# THE INDUCTION OF TYPE-I INTERFERON RESPONSES IN PRIMARY OSTEOBLASTS FOLLOWING STAPHYLOCOCCUS AUREUS INFECTION

by

Kelli Furr

A thesis submitted to the faculty of
The University of North Carolina at Charlotte
in partial fulfillment of the requirements
for the degree of Master of Science in
Biology

Charlotte

2022

Approved by:

_____
Dr. Ian Marriott

_____
Dr. M. Brittany Johnson

_____
Dr. Andrew Truman

# ABSTRACT

KELLI HUTCHESON FURR. Investigating the role of protective type-I interferon responses in primary osteoblasts following *Staphylococcus aureus* infection. (Under the direction of DR. IAN MARRIOTT and DR. M. BRITTANY JOHNSON)

Osteomyelitis is a serious bacterial infection of bone that is associated with progressive inflammatory tissue damage. This condition commonly affects the long bones of children along with the vertebrae and pelvis of adults. *Staphylococcus aureus*, the principal causative agent of osteomyelitis, can enter bone via the bloodstream or surrounding tissues following injury or surgery resulting in disease that is often refractory to therapies such as debridement or antibiotic treatment. Furthermore, the increasing incidence of infections associated with antibiotic resistant strains of *S. aureus* has compounded this problem such that new treatment strategies are needed urgently.

Bone-forming osteoblasts can be infected by *S. aureus* and serve as an intracellular bacterial reservoir for chronic infection. However, this infection can be perceived via an array of microbial pattern recognition receptors that are known to be expressed by osteoblasts, triggering changes in their function. For example, these cells play a critical role in the control of the formation and activity of bone resorbing osteoclasts, and we have shown that they can exacerbate inflammation at sites of infection by producing an array of cytokines, chemokines, and growth factors. As such, infected osteoblasts play an important role in the abnormal bone formation and inflammatory damage associated with *S. aureus* infection.

Type I interferons are best known for their antiviral effects, but it is becoming increasingly apparent that they can impact host susceptibility to a wide range of pathogens including *S. aureus*. Furthermore, two studies have suggested that osteoblasts might be capable

of producing the type I interferon, IFN-β, either constitutively or following exposure to pattern recognition receptor agonists. In the present study, we have assessed the ability of primary murine osteoblasts to produce and respond to IFN-β following infection with *S. aureus* strain UAMS-1. The production of IFN-β and interferon stimulated genes were assessed by specific capture ELISAs and immunoblot analyses.

IFN-β production was confirmed with the demonstration that *S. aureus* induces its rapid and robust release by osteoblasts in a dose-dependent manner. We have also shown increased protein expression of the interferon stimulated gene products IFIT1 and IFIT3 by infected OBs and demonstrated that this occurs secondary to the release of IFN-β by these cells. Finally, we have determined that exposure of *S. aureus*-infected osteoblasts to IFN-β markedly reduces the number of viable bacteria harbored by these cells. Together with studies performed previously by our laboratory, these findings indicate an ability of osteoblasts to respond to bacteria by producing IFN-β that can act in an autocrine and/or paracrine manner to elicit interferon stimulated gene expression and mitigate *S. aureus* infection.

# DEDICATION

*This thesis is dedicated to the man who has always supported my dreams, my father.*

*I love you so much and I could not have done it without you.*

# ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Marriott, who took a chance on me and never gave up. I would also like to thank Dr. Johnson, who taught me the ins and outs of the lab. I would like to thank Dr. Truman for completing my committee, and for always providing positivity and the same shared excitement I have about science. I would like to thank Dr. Reitzel for his continued support and guidance throughout my journey in graduate school. I would like to thank my family and friends for their unwavering patience and support. I would like to thank my husband for staying by side and always offering words of encouragement and love. Finally, I owe all my success to my dad for his infinite words of wisdom, love, and encouragement.

TABLE OF CONTENTS

# LIST OF FIGURES

# CHAPTER 1: INTRODUCTION

## 1.1 Osteomyelitis

Osteomyelitis, a bone disorder that results from bacterial infection, is associated with severe inflammation and progressive bone loss to the affected area (Sax & Lew, 1999). The three types of osteomyelitis are acute, chronic, and vertebral, and can affect all ages and genders. However, this disease typically affects the long bones of children and teens, and the vertebrae and pelvis of adults. Osteomyelitis affects about 2 to 5 in every 10,000 people in the United States annually. There are multiple factors that can contribute to the onset of osteomyelitis such as diabetes or certain fungi; however, the bacterium *S. aureus* is considered the principal causative agent.

The majority of these infections are a result of external factors such as wounds developed from trauma or infections that developed post-operation (Boeck, 2005; Dubey et al., 1988; Mousa, 2003). These post-operative infections have become so common, that the administration of pre-operation antibiotics has become routine. Unfortunately, even with this knowledge and use of precautionary measures, the prevalence of osteomyelitis is still increasing (Jensen et al., 1997; R. et al., 2006). Antibiotic resistance is thought to be one of the main culprits in the rise of cases despite treatment.

The current treatments available for osteomyelitis are expensive and taxing on the body. These treatments can include prolonged (even lifelong in severe cases) antibiotic treatments, surgical debridement (removal) of the necrotic areas of bone, or even amputation. Even with the completion of a specific treatment and the inability to isolate the infection-causing organisms, studies have shown that 17% of cases were not cured and those patients showed recurrent osteomyelitis post-treatment (Craigen et al., 1992; Priest & Peacock, 2005). Therefore, new

treatment strategies are desperately needed to eliminate infections and reduce inflammatory bone loss caused by osteomyelitis.

1.2 *Staphylococcus aureus*

Staphylococci, a spherically shaped bacterium, has traditionally been known to be non-invasive to host bone cells and does damage by adhering to the host cell's extracellular matrix (Nair et al., 1996). *S. aureus* produces an array of factors that contribute to the virulence and evasion capability of the pathogen. These factors include toxins, immune evasion factors, and protein and non-protein factors which all enable host colonization during infection (Cheung et al., 2021). During extracellular infection, *S. aureus* has the ability to produce an array of toxins that are detrimental to the targeted host. Bacterial breach through the epithelial layer of the skin is necessary for systemic *S. aureus* infection. Other than breach through trauma or a surgical procedure, *S. aureus* is also capable of producing alpha toxin which breaks the adherens junction and weakens the actin cytoskeleton (Cheung et al., 2021; Popov et al., 2015). As an entire pathogen, *S. aureus* can also initiate infection simply by entering the host via a contaminated medical device. *S. aureus* accomplishes this route of infection by adhering to both the medical device plastic itself, as well as the matrix molecules established after implantation (Cheung et al., 2021). A third factor is *S. aureus'* ability to be an opportunistic pathogen following other pathogens or conditions. For example, in cases where viral lung infections have occurred, a secondary *S. aureus* infection can develop and ultimately cause host death by the subsequent binding of the already compromised epithelial lining of the lungs. (Cheung et al., 2021; McCullers, 2014).

In addition to the virulence factors previously mentioned, *S. aureus* also has the ability to prolong infection by evading host immune mediators.

Interestingly, although all these factors contribute to successful extracellular *S. aureus* infection, this pathogen has still developed others way to cause infection and avoid host detection.

It is now understood that these bacteria can be internalized by osteoblasts, bone-forming cells, persist within the host cell, and possibly even proliferate within the osteoblasts (Ahmed et al., 2001; Ellington et al., 1999, 2003, 2006; Hudson et al., 1995; Jevon et al., 1999; Mohamed et al., 2014, 2016; Strobel et al., 2016). First, to enter the osteoblasts undetected, *S. aureus* must be taken up by the host cell. The process of internalizing *S. aureus* into osteoblasts is through the β-zipper mechanism (Cheung et al., 2021). First, *S. aureus* targets host fibronectin, an extracellular matrix protein that plays a vital role in host tissue repair. Then, fibronectin binding proteins, primary attachment components located in the bacteria's cell wall, latch onto the host's fibronectin (Schwarz-Linek et al., 2003). In host cells such as OBs, Fn receptors such as α5β1 integrins mediate *S. aureus* internalization into cells, which allows the bacteria-host interaction to continue until the bacteria has been fully internalized (Wen et al., 2020). This stealthy internalization allows the bacteria to then evade the immune system as well as many different antibiotic therapies.

Interestingly, there are also significant time-dependent changes in the structure of *S. aureus* following exposure to an intracellular environment in as little as 12 hours. This exposure causes *S. aureus* to develop small colony variants that have lower cytotoxicity (Ellington et al., 2003; Kalinka et al., 2014). This acquired trait has allowed *S. aureus* to become less sensitive to eukaryotic cell penetrating antibiotics (Ellington et al., 2006; Tuchscherr et al., 2016). It is also relevant that the bacteria released following lysis of *S. aureus* containing human osteoblasts are viable and capable of invading other osteoblasts (Ellington et al., 2003). The survival of this

bacteria, despite the lysis of the host cell, could explain why antibiotic therapies are not as effective as once theorized, given that the antibiotics can eliminate viable bacteria in an osteomyelitis model but does not adequately sterilize infected bone (Monzón et al., 2001; Tuchscherr et al., 2016).

Therefore, *S. aureus* sequestered in osteoblasts potentially provide a bacterial reservoir and contribute to the recurrent staphylococcal osteomyelitis that is resistant to current therapies such as antibiotic treatment. Naturally, the host can combat these extracellular toxins and other evasion mechanisms, as well as the pathogen itself, by using both arms of the immune system. For example, the recruitment of neutrophils, important effector cells in the innate arm of immunity, have the ability to phagocytose said toxins (Tam & Torres, 2019). Therefore, pathogens such as *S. aureus* have had to adapt and find new ways to infect the desired host while also evading the host's various immune responses.

### 1.3 The Role of Osteoblasts in Osteoclastogenesis

Osteoblasts, the bone-forming cells, and osteoclasts, the bone-resorbing cells, work in conjunction with each other to maintain bone homeostasis (Dirckx et al., 2019). The ability to maintain this homeostasis is accomplished by osteoblasts and osteoclasts through bone remodeling. Bone remodeling is the complex process of constantly replacing mature bone with new bone, which is achieved by formation and resorption of osteoblasts and osteoclasts, respectively (Park-Min, 2018). There are three phases of bone remodeling that occur consecutively. First, osteoclasts digest the old or mature bone. Second, mononuclear cells appear on the surface of the area of bone being remodeled to initiate reversal. Finally, osteoblasts form new bone where the resorption took place until the area of resorbed bone is filled back in with new bone (Hadjidakis & Androulakis, 2006).

Osteoclasts have the ability to resorb bone through both acidification and proteolysis of the bone matrix. These processes are accomplished following the attachment of osteoclasts to the bone's surface. Integrins expressed in osteoclasts and specific amino acid sequences within proteins found at the surface of the bone's extracellular matrix is where this attachment occurs. Podosomes, complexes formed by the integrins previously described, allow a constant interaction and subsequent movement of osteoclasts to occur over the surface of bone. However, this bone-resorption performed by osteoclasts must be regulated to prevent overactive osteoclastic activity (Hadjidakis & Androulakis, 2006).

Local regulation of bone resorption is achieved through the OPG/RANK/RANKL system. Osteocytes, osteoblast-lineage cells, express both macrophage-colony stimulating factor and the receptor activator of nuclear factor κB (RANKL), both of which are required for the maturation of osteoclasts (Fumoto et al., 2014). RANKL binds to its receptor, RANK, expressed via the stimulation of macrophage-colony stimulating factor on osteoclast precursor cells. RANK-RANKL signaling then initiates the osteoclast differentiation process which is necessary before the bone resorption step can begin. Finally, once bone resorption has been completed, the differentiation of osteoclasts is regulated by osteoprotegerin, also known as OPG. Osteoprotegerin, a member of the tumor-necrosis factor superfamily, regulates osteoclast differentiation by acting as a decoy receptor and interrupting the RANK-RANKL binding (Maruyama et al., 2006; Park et al., 2017; Yasuda et al., 1998). Osteoprotegerin is produced and released by osteoblasts which ends the osteoclastogenesis portion of the bone remodeling cycle. However, osteoblasts' role in maintaining bone homeostasis is not the only function this bone-forming cell serves.

### 1.4 The Role of Osteoblasts in Immunity and Inflammation

Although the main role of osteoblasts is to synthesize bone components and regulate osteoclast activity and formation, more research is showing that osteoblasts also play a role in the initiation/augmentation of inflammation when in the presence of infection. Previous studies performed by our laboratory have already shown osteoblast's ability, in the presence of infection, to produce various cytokines and chemokines, which can promote osteoclastogenesis and worsen bone loss, while also aiding the recruitment of leukocytes (Johnson, Suptela, et al., 2022).

The human body is constantly defending itself against various and ever-evolving pathogens such a *S. aureus*. Therefore, the body has had to continually adapt to its environment and be able to recognize new, foreign patterns that could cause serious complications. These patterns are known as pathogen-associated molecular patterns (PAMPs). The receptors responsible for the recognition of these PAMPs are known as pattern-recognition receptors (PRRs) (Alonso-Pérez et al., 2018). PAMPs specifically produced by *S. aureus* include lipoproteins, lipopeptides, and peptidoglycans (Soe et al., 2021). The specific PRRs responsible for the recognition of these PAMPs located on osteoblasts are known as toll-like receptors.

An important factor involved in the recognition of a pathogen such as *S. aureus* are the presence of toll-like receptors. Toll-like receptors, specifically TLR2 and TLR4, are members of the innate arm of immunity that recognize a variety of microbial molecules including *S. aureus* bacterial components (Kassem et al., 2016). This recognition of *S. aureus* initiates a signaling cascade that leads to the production of various cytokines that serve either to protect the host, harm the pathogen, or both, all of which are still widely studied. TLR4 is especially important in the regulation of inflammation within the host following pathogen recognition (Alonso-Pérez et al., 2018). These toll-like receptors are found on the surface of osteoblasts, but there are even more receptors located within. Following internalization, osteoblasts have other ways of

recognizing microbial components. For example, a family of nucleotide-binding oligomerization domain-containing (NOD) proteins have been shown to serve as intracellular PRRs in both immune and non-immune cells. NOD1 and NOD2 have both been shown to recognize microbial components of *S. aureus* following bacterial challenge (Marriott et al., 2005). Both NOD1 and NOD2 play a role in the clearance of internalized pathogens and work with toll-like receptors to detect the presence of invading pathogens.

In contrast, intracellular components of osteoblasts have also been shown to, following internalization of the pathogen, lead to the engulfment of the microbial components into the late endosomes or lysosomes. This is accomplished via endocytosis within the osteoblast (Jauregui et al., 2013). This intracellular infection of *S. aureus* into the late endosome and/or lysosome initiates the upregulation of various inflammatory cytokines. Among these cytokines are interleukin-6 (IL-6) and interleukin-12 (IL-12) (Bost et al., 1999). IL-6 functions to stimulate the immune system by stimulating both T- and B-cells following pathogen detection (Tanaka et al., 2014). IL-12 promotes the production of IFN-γ from natural killer (NK) cells and T-lymphocytes (Bost et al., 1999). Finally, a large component of the inflammatory responses produced in response to *S. aureus* infection of osteoblasts are the type I interferon.

### 1.5 Type I Interferons

Interferons are a large group of cytokines that have various roles in innate immunity. Of these, IFN-α and IFN-β are the most versatile members of the type I class of this cytokine family (Parker et al., 2014; Peignier & Parker, 2021). Originally known for their antiviral effects, the type I interferons have now also been shown to play a part in cancer and autoimmunity conditions. More recently, Type I interferons have been shown to initiate intracellular antimicrobial responses via upstream PRR recognition of bacterial pathogen-associated

molecular patterns (PAMPs). However, it is currently unknown whether interferon production and secretion is beneficial or detrimental to the host, especially in *S. aureus* infection, where contradictory effects have been reported (Peignier & Parker, 2021).

IFN-β is a cytokine produced by the immune system that naturally responds to biological and chemical stimuli. One of these many biological stimuli is the detection of certain pathogens such as *S. aureus*. Following pathogen detection and interferon production and secretion, interferon molecules bind to cell surface receptors such as IFNAR1 and IFNAR2 which initiate the Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling cascade (Schneider et al., 2014; Stark & Darnell, 2012).

Binding of the cell surface receptors to specific cytokines such as IFN-β induces the dimerization of the separate subunits that make up the receptor which then activates the receptor associated JAKs. Activated JAKs create sites that allow STAT transcription factors to dock. This docking is created through phosphorylation of tyrosine residues such as tyrosine kinase (TYK2), found on cytokine receptor subunits. Once this JAK-mediated phosphorylation has occurred, the STAT transcription factors STAT1 and STAT2 dimerize and translocate from their receptor docking sites to the nucleus. However, for this translocation to occur, the phosphorylated STAT1/2 dimer must also join with a methylated interferon regulatory factor 9 (IRF9). IRF9 is an integral transcription in the mediation of type I interferon responses (Stark & Darnell, 2012). Translocation to the nucleus allows the STAT transcription to induce the expression of genes containing the appropriate regulatory sequences in their promoter region. The dimer binds to specific adjustment zones in the DNA which in turn regulates the transcription process through the use of negative feedback loops (Xin et al., 2020).

This cascade then leads to the production of interferon stimulated genes. Interferon stimulated genes, transcriptionally induced by interferons, have been studied in the past for their antiviral properties (Borden et al., 2007; Schoggins & Rice, 2011) and are now of interest when in the presence of other pathogens such as bacteria. Currently, the role of type I interferons and their gene products in host susceptibility to *S. aureus* infection of bone tissue has yet to be explored.

1.6 Interferon stimulated genes

Some of the most well-known gene products of type I interferons are interferon stimulated genes. Interferon stimulated genes are capable of interfering with viral and bacterial RNA responses (Carty et al., 2021; Kawasaki & Kawai, 2019). Although interferon stimulated genes are well-known for their ability to combat viral infections, their role in response to specific bacterial pathogens is still mostly a mystery. In various studies, the role interferon stimulated genes play in response to both viral and bacterial challenge starts with assessing the changes in the mRNA in host cells and comparing interferon stimulated gene levels in the absence and presence of infection. In fact, infected osteoblasts have already been shown to have increased levels of interferon stimulated genes following bacterial challenge(Johnson, Furr, et al., 2022). These interferon stimulated genes include members of the IFIT family, SLFN2, IRGM2, MX2, PLSCR1, IFI205, and IGTP.

The interferon-induced protein with tetratricopeptide repeats (IFIT) family is comprised of a group of related genes that have previously been shown to inhibit viral replication. Members of the IFIT family contain multiple tetratricopeptide repeats which serve to regulate transcription, protein transport and folding, and parts of the cell cycle. Interestingly, most cell types do not produce IFIT proteins under basal conditions, but rather are induced rapidly in

response to infection (Diamond, 2014). For example, IFIT1 proteins act as effector molecules to restrict viral translation by blocking the initiation of the viral translation (Diamond, 2014). More recently, IFIT1 protein expression has also been shown to be produced in response to mycobacterium tuberculosis infection which is indicative of interferon stimulated genes role in the immune responses for both viral and bacterial challenges (Andreu et al., 2017). Not surprisingly, there is a similar trend shown with the IFIT protein, IFIT3. IFIT3, also generally poorly expressed in normal cell conditions, shows increased protein expression in the presence of viral infections, interferon treatments, and PAMPs. Also in keeping with the IFIT family trends, IFIT3 functions to inhibit viral replication (Zhang et al., 2023). Similarly, in the presence of bacterial infections such as tuberculosis, IFIT3 protein expression has been shown to be upregulated due to its role in innate immunity (Garlant et al., 2022).

The Schlafen (SLFN) proteins, specifically the ones induced by type I IFNs such as SLFN2, are found to be expressed during thymocyte maturation and T cell differentiation. SLFN2 plays a role in Type I interferon signaling, and has also been shown to be induced by RANKL to promote osteoclastogenesis (Fischietti et al., 2018). There have been various studies suggesting the antiproliferative effects that SLFN2 proteins have on type I interferons (Katsoulidis et al., 2009); however, the connection between the role SLFN2 plays on type I interferons and their respective role in response to both viral and bacterial infections is still being investigated.

The immunity-related GTPase member 2 (IRGM2), is an interferon stimulated gene associated with the protection against sepsis and the regulation of the non-canonical inflammasome response associated with interferon-inducible guanylate binding proteins (GPBs) (Eren et al., 2020). Specifically, following the detection of gram-negative bacteria, IRGM2 is

responsible for the regulation of caspase-11, which detects the gram-negative bacterial cell wall (Pilla-Moffett et al., 2016). Although the role of IRGM2 has been studied in gram-negative bacteria, their role in the presence of gram-positive bacteria has yet to be explored.

The Mx GTPases, such as MX2, are known as key antiviral effector proteins. They have been shown to inhibit viral infections by blocking the initial steps in the viral replication process (Haller et al., 2015). MX2 has even been shown to limit nuclear entry by expressing type I interferon-inducible antiviral effectors following to inhibit HIV infection (Bhargava et al., 2018). In regards to bacterial infection, MX2 mRNA expression was shown to be upregulated following tuberculosis infection which suggests a possible immune response associated with bacterial challenge as well (Yi et al., 2021).

Phospholipid scramblases (PLSCRs) are a family of single pass transmembrane proteins responsible for Ca2+ mediated bidirectional phospholipid translocation in plasma membranes. One of the family members, PLSCR1, is the most extensively studied PLSCR due its role in apoptosis (Sivagnanam et al., 2017). Following viral infection, PLSCR1 has been shown to play a significant role in interferon-mediated antiviral defense and apoptosis via the JAK-STAT pathway (Sivagnanam et al., 2017). In fact, PLSCR1 has been reported to have a protective role in the presence of certain bacterial pathogens. For example, in the presence of the alpha-toxin produced by *S. aureus*, interferon-induced PLSCR1 was shown to protect the host cells from the toxin as well as reduce intracellular leakage of host cell ATP into the extracellular space (Lizak & Yarovinsky, 2012; Yarovinsky, 2012).

Both IFI205 and IGTP are considered interferon inducible proteins. The interferon-inducible p205 protein (IFI205), is named/defined for having at least one 200 amino acid region and is responsible for the slowing down of bone marrow cell growth. IFI205 has been shown to

inhibit cell proliferation, suppress tumor growth, and cause inflammasome activation in bone marrow derived macrophages (Ghosh et al., 2017). Interestingly, IFI205 has also been discovered to enhance osteoblast differentiation when overexpressed (Li et al., 2016). IGTP is a member of the interferon induced GTPases and has previously been shown to be critical for host resistance against pathogens (Collazo et al., 2002). More recently, IGTP has also been shown to help regulate antimicrobial autophagy induction and autophagosome maturation in response to infections (Coers et al., 2018).

All the interferon stimulated genes either play a part in the innate immune system regarding host responses, or responses towards infection, both viral and/or bacterial. However, whether the roles of these interferon stimulated genes in response to bacterial infection, such as during *S. aureus* infection, is playing a harmful or protective role is still being investigated.

### 1.7 Bacterial Survival

*S. aureus* is still regarded as an extracellular pathogen that damages host cells by binding to the extracellular matrix. This is despite the knowledge that various mammalian cells have now been shown to internalize *S. aureus*, including osteoblasts (Ahmed et al., 2001; Ellington et al., 1999, 2003, 2006; Hudson et al., 1995; Jevon et al., 1999; Mohamed et al., 2016; Strobel et al., 2016). Importantly, *S. aureus* can persist within osteoblasts once internalized and undergo rapid small colony variant phenotypic changes. These phenotypic changes allow *S. aureus* to be less sensitive to both bacteriostatic and bactericidal antibiotics. Subsequently, bacteria that is released from lysed human cells have been shown to be viable and capable of invasion into other osteoblasts (Ellington et al., 2003). *S. aureus*' ability to create these reservoirs of infection may explain how staphylococcal osteomyelitis persists despite recurrent antibiotic therapies and seemingly sufficient humoral responses.

## 1.8 Hypothesis of the Present Study

In the present study, we have tested the hypothesis that osteoblasts infected with *S. aureus* infection will directly stimulate the expression of type I interferons, and that this expression of type I interferons will cause the upregulation of various interferon stimulated genes (Figure 1). We have also tested the hypothesis that type I interferons function to limit bacterial burden associated with osteomyelitis (Figure 1). In previous studies, Type I interferons have been reported to have both protective and deleterious effects on *S. aureus* infections depending on the model and/or the pathogen (Martin et al., 2009; Parker et al., 2014; Parker & Prince, 2012). Since the expression of IFN-β has previously been shown to have both antiviral and antibacterial properties, we sought to determine if IFN-β plays the same role in osteoblasts when in the presence of infection.

Given the known importance of IFN-β in establishing an antiviral state within host cells, we sought to test the hypothesis that treatment of osteoblasts with IFN-β will show a decrease in the number of live intracellular bacteria following infection. This could be due to IFN-β also having antibacterial properties in the presence of infection. We sought to demonstrate that IFN-β treatment may play a protective role in osteoblasts during bacterial challenge (Figure 1).

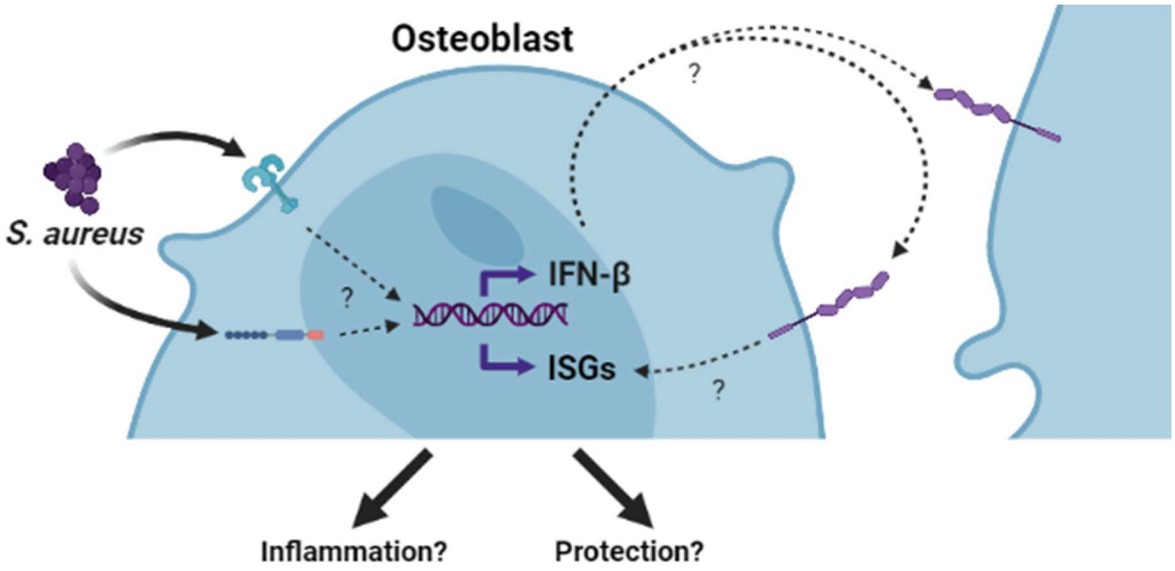


Figure 1: Schematic indicating proposed mechanism for type I interferon and interferon stimulated gene production following *S. aureus* infection. *S. aureus* PAMPs are recognized either by membrane associated or cytosolic PRRs. The recognition and subsequent bacterial invasion of these microbial components trigger osteoblasts to initiate gene transcription of the type I interferon, IFN-β or interferon stimulated genes. IFN-β protein production and release then either acts back on osteoblasts in an autocrine and/or paracrine manner. IFN-β is perceived by an interferon receptor, which then could initiate interferon stimulated gene transcription and protein production. IFN-β and interferon stimulated gene production following *S. aureus* infection has the potential to affect levels of inflammation and the overall protection of the host following bacterial challenge.

# CHAPTER 2: MATERIALS AND METHODS

## 2.1 *S. aureus* propagation

*S. aureus* strain UAMS-1 was grown on Luria broth (LB) agar plates overnight at 37□ in a 5% CO2 atmosphere and was cultured in Luria broth on an orbital rocker at 37□ in 5% CO2 overnight. Prior to use, the cultures were grown to mid-log phase in Luria broth at 37□ with 5% CO2 and the number of colony forming units was determined by spectrophotometry using a Genespec3 spectrophotometer (MiraiBio Inc.).

## 2.2 Isolation and Culture of Mouse Calvaria and Osteoblasts

Murine osteoblast cultures were obtained from the skull caps of murine neonates 2-3 days after birth. Skulls from C57BL/6J neonates were surgically detached from the body with sterile scissors. Sterile scissors and forceps were used to remove the skull cap from the rest of the detached skull. Surgical forceps were used to remove any remaining tissue from the skull caps before digestion. The prepared skull caps were then placed in digest media containing low glucose Dulbecco's Modified Eagle Medium (DMEM), collagenase P, and 0.25% Trypsin. Skull caps are rocked in 2mLs of digest media for 15 minutes per well in a 6-well plate. The supernatant in the first well is discarded, and the remaining wells supernatants (10mLs total) are collected into a conical where an equal amount of plating media containing Dulbecco's Modified Eagle Medium (DMEM), 10% Fetal Bovine Serum (FBS), and 1% penicillin/streptomycin is added. The total 20mLs of media containing the digested osteoblasts are then spun down at 1000 RPM for 5 minutes. The supernatant is discarded, and the primary osteoblasts are resuspended in fresh plating media and stored at 37°C in a 5% $CO_2$ atmosphere. At 24 to 72 hours, primary OBs were plated at a density of 2 x $10^5$ cells per well in a 6-well plate and differentiated in alpha minimum essential medium (αMEM) supplemented with 10% FBS, 0.1M ascorbic acid, 1M β-

glycerol phosphate, and 1% penicillin/streptomycin at 37°C in a 5% $CO_2$ atmosphere. The differentiation media was changed every other day until day 10, the day of experiment/infection. The presence of mature osteoblasts was confirmed using an alkaline phosphatase staining kit (abcam) on day 10. Positive staining was assessed by light field microscopy.

2.3 In vitro *S. aureus* infection of murine osteoblasts

Differentiated osteoblasts were infected with *S. aureus* at multiplicities of infection (MOI) between 50:1 and 150:1 bacteria to each bone cell in antibiotic-free media at 37°C in a 5% $CO_2$ atmosphere for 2 hours, and then the media was discarded. A quick wash of 1X phosphate buffered saline (PBS) was used to remove any residual media. Antibiotic containing media was placed in each well to kill residual extracellular bacteria. At the indicated time points following infection with *S. aureus*, cell supernatants and whole cell protein lysates were collected. In the studies evaluating interferon stimulated gene expression following *S. aureus* infection, osteoblasts were lysed with either 1X triton-lysis buffer or tissue protein extraction reagent (T-PER).

2.4 Enzyme-linked immunosorbent assays

Specific capture ELISA was performed to quantify the production of IFN-β following *S. aureus* infection. An IFN-β DuoSet kit provided the capture and detection antibodies (R&D Systems; Cat # DY8234-05; Lot # P332744). Streptavidin-HRP (R&D Systems; Lot # P198628) was added prior to the addition of tetramethylbenzidine substrate. The reaction was stopped with 1:30 $H_2SO_4$ and the absorbance was measured at 450 nm. Recombinant murine IFN-β (R&D Systems; Lot # P296488) was utilized to generate standard curves. Extrapolation of the absorbance of the to the standard curve was used to determine the concentration of IFN-β in the cell supernatants.

2.5 Immunoblot analyses

Total cell lysates were evaluated using immunoblot analysis for the presence of IFIT, IFIT3, and PLSCR1. After blocking in 5% milk in tris-buffered saline with 0.1% Tween (TBS-T) for at least 1 hour, immunoblots were incubated with a monoclonal antibody directed against IFIT1 (Novus Biologicals, clone OTI3G8), a polyclonal antibody directed against IFIT3 (Invitrogen), or a monoclonal antibody directed against PLSCR1 (Invitrogen, clone ARC2028), overnight at 4°C. Immunoblots were then washed and incubated with a horseradish peroxidase (HRP)-conjugated anti-mouse or anti-rabbit secondary antibody. Bound antibody was detected with a Pierce enhanced chemiluminescence (ECL) immunoblotting substrate (ThermoFisher Scientific). The HRP-conjugated anti-mouse or anti-rabbit secondary antibody was then deactivated by washing in 30% hydrogen peroxide for 20 minutes. Immunoblots were then reprobed with a mouse monoclonal antibody against β-actin (Abcam, Cat# 49900; 13μg/ml) to assess total protein loading. The immunoblots shown are representative of at least three separate experiments. Densitometric analysis was performed using ImageLab software (BioRad). IFIT1, IFIT3, and PLSCR1 protein levels were normalized to β-actin expression.

2.6 Bacterial Viability Assays

Differentiated osteoblasts were infected with *S. aureus* at multiplicities of infection (MOI) of 75:1 bacteria to each bone cell in antibiotic-free media at 37°C in a 5% $CO_2$ atmosphere for 2 hours, and then the media was discarded. A quick wash of 1X phosphate buffered saline (PBS) was used to remove any residual media. 400 microliters of a 1% Saponin solution was added to each well of the 6-well plate, and the plate was incubated at 37°C in a 5% $CO_2$ atmosphere for 10 minutes. While the plate was incubating, a 96-well plate was set up for the bacterial dilution by adding 90 microliters of Luria broth to each well. Following the 10-

minute incubation of the 6-well plate, 600 microliters of Luria broth was added to each well to bring the volume in each well up to 1mL. The contents of each well were rhythmically pipetted up and down. 10 microliters were retrieved from each well and placed in one of the top wells in the 96-well plate. A serial dilution was performed for each treatment until a dilution of $10^{-6}$ had been achieved. Each treatment condition's serial dilution was then pipetted onto Luria broth agar plates in triplicate and incubated at 37°C in a 5% $CO_2$ atmosphere for 24 hours. The surviving bacteria were enumerated at the highest dilution factor where it was still possible to get an accurate count.

2.7 Statistical analysis

Data is expressed as the mean ± standard error of the mean (SEM). Commercially available software (GraphPad Prism, Graphpad Software, La Jolla, CA) was used to conduct statistical analyses including Student's *t* test, one-way analysis of variance (ANOVA) with Dunnet's post hoc test, or two-way ANOVA with Tukey's post hoc test. For all experiments, a p-value of less than 0.05 was considered statistically significant.

# CHAPTER 3: RESULTS

## 3.1 *S. aureus* induces the expression of IFN-β by isolated primary murine osteoblasts.

Recent studies performed by our laboratory determined the contribution made by resident bone cells to IFN-β production by assessing the changes in mRNA expression by primary murine osteoblasts following *S. aureus* challenge via RNA Tag-Seq analysis. This analysis demonstrated that osteoblasts displayed increased levels of gene products associated with type I interferons, interferon stimulated genes, and/or interferon-mediated cellular effects, following *S. aureus* challenge (Sipprell et al, Under Revision). Specifically, this analysis revealed that osteoblasts showed a marked increase in the expression of mRNA encoding IFN-β as early as 4 hours post infection. Importantly, we have confirmed that such upregulation results in this type I interferon protein production with the demonstration that *S. aureus* challenge elicits the rapid release of IFN-β by osteoblasts and does so in a dose-dependent manner (Figure 2).

## 3.2 Murine osteoblasts express interferon stimulated genes in response to *S. aureus* infection

The RNA Taq-Seq analysis previously performed by our laboratory also indicated the upregulated expression of an array of interferon stimulated genes in primary murine osteoblasts following S. *aureus* infection. Specifically, osteoblasts showed marked increases in the expression of mRNA encoding IFIT1, IFIT3, SLFN2, IRGM2, MX2, PLSCR1, IFI205, and IGTP, as early as 4 hours post-infection. In the current study, we determined whether such upregulation results in increased interferon stimulated gene protein product levels in infected osteoblasts. As shown in Figure 3, *S. aureus* challenge induced the production of IFIT1 by osteoblasts in a time and dose-dependent manner. Similarly, bacterial challenge also elicited an increase in IFIT3 protein expression by osteoblasts over constitutive levels as determined by immunoblot analysis (Figure 4). In contrast, *S. aureus* infection failed to elevate PLSCR1

expression in osteoblasts above the robust levels seen in uninfected cells (Figure 5) despite an apparent increase in mRNA expression encoding this interferon stimulated gene product in the previously performed RNA Tag-Seq analysis.

3.3 IFN-β can limit intracellular bacterial burden in *S. aureus*-infected osteoblasts

The ability of osteoblasts to produce IFN-β in sufficient quantities to induce interferon stimulated gene expression in an autocrine and/or paracrine manner following bacterial challenge raises the possibility that such responses serve to limit *S. aureus* infections. To begin to test this hypothesis and assess the relevance of osteoblast type I interferon responses to this bacterium, we have determined whether IFN-β impacts the ability of *S. aureus* to persist/replicate intracellularly in these bone cells. As shown in Figure 6, treatment of *S. aureus*-infected osteoblasts with recombinant IFN-β results in rapid and time-dependent reductions in the number of viable intracellular bacteria. These results support the contention that osteoblasts produce IFN-β in response to *S. aureus* to induce antibacterial responses that limit intracellular bacterial burden.

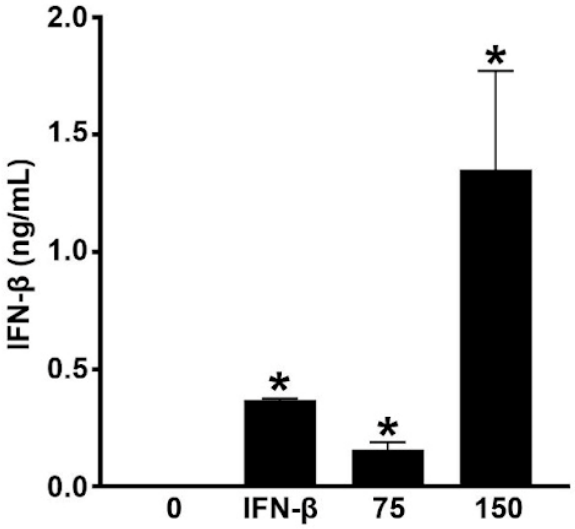


**Figure 2: Osteoblasts produce the type I interferon IFN-β following *S. aureus* challenge.** Murine osteoblasts were either uninfected or infected with *S. aureus* (bacteria: OB 75:1 or 1:150). Treatment with IFN-β was used as a positive control. Supernatants were collected at 4 hours and IFN-β secretion was determined by specific capture ELISA. Data are expressed as mean ± SEM for a minimum of at least three independent experiments. A two-way ANOVA was performed. Asterisks denote statistical significance compared to unchallenged cells as determined by Tukey's multiple comparison test ($p < 0.05$).

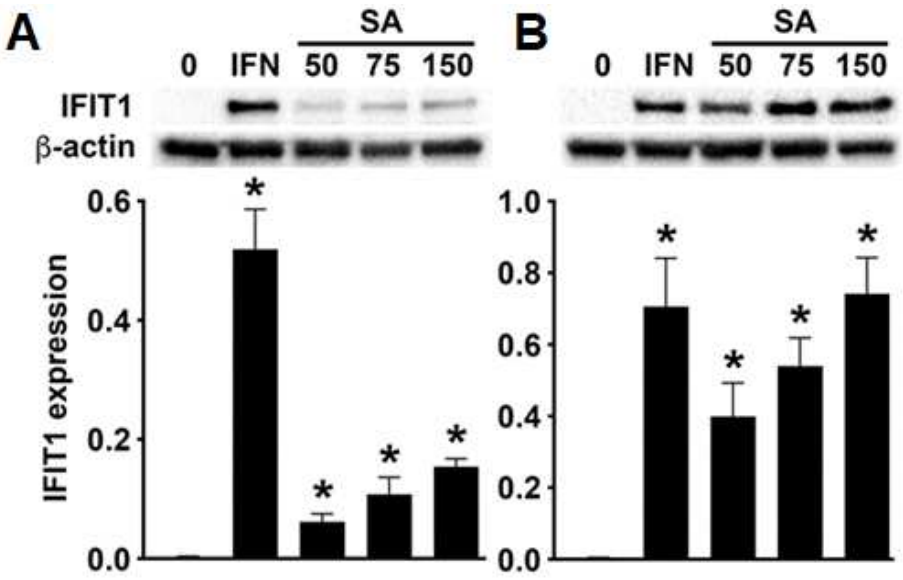


**Figure 3: Murine osteoblasts show increased levels of IFIT1 protein expression following bacterial challenge.** Murine osteoblasts were stimulated with 1 ng/mL of murine IFN-β, uninfected, or infected with *S. aureus* (OB: bacteria 1:50, 1:75, and 1:150) and lysates were collected 8 (A) or 20 (B) hours post infection. Cell lysates were analyzed for protein expression of IFIT1 and the housekeeping gene, β-actin, via immunoblot analysis. Relative IFIT1 protein expression normalized to β-actin is displayed graphically below the representative immunoblot. Data are expressed as mean ± SEM for six independent experiments. Asterisks denote statistical significance compared to unchallenged cells as determined by Student's t test ($p < 0.05$).

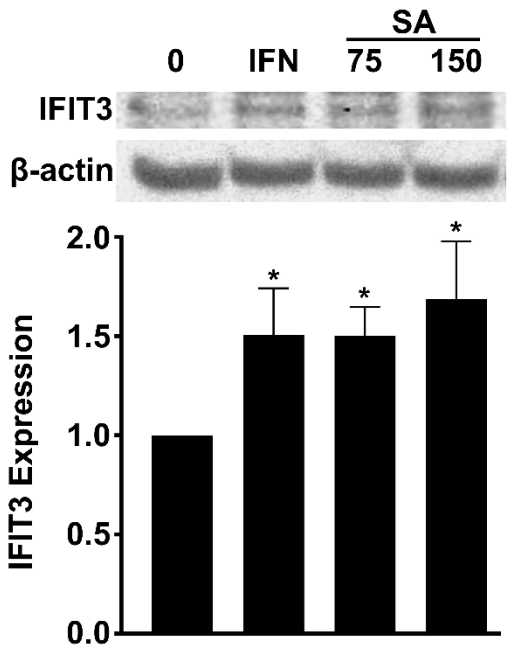


**Figure 4: Murine osteoblasts show increased levels of IFIT3 protein expression following bacterial challenge**. Murine osteoblasts were stimulated with 1 ng/mL of murine IFN-β, uninfected, or infected with *S. aureus* (OB: bacteria, 1:75 and 1:150) and lysates were collected at 8 hours post infection. Cell lysates were analyzed for protein expression of IFIT3 and the housekeeping gene, β-actin, via immunoblot analysis. Relative IFIT1 protein expression normalized to β-actin is displayed graphically below the representative immunoblot. Data are expressed as mean ± SEM for four independent experiments. Asterisks denote statistical significance compared to unchallenged cells as determined by Student's t test ($p < 0.05$).

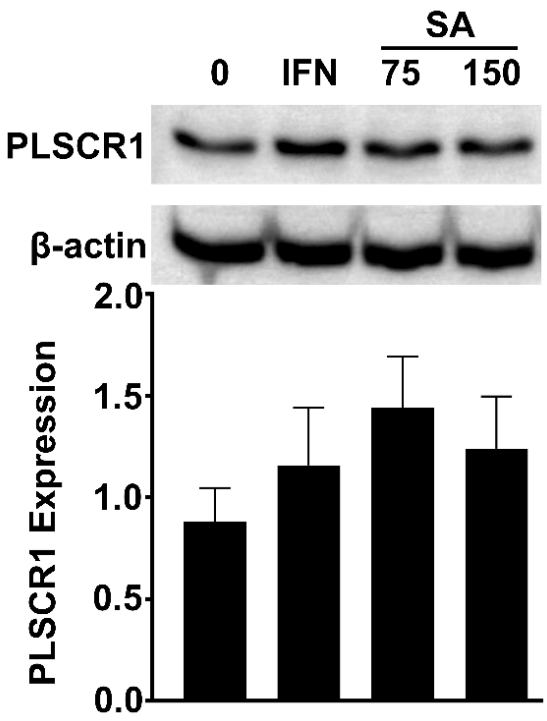


**Figure 5: Murine osteoblasts show no significant increase of PLSCR1 protein expression following bacterial challenge**. Murine osteoblasts were stimulated with 1 ng/mL of murine IFN-β, uninfected, or infected with *S. aureus* (OB: bacteria, 1:75 and 1:150) and lysates were collected at 8 hours post infection. Cell lysates were analyzed for protein expression of PLSCR1 and the housekeeping gene, β-actin, via immunoblot analysis. Relative PLSCR1 protein expression normalized to β-actin is displayed graphically below the representative immunoblot. Data are expressed as mean ± SEM for three independent experiments. Asterisks denote statistical significance compared to unchallenged cells as determined by Student's t test ($p < 0.05$).

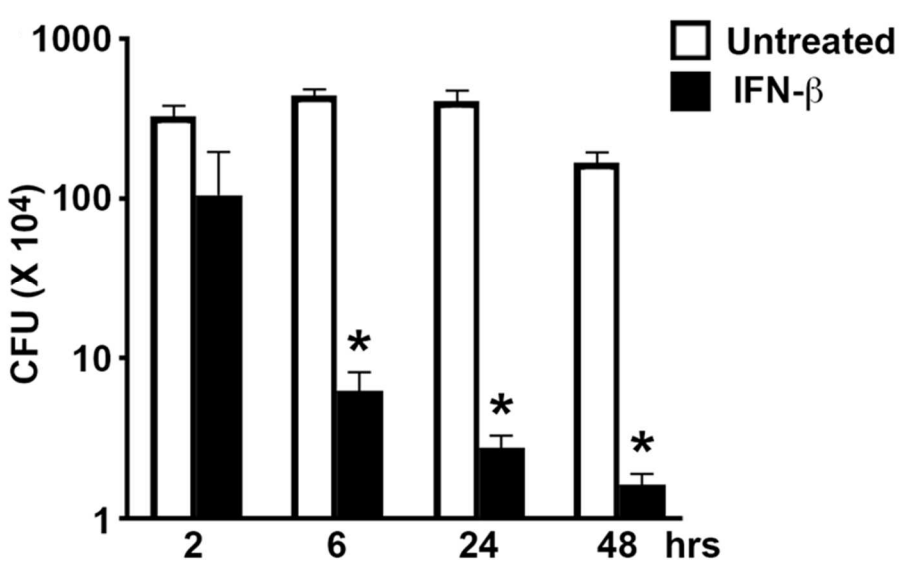


**Figure 6: IFN-β can limit bacterial burden in *S. aureus* challenged osteoblasts.** Osteoblasts were infected with *S. aureus* (MOI of 75:1) in the absence or presence of recombinant IFN-β (1ng/ml). At 2-, 6-, 24-, and 48-hours following infection, cells were lysed, and the number of viable intracellular bacteria were determined by colony count. Data is shown as the mean +/- SEM for six independent experiments and asterisks indicate a statistically significant difference from untreated cells ($p < 0.05$).

# CHAPTER 4: DISCUSSION

It is becoming increasingly apparent that type I IFNs, including IFN- β can impact host susceptibility to a wide range of pathogens including bacteria (Peignier & Parker, 2021). Type I interferons have been reported to have both protective and deleterious effects on *S. aureus* infections, with decreased mortality observed with IFNAR deficient mice in a staphylococcal pneumonia model (Martin et al., 2009; Parker et al., 2014; Parker & Prince, 2012), while an anti-IFNAR1 subunit antibody has been reported to increase lung *S. aureus* burden (Spolski et al., 2019). In support of our hypothesis that type I interferons are produced in response to *S. aureus* infection and impact subsequent disease progression and severity, our laboratory has now demonstrated the local production of IFN-β in infected bone tissue in a mouse model of staphylococcal osteomyelitis (Johnson, Furr, et al., 2022).

In keeping with our hypothesis, we have now shown *S. aureus* PAMPs are recognized either by membrane associated or cytosolic PRRs. The recognition and subsequent bacterial invasion of these microbial components trigger osteoblasts to initiate gene transcription of the type I interferon, IFN-β. IFN-β protein production and release then either acts back on osteoblasts in an autocrine and/or paracrine manner. IFN-β is perceived by an interferon receptor, which then initiates interferon stimulated gene transcription and protein production. Finally, IFN-β and interferon stimulated gene production following *S. aureus* infection has the potential to affect the overall protection of the host following bacterial challenge (Figure 7).

Elevated IFN-β expression in *S. aureus* infected bone tissue may be attributable to production by infiltrating leukocytes. However, both our laboratory and others have demonstrated that resident osteoblasts produce an array of products in response to *S. aureus* challenge that can regulate both bone homeostasis and inflammation (Sipprell et. al, Under

Revision). Interestingly, a murine osteoblast-like cell line has been shown to express mRNA encoding IFN- β in response to the TLR3 agonist poly(I:C), and the ability of an IFN- β neutralizing antibody to partially inhibit their responses to this stimulus appears to support the ability of activated osteoblasts to release this cytokine (Nakamura et al., 2007). In addition, constitutive IFN- β production has recently been reported by mature osteoblasts/osteocytes in a 3D in vitro co-culture system (Hayashida et al., 2014). Recent findings by our laboratory have described the upregulated expression of 122 genes in murine osteoblasts following *S. aureus* challenge via RNA sequencing and gene ontology analysis (Sipprell et al, Under Revision) and our laboratory recently reported that these genes include those encoding type I interferon proteins, IFNAR and associated signaling components, and interferon stimulated genes (Johnson, Furr, et al., 2022). Previous findings from our laboratory have also shown that these cells display increased expression of mRNA encoding IFN- β, IFNAR1, and STAT1/2, following infection (Johnson, Furr, et al., 2022; Johnson, Suptela, et al., 2022). Importantly, we subsequently confirmed the significant release of IFN-β by infected osteoblasts and showed that it occurred in a bacterial dose-dependent manner. As such, these findings represent the first demonstration of *S. aureus*-induced production of IFN-β by osteoblasts.

While the mechanisms underlying IFN- β induction by *S. aureus* remain to be determined, internalized *S. aureus* can escape to the cytoplasm via the production of cytotoxic phenol-soluble modulins (El-Aouar Filho et al., 2017). There, they could be detected NOD2, that we have shown to be functionally expressed by osteoblasts (Chauhan & Marriott, 2010; McCall et al., 2007), which can trigger interferon regulatory factor activation and initiate type I interferon gene expression (Stark & Darnell, 2012).

The potential importance of osteoblast-mediated IFN-β production is underscored by our laboratories previous demonstration that levels of mRNA encoding the ISGs, IFIT1, IFIT3, SLFN2, IRGM2, MX2, PLSCR1, IFI205, and IGTP, are markedly elevated in these cells following *S. aureus* challenge due, in large part, to autocrine and/or paracrine IFN-β signaling via IFNAR (Figure 7). While IFIT1 is an RNA-binding protein that can act as a sensor of viral single-stranded RNAs and inhibit viral mRNA expression, it has also been suggested to function as a negative regulator of inflammatory mediator production and a defective IFIT1-dependent interferon response has been shown to increase susceptibility to intracellular bacterial infection (John et al, 2018). Similarly, other interferons, including SLFN2, have been reported to be protective against bacterial infection (Mavrommatis et al., 2013). As such, interferon stimulated gene upregulation could serve to limit intracellular bacterial burden and this possibility is supported by the present demonstration that exposure of *S. aureus* infected osteoblasts to exogenous IFN- β markedly decreases the number of viable bacteria within these cells.

Interestingly, IFN-β can also have major effects on bone cell formation and/or function (Amarasekara et al., 2018, 2021). IFN-β is a potent inhibitor of osteoclastogenesis (Amarasekara et al., 2018) and has been reported to inhibit RANKL-induced osteoclastogenesis via a reduction in the expression of the critical signal transduction component c-Fos (Avnet et al., 2007; Ha et al., 2008; Takayanagi et al., 2005). This suggests that type I interferons may serve as a negative feedback inhibitor of RANKL-induced osteoclastogenesis (Takayanagi et al., 2005). However, ascribing a bone-preserving anti-osteoclastogenic role to type I interferons is complicated by the fact that IFN- β can directly augment pro-osteoclastogenic inflammatory cytokine production. Additionally, IFN- β has also been reported to be anti-osteoblastogenic (Amarasekara et al., 2021), and can inhibit bone formation and matrix mineralization by

reducing the expression of multiple matrix components by osteoblasts (Deng et al., 2020; Woeckel et al., 2012). Furthermore, the finding that RANKL deficient mice show increased susceptibility to endotoxic shock while exogenous RANKL administration is protective (Maruyama et al., 2006), suggests that IFN- β mediated decreases in RANKL production could exacerbate detrimental inflammation. As such, the net impact of type I interferon production by resident bone cells on the progressive inflammatory damage associated with staphylococcal osteomyelitis remains to be determined.

Collectively, the present study has identified a previously unknown type I interferon response as well as the upregulation in subsequent interferon stimulated gene production by osteoblasts to *S. aureus* challenge, one that could have protective effects by limiting intracellular bacterial survival/propagation within these resident bone cells and has the potential to mitigate the pro-osteoclastogenic effects of mediators such as RANKL that are produced at sites of bone infection.

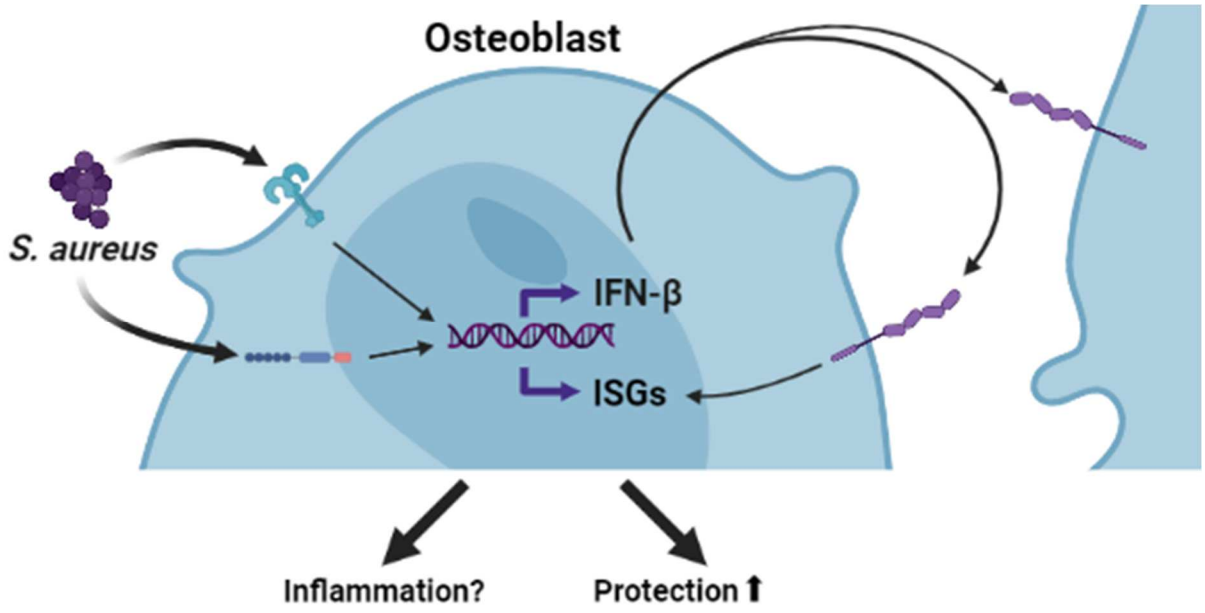


Figure 7: Schematic showing the mechanism underlying interferon responses in *S. aureus*-infected osteoblasts based upon the results of the present study. *S. aureus* PAMPs are recognized either by membrane associated or cytosolic PRRs. The recognition and subsequent bacterial invasion of these microbial components trigger osteoblasts to initiate gene transcription of the type I interferon, IFN-β. IFN-β protein production and release then either acts back on osteoblasts in an autocrine and/or paracrine manner. IFN-β is perceived by an interferon receptor, which then initiates interferon stimulated gene transcription and protein production. IFN-β and interferon stimulated gene production following *S. aureus* infection has the potential to affect the overall protection of the host following bacterial challenge.

# REFERENCES


Ahmed, S., Meghji, S., Williams, R. J., Henderson, B., Brock, J. H., & Nair, S. P. (2001). *Staphylococcus aureus* Fibronectin Binding Proteins Are Essential for Internalization by Osteoblasts but Do Not Account for Differences in Intracellular Levels of Bacteria. *Infection and Immunity*, *69*(5), 2872–2877. https://doi.org/10.1128/IAI.69.5.2872-2877.2001

Alonso-Pérez, A., Franco-Trepat, E., Guillán-Fresco, M., Jorge-Mora, A., López, V., Pino, J., Gualillo, O., & Gómez, R. (2018). Role of Toll-Like Receptor 4 on Osteoblast Metabolism and Function. *Frontiers in Physiology*, *9*. https://doi.org/10.3389/fphys.2018.00504

Amarasekara, D. S., Kim, S., & Rho, J. (2021). Regulation of Osteoblast Differentiation by Cytokine Networks. *International Journal of Molecular Sciences*, *22*(6), 2851. https://doi.org/10.3390/ijms22062851

Amarasekara, D. S., Yun, H., Kim, S., Lee, N., Kim, H., & Rho, J. (2018). Regulation of Osteoclast Differentiation by Cytokine Networks. *Immune Network*, *18*(1). https://doi.org/10.4110/in.2018.18.e8

Andreu, N., Phelan, J., de Sessions, P. F., Cliff, J. M., Clark, T. G., & Hibberd, M. L. (2017). Primary macrophages and J774 cells respond differently to infection with Mycobacterium tuberculosis. *Scientific Reports*, *7*(1), 42225. https://doi.org/10.1038/srep42225

Avnet, S., Cenni, E., Perut, F., Granchi, D., Brandi, M. L., Giunti, A., & Baldini, N. (2007). Interferon-alpha inhibits in vitro osteoclast differentiation and renal cell carcinoma-induced angiogenesis. *International Journal of Oncology*, *30*(2), 469–476. http://www.ncbi.nlm.nih.gov/pubmed/17203230

Bhargava, A., Lahaye, X., & Manel, N. (2018). Let me in: Control of HIV nuclear entry at the nuclear envelope. *Cytokine & Growth Factor Reviews*, *40*, 59–67. https://doi.org/10.1016/j.cytogfr.2018.02.006

Boeck, H. De. (2005). Osteomyelitis and septic arthritis in children. *Acta Orthopaedica Belgica*, *71*(5), 505–515.

Borden, E. C., Sen, G. C., Uze, G., Silverman, R. H., Ransohoff, R. M., Foster, G. R., & Stark, G. R. (2007). Interferons at age 50: past, current and future impact on biomedicine. *Nature Reviews Drug Discovery*, *6*(12), 975–990. https://doi.org/10.1038/nrd2422

Bost, K. L., Ramp, W. K., Nicholson, N. C., Bento, J. L., Marriott, I., & Hudson, M. C. (1999).

*Staphylococcus aureus* Infection of Mouse or Human Osteoblasts Induces High Levels of Interleukin□6 and Interleukin□12 Production. *The Journal of Infectious Diseases*, *180*(6), 1912–1920. https://doi.org/10.1086/315138

Carty, M., Guy, C., & Bowie, A. G. (2021). Detection of Viral Infections by Innate Immunity. *Biochemical Pharmacology*, *183*, 114316. https://doi.org/10.1016/j.bcp.2020.114316

Chauhan, V. S., & Marriott, I. (2010). Differential roles for NOD2 in osteoblast inflammatory immune responses to bacterial pathogens of bone tissue. *Journal of Medical Microbiology*, *59*(7), 755–762. https://doi.org/10.1099/jmm.0.015859-0

Cheung, G. Y. C., Bae, J. S., & Otto, M. (2021). Pathogenicity and virulence of Staphylococcus aureus. *Virulence*, *12*(1), 547–569. https://doi.org/10.1080/21505594.2021.1878688

Coers, J., Brown, H. M., Hwang, S., & Taylor, G. A. (2018). Partners in anti-crime: how interferon-inducible GTPases and autophagy proteins team up in cell-intrinsic host defense. *Current Opinion in Immunology*, *54*, 93–101. https://doi.org/10.1016/j.coi.2018.06.008

Collazo, C. M., Yap, G. S., Hieny, S., Caspar, P., Feng, C. G., Taylor, G. A., & Sher, A. (2002). The function of gamma interferon-inducible GTP-binding protein IGTP in host resistance to Toxoplasma gondii is Stat1 dependent and requires expression in both hematopoietic and nonhematopoietic cellular compartments. *Infection and Immunity*, *70*(12), 6933–6939. https://doi.org/10.1128/IAI.70.12.6933-6939.2002

Craigen, M. A., Watters, J., & Hackett, J. S. (1992). The changing epidemiology of osteomyelitis in children. *The Journal of Bone and Joint Surgery. British Volume*, *74-B*(4), 541–545. https://doi.org/10.1302/0301-620X.74B4.1624513

Deng, Z., Ng, C., Inoue, K., Chen, Z., Xia, Y., Hu, X., Greenblatt, M., Pernis, A., & Zhao, B. (2020). Def6 regulates endogenous type-I interferon responses in osteoblasts and suppresses osteogenesis. *ELife*, *9*. https://doi.org/10.7554/eLife.59659

Diamond, M. S. (2014). IFIT1: A dual sensor and effector molecule that detects non-2′-O methylated viral RNA and inhibits its translation. *Cytokine & Growth Factor Reviews*, *25*(5), 543–550. https://doi.org/10.1016/j.cytogfr.2014.05.002

Dirckx, N., Moorer, M. C., Clemens, T. L., & Riddle, R. C. (2019). The role of osteoblasts in energy homeostasis. *Nature Reviews Endocrinology*, *15*(11), 651–665. https://doi.org/10.1038/s41574-019-0246-y

Dubey, L., Krasinski, K., & Hernanz-Schulman, M. (1988). Osteomyelitis secondary to trauma

or infected contiguous soft tissue. *The Pediatric Infectious Disease Journal*, 26–33.

El-Aouar Filho, R. A., Nicolas, A., De Paula Castro, T. L., Deplanche, M., De Carvalho Azevedo, V. A., Goossens, P. L., Taieb, F., Lina, G., Le Loir, Y., & Berkova, N. (2017). Heterogeneous Family of Cyclomodulins: Smart Weapons That Allow Bacteria to Hijack the Eukaryotic Cell Cycle and Promote Infections. *Frontiers in Cellular and Infection Microbiology*, *7*. https://doi.org/10.3389/fcimb.2017.00208

Ellington, J. K., Harris, M., Hudson, M. C., Vishin, S., Webb, L. X., & Sherertz, R. (2006). IntracellularStaphylococcus aureus and antibiotic resistance: Implications for treatment of staphylococcal osteomyelitis. *Journal of Orthopaedic Research*, *24*(1), 87–93. https://doi.org/10.1002/jor.20003

Ellington, J. K., Harris, M., Webb, L., Smith, B., Smith, T., Tan, K., & Hudson, M. (2003). Intracellular Staphylococcus aureus. A mechanism for the indolence of osteomyelitis. *The Journal of Bone and Joint Surgery. British Volume*, *85*(6), 918–921.

Ellington, J. K., Reilly, S. S., Ramp, W. K., Smeltzer, M. S., Kellam, J. F., & Hudson, M. C. (1999). Mechanisms ofStaphylococcus aureusinvasion of cultured osteoblasts. *Microbial Pathogenesis*, *26*(6), 317–323. https://doi.org/10.1006/mpat.1999.0272

Eren, E., Planès, R., Bagayoko, S., Bordignon, P., Chaoui, K., Hessel, A., Santoni, K., Pinilla, M., Lagrange, B., Burlet□Schiltz, O., Howard, J. C., Henry, T., Yamamoto, M., & Meunier, E. (2020). Irgm2 and Gate□16 cooperatively dampen Gram□negative bacteria□induced caspase□11 response. *EMBO Reports*, *21*(11). https://doi.org/10.15252/embr.202050829

Fischietti, M., Arslan, A. D., Sassano, A., Saleiro, D., Majchrzak-Kita, B., Ebine, K., Munshi, H. G., Fish, E. N., & Platanias, L. C. (2018). Slfn2 Regulates Type I Interferon Responses by Modulating the NF-κB Pathway. *Molecular and Cellular Biology*, *38*(16). https://doi.org/10.1128/MCB.00053-18

Fumoto, T., Takeshita, S., Ito, M., & Ikeda, K. (2014). Physiological Functions of Osteoblast Lineage and T Cell-Derived RANKL in Bone Homeostasis. *Journal of Bone and Mineral Research*, *29*(4), 830–842. https://doi.org/10.1002/jbmr.2096

Garlant, H. N., Ellappan, K., Hewitt, M., Perumal, P., Pekeleke, S., Wand, N., Southern, J., Kumar, S. V., Belgode, H., Abubakar, I., Sinha, S., Vasan, S., Joseph, N. M., & Kempsell, K. E. (2022). Evaluation of Host Protein Biomarkers by ELISA From Whole Lysed Peripheral Blood for Development of Diagnostic Tests for Active Tuberculosis. *Frontiers in*

*Immunology*, *13*. https://doi.org/10.3389/fimmu.2022.854327

Ghosh, S., Wallerath, C., Covarrubias, S., Hornung, V., Carpenter, S., & Fitzgerald, K. A. (2017). The PYHIN Protein p205 Regulates the Inflammasome by Controlling Asc Expression. *The Journal of Immunology*, *199*(9), 3249–3260. https://doi.org/10.4049/jimmunol.1700823

Ha, H., Lee, J.-H., Kim, H.-N., Kwak, H. B., Kim, H.-M., Lee, S. E., Rhee, J. H., Kim, H.-H., & Lee, Z. H. (2008). Stimulation by TLR5 Modulates Osteoclast Differentiation through STAT1/IFN-β. *The Journal of Immunology*, *180*(3), 1382–1389. https://doi.org/10.4049/jimmunol.180.3.1382

Hadjidakis, D. J., & Androulakis, I. I. (2006). Bone remodeling. *Annals of the New York Academy of Sciences*, *1092*, 385–396. https://doi.org/10.1196/annals.1365.035

Haller, O., Staeheli, P., Schwemmle, M., & Kochs, G. (2015). Mx GTPases: dynamin-like antiviral machines of innate immunity. *Trends in Microbiology*, *23*(3), 154–163. https://doi.org/10.1016/j.tim.2014.12.003

Hayashida, C., Ito, J., Nakayachi, M., Okayasu, M., Ohyama, Y., Hakeda, Y., & Sato, T. (2014). Osteocytes Produce Interferon-β as a Negative Regulator of Osteoclastogenesis. *Journal of Biological Chemistry*, *289*(16), 11545–11555. https://doi.org/10.1074/jbc.M113.523811

Hudson, M. C., Ramp, W. K., Nicholson, N. C., Williams, A. S., & Nousiainen, M. T. (1995). Internalization ofStaphylococcus aureusby cultured osteoblasts. *Microbial Pathogenesis*, *19*(6), 409–419. https://doi.org/10.1006/mpat.1995.0075

Jauregui, C. E., Mansell, J. P., Jepson, M. A., & Jenkinson, H. F. (2013). Differential interactions of *Streptococcus gordonii* and *Staphylococcus aureus* with cultured osteoblasts. *Molecular Oral Microbiology*, *28*(4), 250–266. https://doi.org/10.1111/omi.12022

Jensen, A. G., Espersen, F., Skinhøj, P., Rosdahl, V. T., & Frimodt-Møller, N. (1997). Increasing frequency of vertebral osteomyelitis following Staphylococcus aureus bacteraemia in Denmark 1980–1990. *Journal of Infection*, *34*(2), 113–118. https://doi.org/10.1016/S0163-4453(97)92395-1

Jevon, M., Guo, C., Ma, B., Mordan, N., Nair, S. P., Harris, M., Henderson, B., Bentley, G., & Meghji, S. (1999). Mechanisms of Internalization of *Staphylococcus aureus* by Cultured Human Osteoblasts. *Infection and Immunity*, *67*(5), 2677–2681. https://doi.org/10.1128/IAI.67.5.2677-2681.1999

Johnson, M. B., Furr, K. H., Suptela, S. R., Leach, W., & Marriott, I. (2022). Induction of protective interferon-β responses in murine osteoblasts following Staphylococcus aureus infection. *Frontiers in Microbiology*, *13*. https://doi.org/10.3389/fmicb.2022.1066237

Johnson, M. B., Suptela, S. R., Sipprell, S. E., & Marriott, I. (2022). Substance P Exacerbates the Inflammatory and Pro-osteoclastogenic Responses of Murine Osteoclasts and Osteoblasts to Staphylococcus aureus. *Inflammation*. https://doi.org/10.1007/s10753-022-01731-z

Kalinka, J., Hachmeister, M., Geraci, J., Sordelli, D., Hansen, U., Niemann, S., Oetermann, S., Peters, G., Löffler, B., & Tuchscherr, L. (2014). Staphylococcus aureus isolates from chronic osteomyelitis are characterized by high host cell invasion and intracellular adaptation, but still induce inflammation. *International Journal of Medical Microbiology*, *304*(8), 1038–1049. https://doi.org/10.1016/j.ijmm.2014.07.013

Kassem, A., Lindholm, C., & Lerner, U. H. (2016). Toll-Like Receptor 2 Stimulation of Osteoblasts Mediates Staphylococcus Aureus Induced Bone Resorption and Osteoclastogenesis through Enhanced RANKL. *PLOS ONE*, *11*(6), e0156708. https://doi.org/10.1371/journal.pone.0156708

Katsoulidis, E., Carayol, N., Woodard, J., Konieczna, I., Majchrzak-Kita, B., Jordan, A., Sassano, A., Eklund, E. A., Fish, E. N., & Platanias, L. C. (2009). Role of Schlafen 2 (SLFN2) in the Generation of Interferon α-induced Growth Inhibitory Responses. *Journal of Biological Chemistry*, *284*(37), 25051–25064. https://doi.org/10.1074/jbc.M109.030445

Kawasaki, T., & Kawai, T. (2019). *Discrimination Between Self and Non-Self-Nucleic Acids by the Innate Immune System* (pp. 1–30). https://doi.org/10.1016/bs.ircmb.2018.08.004

Li, H., Jiao, Y., Zhang, L., Wang, C., Zhang, X., Guo, H., & Xu, H. (2016). The interferon-inducible protein p205 acts as an activator in osteoblast differentiation of mouse BMSCs. *Differentiation*, *92*(5), 318–325. https://doi.org/10.1016/j.diff.2016.02.006

Lizak, M., & Yarovinsky, T. O. (2012). Phospholipid Scramblase 1 Mediates Type I Interferon-Induced Protection against Staphylococcal α-Toxin. *Cell Host & Microbe*, *11*(1), 70–80. https://doi.org/10.1016/j.chom.2011.12.004

Marriott, I., Rati, D. M., McCall, S. H., & Tranguch, S. L. (2005). Induction of Nod1 and Nod2 Intracellular Pattern Recognition Receptors in Murine Osteoblasts following Bacterial Challenge. *Infection and Immunity*, *73*(5), 2967–2973. https://doi.org/10.1128/IAI.73.5.2967-2973.2005

Martin, F. J., Gomez, M. I., Wetzel, D. M., Memmi, G., O'Seaghdha, M., Soong, G., Schindler, C., & Prince, A. (2009). Staphylococcus aureus activates type I IFN signaling in mice and humans through the Xr repeated sequences of protein A. *Journal of Clinical Investigation*. https://doi.org/10.1172/JCI35879

Maruyama, K., Takada, Y., Ray, N., Kishimoto, Y., Penninger, J. M., Yasuda, H., & Matsuo, K. (2006). Receptor Activator of NF-κB Ligand and Osteoprotegerin Regulate Proinflammatory Cytokine Production in Mice. *The Journal of Immunology*, *177*(6), 3799–3805. https://doi.org/10.4049/jimmunol.177.6.3799

Mavrommatis, E., Arslan, A. D., Sassano, A., Hua, Y., Kroczynska, B., & Platanias, L. C. (2013). Expression and Regulatory Effects of Murine Schlafen (Slfn) Genes in Malignant Melanoma and Renal Cell Carcinoma. *Journal of Biological Chemistry*, *288*(46), 33006–33015. https://doi.org/10.1074/jbc.M113.460741

McCall, S. H., Sahraei, M., Young, A. B., Worley, C. S., Duncan, J. A., Ting, J. P.-Y., & Marriott, I. (2007). Osteoblasts Express NLRP3, a Nucleotide-Binding Domain and Leucine-Rich Repeat Region Containing Receptor Implicated in Bacterially Induced Cell Death. *Journal of Bone and Mineral Research*, *23*(1), 30–40. https://doi.org/10.1359/jbmr.071002

McCullers, J. A. (2014). The co-pathogenesis of influenza viruses with bacteria in the lung. *Nature Reviews Microbiology*, *12*(4), 252–262. https://doi.org/10.1038/nrmicro3231

Mohamed, W., Domann, E., Chakraborty, T., Mannala, G., Lips, K. S., Heiss, C., Schnettler, R., & Alt, V. (2016). TLR9 mediates S. aureus killing inside osteoblasts via induction of oxidative stress. *BMC Microbiology*, *16*(1), 230. https://doi.org/10.1186/s12866-016-0855-8

Mohamed, W., Sommer, U., Sethi, S., Domann, E., Thormann, U., Schütz, I., Lips, K. S., Chakraborty, T., Schnettler, R., & Alt, V. (2014). Intracellular proliferation of S. aureus in osteoblasts and effects of rifampicin and gentamicin on S. aureus intracellular proliferation and survival. *European Cells and Materials*, *28*, 258–268. https://doi.org/10.22203/eCM.v028a18

Monzón, M., García-Álvare, F., Laclériga, A., Gracia, E., Leiva, J., Oteiza, C., & Amorena, B. (2001). A simple infection model using pre-colonized implants to reproduce rat chronicStaphylococcus aureus osteomyelitis and study antibiotic treatment. *Journal of Orthopaedic Research*, *19*(5), 820–826. https://doi.org/10.1016/S0736-0266(00)00076-0

Mousa, H. A. (2003). Bone infection. *Eastern Mediterranean Health Journal*, *9*(1–2), 208–214.

Nair, S. P., Meghji, S., Wilson, M., Reddi, K., White, P., & Henderson, B. (1996). Bacterially induced bone destruction: mechanisms and misconceptions. *Infection and Immunity*, *64*(7), 2371–2380. https://doi.org/10.1128/iai.64.7.2371-2380.1996

Nakamura, K., Deyama, Y., Yoshimura, Y., Suzuki, K., & Morita, M. (2007). Toll-like receptor 3 ligand-induced antiviral response in mouse osteoblastic cells. *International Journal of Molecular Medicine*, *19*(5), 771–775.

Park-Min, K.-H. (2018). Mechanisms involved in normal and pathological osteoclastogenesis. *Cellular and Molecular Life Sciences*, *75*(14), 2519–2528. https://doi.org/10.1007/s00018-018-2817-9

Park, J. H., Lee, N. K., & Lee, S. Y. (2017). Current Understanding of RANK Signaling in Osteoclast Differentiation and Maturation. *Molecules and Cells*, *40*(10), 706–713. https://doi.org/10.14348/molcells.2017.0225

Parker, D., Planet, P. J., Soong, G., Narechania, A., & Prince, A. (2014). Induction of Type I Interferon Signaling Determines the Relative Pathogenicity of Staphylococcus aureus Strains. *PLoS Pathogens*, *10*(2), e1003951. https://doi.org/10.1371/journal.ppat.1003951

Parker, D., & Prince, A. (2012). *Staphylococcus aureus* Induces Type I IFN Signaling in Dendritic Cells Via TLR9. *The Journal of Immunology*, *189*(8), 4040–4046. https://doi.org/10.4049/jimmunol.1201055

Peignier, A., & Parker, D. (2021). Impact of Type I Interferons on Susceptibility to Bacterial Pathogens. *Trends in Microbiology*, *29*(9), 823–835. https://doi.org/10.1016/j.tim.2021.01.007

Pilla-Moffett, D., Barber, M. F., Taylor, G. A., & Coers, J. (2016). Interferon-Inducible GTPases in Host Resistance, Inflammation and Disease. *Journal of Molecular Biology*, *428*(17), 3495–3513. https://doi.org/10.1016/j.jmb.2016.04.032

Popov, L. M., Marceau, C. D., Starkl, P. M., Lumb, J. H., Shah, J., Guerrera, D., Cooper, R. L., Merakou, C., Bouley, D. M., Meng, W., Kiyonari, H., Takeichi, M., Galli, S. J., Bagnoli, F., Citi, S., Carette, J. E., & Amieva, M. R. (2015). The adherens junctions control susceptibility to Staphylococcus aureus α-toxin. *Proceedings of the National Academy of Sciences*, *112*(46), 14337–14342. https://doi.org/10.1073/pnas.1510265112

Priest, D. H., & Peacock, J. E. (2005). Hematogenous Vertebral Osteomyelitis Due to

Staphylococcus aureus in the Adult: Clinical Features and Therapeutic Outcomes. *Southern Medical Journal*, *98*(9), 854–862. https://doi.org/10.1097/01.smj.0000168666.98129.33

R., A. S., Elias, D., Buckingham, S. C., Thomas, E. D., Novais, E., Arkader, A., & Howard, C. (2006). Changing Patterns of Acute Hematogenous Osteomyelitis and Septic Arthritis: Emergence of Community-associated Methicillin-resistant Staphylococcus aureus. *Journal of Pediatric Orthopedics*, *26*(6), 703–708.

Sax, H., & Lew, D. (1999). Osteomyelitis. *Current Infectious Disease Reports*, *1*(3), 261–266.

Schneider, W. M., Chevillotte, M. D., & Rice, C. M. (2014). Interferon-Stimulated Genes: A Complex Web of Host Defenses. *Annual Review of Immunology*, *32*(1), 513–545. https://doi.org/10.1146/annurev-immunol-032713-120231

Schoggins, J. W., & Rice, C. M. (2011). Interferon-stimulated genes and their antiviral effector functions. *Current Opinion in Virology*, *1*(6), 519–525. https://doi.org/10.1016/j.coviro.2011.10.008

Schwarz-Linek, U., Werner, J. M., Pickford, A. R., Gurusiddappa, S., Kim, J. H., Pilka, E. S., Briggs, J. A. G., Gough, T. S., Höök, M., Campbell, I. D., & Potts, J. R. (2003). Pathogenic bacteria attach to human fibronectin through a tandem β-zipper. *Nature*, *423*(6936), 177–181. https://doi.org/10.1038/nature01589

Sivagnanam, U., Palanirajan, S. K., & Gummadi, S. N. (2017). The role of human phospholipid scramblases in apoptosis: An overview. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1864*(12), 2261–2271. https://doi.org/10.1016/j.bbamcr.2017.08.008

Soe, Y. M., Bedoui, S., Stinear, T. P., & Hachani, A. (2021). Intracellular <scp> *Staphylococcus aureus* </scp> and host cell death pathways. *Cellular Microbiology*, *23*(5). https://doi.org/10.1111/cmi.13317

Spolski, R., West, E. E., Li, P., Veenbergen, S., Yung, S., Kazemian, M., Oh, J., Yu, Z.-X., Freeman, A. F., Holland, S. M., Murphy, P. M., & Leonard, W. J. (2019). IL-21/type I interferon interplay regulates neutrophil-dependent innate immune responses to Staphylococcus aureus. *ELife*, *8*. https://doi.org/10.7554/eLife.45501

Stark, G. R., & Darnell, J. E. (2012). The JAK-STAT pathway at twenty. *Immunity*, *36*(4), 503–514. https://doi.org/10.1016/j.immuni.2012.03.013

Strobel, M., Pförtner, H., Tuchscherr, L., Völker, U., Schmidt, F., Kramko, N., Schnittler, H.-J., Fraunholz, M. J., Löffler, B., Peters, G., & Niemann, S. (2016). Post-invasion events after

infection with Staphylococcus aureus are strongly dependent on both the host cell type and the infecting S. aureus strain. *Clinical Microbiology and Infection*, *22*(9), 799–809. https://doi.org/10.1016/j.cmi.2016.06.020

Takayanagi, H., Sato, K., Takaoka, A., & Taniguchi, T. (2005). Interplay between interferon and other cytokine systems in bone metabolism. *Immunological Reviews*, *208*(1), 181–193. https://doi.org/10.1111/j.0105-2896.2005.00337.x

Tam, K., & Torres, V. J. (2019). Staphylococcus aureus Secreted Toxins and Extracellular Enzymes. *Microbiology Spectrum*, *7*(2). https://doi.org/10.1128/microbiolspec.GPP3-0039-2018

Tanaka, T., Narazaki, M., & Kishimoto, T. (2014). IL-6 in Inflammation, Immunity, and Disease. *Cold Spring Harbor Perspectives in Biology*, *6*(10), a016295–a016295. https://doi.org/10.1101/cshperspect.a016295

Tuchscherr, L., Kreis, C. A., Hoerr, V., Flint, L., Hachmeister, M., Geraci, J., Bremer-Streck, S., Kiehntopf, M., Medina, E., Kribus, M., Raschke, M., Pletz, M., Peters, G., & Löffler, B. (2016). *Staphylococcus aureus* develops increased resistance to antibiotics by forming dynamic small colony variants during chronic osteomyelitis. *Journal of Antimicrobial Chemotherapy*, *71*(2), 438–448. https://doi.org/10.1093/jac/dkv371

Wen, Q., Gu, F., Sui, Z., Su, Z., & Yu, T. (2020). The Process of Osteoblastic Infection by Staphylococcus Aureus. *International Journal of Medical Sciences*, *17*(10), 1327–1332. https://doi.org/10.7150/ijms.45960

Woeckel, V. J., Eijken, M., van de Peppel, J., Chiba, H., van der Eerden, B. C. J., & van Leeuwen, J. P. T. M. (2012). IFNβ impairs extracellular matrix formation leading to inhibition of mineralization by effects in the early stage of human osteoblast differentiation. *Journal of Cellular Physiology*, *227*(6), 2668–2676. https://doi.org/10.1002/jcp.23009

Xin, P., Xu, X., Deng, C., Liu, S., Wang, Y., Zhou, X., Ma, H., Wei, D., & Sun, S. (2020). The role of JAK/STAT signaling pathway and its inhibitors in diseases. *International Immunopharmacology*, *80*, 106210. https://doi.org/10.1016/j.intimp.2020.106210

Yarovinsky, T. O. (2012). Role of phospholipid scramblase 1 in type I interferon-induced protection from staphylococcal α-toxin. *Virulence*, *3*(5), 457–458. https://doi.org/10.4161/viru.21329

Yasuda, H., Shima, N., Nakagawa, N., Yamaguchi, K., Kinosaki, M., Mochizuki, S., Tomoyasu,

A., Yano, K., Goto, M., Murakami, A., Tsuda, E., Morinaga, T., Higashio, K., Udagawa, N., Takahashi, N., & Suda, T. (1998). Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastogenesis-inhibitory factor and is identical to TRANCE/RANKL. *Proceedings of the National Academy of Sciences*, *95*(7), 3597–3602. https://doi.org/10.1073/pnas.95.7.3597

Yi, F., Hu, J., Zhu, X., Wang, Y., Yu, Q., Deng, J., Huang, X., Ma, Y., & Xie, Y. (2021). Transcriptional Profiling of Human Peripheral Blood Mononuclear Cells Stimulated by Mycobacterium tuberculosis PPE57 Identifies Characteristic Genes Associated With Type I Interferon Signaling. *Frontiers in Cellular and Infection Microbiology*, *11*. https://doi.org/10.3389/fcimb.2021.716809

Zhang, W., Li, Y., Xin, S., Yang, L., Jiang, M., Xin, Y., Wang, Y., Cao, P., Zhang, S., Yang, Y., & Lu, J. (2023). The emerging roles of IFIT3 in antiviral innate immunity and cellular biology. *Journal of Medical Virology*, *95*(1). https://doi.org/10.1002/jmv.28259