	ST1	ST1										
D	ST2	ST2										
E	ST3	ST3										
F	ST4	ST4										
G	ST5	ST5										
H	ST6	ST6										

Sample Dilution: None

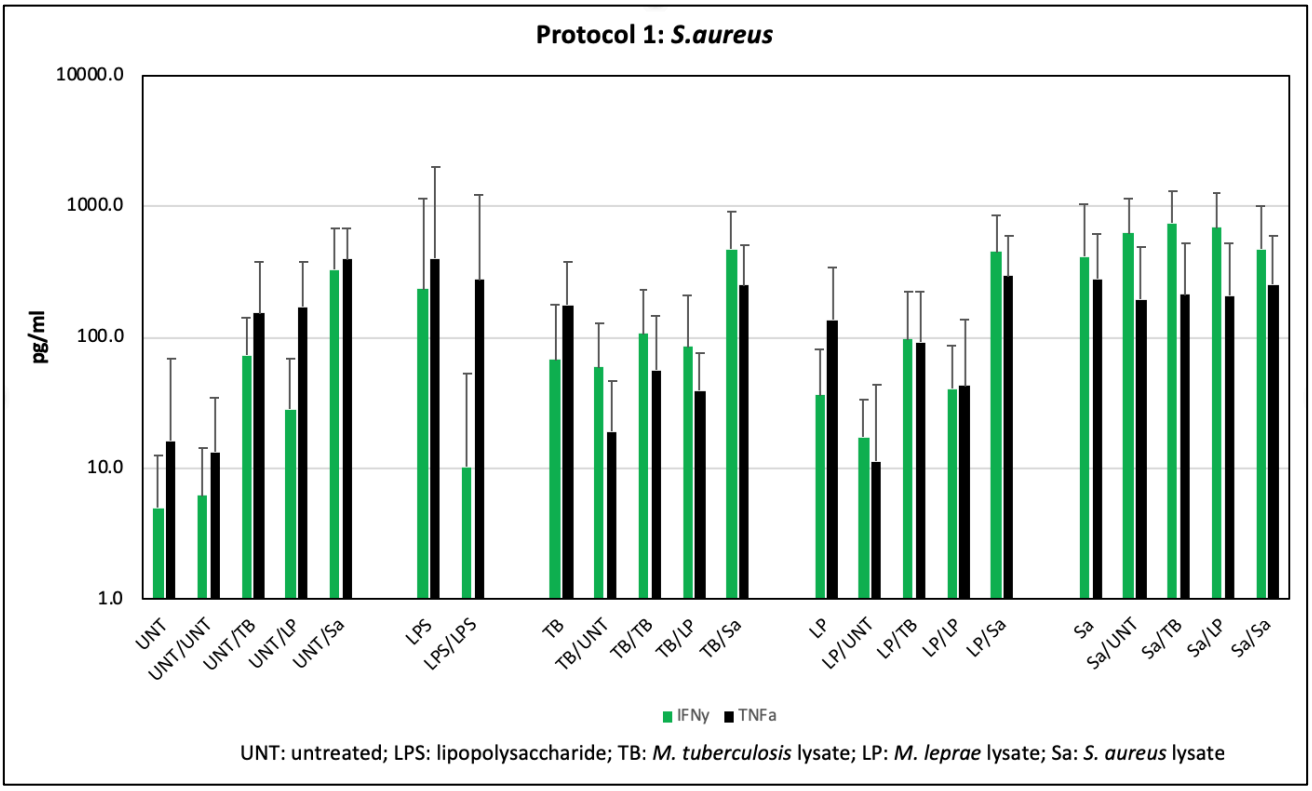
Sample diluent: None

Standard diluent: eBioscience Assay Diluent

Standards (concentrations):

Observations:

3. Protocol 1 Results & Adjusted P values



Adjusted P values TNFα – *S. aureus*

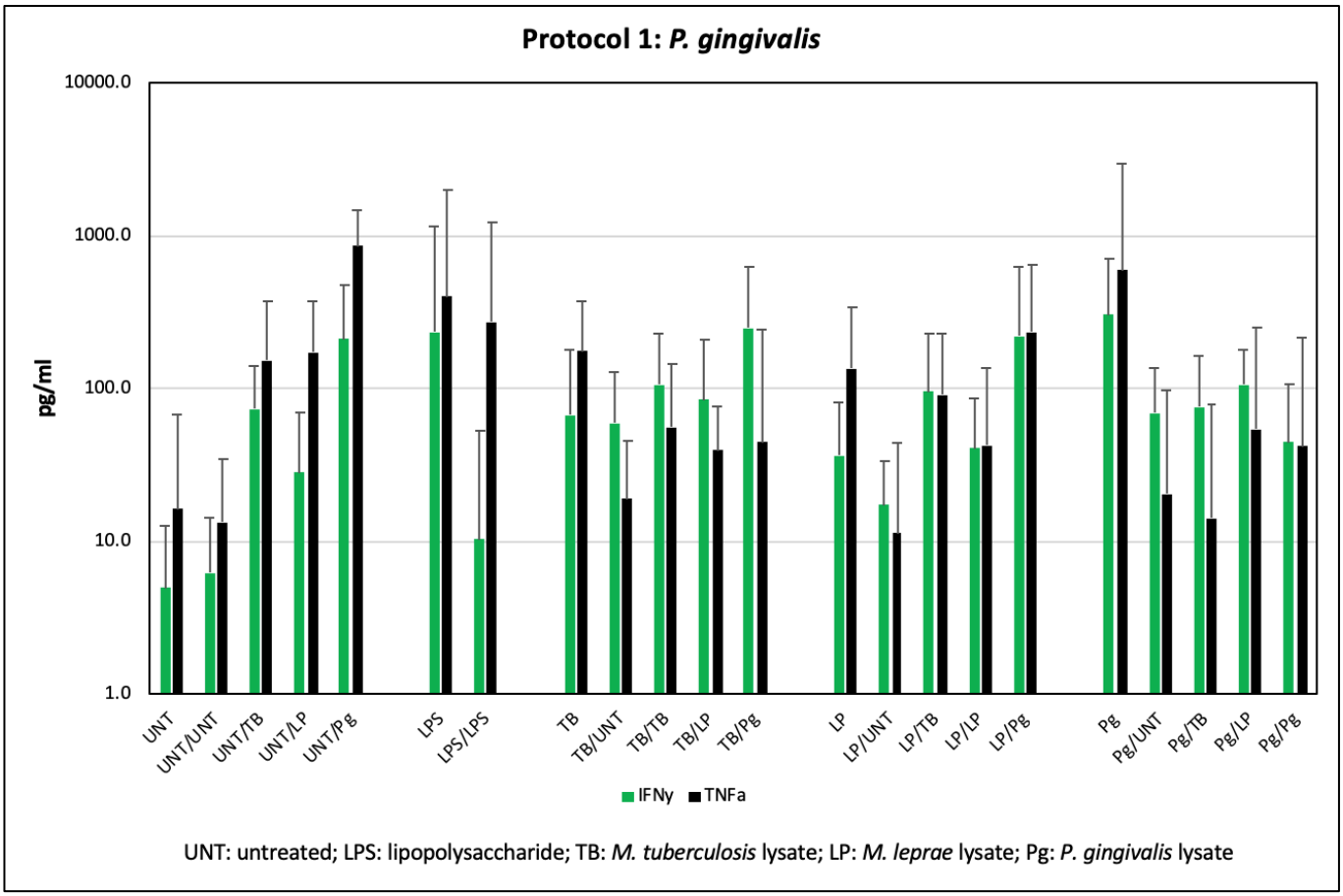
Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	TB/UNT vs. TB/TB	0.5656
UNT vs. UNT/TB	0.0514	TB/UNT vs. TB/LP	0.5161
UNT vs. UNT/LP	0.005	TB/UNT vs. TB/Sa	0.0003
UNT vs. UNT/Sa	<0.0001	TB/TB vs. TB/LP	>0.9999
UNT/UNT vs. UNT/TB	0.1432	TB/TB vs. TB/Sa	0.0654
UNT/UNT vs. UNT/LP	0.0165	TB/LP vs. TB/Sa	0.1669
UNT/UNT vs. UNT/Sa	<0.0001	LP vs. LP/UNT	0.0001
UNT/TB vs. UNT/LP	>0.9999	LP vs. LP/TB	>0.9999
UNT/TB vs. UNT/Sa	0.3306	LP vs. LP/LP	0.1377
UNT/LP vs. UNT/Sa	>0.9999	LP vs. LP/Sa	0.3736
TB vs. TB/UNT	0.0003	LP/UNT vs. LP/TB	0.0017
TB vs. TB/TB	0.2353	LP/UNT vs. LP/LP	0.4082
TB vs. TB/LP	0.6548	LP/UNT vs. LP/Sa	<0.0001
TB vs. TB/Sa	>0.9999	LP/TB vs. LP/LP	0.2591

Dunn's multiple comparisons test	Adjusted P Value
LP/TB vs. LP/Sa	>0.9999
LP/LP vs. LP/Sa	0.0009
Sa vs. Sa/UNT	>0.9999
Sa vs. Sa/TB	>0.9999
Sa vs. Sa/LP	>0.9999
Sa vs. Sa/Sa	>0.9999
Sa/UNT vs. Sa/TB	>0.9999
Sa/UNT vs. Sa/LP	>0.9999
Sa/UNT vs. Sa/Sa	>0.9999
Sa/TB vs. Sa/LP	>0.9999
Sa/TB vs. Sa/Sa	>0.9999
Sa/LP vs. Sa/Sa	>0.9999
UNT/Sa vs. TB/Sa	0.7541
UNT/Sa vs. LP/Sa	>0.9999
UNT/Sa vs. Sa/Sa	0.3005
TB/Sa vs. LP/Sa	>0.9999
TB/Sa vs. Sa/Sa	>0.9999
LP/Sa vs. Sa/Sa	>0.9999

Adjusted P values IFNγ – *S. aureus*

Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	TB/UNT vs. TB/Sa	0.0007
UNT vs. UNT/TB	0.0035	TB/TB vs. TB/LP	>0.9999
UNT vs. UNT/LP	0.2244	TB/TB vs. TB/Sa	0.0135
UNT vs. UNT/Sa	<0.0001	TB/LP vs. TB/Sa	0.0007
UNT/UNT vs. UNT/TB	0.0116	LP vs. LP/UNT	>0.9999
UNT/UNT vs. UNT/LP	0.5286	LP vs. LP/TB	0.9669
UNT/UNT vs. UNT/Sa	<0.0001	LP vs. LP/LP	>0.9999
UNT/TB vs. UNT/LP	>0.9999	LP vs. LP/Sa	<0.0001
UNT/TB vs. UNT/Sa	0.9752	LP/UNT vs. LP/TB	0.0795
UNT/LP vs. UNT/Sa	0.0521	LP/UNT vs. LP/LP	0.9363
TB vs. TB/UNT	>0.9999	LP/UNT vs. LP/Sa	<0.0001
TB vs. TB/TB	0.52	LP/TB vs. LP/LP	>0.9999
TB vs. TB/LP	>0.9999	LP/TB vs. LP/Sa	0.1422
TB vs. TB/Sa	<0.0001	LP/LP vs. LP/Sa	0.0003
TB/UNT vs. TB/TB	>0.9999	Sa vs. Sa/UNT	0.9051
TB/UNT vs. TB/LP	>0.9999	Sa vs. Sa/TB	0.3737

Dunn's multiple comparisons test	Adjusted P Value
Sa vs. Sa/Sa	>0.9999
Sa/UNT vs. Sa/TB	>0.9999
Sa/UNT vs. Sa/LP	>0.9999
Sa/UNT vs. Sa/Sa	>0.9999
Sa/TB vs. Sa/LP	>0.9999
Sa/TB vs. Sa/Sa	>0.9999
Sa/LP vs. Sa/Sa	>0.9999
UNT/Sa vs. TB/Sa	>0.9999
UNT/Sa vs. LP/Sa	>0.9999
UNT/Sa vs. Sa/Sa	>0.9999
TB/Sa vs. LP/Sa	>0.9999
TB/Sa vs. Sa/Sa	>0.9999
LP/Sa vs. Sa/Sa	>0.9999



Adjusted P values TNFα – *P. gingivalis*

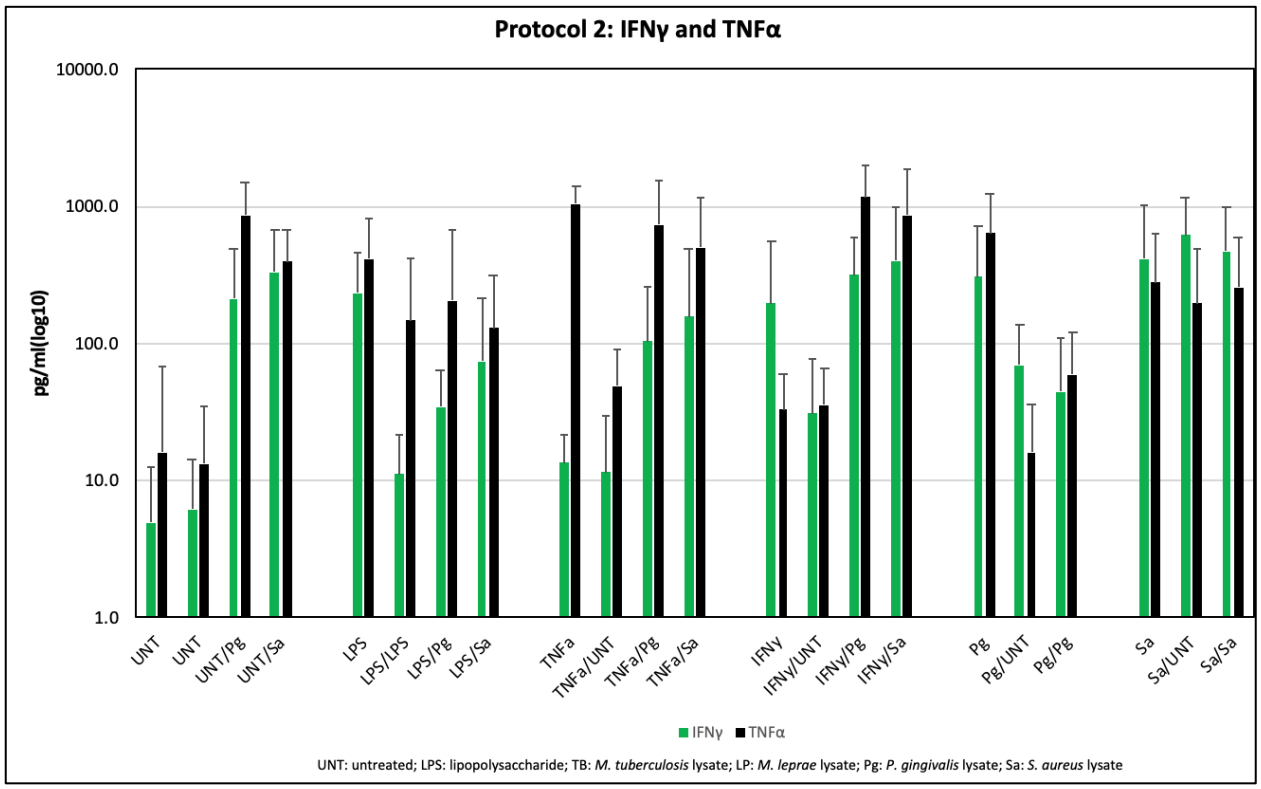
Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	LP/UNT vs. LP/LP	0.3535
UNT vs. UNT/TB	0.0711	LP/UNT vs. LP/Pg	<0.0001
UNT vs. UNT/LP	0.0085	LP/TB vs. LP/LP	0.2054
UNT vs. UNT/Pg	<0.0001	LP/TB vs. LP/Pg	>0.9999
UNT/UNT vs. UNT/TB	0.1947	LP/LP vs. LP/Pg	0.0167
UNT/UNT vs. UNT/LP	0.0273	Pg vs. Pg/UNT	<0.0001
UNT/UNT vs. UNT/Pg	<0.0001	Pg vs. Pg/TB	0.0002
UNT/TB vs. UNT/LP	>0.9999	Pg vs. Pg/LP	0.0002
UNT/TB vs. UNT/Pg	0.0584	Pg vs. Pg/Pg	<0.0001
UNT/LP vs. UNT/Pg	0.2907	Pg/UNT vs. Pg/TB	>0.9999
TB vs. TB/UNT	0.0001	Pg/UNT vs. Pg/LP	>0.9999
TB vs. TB/TB	0.1708	Pg/UNT vs. Pg/Pg	0.4163
TB vs. TB/LP	0.525	Pg/TB vs. Pg/LP	>0.9999
TB vs. TB/Pg	>0.9999	Pg/TB vs. Pg/Pg	>0.9999
TB/UNT vs. TB/TB	0.4697	Pg/LP vs. Pg/Pg	>0.9999
TB/UNT vs. TB/LP	0.4227	UNT/Pg vs. TB/Pg	0.0008
TB/UNT vs. TB/Pg	0.018	UNT/Pg vs. LP/Pg	0.0388
TB/TB vs. TB/LP	>0.9999	UNT/Pg vs. Pg/Pg	<0.0001
TB/TB vs. TB/Pg	>0.9999	TB/Pg vs. LP/Pg	>0.9999
TB/LP vs. TB/Pg	>0.9999	TB/Pg vs. Pg/Pg	>0.9999
LP vs. LP/UNT	<0.0001	LP/Pg vs. Pg/Pg	0.5901
LP vs. LP/TB	>0.9999		
LP vs. LP/LP	0.1095		
LP vs. LP/Pg	>0.9999		

Adjusted P values IFNγ – *P. gingivalis*

Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	UNT/UNT vs. UNT/Pg	<0.0001
UNT vs. UNT/TB	0.003	UNT/TB vs. UNT/LP	>0.9999
UNT vs. UNT/LP	0.1654	UNT/TB vs. UNT/Pg	>0.9999
UNT vs. UNT/Pg	<0.0001	UNT/LP vs. UNT/Pg	0.6719
UNT/UNT vs. UNT/TB	0.0112	TB vs. TB/UNT	>0.9999
UNT/UNT vs. UNT/LP	0.4336	TB vs. TB/TB	0.4123

Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
TB vs. TB/Pg	0.0236	Pg vs. Pg/UNT	>0.9999
TB/UNT vs. TB/TB	>0.9999	Pg vs. Pg/TB	>0.9999
TB/UNT vs. TB/LP	>0.9999	Pg vs. Pg/LP	>0.9999
TB/UNT vs. TB/Pg	0.258	Pg vs. Pg/Pg	0.0216
TB/TB vs. TB/LP	>0.9999	Pg/UNT vs. Pg/TB	>0.9999
TB/TB vs. TB/Pg	>0.9999	Pg/UNT vs. Pg/LP	>0.9999
TB/LP vs. TB/Pg	0.217	Pg/UNT vs. Pg/Pg	>0.9999
LP vs. LP/UNT	>0.9999	Pg/TB vs. Pg/LP	>0.9999
LP vs. LP/TB	0.8453	Pg/TB vs. Pg/Pg	>0.9999
LP vs. LP/LP	>0.9999	Pg/LP vs. Pg/Pg	0.2723
LP vs. LP/Pg	0.1409	UNT/Pg vs. TB/Pg	>0.9999
LP/UNT vs. LP/TB	0.0537	UNT/Pg vs. LP/Pg	>0.9999
LP/UNT vs. LP/LP	0.7812	UNT/Pg vs. Pg/Pg	0.2364
LP/UNT vs. LP/Pg	0.007	TB/Pg vs. LP/Pg	>0.9999
LP/TB vs. LP/LP	>0.9999	TB/Pg vs. Pg/Pg	0.0414
LP/TB vs. LP/Pg	>0.9999	LP/Pg vs. Pg/Pg	0.5065
LP/LP vs. LP/Pg	0.2831		

4. Protocol 2 Results & Adjusted P values



Adjusted P values TNFα

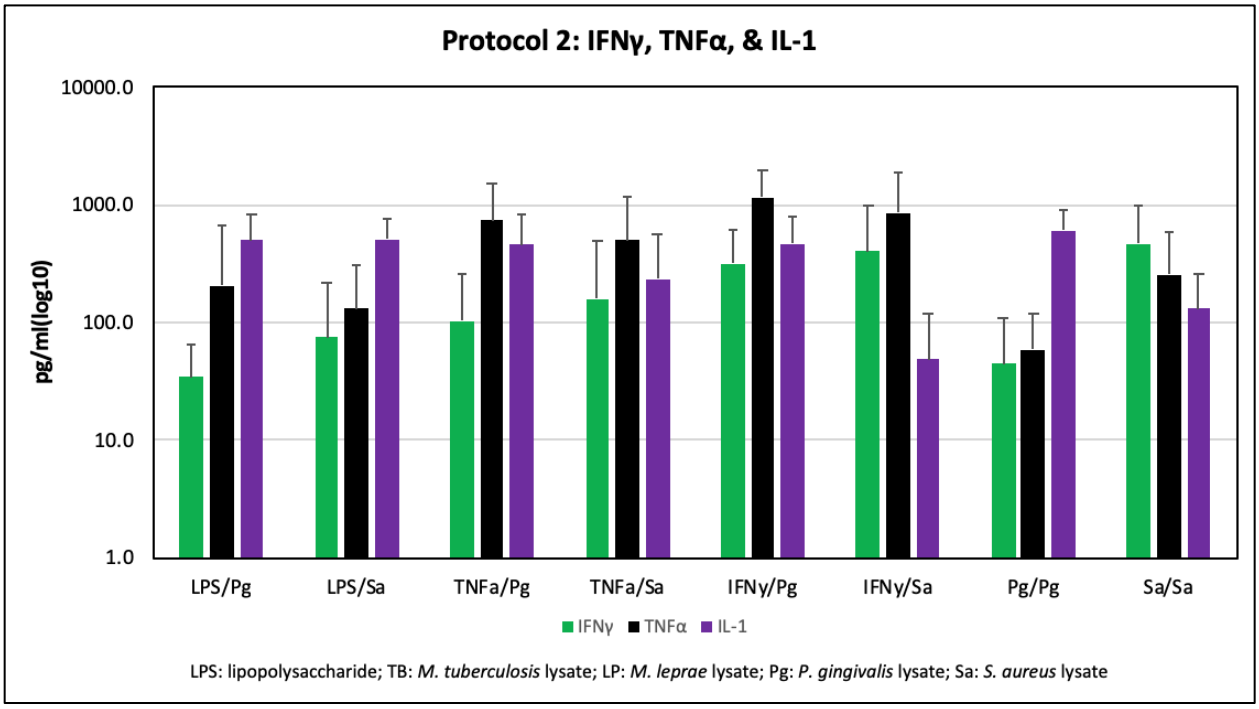
Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	LPS/Pg vs. LPS/Sa	>0.9999
UNT vs. UNT/Pg	<0.0001	TNFα vs. TNFα/UNT	0.0002
UNT vs. UNT/Sa	<0.0001	TNFα vs. TNFα/Pg	0.7501
UNT/UNT vs. UNT/Pg	<0.0001	TNFα vs. TNFα/Sa	0.0535
UNT/UNT vs. UNT/Sa	<0.0001	TNFα/UNT vs. TNFα/Pg	0.0274
UNT/Pg vs. UNT/Sa	>0.9999	TNFα/UNT vs. TNFα/Sa	0.3691
LPS vs. LPS/LPS	0.0228	TNFα/Pg vs. TNFα/Sa	>0.9999
LPS vs. LPS/Pg	0.9006	IFNγ vs. IFNγ/UNT	>0.9999
LPS vs. LPS/Sa	0.6355	IFNγ vs. IFNγ/Pg	0.0007
LPS/LPS vs. LPS/Pg	0.8049	IFNγ vs. IFNγ/Sa	0.0109
LPS/LPS vs. LPS/Sa	0.9608	IFNγ/UNT vs. IFNγ/Pg	0.0017

IFNγ/UNT vs. IFNγ/Sa	0.0207
IFNγ/Pg vs. IFNγ/Sa	>0.9999
Pg vs. Pg/UNT	<0.0001
Pg vs. Pg/Pg	<0.0001
Pg/UNT vs. Pg/Pg	0.1934
Sa vs. Sa/UNT	>0.9999
Sa vs. Sa/Sa	>0.9999
Sa/UNT vs. Sa/Sa	>0.9999
LPS/Pg vs. TNFα/Pg	0.1798
LPS/Pg vs. IFNγ/Pg	0.0163
LPS/Pg vs. Pg/Pg	>0.9999
TNFα/Pg vs. IFNγ/Pg	>0.9999
TNFα/Pg vs. Pg/Pg	0.0011
IFNγ/Pg vs. Pg/Pg	<0.0001
LPS/Sa vs. TNFα/Sa	>0.9999
LPS/Sa vs. IFNγ/Sa	0.1242
LPS/Sa vs. Sa/Sa	>0.9999
TNFα/Sa vs. IFNγ/Sa	>0.9999
TNFα/Sa vs. Sa/Sa	>0.9999
IFNγ/Sa vs. Sa/Sa	0.2878

Adjusted P values IFNγ

Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	IFNγ/UNT vs. IFNγ/Pg	0.0531
UNT vs. UNT/Pg	<0.0001	IFNγ/UNT vs. IFNγ/Sa	0.3179
UNT vs. UNT/Sa	<0.0001	IFNγ/Pg vs. IFNγ/Sa	>0.9999
UNT/UNT vs. UNT/Pg	<0.0001	Pg vs. Pg/UNT	0.6754
UNT/UNT vs. UNT/Sa	<0.0001	Pg vs. Pg/Pg	0.0156
UNT/Pg vs. UNT/Sa	>0.9999	Pg/UNT vs. Pg/Pg	0.9877
LPS vs. LPS/LPS	0.0005	Sa vs. Sa/UNT	0.2327
LPS vs. LPS/Pg	0.094	Sa vs. Sa/Sa	0.7335
LPS vs. LPS/Sa	0.248	Sa/UNT vs. Sa/Sa	>0.9999
LPS/LPS vs. LPS/Pg	0.4957	LPS/Pg vs. TNFα/Pg	>0.9999
LPS/LPS vs. LPS/Sa	0.202	LPS/Pg vs. IFNγ/Pg	0.0991
LPS/Pg vs. LPS/Sa	>0.9999	LPS/Pg vs. Pg/Pg	>0.9999
TNFα vs. TNFα/UNT	0.9499	TNFα/Pg vs. IFNγ/Pg	0.1663
TNFα vs. TNFα/Pg	0.3986	TNFα/Pg vs. Pg/Pg	>0.9999

TNFα vs. TNFα/Sa	0.3711	IFNγ/Pg vs. Pg/Pg	0.0268
TNFα/UNT vs. TNFα/Pg	0.0136	LPS/Sa vs. TNFα/Sa	>0.9999
TNFα/UNT vs. TNFα/Sa	0.0124	LPS/Sa vs. IFNγ/Sa	>0.9999
TNFα/Pg vs. TNFα/Sa	>0.9999	LPS/Sa vs. Sa/Sa	0.0807
IFNγ vs. IFNγ/UNT	0.9792	TNFα/Sa vs. IFNγ/Sa	>0.9999
IFNγ vs. IFNγ/Pg	>0.9999	TNFα/Sa vs. Sa/Sa	0.0875
IFNγ vs. IFNγ/Sa	>0.9999	IFNγ/Sa vs. Sa/Sa	>0.9999



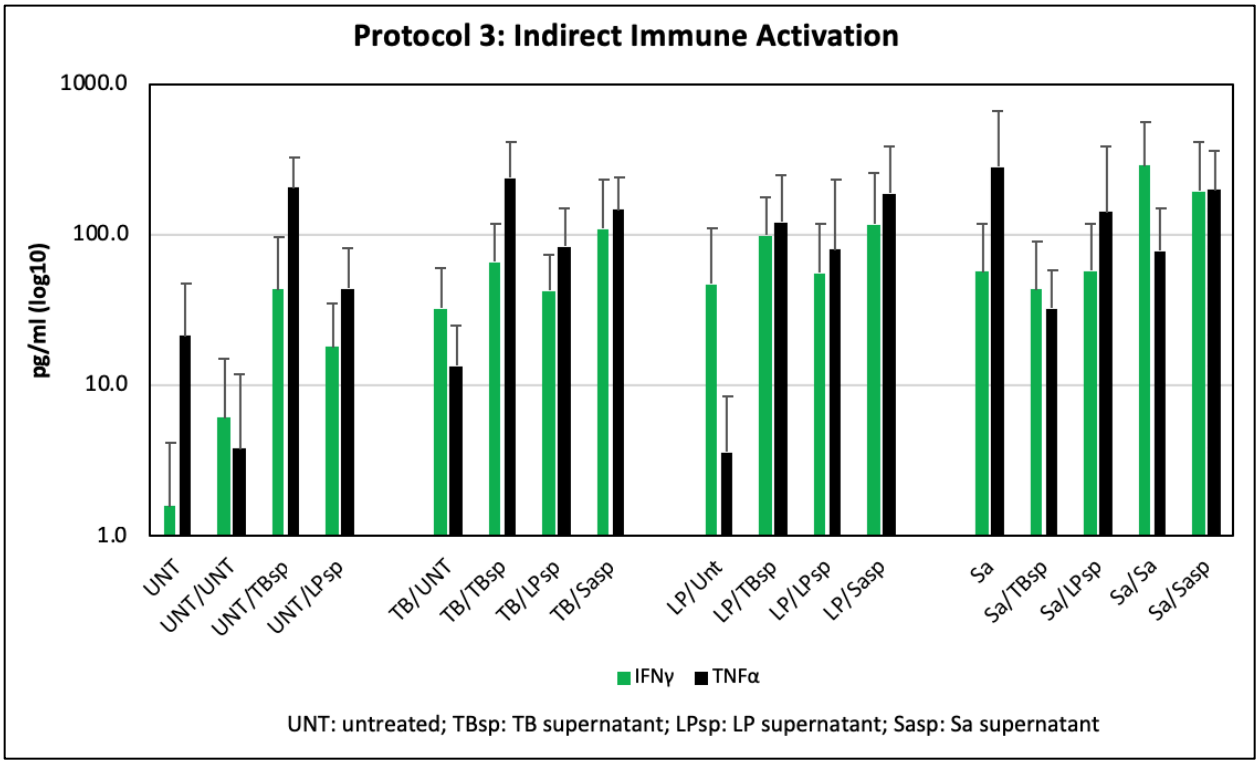
Adjusted P values IL-1

Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
Pg/Pg vs. LPS/Pg	>0.9999	Sa/Sa vs. IFNγ/Sa	>0.9999
Pg/Pg vs. IFNγ/Pg	>0.9999	LPS/Sa vs. TNFα/Sa	0.2443
Pg/Pg vs. TNFα/Pg	>0.9999	LPS/Sa vs. IFNγ/Sa	0.0112
LPS/Pg vs. IFNγ/Pg	>0.9999	TNFα/Sa vs. IFNγ/Sa	>0.9999
LPS/Pg vs. TNFα/Pg	>0.9999	LPS/Sa vs. LPS/Pg	0.9452
IFNγ/Pg vs. TNFα/Pg	>0.9999	IFNγ /Sa vs. IFNγ/Pg	0.0317
Sa/Sa vs. LPS/Sa	0.0986	TNFα/Sa vs. TNFα/Pg	0.2465
Sa/Sa vs. TNFα/Sa	>0.9999		

Adjusted P values cross cytokine comparison

Dunn's multiple comparisons test	Adjusted P Value
TNFα Pg/Pg vs. IL1 Pg/Pg	0.0037
TNFα Pg/Pg vs. IFNγ Pg/Pg	0.781
IL1 Pg/Pg vs. IFNγ Pg/Pg	0.0002
IL1LPS/Pg vs. TNFα LPS/Pg	0.4189
IL1LPS/Pg vs. LPS/Pg	0.0042
TNFα LPS/Pg vs. LPS/Pg	0.1218
IL1 IFNγ /Pg vs. TNFα IFNγ /Pg	0.4299
IL1 IFNγ /Pg vs. IFNγ IFNγ /Pg	>0.9999
TNFα IFNγ /Pg vs. IFNγ IFNγ /Pg	0.0225
IL1 TNFα /Pg vs. TNFα TNFα /Pg	>0.9999
IL1 TNFα /Pg vs. IFNγ TNFα /Pg	0.0361
TNFα TNFα /Pg vs. IFNγ TNFα /Pg	0.0046
IL1Sa/Sa vs. TNFα Sa/Sa	>0.9999
IL1Sa/Sa vs. IFNγ Sa/Sa	0.5415
TNFα Sa/Sa vs. IFNγ Sa/Sa	0.6938
IL1LPS/Sa vs. TNFα LPS/Sa	0.0339
IL1LPS/Sa vs. IFNγ LPS/Sa	0.0009
TNFα LPS/Sa vs. IFNγ LPS/Sa	0.626
IL1 TNFα /Sa vs. TNFα TNFα /Sa	0.5426
IL1 TNFα /Sa vs. IFNγ TNFα /Sa	>0.9999
TNFα TNFα /Sa vs. IFNγ TNFα /Sa	0.0787
IL1 IFNγ /Sa vs. TNFα IFNγ /Sa	0.022
IL1 IFNγ /Sa vs. IFNγ IFNγ /Sa	0.3713
TNFα IFNγ /Sa vs. IFNγ IFNγ /Sa	0.4857

5. Protocol 3 Results & Adjusted P values



Protocol 3 Adjusted P values TNFα

Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	TB/TB vs. LP/TB	0.927
UNT vs. UNT/TB	0.1512	TB/TB vs. Sa/TB	0.1624
UNT vs. UNT/LP	>0.9999	LP/TB vs. Sa/TB	0.7918
UNT/UNT vs. UNT/TB	0.0017	LP/LP vs. TB/LP	0.9942
UNT/UNT vs. UNT/LP	0.1644	LP/LP vs. Sa/LP	>0.9999
UNT/TB vs. UNT/LP	>0.9999	TB/LP vs. Sa/LP	>0.9999
TB/UNT vs. TB/TB	0.0367	LP/Sa vs. TB/Sa	>0.9999
TB/UNT vs. TB/LP	0.864	LP/Sa vs. Sa/Sasp	>0.9999
TB/UNT vs. TB/Sa	0.1704	LP/Sa vs. Sa/Sa	>0.9999
TB/TB vs. TB/LP	>0.9999	TB/Sa vs. Sa/Sasp	>0.9999
TB/TB vs. TB/Sa	>0.9999	TB/Sa vs. Sa/Sa	>0.9999
TB/LP vs. TB/Sa	>0.9999	Sa/Sasp vs. Sa/Sa	0.8938
LP/UNT vs. LP/SA	0.0299	LP/Sa vs. LP/LP	>0.9999
LP/UNT vs. LP/LP	0.5471	LP/Sa vs. LP/TB	>0.9999

LP/Unt vs. LP/TB	0.0419	LP/LP vs. LP/TB	>0.9999
Sa vs. Sa/TB	0.8543		
Sa vs. Sa/LP	>0.9999		
Sa vs. Sa/Sasp	>0.9999		
Sa vs. Sa/Sa	>0.9999		
Sa/TB vs. Sa/LP	>0.9999		
Sa/TB vs. Sa/Sasp	0.7092		
Sa/TB vs. Sa/Sa	>0.9999		
Sa/LP vs. Sa/Sasp	>0.9999		
Sa/LP vs. Sa/Sa	>0.9999		
Sa/Sasp vs. Sa/Sa	>0.9999		

Adjusted P values IFNγ

Dunn's multiple comparisons test	Adjusted P Value	Dunn's multiple comparisons test	Adjusted P Value
UNT vs. UNT/UNT	>0.9999	LP/TB vs. TB/TB	>0.9999
UNT vs. UNT/TB	0.0081	LP/TB vs. Sa/TB	0.4368
UNT vs. UNT/LP	0.0776	TB/TB vs. Sa/TB	>0.9999
UNT/UNT vs. UNT/TB	0.3182	LP/LP vs. TB/LP	>0.9999
UNT/UNT vs. UNT/LP	>0.9999	LP/LP vs. Sa/LP	>0.9999
UNT/TB vs. UNT/LP	>0.9999	TB/LP vs. Sa/LP	>0.9999
TB/UNT vs. TB/TB	>0.9999	LP/Sa vs. TB/Sa	>0.9999
TB/UNT vs. TB/LP	>0.9999	LP/Sa vs. Sa/Sasp	>0.9999
TB/UNT vs. TB/Sa	>0.9999	LP/Sa vs. Sa/Sa	>0.9999
TB/TB vs. TB/LP	>0.9999	TB/Sa vs. Sa/Sasp	>0.9999
TB/TB vs. TB/Sa	>0.9999	TB/Sa vs. Sa/Sa	>0.9999
TB/LP vs. TB/Sa	>0.9999	Sa/Sasp vs. Sa/Sa	>0.9999
LP/UNT vs. LP/Sa	>0.9999	Sa vs. Sa/Sa	>0.9999
LP/UNT vs. LP/LP	>0.9999	Sa vs. Sa/Sasp	>0.9999
LP/UNT vs. LP/TB	0.4993	Sa/TB vs. Sa/LP	>0.9999
LP/Sa vs. LP/LP	>0.9999	Sa/TB vs. Sa/Sasp	>0.9999
LP/Sa vs. LP/TB	>0.9999	Sa/TB vs. Sa/Sa	>0.9999
LP/LP vs. LP/TB	>0.9999	Sa/LP vs. Sa/Sasp	>0.9999
Sa vs. SA/TB	>0.9999	Sa/LP vs. Sa/Sa	>0.9999
Sa vs. SA/LP	>0.9999	Sa/Sasp vs. Sa/ Sa	>0.9999

6. Sample Sizes

Total initial sample sizes are reported here. Experimental conditions with less than a minimum of four were automatically excluded from analysis. These sample sizes do not account for the removal of outliers.

	TNFα (n)	IFNγ (n)		TNFα (n)	IFNγ (n)
UNT	31	30	**Pg/Pg**	22	23
LPS	15	14	**Pg/UNT**	11	11
TNFα	12	12	**Pg/TB**	8	8
IFNγ	10	10	**Pg/LP**	8	8
Pg	29	29	**Sa/UNT**	8	7
Sa	26	25	**Sa/Sa**	20	19
TB	36	36	**Sa/TB**	8	7
LP	38	36	**Sa/LP**	8	7
UNT	37	36	**TB/UNT**	30	26
LPS/LPS	8	7	**TB/PG**	14	14
UNT/TNFα	2	2	**TB/SA**	14	13
UNT/IFNγ	2	2	**TB/TB**	32	28
UNT/Pg	17	17	**TB/LP**	22	21
UNT/Sa	16	15	**LP/LP**	38	35
UNT/TB	13	12	**LP/UNT**	29	27
UNT/LP	13	12	**LP/PG**	12	12
LPS/Pg	11	11	**LP/SA**	14	13
LPS/Sa	12	12	**LP/TB**	16	15
TNFα/UNT	8	8	**IFNγ/UNT**	8	8
TNFα/Pg	12	12	**IFNγ/Pg**	10	10
TNFα/Sa	12	12	**IFNγ/Sa**	10	10

IL-1β

UNT	7	**IFNγ/Pg**	5
UNT/TNFα	2	**TNFα/Pg**	7
TNF/U	3	**Sa/Sa**	7
UNT/IFNγ	2	**LPS/Sa**	7
IFNγ/U	3	**TNFα/Sa**	7
Pg/Pg	7	**IFNγ/Sa**	5
LPS/Pg	6		

Supernatants

	TNFα SP (n)	IFNγ SP (n)
UNT	5	7
LPS	5	4
Sa	5	4
UNT/UNT	8	4
LPS/LPS	5	4
UNT/TB	5	5
UNT/LP	5	5
LP/UNT	7	8
LP/Sa	5	4
LP/LP	8	8
LP/TB	7	8
TB/UNT	5	5
TB/TB	4	5
TB/LP	5	5
TB/Sa	5	4
Sa/TB	5	4
Sa/LP	5	4
Sa/Sasp	5	4
Sa/Sa	5	4

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Reconstructing host immune status in acquired syphilis: a bioarchaeological and immunological approach (Coauthor)
Megan Duncanson, Molly Zuckerman, Sharon DeWitte, and Fabián Crespo
American Association of Physical Anthropologists – March 30, 2019, Cleveland, Ohio

Skeletal inflammatory index: Pursuing experimental evidence
Megan Duncanson and Fabián Crespo
American Association of Physical Anthropologists – April 13, 2018, Austin, Texas

Effect of Mycobacterial Species on Immune Cells and its Potential Impact on Inflammatory Responses in Periosteal Lesions
Megan Duncanson, Sharon DeWitte, and Fabián Crespo
American Association of Physical Anthropologists – April 22, 2017, New Orleans, Louisiana

Tuberculosis and Leprosy Cross-Immunity Hypothesis: Considering the Potential Role of Other Mycobacterial Species (Coauthor)
Haleigh Mitchell, Jacob White, Megan Duncanson, and Fabián Crespo
American Association of Physical Anthropologists – Undergraduate Research Symposium
April 19, 2017, New Orleans, Louisiana

Periosteal Lesions As Marker For Systemic Inflammatory Shifts In Tuberculosis And Leprosy Infections: An Invitation For A New Dialogue Between Experimental Immunology And Paleopathology.
Megan Duncanson, Christopher Klaes, Sharon DeWitte, and Fabián Crespo
Research! Louisville– October 11, 2016, Louisville, Kentucky

Periosteal Lesions as Marker for Systemic Inflammatory Shifts in Tuberculosis and Leprosy Infections: An in vitro analysis
Megan Duncanson, Christopher Klaes, Sharon DeWitte, and Fabián Crespo
Paleopathology Association – April 13, 2016, Atlanta, Georgia

Impact of Gestational Alcohol Exposure on Offspring Glucose Availability
Megan Duncanson, Caitlin Gambrell, Rachel E. Neal
American Association of Physical Anthropologists – Undergraduate Research Symposium-
March 25, 2015, St. Louis, Missouri

Impact of Gestational Alcohol Exposure on Offspring Glucose Availability
Megan Duncanson, Caitlin Gambrell, Rachel E. Neal
Ohio Valley Chapter of the Society of Toxicology – September 26, 2014, Dayton, Ohio

Impact of Gestational Alcohol Exposure on Offspring Glucose Availability
Megan Duncanson, Lorena Canlaes, Caitlin Gambrell1, Rachel E. Neal
Summer Research Opportunity Program – August 31, 2014, Louisville, Kentucky