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Infection Models of Intracellular Staphylococcus aureus and The Impact of Interleukin 12 on Macrophage Killing Activity

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Infection Models of Intracellular *Staphylococcus aureus* and The Impact of Interleukin 12 on Macrophage Killing Activity

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Dissertation submitted to the School of Pharmacy at West Virginia University in partial
fulfillment of the requirements for the degree of

Doctor of Philosophy

In

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Keywords: osteomyelitis, *Staphylococcus aureus*, infection, interleukin 12, cytokine, osteoblast, macrophage, rat, intracellular *S. aureus*. Copyright 2012 Therwa Hamza.

ABSTRACT

Infection Models of Intracellular *Staphylococcus aureus* and the Impact of Interleukin 12 on Macrophage Killing Activity

Therwa Hamza

Staphylococcus aureus (*S. aureus*) is the predominant cause of bone and joint infections worldwide. These infections may lead to severe bone and joint destruction and high mortality. Treatment of bone infections frequently requires long-term hospitalization for repeated debridement and prolonged antibiotic treatment. Bacteria including *S. aureus*, however, are often surprisingly successful in overcoming both host defense mechanisms and antibiotic treatments resulting in chronic and recurrent bone infections. Indeed, a high rate of recurrence or relapse of bone infections has been reported where ~17% of infections related to traumatic extremity injuries recur or relapse. One thought is that bacteria like *S. aureus* used to elude host defenses and antibiotic treatments are able to “hide” themselves intracellularly within host cells.

S. aureus has conventionally been considered as an extracellular pathogen, but there is growing evidence showing that it can survive intracellularly within host cells. Once internalized into host cells, *S. aureus* may elicit a pro-inflammatory response often characterized by the secretion of interleukin 1 β (IL-1 β), IL-6, IL-12p70 (IL-12), and tumor necrosis factor- α (TNF- α) among other cytokines. Production of pro-inflammatory cytokines from infected osteoblasts may disrupt the balance of the immune system thereby leading to uncontrolled inflammation and bone destruction. On the other hand, IL-12 is released primarily by antigen-presenting cells and acts as a link between innate

and acquired immunities by inducing the differentiation of the T helper 1 (Th1) phenotype and the release of interferon gamma (IFN- γ) from activated T-cells as well as natural killer cells. T-cell-derived IFN- γ is considered crucial to the activation of macrophages, the ultimate effector cells in host defense against intracellular infection. Studies have shown that both IL-12 and IFN- γ are up-regulated in infection models of other bacteria, parasites, and viruses but little is known about how they may contribute to the outcome of infection.

The overall goal of this dissertation is to develop *in vitro* and *in vivo* infection models of intracellular *S. aureus* that can aid in understanding the pathogenesis of chronic and recurrent infections and to develop effective immunotherapy regimens to prevent or treat such infections. This dissertation starts with an overall literature review in **Chapter 1** about the bacterium *S. aureus*, implant-associated infection, and finally IL-12 as a potential therapeutic agent against infections. **Chapter 2** establishes and compares two *S. aureus* infection models utilizing a non-phagocytic cell (osteoblast) and a phagocyte (macrophage) and the corresponding cellular responses of each cell type upon infection. In **Chapter 3**, *S. aureus* within osteoblasts is used to infect an open femur fracture rat model. We have presented the first direct *in vivo* evidence that intracellular *S. aureus* could be sufficient to induce bone infection in animals and intracellular *S. aureus* could play an important role in the pathogenesis of *S. aureus* infections. Finally, a potential approach using IL-12 as a therapeutic agent to improve macrophage killing activities against intracellular *S. aureus* is investigated in **Chapter 4**.

Keywords: osteomyelitis, *Staphylococcus aureus*, infection, interleukin 12, cytokine, osteoblast, macrophage, rat, intracellular *S. aureus*.

DEDICATION

To my family:

Mohammad Riyad Hamza

Neeran Al-Taiyar

Raniya Hamza

Bashar Hamza

Sarah Hamza

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Science is not an individual pursuit; it is a team effort, a significant collaboration in which we learn something wonderful, not only about the world around us, but also about ourselves as human beings in that world. The work presented here would never have been possible without the help and support of a large community of family, friends, and colleagues.

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SPECIFIC AIMS

For decades, *S. aureus* has been recognized as one of the most important leading cause of hospital- and community-acquired infections worldwide. Controlling *S. aureus* infections, especially chronic and recurrent infections, is a growing clinical challenge, and a better understanding of *S. aureus* pathogenesis and development of new therapeutic strategies are of importance. To achieve this long-term goal, my project has three specific aims:

- 1) Establish and compare *in vitro* *S. aureus* infection models using phagocytic (macrophage) and non-phagocytic cells (osteoblast)
- 2) Determine whether intracellular *S. aureus* can induce bone infection *in vivo*
- 3) Introduce IL-12 as a therapeutic agent to improve the killing efficacy of macrophages against intracellular *S. aureus*

CHAPTER 1

Literature Review

1.1 *Staphylococcus aureus*

1.1.1. Biology and Virulence Factors

Staphylococcus aureus (*S. aureus*) is a Gram-positive spherical bacterium, between 0.5 to 1.7 μm in diameter, non-motile, facultative anaerobic, catalase and coagulase positive that is able to induce β -haemolysis [1]. Traditionally, *S. aureus* is considered to be an extracellular pathogen [2, 3], but increasing evidence indicates that *S. aureus* might be a facultative intracellular pathogen [4-9]. The species name “aureus”, which means “golden” in Latin, is due to the yellow-golden color of the colonies. The color is imparted by carotenoid pigments that support the bacteria in the prevention of oxidant killing by phagocytes. Approximately, 20-30% of human beings are persistently colonized with *S. aureus* in the nose, skin, mucosa, the axilla region, or gastrointestinal tract [10].

The remarkable success of *S. aureus* as a pathogen is due to the expression of a wide array of virulence factors including a capsule as well as surface-exposed and secreted proteins [11, 12]. A schematic representation of *S. aureus* virulence factors is illustrated in Figure 1. Protein A is the hallmark of *S. aureus* which is able to bind immunoglobulins through their fragment crystallizable region (Fc) thereby hampering Fc receptor-mediated opsonophagocytosis [13, 14]. Protein A can also induce T-cell-independent proliferation and apoptosis of B cells [15]. Coagulase is an extracellular protein that results in clotting of the blood that might protect the bacterium from

Figure 1. Pathogenic factors of *Staphylococcus aureus*, with structural and secreted products both playing roles as virulence factors.

phagocytic uptake by immune cells [16]. The clumping factor A and B bind serum fibrin/fibrinogen and reduce the activity of phagocytic immune cells [17]. The collagen-binding and fibronectin-binding proteins support the adhesion of *S. aureus* to connective tissue, cartilage, and extracellular matrix [18, 19]. Enterotoxin B and toxic-shock-syndrome-toxin 1 (TSST-1) are bacterial superantigens that bind major histocompatibility complex II (MHC II) complexes on antigen-presenting cells leading to immunosuppression [20-22]. The α -toxin is the best-characterized and more potent cytotoxin of *S. aureus*. It binds to cell surface receptors and lipid bilayers on host cells and forms pores that eventually cause cell death [23, 24].

1.1.2. Intracellular *S. aureus*

S. aureus has long been considered an extracellular pathogen [5]. However, many studies have demonstrated its remarkable ability to survive and replicate intracellularly [25]. The ability of *S. aureus* to escape host immunity and then establish a cell-niche are actually crucial steps in its pathogenesis [3, 5].

S. aureus possesses several cell-surface adhesion molecules that facilitate its binding to extracellular matrix components. Binding involves a family of adhesins known as microbial surface components recognizing adhesive matrix molecules (MSCRAMMs). MSCRAMMs recognize proteins such as fibronectin, collagen, and fibrinogen to mediate adherence of *S. aureus* to epithelium, endothelium, bone, and biomaterials coated with host proteins [26]. Among all MSCRAMMs, fibronectin-binding proteins (FnBPs) are the most critical for adherence and internalization of *S. aureus*. The schematic representation in Figure 2 shows *S. aureus* attached to $\alpha_5\beta_1$ -integrins on the surface of a non-phagocytic cells, such as epithelial cells, endothelial cells, or osteoblasts, via fibronectin fibrils that are bound to FnBP (attachment). The host cell integrins are associated with myosin bound tensins, and by contraction of the myosin, the bacteria are transported toward the cell center. The uptake is performed by tensin-mediated actin reorganization [27, 28].

Currently, the mechanisms allowing for the intracellular survival of *S. aureus* have not yet been fully understood [5, 29]. In some non-phagocytic cells, *S. aureus* is able to escape from phagolysosomes and multiply in the cytosol. It also leads to the death

Figure 2. Schematic diagram of some of the signaling events involved in invasion of ‘non-professional phagocytic’ host cells by *S. aureus*. Adapted from Wright and Nair (28).

of these cells by apoptosis [30-32]. In contrast, in phagocytic cells, *S. aureus* seems to remain trapped within vacuoles [33, 34] where its multiplication is slowed down but not impaired by the acidic pH [35]. *S. aureus* is also known to produce enzyme degrading oxidant species (catalase, hydroperoxide reductase, and superoxide dismutase) [36], which may contribute to protecting it intracellularly.

Within the cell, *S. aureus* can switch to a phenotype known as small-colony variants (SCVs) that provides several advantages for its intracellular survival, among which are smaller colony size and reduced growth rate [37-39, 5]. SCVs demonstrate a number of characteristics that are different from *S. aureus* and are linked to interruption of electron transport [40]; they also present an altered antibiotic sensitivity [38], being most notably resistant to aminoglycosides [41]. SCVs are auxotrophic and can revert to

normal growth and morphology in the presence of menadione and hemin, thiamine, or CO₂-rich culture medium [42].

1.1.3. Host Immune Response

Normally, macrophages and other leukocytes in the host eliminate invading bacteria by a process called phagocytosis. Bacteria are engulfed into phagosomes and eventually fuse with lysosomes forming a phagolysosome where the microorganisms are destined for destruction. There, the bacteria are exposed to a number of toxic chemicals and enzymatic agents, some of which are reactive oxidative species such as superoxide anions and hydrogen peroxide, acidic pH, and degradative proteases. The pathogenicity of *S. aureus* is mainly mediated by a variety of secreted enzymes, adhesins, immunomodulating proteins, and the presence of cell wall compounds [43]. Recognition of *S. aureus* components by innate immune cells is the first step of host defense. Toll-like receptors (TLRs) are a class of receptors that recognize pathogen-associated molecular patterns (PAMPs) such as LPS, peptidoglycan, and lipoprotein [44, 45]. TLRs that are involved in the recognition of *S. aureus* include TLR2 which binds bacterial peptidoglycan and TLR9 which binds bacterial CpG DNA. Triggering of TLR signaling after pathogen recognition leads to the activation of transcription factors that induce transcription of genes coding for various proteins including cytokines and inflammatory mediators [46]. This process results in a massive invasion of immune cells to the site of infection. Several studies using knockout mice have shown that TLRs play a critical role in the innate immune response to *Staphylococcal* infections as mice deficient in the expression of myeloid differentiation factor 88 (MyD88), a cytoplasmic adaptor molecule

essential for the signaling of IL-1R/TLR family, are highly susceptible to this pathogen [47].

An effective immune response against *S. aureus* involves polymorphonuclear cell (PMN) recruitment such as neutrophils [48, 49]. The importance of neutrophils in host defense against *S. aureus* infection has been demonstrated by recurrent *S. aureus* infections in patients with chronic granulomatous disease, who have a defect in the NADPH oxidase and respiratory burst [48, 50].

1.1.4. Treatment for *S. aureus* Infections

Despite increasing knowledge on this bacterium, it is still difficult to prevent or effectively treat *Staphylococcal* infections in many cases. Developing new therapeutic strategies (including antibiotic and alternative therapies) as well as prophylaxis strategies, which are important for patients before invasive medical procedures, is an essential topic in research [51-54].

Treatment of *S. aureus* infections is commonly done using antibiotics, especially β -lactams [55]. The hospital-acquired strain of methicillin-resistant *S. aureus* can be susceptible to rifampicin, fusidic acid, and co-trimoxazole but they are rarely used because this strain can easily acquire resistance [56].

Cationic antimicrobial peptides (CAMPs) such as defensins, cathelicidin, and thrombocidins are positively charged (+2 to +7) amphipathic molecules with antibacterial properties [57-59]. Produced by the innate immune system, they protect the skin and epithelia from bacterial invasion [60].

There is still a problem with the introduction of an efficient vaccine in the prevention of infections caused by *S. aureus*. This can be associated with the fact that, throughout its life, a human organism is in contact with this bacterium as many people are constant or transient carriers of *S. aureus* on the mucus of the nasal or oral cavity. Earlier, the whole bacterial cell was used as an antigen, but recent research is aimed at obtaining antibodies against specific virulence factors such as adhesins, toxins, or invasins [61].

FnBPs are responsible for adherence of *S. aureus* to and subsequent internalization by phagocytes. Intramuscular application of a recombinant fragment of the fibronectin-binding domain (rFnBF), which potentially inhibits the entrance of *S. aureus* to host cells, counteracted the formation of abscesses in guinea pigs. The protective effect was dose-dependent and increased the efficacy of preventive treatment if applied simultaneously with cefazolin. The efficacy of this method relies on competition in the adhesion of exogenic *S. aureus* rFnBF with FnBP, which causes infection [62].

1.2 Implant-Associated Infections and Osteomyelitis

1.2.1. General Concepts

Bone turnover is a highly regulated process. New bone is formed by osteoblasts, which are cells of mesenchymal lineage derived from osteoprogenitor cells located in the periosteum or bone marrow. Osteoblasts form new bone matrix, called osteoid, which is later mineralized as osteoblasts mature. When osteoblasts become surrounded by mineralized matrix, they become osteocytes. Old or damaged bone is resorbed by osteoclasts via a process called bone resorption. Osteoclasts are large multinucleated cells

of hematopoietic lineage derived from the fusion of monocytes and macrophages. Osteoclasts are activated and recruited to sites of microfracture by factors such as macrophage colony stimulating factor (M-CSF) and receptor activator of nuclear factor $\kappa\beta$ ligand (RANKL). The balance in osteoblast and osteoclast activities is critical for healthy bone environment and successful implant procedures.

Orthopaedic implants are commonly used in treating patients with pain and compromised mobility or for fracture fixation. The risk of infection is increased by the presence of foreign materials in the body. The most commonly cultured pathogens isolated from prosthetic implant-associated infections are *S. epidermidis* (30 to 43%) and *S. aureus* (12 to 23%), followed by mixed bacterial flora (10 to 11%), streptococci (9 to 10%), Gram-negative bacilli (3 to 6%), enterococci (3 to 7%), and anaerobes (2 to 4%) [63]. Recently, *S. aureus* SCVs, which have a slower metabolism, do not produce toxins, and are more resistant to antibiotics, have been isolated from implant-associated infections and represent a risk factor for treatment failure, and persistence and recurrence of infection [64].

Implant-associated infections occur by bacterial contamination of the surgical site during surgery or immediately after, or they result from haematogenous spreading from distinct infections in the nose or on the skin. The resulting infection of the bone and soft tissue is called osteomyelitis, which is an inflammatory process characterized by massive bone destruction, or osteolysis (5). The disease can be acute or chronic with periodic recurrence [65, 66]. Chronic osteomyelitis is usually defined as osteomyelitis occurring in a patient who cannot be cured by medical means within six weeks [67]. The hallmarks

of chronic osteomyelitis include the presence of pus, persisting microorganisms, low-grade inflammation, and dead bone without a blood supply (sequestra) [68].

There are three types of osteomyelitis: haematogenous osteomyelitis which is commonly found in children and in elderly patients, osteomyelitis secondary to diabetic foot infection, and osteomyelitis caused by local spread from an adjacent infection after trauma, surgery, or joint replacement, while the incidence of the latter is the highest [5].

1.2.2. Pathogenesis

Staphylococci have a strong ability to adhere to plastic surfaces, a fact that has been used in basic *in vitro* assays. However, during colonization on medical devices, the additional interaction of MSCRAMMs and host proteins most likely cause a stronger attachment [61, 69]. This bacterial colonization forms what is called a biofilm, which is a microbial community covered by a thin three-dimensional structure matrix [70, 71]. The host immune system responds to foreign bodies with a localized inflammation leading first to the formation of a capsule-like membrane of fibrinogen, fibronectin, and collagen on the surface [72]. Extracellular proteins such as fibronectin and fibrinogen mediate bacterial adhesion to medical devices and host tissue. The localized inflammation causes a secondary phenomenon known as “frustrated phagocytosis”, which results from granulocyte contact with the foreign body and causes a functional granulocyte defect with reduced oxygen burst and chemotaxis in the presence of foreign bodies (Figure 3). This process may be partly responsible for the high susceptibility of implants to infections [74].

Figure 3. Pathogenesis of implant-associated infection. Adapted from Biomaterials (Biomaterials Science: An Introduction to Materials in Medicine, 2nd edition).

1.2.3. Therapy

The goal of implant-associated infection therapy is a long-term application and functional joint and is achieved by elimination of the infection. But, implant-associated infections are difficult to treat due to lack of microcirculation, which is crucial for host defense and for the delivery of antibiotics. Five treatment approaches utilizing appropriate surgical treatment and antimicrobial therapy are available. (I) Long-term antimicrobial therapy alone does not eradicate the infection. Additionally, clinical symptoms re-appear during or after antimicrobial breaks. (II) Debridement with retention of the prosthesis means removal of tissue around a stable implant for patients with an early postoperative or acute haematogenous infection (<3 weeks). Furthermore, an agent with activity against biofilm bacteria should be available. (III) and (IV) One- or two-stage revision includes removal of all devices, debridement, and replacement of a new prosthesis during the same procedure or after a period of time, respectively. (V) Finally, resection is defined as permanent removal and debridement without replacement [74, 63].

Surgical intervention as well as antibiotic therapy using cephalosporins and penicillins remains the most effective means of treatment for osteomyelitis [75]. However, osteomyelitis cases caused by *S. aureus* are especially chronic in nature and recalcitrant to therapy. In addition, the effectiveness of traditional antibacterial agents is becoming increasingly limited as the incidence of methicillin-resistant *S. aureus* (MRSA) has increased sharply in recent years. The wide array of virulence factors in *S. aureus*, including secreted products for host damage and immune evasion, coupled with its ability to invade and reside inside host cells using cell surface adherence factors, can protect

these bacteria from host defense mechanisms and antibiotics which can account for the persistence of disease despite what may be considered adequate surgical and antibiotic management [76].

1.3 Interleukin 12: a Key Immunoregulatory Cytokine in Infection Applications

It is well-established that $CD4^{+}$ T helper (Th) cells can be divided, besides T regulatory and Th17, into two major subsets: Th1 and Th2, based on their patterns of cytokine production. Th1 cells mainly secrete interleukin 2 (IL-2), IFN- γ , and IL-12, whereas Th2 cells secrete IL-4, IL-5, and IL-10 [77]. Functionally, Th1 cells predominantly promote cell-mediated immunity and help in clearance of intracellular pathogens, whereas Th2 cells are responsible for humoral immunity protecting against extracellular invaders. The balance between IL-12, favoring Th1 responses, and IL-4, favoring Th2 responses, determines the early preference expressed in the immune response.

IL-12 has multiple biological functions; importantly, it bridges the early nonspecific innate resistance and subsequent antigen-specific adaptive immunity [78]. IL-12 was first identified as a product of Epstein-Barr virus (EBV)-transformed human B cell lines. IL-12 was previously known as T-cell differentiation factor (TCDF), or natural killer cell stimulatory factor (NKSF). IL-12 was used to be the only known heterodimeric cytokine, but it is now part of a family consisting of several other members including IL-23, IL-27, and the recently identified IL-35. These new molecules have been found to play distinct cellular and functional roles in Th1 development [79].

1.3.1 IL-12 Molecular Structure

IL-12 is a key immunoregulatory cytokine [78, 80, 81] with a molecular weight of 70 kDa; it is composed of two covalently-linked subunits, IL-12p35 (35 kDa) and IL-12p40 (40 kDa), each of which is expressed on different chromosomes. The sequence of the p35 gene is homologous similar to that of IL-6 and granulocyte-colony stimulating factor [82]. The sequence of the p40 chain has a homology to the extracellular domain of IL-6 receptor (IL-6R) α -chain and the ciliary neurotropic factor [83]. This homology explains some of the redundant actions applied by these cytokines.

Although p35 transcripts are found in many cell types, free p35 is not secreted without the p40 subunit. IL-12p40 is produced predominantly by activated monocytes, macrophages (MΦs), neutrophils, and dendritic cells (DCs). It has been suggested that in mice, but not in humans, IL-12p40 homodimers antagonize IL-12p70 activity by binding to the β 1 subunit of the IL-12 receptor [84-86]. Also, IL-12p40 has been shown to act as a chemoattractant for MΦs and promotes the migration of stimulated dendritic cells [87]. The p40 subunit is associated with several pathogenic inflammatory responses such as silicosis, graft rejection, and asthma, but it is also found to be protective in a mycobacterial infection model [87].

1.3.2 IL-12 Receptor and Signaling Pathway

The biological activities of IL-12 are mediated via binding to a membrane receptor complex which is also composed of two subunits: IL-12R β 1 and IL-12R β 2. Both of the subunits are members of the class I cytokine receptor family, which includes IL-6, IL-11, and leukocyte inhibitory factor related to glycoprotein gp130 [88, 89]. There

Figure 4. IL-12 signaling pathway. IL-12 activates the Jak/STAT pathway. Following binding of IL-12p40 and IL-12p35 to IL-12R β1 and IL-12R β2, respectively, Jak-2 and Tyk-2 get transphosphorylated. Phosphorylated IL-12R β2 binds to STAT4 which will then dimerize with another STAT4 molecule. STAT4 homodimers translocate to the nucleus and promote IFN-γ gene transcription. T-bet is another transcription factor that plays a role in Th1 development and IFN-γ production. The IL-12 and IFN-γ produced induce the activity and proliferation of MΦs, NK cells, and T-cells, which also secrete IL-12. Modified from [97]. Reprinted from *Cytokine & Growth Factor Rev.*, 14(5), Watford, W.T.; Moriguchi, M.; Morinobu, A.; O'Shea, J.J. The biology of IL-12: coordinating innate and adaptive immune responses, 361-368, Copyright (2003), with permission from Elsevier.

is a high level of conservation, with a 68% amino acid sequence homology, between mouse and human IL-12R β2 proteins, and a 54% sequence identity homology between the mouse and human IL-12R β1 [88]. Both receptor chains are required to mediate maximal signaling; however, the two chains have independent roles. IL-12R β1 is required for high-affinity binding to the IL-12p40 subunit and is associated with the

Janus kinase (Jak) family member Tyk-2. The IL-12R β 2 chain mediates signal transduction via three tyrosine residues that act as a docking site for STAT4; it is associated with Jak-2 (Figure 4). IL-12R β 2 recognizes either the heterodimer IL-12 or IL-12p35 subunit and is expressed, not on naive T-cells, at low levels after T-cell receptor stimulation. Expression of this receptor subunit is critically influenced by IL-12 and type 1 IFNs [81]. The initial expression of functional IL-12Rs is further enhanced when IL-12 is present at the time of priming, working as a positive feedback loop regulator [90].

The signaling pathway of IL-12 is described in Figure 4. On binding of IL-12 to the IL-12R complex, activation of Jak kinases (Tyk-2 and Jak-2) occurs, leading to phosphorylation of the receptor, which becomes a binding site for STAT4 proteins that are rapidly recruited to the receptor and phosphorylated on their tyrosine residues by the Jak kinases. Tyrosine phosphorylation of STAT4 proteins induces their homodimerization and translocation to the nucleus where they bind to specific sequences and regulate gene transcription (Figure 4). In Th1 and NK cells, IL-12 mainly induces STAT4 activation. In addition, STAT1, STAT3, and STAT5 can also be activated by IL-12 signaling [89, 91]. Studies have shown that IFN- γ increases the transcription factor T-bet activity, which was originally shown to be induced in Th1 cells [92]. T-bet response to IFN- γ leads to the up-regulation of IL-12R β 2 surface expression and allows for Th1 cell responsiveness to IL-12 [92, 93]. More recent studies showed that T-bet negatively regulates GATA-3 expression, the main regulator of Th2 cell axis [94]. In contrast, the Th2 cytokine IL-4 reduces IL-12R β 2 expression and thus leaves Th2 cells non-responsive [95]. The opposite effects of IFN- γ and IL-4 on IL-12R β 2 expression may

contribute to the commitment of Th1/Th2 differentiation. Priming of macrophages with IFN- γ increases cellular responsiveness to inflammatory stimuli, including IFN- γ itself, which activates direct microbicidal functions including the production of reactive oxygen species and TNF- α and promotes the Ag processing and presentation capacities of macrophages. Also, IFN- γ plays a role in the regulation of T-cell proliferation and activity [96].

1.3.3 IL-12 Family Members

The IL-12 family has expanded recently to include members including IL-23, IL-27, and IL-35. IL-12 and these new family members play critical roles in Th1 cell development [80, 98]. Table 1 summarizes the structure and biological properties of these major IL-12 family members.

A. Interleukin 23

IL-23 is a proinflammatory cytokine that is closely related to IL-12 in structure; they both share the p40 subunit that is able to build a disulfide bridge complex with the p19 subunit to form the biologically active molecule. The p40-p19 complex is mainly secreted by activated intestinal antigen presenting cells, such as DCs and monocytes [98]. Similar to IL-12 signaling, IL-12R β 1 is required for high affinity binding to p40, whereas IL-23R has a cytoplasmic STAT4 binding domain for signal transduction. However, STAT3/STAT4 heterodimers in IL-23 instead of STAT4 homodimers in IL-12 are translocated to the nucleus to induce specific gene expression [99].

	IL-12	IL-23	IL-27	IL-35
Cytokines				
Receptors				
Kinases				
Vital STATs				
Also phosphorylated	STAT 1/3/5	STAT 1/5	STAT 4/5	?
Cells that Produce	DCs, monocytes macrophages, B cells	DCs, monocytes macrophages, B cells	DCs, monocytes macrophages, B cells	Foxp3 ⁺ T _{regs} , Others?
Cells that Respond	Naïve T cells Th1 cells NK cells	Memory T cells Th1 & Th17 cells NK cells	T cells Th1 cells NK cells	T cells, Others?
Function	Th1 activation Th1 maintenance Blocks Th2	Th1 activation Th17 polarization & proliferation	Th1 cell proliferation Skewing effector T cell lineages Blocks Th17	Inhibits T cell proliferation Additional?

Table 1. The IL-12 cytokine family; structural and biological characteristics.

The main role of IL-23 involves the stimulation of Th17 cells to produce IL-17 [79] and it induces the proliferation of memory T-cells [100]. Unlike IL-12, IL-23 does not induce significant production of IFN- γ , and in the absence of IL-23, IFN- γ production and Th1 differentiation are still found to be normal [98].

B. Interleukin 27

IL-27 is another heterodimeric cytokine composed of the p28 subunit and the p40-related protein Epstein-Barr virus-induced gene 3 (EBi3). IL-27 activities are driven via one receptor that was described recently with some homology to gp130, known as TCCR or WSX-1 [101]. Signaling through TCCR or WSX-1 is required for the early initiation of a Th1 response, but not necessary for the maintenance of Th1 responses [101]. The signaling pathway initiated by IL-27 activates Jak-1 and Jak-2 molecules. However, only STAT1 and STAT3 are critical to IL-27 bioactivities, and STAT3 rather than STAT1 is required for IL-27-induced proliferation [102].

IL-27 is rapidly produced by human phagocytic cells and DCs after their activation [101]. IL-27 is involved in early Th1 initiation, is able to induce proliferation of naive but not memory T-cells, and can synergize with IL-12 in IFN- γ production [102].

C. Interleukin 35

IL-35 was discovered very recently [103]; this heterodimeric cytokine is composed of the p35 subunit and the p40-related protein EBi3. IL-35R complex has not been characterized yet, but it is possible that it may be composed of the receptors known for IL-27 and IL-12, either gp130 or WSX-1 and IL-12R β 2.

Within the CD4⁺ T-cell population, IL-35 is expressed by resting and activated T regulatory cells (Tregs) but not activated T-cells. Loss of IL-35 expression results in reduced immune suppression by Tregs. IL-35 appears to function as an anti-inflammatory molecule by inhibiting T-cell proliferation. It is suggested that IL-35 can suppress Th17

development *in vivo* [104]; however, further studies are required to fully understand the exact mechanism behind these functions.

1.3.4 IL-12 Biological Activities

IL-12 is produced mainly by DCs, MΦs, monocytes, neutrophils, microglia cells, and, to a lesser extent, B-cells (Figure 5); human but not murine B cells were found to produce IL-12 following CD40 ligation [105]. Non-immune cells such as infected-keratinocytes and osteoblasts, epithelial, and endothelial cells have also been shown to produce some amounts of this cytokine [106, 107]. Pathogen-associated molecular patterns such as LPS, teichoic acid, peptidoglycan, and bacterial CpG DNA, can induce IL-12 production. The production of IL-12 is regulated by positive and negative feedback mechanisms involving Th1 cytokines (e.g. IFN- γ), Th2 cytokines (e.g. IL-10), and type 1 IFN (Figure 5) [108, 109].

IL-12 has multiple biological functions (Table 2) and it bridges innate and adaptive immunity. IL-12 induces differentiation of naive CD4⁺ T-cells to Th1 cells and activates NK cells. Upon activation, these cells produce IFN- γ and other type-1 cytokines [110]. IL-12 also protects CD4⁺ Th1 cells from antigen-induced apoptotic death [111]. IL-12 was found to have synergistic effects with IL-18 in developing Th1 cells, and IL-12 and IL-18 reciprocally upregulate each other's receptors [81]. In addition, IL-12 plays a role in T-cell trafficking and migration by inducing functional adhesion molecules such as P- and E-selectin ligand expression on Th1 cells but not Th2 cells; therefore, these cells are selectively recruited to sites where Th1 immune responses are needed [112]. Studies also reported selective expression of CCR5 and CXCR3 when naive T-cells were primed in

the presence of IL-12 [110, 113]. Stimulation of MΦ-derived IL-12 also plays a major role in the induction of resistance in parasitic infestation [114].

Figure 5. Summary of the biology of IL-12 [90]. Reprinted by permission from Macmillan Publishers Ltd: *Nat Rev Immunol.*, 3, Trinchieri, G. Interleukin-12 and the regulation of innate resistance and adaptive immunity, 133-146, Copyright (2003).

<ul style="list-style-type: none"> • Promote naive CD4⁺T-cells to differentiate into Th1 • Enhance the generation and activity of cytotoxic T lymphocytes • Induce IFN-γ production by (i) NK cell, T-cell, DC, and MΦ, (ii) cooperating with B7/CD28 interaction, and (iii) synergizing with IL-18
<ul style="list-style-type: none"> • Increase MΦ antimicrobial activity
<ul style="list-style-type: none"> • Prime DC activation to induce more IL-12 production
<ul style="list-style-type: none"> • Induce functional adhesion molecule expression on Th1 cells and influence T-cell trafficking

Table 2. Main immunological functions of IL-12.

The biological activity and quantity of IL-12 can be determined using molecular approaches. One of the first approaches for quantifying IL-12 was based on IL-12-induced proliferation of phytohemagglutinin (PHA)-stimulated lymphocytes [115]. Peripheral blood mononuclear cells activated with PHA along with IL-2 proliferate in response to IL-12. Enzyme-linked immunosorbent assay (ELISA) was also applied to quantify specifically IL-12p40 and IL-12p70 [116]. In combination with ELISA, another approach, based on the ability of IL-12 to induce IFN- γ secretion from activated T-lymphoblasts, was developed and has been used to measure the biological activity of IL-12 [117, 118].

1.3.5 IL-12 Therapeutic Applications in Infections

IL-12 holds considerable promise as an immunotherapeutic agent because it plays a central role in regulating innate and adaptive immune responses and synergizes with several other cytokines for increased immunoregulatory activities. Animal and human studies have shown improved outcomes in treating or preventing infections based on the mechanisms of IL-12-dependent therapies.

1.3.5.1 Viral Infections

The role of IL-12 in promoting endogenous protective immune responses to viral infections has been attracting more attention with time. Many experimental models have been developed to test the potential effects of IL-12 and other immune mediators on herpes simplex virus, influenza virus, human immunodeficiency virus (HIV), lymphocytic choriomeningitis virus, mouse cytomegalovirus, and many others [119].

A. Herpes simplex virus

In an *in vivo* herpes simplex virus-1 (HSV-1) mouse model, sustained expression of IL-12 was induced by the virus. This local IL-12 production was considered to cause an immunopathological response in the mouse eye environment due to Th1 immunity [120]. However, exogenous IL-12 showed a protective role and increased the resistance to infection in HSV-1-thermally injured mice, possibly resulting from the activation of viral-specific Th1 immunity [121]. Also, IL-12 and IL-18 were shown to play an important role in innate immunity against genital HSV-2 infection in a mouse model; the protection was mainly mediated by IFN- γ production [122].

B. Influenza virus

CD4⁺ T-cells are important in controlling influenza A virus infection. CD4⁺ T-cells drive the induction and expansion of cytotoxic T lymphocyte (CTL) against such viral pathogens. Earlier studies using the influenza virus system as a model showed that DCs induced effective CTL response without increasing endogenous IL-12 levels. Exogenous application of IL-12 was found to significantly enhance CTL activity [123]. Studies also demonstrated a combined effective role of IL-12 and DCs together in enhancing CTL immunity to viral infection [123]. The influenza virus vaccination effectively protects individuals against serious complications through induction of humoral and cellular responses [124]. Interestingly, patients identified with genetic IL-12/23R β 1 or IFN- γ R deficiencies were found to develop immune responses to childhood vaccination [123]. Also, influenza vaccination could induce humoral immunity and IFN- γ production in IL-12/23R β 1 deficient patients [125]. These studies may imply that both IL-12 and IFN- γ play a significant role in fighting viral infections.

C. Human immunodeficiency virus (HIV)

HIV infection mainly induces Th2 immunity which is unable to clear the viral infection most of the time. Glycoprotein gp120 of HIV has been shown to induce IFN- γ dependent production of IL-12 in cultured M Φ s. The use of IL-12 production and Th1 activation might contribute to decreasing viral load and increasing clearance of the infection [126]. However, phase I trials in HIV patients have shown moderate effects accompanied by some toxic responses [127].

1.3.5.2 Bacterial Infections

As a potent inducer of Th1 immune response and an important mediator between innate and adaptive immunity, IL-12 has potential clinical uses in treating and preventing bacterial infections.

A. Bone infection

Osteomyelitis is a bone infection characterized by the presence of necrotic bone tissue and increased osteoclast activity. It is commonly caused by *Staphylococcus aureus* (*S. aureus*), which is traditionally known as an extracellular pathogen; however, it has been shown that *S. aureus* can be internalized and live within a variety of host cells [128, 129]. Internalization of *S. aureus* into the host cells could be an important pathogenicity factor for escaping the host immune system leading to persistence of infection [130]. It was reported that *in vitro* mouse and human osteoblasts infected with *S. aureus* express high levels of IL-12 [107]; IL-12 and/or IL-23 knockout mice showed an increased susceptibility to bacterial, parasitic, and fungal infections [131]. Therefore, IL-12 may play a critical role in the downregulation of *S. aureus* growth *in vivo* [132].

Figure 6. Local IL-12 therapy stimulates Th cells to secrete Th1 cytokines [63]. Exogenous IL-12 application creates an environment rich in IL-12 around the infection site. In such a local environment, newly activated Th cells, responding to the presence of bacteria, exit the blood and are influenced to become Th1 cells and secrete more Th1 cytokines and activate macrophages and NK cells. Activated macrophages will produce more IL-12 via a positive feedback; cell-mediated immunity can be promoted to battle bacteria thereby leading to the prevention of infection. Reprinted from *Biomaterials*, 30, Li, B.; Jiang, B.; Boyce, B.M.; Lindsey, B.A. Multilayer polypeptide nanoscale coatings incorporating IL-12 for the prevention of biomedical device-associated infections, 2552-2558, Copyright (2009), with permission from Elsevier.

More recently, studies have shown that IL-12 may play a significant role in preventing osteomyelitis. A decrease in IL-12 production was observed upon pathogen-mediated ligation of MΦ Fcγ or complement receptors [133, 134]. This finding may indicate that there are cases in which regulation of IL-12 production can be affected by

pathogens. Perhaps down-regulation of IL-12 has the potential of providing pathogens with a means of suppressing cell-mediated immunity which would make pathogen clearance takes longer and increase the risk of bacterial infections. Meanwhile, IL-12 production was shown to decrease following trauma or major burn resulting in decreased or delayed cell-mediated immune response and decreased resistance to infection [135-138]. Exogenous application of IL-12, combined with nanotechnology drug delivery systems, may have restored the decreased IL-12 level and recovered the host's cell-mediated immune response; the combination has been found to be effective in preventing open fracture-associated osteomyelitis [139, 140]. The mechanism of protection is related to the enhancement of Th1 reactivity and activation of MΦs at an early stage following surgery (Figure 6) [139]. The activation of MΦs may lead to enhanced antigen presentation, phagocytosis, Fc receptor expression, and nitric oxide and superoxide production [141]. As a result, these changes may substantially increase the ability of MΦs to kill a wide variety of intracellular and extracellular bacteria and enhance the development of long-term immunity to those pathogens [141].

B. Tuberculosis

Tuberculosis (TB) is a common and often deadly infectious disease caused by mycobacteria including *Mycobacterium tuberculosis*, *Mycobacterium bovis*, *Mycobacterium africanum*, *Mycobacterium canetti*, and others. The chronic nature of *Mycobacterium tuberculosis* (Mtb) infection has led researchers to investigate the host's immunity to respond to and control this disease. Previous reports involving TB infection models showed that the development of Th1 cells in response to IL-12 production and

subsequent induction of IFN- γ are key players in immunity to TB [142]. Exogenous IL-12 application was found to result in lower bacterial load and increased incidence of survival [143]. DC and Th1 activation and migration to the infected lungs were shown to control bacterial growth via IFN- γ -induced activation of phagocytes [144]. This immune response, however, may not be solely mediated by IL-12, as the other IL-12 family members (IL-23, IL-27, and IL-35) may also participate as well [87].

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CHAPTER 2

***Staphylococcus aureus* Internalization and Associated Biological**

Responses of Osteoblasts and Macrophages

Abstract

The aims of this study were to compare *Staphylococcus aureus* (*S. aureus*) internalization in a phagocytic cell (i.e. macrophage) to a non-phagocytic cell (i.e. osteoblast), and to investigate the cells' responses upon infection. We found that *S. aureus* could internalize within macrophages and osteoblasts, and upon infection, a significantly higher number of live intracellular *S. aureus* was observed in macrophages compared to osteoblasts. The viability of macrophages and osteoblasts both decreased with increasing infection time, and macrophages had significantly lower viability during 2 h infection and significantly higher viability during 8 h infection compared to osteoblasts. Moreover, intracellular *S. aureus* was found to survive within macrophages and osteoblasts for approximately 5 and 7 days, respectively. The percentage of *S. aureus* survival within macrophages and osteoblasts decreased with increasing post-infection time, and the percentage of *S. aureus* survival within macrophages was significantly lower compared to that within osteoblasts. More importantly, compared to non-infected controls, *S. aureus* infection resulted in (i) significantly increased hydrogen peroxide production in macrophages and osteoblasts, (ii) significantly increased superoxide anion production in macrophages but not in osteoblasts, (iii) relatively lower alkaline phosphatase activity in infected osteoblasts, and (iv) higher phagocytosis activity in infected macrophages.

Keywords: Infection; internalization; intracellular bacteria; *S. aureus*; chronic infection; recurrent infection.

1. Introduction

Staphylococcus aureus (*S. aureus*) is one of the primary causes of bone infections [1-3]. These infections are often chronic, difficult to eradicate, and cause high morbidity rates [4]. *S. aureus* can gain access deep into bone and soft tissue as a result of severe trauma or foreign body implants [5]. Although *S. aureus* has been traditionally considered as an extracellular pathogen, it has been reported by several groups that this bacterium can invade and survive within a variety of cells such as neutrophils, macrophages, T-lymphocytes, epithelial cells, endothelial cells, fibroblasts, and osteoblasts [6-15]. One hypothesis, not yet proven, about chronic and recurrent infections is that bacteria internalize into the host cells and such intracellular bacteria may potentially lead to their evasion of the host's immune responses and protection from most conventional antibiotics [16].

The main role of osteoblasts is to synthesize bone components and induce bone matrix mineralization [17]. Osteoblasts are not traditionally considered as part of the immune system. However, osteoblasts were recently found to be able to induce inflammatory cytokines and chemokines upon *S. aureus* internalization [18,19]. This finding may suggest an important role for osteoblasts in triggering immune responses after *S. aureus* infection. *S. aureus* can be internalized into osteoblasts and its internalization is believed to be mediated by binding of fibronectins on osteoblast surfaces, which is connected to the integrin dimer $\alpha_5\beta_1$ molecule and fibronectin-binding

proteins on *S. aureus* surfaces [6]. Protein-ligand interaction leads to *S. aureus* adhesion and invasion by a “zipper-like” mechanism [15]. Eventually, internalized bacteria escape into the cytoplasm and may lead to host cell death by apoptosis [20]. In addition, live osteoblasts are necessary for *S. aureus* internalization as formalin-fixed osteoblasts could not internalize *S. aureus* [10,21].

The major role of phagocytes, such as macrophages, neutrophils, and dendritic cells is to engulf and eliminate a wide variety of invading pathogens. As a result, high influxes of such phagocytes are expected at the infection site upon pathogen invasion. For instance, a high influx of neutrophils was detected at the infection site of *S. aureus* bone infection [22]. Unfortunately, some pathogens can survive within these phagocytes after being phagocytized which may lead to chronic diseases [23,24]. It was reported that *S. aureus* can survive within neutrophils and its survival may have contributed to infection persistence as well as dissemination *in vivo* [7]. Neutrophils are short-lived and are unlikely to carry intracellular pathogens for long [25]. Macrophages, however, are long lived and may possibly allow surviving pathogens to invade the circulatory system from localized infection sites [26] and thereby may be more likely contribute to chronic and recurrent infections.

The aims of this study were to compare *S. aureus* internalization in a phagocytic cell (i.e. macrophage) and a non-phagocytic cell (i.e. osteoblast), and to investigate macrophage and osteoblast responses upon *S. aureus* infection. We hypothesized that *S. aureus* can internalize into macrophages and osteoblasts and survive for at least days, and its internalization may lead to changes in macrophage and osteoblast activities.

2. Materials and Methods

2.2.1. Reagents

Tryptic soy agar (TSA, w/5% sheep blood) plates, tryptic soy broth (TSB), phosphate buffered saline (PBS), fetal bovine serum (FBS), 0.25% trypsin/2.21 mM ethylenediaminetetraacetic acid (EDTA) solution, 45% glucose solution, 7.5% sodium bicarbonate, sodium pyruvate, and HEPES buffer were all obtained from Fisher Scientific (Pittsburgh, PA). Dulbecco's Modified Eagle Media: Nutrient Mixture F-12 (DMEM/F12) and RPMI-1640 media were purchased from LONZA (Walkersville, MD). DiI fluorescent dye, Syto-9, propidium iodide (PI), and 100 U/mL penicillin/100 mg/mL streptomycin solution were from Invitrogen (Carlsbad, CA). Gentamicin, Triton X-100, cytochalasin D, dimethyl sulfoxide (DMSO), bovine serum albumin (BSA), lysostaphin, fluorescein isothiocyanate (FITC), paraformaldehyde, and glutaraldehyde were obtained from Sigma (St. Louis, MO). Primary antibody Ab20920 and goat polyclonal to rabbit IgG secondary antibody-Cy5 ab6564 were obtained from Abcam (Cambridge, MA). Dichlorofluorescein diacetate (H₂DCF-DA) and dihydroethidium (DHE) were from Life Technologies (Grand Island, NY).

2.2.2. Bacteria

S. aureus (ATCC 25923) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). A fresh inoculum was prepared by suspending 5 colonies of *S. aureus*, grown on a blood agar plate, in 5 mL TSB and incubated at 37°C for 18 h. After incubation, the *S. aureus* inoculum was centrifuged at 3750 rpm for 15 min at 4°C and washed once with 10 mL PBS to obtain an optical density of 0.80 which yields (6-8)×10⁸ colony forming units/mL or CFU/mL. The bacteria pellet was then re-

suspended in either DMEM/F12 for the infection of osteoblasts or in RPMI-1640 medium for the infection of macrophages; both cell culture media were free from streptomycin/penicillin and FBS.

2.3. Infection of osteoblasts with *S. aureus*

Rat osteoblasts (UMR-106) were obtained from ATCC and grown in full-supplemented DMEM/F12 medium containing 10% FBS and 1% of penicillin/streptomycin solution. In 12-well plates (Fisher Scientific), 3×10^5 cells/mL were seeded and cultured in full-supplemented DMEM/F12 medium for at least 24 h at 37°C in a 5% CO₂ incubator until they reached ~ 80% confluence. Osteoblasts were infected and the effects of multiplicity of infection (MOI), which represents *S. aureus* to osteoblast ratio, and infection time on osteoblast infection were investigated: (1) To examine the effect of MOI on osteoblast infection, the osteoblast monolayer was washed 3 times with PBS and then fresh DMEM/F12 media was added (free from streptomycin/penicillin and FBS). Immediately, *S. aureus* was added at MOIs of 100:1, 500:1, and 1000:1 and incubated for 2 h. (2) To examine the effect of infection time on osteoblast infection, the osteoblast monolayer was washed 3 times with PBS and then fresh DMEM/F12 media (free from streptomycin/penicillin and FBS) was added. *S. aureus* was added at an MOI of 500:1 and incubated for different times, i.e. infection times, of 0.5, 2, 4, 6, and 8 h. After each time point, the osteoblast monolayer was washed 3 times with PBS and treated with 100 µg/mL gentamicin (an antibiotic known not to penetrate mammalian cell membranes within a few hours [27,28]) for 2 h at 37°C in a 5% CO₂ incubator. Osteoblasts were then washed 3 times with PBS and immediately lysed with 0.1% Triton X-100 in PBS for 10 min at 37°C; the cell lysates were diluted in

PBS and plated on blood agar plates overnight. To determine viability, osteoblasts were detached by incubating them at 37°C for 3 min in a 0.25% trypsin/2.21 mM EDTA solution; trypsinization was stopped by adding DMEM/F12 medium supplemented with 10% FBS. Viable osteoblasts were counted using a hemocytometer (Bright-Line Hausser Scientific, Horsham, PA) following the trypan-blue exclusion assay which depends on cellular membrane integrity; dead cells allow the stain to penetrate while live cells do not.

2.4. Infection of macrophages with S. aureus

Rat alveolar macrophage cell-line (NR 8383) was obtained from ATCC and grown in full-supplemented RPMI-1640 medium containing 10% FBS, 1% streptomycin/penicillin, 45% glucose solution, 7.5% sodium bicarbonate, and sodium pyruvate. The infection of macrophages with *S. aureus* was studied at different MOIs and infection times. The protocols for infecting macrophages were similar to those of infecting osteoblasts as described previously. In brief, 3×10^5 cells/mL were seeded in 12-well plates and cultured in full-supplemented RPMI-1640 medium for at least 24 h to achieve adherence at 37°C in a 5% CO₂ incubator. Cultured macrophages were washed 3 times with PBS and then infected with *S. aureus* at different MOIs (100:1, 500:1, and 1000:1) or infection times (0.5-8 h). Infected macrophages were washed, treated with gentamicin, washed again, and then lysed to determine the number of live intracellular *S. aureus*. To determine the viability of macrophages, adherent macrophages were scraped using a cell scraper (Fisher Scientific) and combined with floating macrophages from the same sample for trypan-blue exclusion assay and hemocytometry.

The viability of osteoblasts and macrophages after infection with *S. aureus* was calculated relevant to their control (non-infected) cells according to the following equation:

$$\text{Viability (\%)} = \frac{\frac{\text{Number of live cells in infected sample}}{\text{Number of live and dead cells in infected sample}}}{\frac{\text{Number of live cells in control sample}}{\text{Number of live and dead cells in control sample}}} \times 100\%$$

Note that the total cell numbers in the infected and control samples were the same at the beginning of the infection (i.e. infection time = 0 h) but were different at later infection time periods (i.e. 0.5-8 h).

2.5. Inhibition of *S. aureus* internalization in osteoblasts

Cytochalasin D was re-constituted in 1% DMSO; 3×10^5 cells/mL were seeded in 12-well plates and cultured in full-supplemented DMEM/F12 medium to reach ~ 80% confluence. The osteoblast monolayer was washed 3 times with PBS and then fresh DMEM/F12 media was added (free from streptomycin/penicillin and FBS) together with cytochalasin D (0.5, 1, 5, 10, and 20 $\mu\text{g/mL}$). After culturing for 30 min, *S. aureus* was added at an MOI of 500:1 and further incubated for 2 h. Following treatment with gentamicin, infected osteoblasts were lysed and plated to determine the number of live intracellular bacteria.

2.6. Survival of *S. aureus* within osteoblasts or macrophages

Osteoblasts or macrophages were infected with *S. aureus* at an MOI of 500:1 for 2 h, treated with gentamicin, washed, and cultured for up to a week in DMEM/F12 (for

osteoblasts) or RPMI-1640 (for macrophages) supplemented with 5% FBS and 5 µg/mL lysostaphin; lysostaphin does not penetrate mammalian cell membranes for long time periods, e.g. weeks [29-31]. The cell culture media was changed every 3 days. At post-infection days 0, 1, 3, 5, 6, 7, and/or 8 and 9, independent samples of infected cells were washed with PBS, lysed with 0.1% Triton X-100, and plated on blood agar plates to determine the number of live intracellular *S. aureus*. Percentage of live intracellular CFU at different times following infection was calculated based on the live intracellular CFU immediately after infection (i.e. post-infection day 0).

2.7. Confocal microscopy

A dual staining approach [32,33] was adopted to visualize intracellular *S. aureus*. Osteoblasts or macrophages were cultured on rounded cover-slips for at least 24 h in full-supplemented DMEM/F12 or RPMI-1640, respectively. Fresh *S. aureus* was cultured for 18 h at 37°C in a 5% CO₂ incubator. After washing the bacteria once with PBS, the pellet was stained with 100 µg/mL FITC in PBS for 30 min at room temperature prior to infection. Excess FITC was removed by washing with PBS and centrifugation at 3750 rpm for 15 min at 4°C. After infecting with the stained *S. aureus* for 2 h at an MOI of 500:1, osteoblasts were trypsinized using a 0.25% trypsin/2.21 mM EDTA solution for 30 seconds at room temperature to remove adherent extracellular *S. aureus*; no trypsinization was used in the macrophage samples. Osteoblasts or macrophages were then fixed with 4% paraformaldehyde in distilled water for 30 min at room temperature. Fixed cells were washed 3 times with PBS and blocked with 5% BSA for 1 h at room temperature. To further label the extracellular *S. aureus*, the fixed cells were incubated overnight at 4°C with a primary antibody Ab20920S in 5% BSA, washed 3 times with

PBS (to remove excess free primary antibody), and then incubated in the dark with a secondary antibody-conjugated Cy5 fluorescent dye in 2.5% BSA for 45 min at room temperature. After washing the excess secondary antibody with PBS, the cover-slips were flipped onto microscopic glass slides and used for image observation; macrophage samples were mounted in the presence of 4',6-diamidino-2-phenylindole (DAPI) fluorescent dye to visualize the nuclei of macrophages. Slides were visualized using a 159 Plan-Apochromat 63x/1.40 oil objective on an LSM 510 confocal microscope (Zeiss, Jena, Germany).

To confirm the presence of live intracellular *S. aureus* and the efficacy of gentamicin killing of extracellular *S. aureus*, osteoblasts were seeded on a rounded cover-slip overnight. The monolayer was then stained with DiI fluorescent dye for 20 min at 37°C in the dark. The monolayer was washed once with PBS and infected with Syto-9 labeled *S. aureus* as aforementioned. After gentamicin treatment, infected osteoblasts were washed 3 times with HEPES buffer and PI stain was added for 15 min at room temperature in the dark. Immediately after washing off the excess PI, the slides were examined under the LSM 510 confocal microscope and images of Z-stack sections were taken to confirm the live intracellular *S. aureus*. Z-stack sections were generated and the X-Y planes showed that all live (green) *S. aureus* was inside the osteoblasts.

2.8. Transmission electron microscopy (TEM)

Osteoblasts were infected with *S. aureus* at an MOI of 500:1 for 2 h, washed once with PBS, and detached using trypsin-EDTA. Osteoblasts were then collected by centrifugation at 1200 rpm at 4°C for 7 min, and the pellet was washed twice with PBS then fixed with 2% paraformaldehyde and 4% glutaraldehyde mixed with 0.075 M PBS

for 30 min at room temperature. The fixed cell mass was collected in 1.5 mL Eppendorf tubes. The cell pellet was washed 3 times with PBS, post-fixed in 1% osmium tetroxide for 2 h at room temperature, washed 3 times with PBS, treated with aqueous 1% tannic acid for 1 h at room temperature, and then dehydrated in a gradient ethanol series. The cells were embedded in pure LR white resin solution and polymerized at 60°C for 24-48 h. Thin (0.1 μm) sections were cut and placed on nickel grids, stained with 2% uranyl acetate and lead citrate, and viewed using TEM (JEOL, Peabody, MA).

2.9. Reactive oxygen species production

Osteoblasts and macrophages were infected with *S. aureus* at an MOI of 500:1. At pre-determined time points (0.5, 1, and 2 h), samples of infected osteoblasts or macrophages were taken, washed once with PBS, and then incubated with H₂DCF-DA or DHE at 37°C for 1 h in the dark; separate samples were used for the staining of H₂DCF-DA and DHE. Non-infected osteoblasts and macrophages were used as controls and were treated the same as the infected cells except no *S. aureus* was added. Viable cells of infected and control samples at the pre-determined time points were obtained using hemocytometry and were used to analyze the final fluorescent data. The fluorescence intensity was measured using a fluorescent microplate reader (BioTek Instrument, Inc., Winooski, VT) at 492 nm/520 nm for 2',7'-dichlorofluorescein (DCF), converted intracellularly from H₂DCF-DA, and 492 nm/620 nm for DHE. H₂DCF-DA and DHE are commonly used to stain intracellular hydrogen peroxide (H₂O₂) and superoxide anion (O₂⁻), respectively [34]. The acetate groups of H₂DCF-DA are cleaved by intracellular esterases and oxidation and convert to highly fluorescent DCF.

2.10. Osteoblast alkaline phosphatase (ALP) activity

Osteoblasts were cultured in 12-well plates at a density of 5×10^4 cells/mL, infected at an MOI of 500:1 for 2 h following the aforementioned infection protocol. Non-infected (control) and infected osteoblasts were cultured in DMEM/F12 supplemented with 5% FBS and 5 μ g/mL lysostaphin solution for a week; media was changed every 3 days. On post-infection days 1, 4, and 7, osteoblast monolayers were washed with PBS once and the ALP activity was determined using an ALP assay kit (Abcam) and expressed as Unit/mL.

2.11. Macrophage phagocytosis assay

Macrophage phagocytosis (ingestion) activity was tested by measuring the uptake of FITC-labeled *S. aureus* by non-infected (control) macrophages and macrophages infected with *S. aureus* (unlabeled) at an MOI of 500:1 for 2 h. After incubating 5×10^5 cells/mL non-infected (control) and infected macrophages separately with FITC-labeled *S. aureus* at 10:1 MOI for 2 h, macrophages (infected and non-infected) were treated with 100 μ g/mL gentamicin for 2 h at 37°C in a 5% CO₂ incubator. Macrophages were then scraped and collected for flow cytometry analysis using BD-FACS Calibur (BD, Franklin Lakes, NJ); 10,000 events were collected. Data were acquired in logarithmic mode for the forward scatter (FSC), side scatter (SSC), and green fluorescence channel FL-1H (i.e. FITC). Control macrophages were subjected to the same experimental protocols as the infected cells but without infection with *S. aureus*. The percentage of macrophages with FITC fluorescent intensity corresponds to the ingestion activity of macrophages.

2.12. Statistical analysis

Statistical analyses were performed using JMP Statistical Visualization Software (SAS Institute, Cary, NC). Experiments were repeated at least twice on separate days to verify reproducibility. All data were expressed as mean $\pm$ SD and analyzed using one-way analysis of variance (ANOVA). Statistical significance was set at $p < 0.05$.

3. Results

3.1. Characterization of *S. aureus* infection of osteoblasts and macrophages

After incubating with *S. aureus* for 2 h at an MOI of 100:1 to 1000:1, osteoblasts and macrophages were both found to be infected. However, significantly higher (~ 2 orders) numbers of intracellular *S. aureus* were found within macrophages compared to osteoblasts (Figure 2A); the intracellular CFUs for infected macrophages and osteoblasts were approximately 3.5×10^6 and 3.1×10^4 CFU/(10^5 cells), respectively. No significant differences were observed in the same cell type at the various MOIs studied (i.e. 100:1, 500:1, and 1000:1). By contrast, significantly lower viability was observed in macrophages compared to osteoblasts at 2 h infection; the viability of macrophages and osteoblasts were 62-78% and 90-95%, respectively (Figure 2B). No significant differences in viability for the same cell type at the MOIs investigated (i.e. 100:1, 500:1, and 1000:1) were noticed following the 2 h infection.

At an MOI of 500:1, the number of intracellular *S. aureus* for both macrophages and osteoblasts increased with increasing infection time and reached a plateau at 2 h, at which point the intracellular CFUs for macrophages and osteoblasts were 5.0×10^6 and 3.9×10^4 CFU/(10^5 cells), respectively (Figure 2C). At the infection times of 2-8 h, the intracellular CFUs for macrophages were significantly (about 2 orders) higher than those of osteoblasts. At an MOI of 500:1, the viability of macrophages and osteoblasts decreased approximately linearly with increasing infection time. The viability of macrophages at infection times of 2, 4, 6, and 8 h was significantly lower than that of both macrophage control and at infection time of 0.5 h. The viability of osteoblasts at

Figure 2 (A)

Figure 2 (B)

Figure 2. *S. aureus* infection of osteoblasts and macrophages. (A) Live intracellular *S. aureus* and (B) viability of osteoblasts and macrophages at different MOIs (100:1, 500:1, and 1000:1) for 2 h. * $p < 0.05$ and ** $p < 0.001$ compared to osteoblasts at the same MOI.

infection times of 4, 6, and 8 h was significantly lower than that of both osteoblast control and at infection time of 0.5 h (Figure 2D). In addition, the viability of macrophages was significantly lower at 2 h infection but significantly higher at 8 h infection compared to osteoblasts at corresponding infection time periods (Figure 2D).

The *S. aureus* infection of osteoblasts was also found to be significantly inhibited by the addition of cytochalasin D. The intracellular CFUs of *S. aureus* decreased significantly with increasing cytochalasin D at the dose range studied (0.5-20 $\mu\text{g/mL}$), reaching 50% inhibition at 20 $\mu\text{g/mL}$ (Figure 2E). Relatively higher cytochalasin D doses of 10 and 20 $\mu\text{g/mL}$ also led to significantly lower intracellular CFUs of *S. aureus* compared to the doses of 0.5, 1, and 5 $\mu\text{g/mL}$ (Figure 2E).

Figure 2 (C). *S. aureus* infection of osteoblasts and macrophages. Live intracellular *S. aureus* and at an MOI of 500:1 for various infection times. ** $p<0.001$ compared to osteoblasts at the same infection time.

Figure 2 (D). *S. aureus* infection of osteoblasts and macrophages. Viability of osteoblasts and macrophages at an MOI of 500:1 for various infection times. [&] $p < 0.01$ compared to macrophages at infection times 0 and 0.5 h, [^] $p < 0.01$ compared to osteoblasts at the same infection times, and [#] $p < 0.05$ compared to osteoblasts at infection times 0 and 0.5 h

Figure 2 (E). *S. aureus* infection of osteoblasts and macrophages. Effect of cytochalasin D on *S. aureus* internalization in osteoblasts. ^{**} $p < 0.001$ compared to the controls and ^{^^} $p < 0.001$ compared to 0.5, 1, and 5 µg/mL.

S. aureus was found to be able to survive within macrophages and osteoblasts for approximately a week; live intracellular *S. aureus* was found in macrophages and osteoblasts for 5 and 7 days, respectively (Figure 3). The percentage of live intracellular *S. aureus* for both macrophages and osteoblasts decreased continuously with increasing culturing time after infection, and significantly reduced survival of *S. aureus* was found in macrophages compared to osteoblasts at the same post-infection time period (Figure 3). In addition, no differences in osteoblast proliferation were observed between infected and non-infected osteoblasts within one week post-infection (data not shown).

Figure 3. Survival of intracellular *S. aureus* within osteoblasts and macrophages after infection at an MOI of 500:1 for 2 h. ** $p < 0.001$ compared to osteoblasts at the same post-infection time.

Confocal microscopy and TEM images confirmed that *S. aureus* was internalized and could survive within macrophages and osteoblasts (Figure 4). Meanwhile, substantially more (likely two orders) *S. aureus* was internalized (not necessarily alive) by macrophages compared to osteoblasts (Figure 4A and B). Confocal microscopy imaging also found that all live *S. aureus* was inside the osteoblasts and there were no live *S. aureus* on the surface of the osteoblasts after gentamicin treatment (Figure 4C). The internalization of *S. aureus* within osteoblasts was found to be non-uniform as some osteoblasts had multiple *S. aureus* bacteria while others had none (Figure 4D).

Figure 4 (A)

Figure 4 (B)

Figure 4. Visualization of intracellular *S. aureus* within (A) macrophages and (B) osteoblasts. Osteoblasts and macrophages were infected with *S. aureus* at an MOI of 500:1 for 2 h. (A and B) *S. aureus* was stained with FITC before infection. Infected osteoblasts and macrophages were fixed, blocked, stained first with primary antibody to *S. aureus* surface protein A, and then secondary antibody conjugated to Cy-5. The nuclei of the macrophages were additionally stained with DAPI. Visualized at (I) 488 nm, (II) 633 nm, and (III) 405 nm. (IV) Merged images of (I), (II), and (III). As a result, intracellular *S. aureus* is shown in green (FITC) and extracellular *S. aureus* is co-localized with both red (Cy-5) and green (FITC).

Figure 4. Visualization of intracellular *S. aureus* within osteoblasts. (C) Z-stack sections were used to confirm that all live *S. aureus* was inside osteoblasts as determined by the X-Y planes. Live *S. aureus* are green (Syto 9) and dead *S. aureus* are red (PI). Osteoblasts were infected with Syto 9-labeled *S. aureus*, then extracellular bacteria were killed with gentamicin and washed. Osteoblasts were detached from the wells and stained with PI. Approximately 20 cells (randomly selected) were examined.

3.2. Biological responses of osteoblasts and macrophages upon *S. aureus* infection

Significantly higher H_2O_2 levels were observed at 0.5 and 1 h in infected osteoblasts compared to non-infected osteoblasts, i.e. control (Figure 5A). Significantly higher H_2O_2 levels were also observed following a 1 h infection in macrophages compared to non-infected control (Figure 5A). No significant changes in O_2^- production in osteoblasts were observed at the infection time periods studied (i.e. 0.5, 1, and 2 h),

while significantly higher O_2^- levels were found in macrophages at 0.5 and 1 h infection (Figure 5B).

Figure 4 (D). Visualization of intracellular *S. aureus* within osteoblasts. Confirmation of intracellular *S. aureus* within osteoblasts using TEM.

Figure 5 (A)

Figure 5 (B)

Figure 5. Cellular and molecular responses of osteoblasts and macrophages infected with *S. aureus* at an MOI of 500:1 for 2 h. (A) DCF fluorescence intensity as an indicator of H_2O_2 production by non-infected controls and *S. aureus*-infected osteoblasts and macrophages. (B) DHE fluorescence intensity as an indicator of O_2^- production by non-infected controls and *S. aureus*-infected osteoblasts and macrophages. * $p < 0.01$, ** $p < 0.001$, and *** $p < 0.0001$ compared to control.

No significant changes in ALP enzyme activity were found between infected and non-infected osteoblasts at post-infection days 1, 4, and 7, although the ALP activity was lower for the infected cells at days 4 and 7 (Figure 5C). The macrophage phagocytosis activity studies showed that the ability to ingest bacteria was much higher for infected macrophages (83%) compared to non-infected ones (44%) (Figure 5D).

Figure 5 (C). Cellular and molecular responses of osteoblasts and macrophages infected with *S. aureus* at an MOI of 500:1 for 2 h. Osteoblast ALP activity measured at post-infection days 1, 4, and 7.

Figure 5 (D)

Figure 5 (D). Cellular and molecular responses of osteoblasts and macrophages infected with *S. aureus* at an MOI of 500:1 for 2 h. Macrophage phagocytosis activity (ingestion). Light gray area: control macrophages without incubation with *S. aureus*; dark gray area: non-infected macrophages; black area: infected macrophages. * $p<0.01$, ** $p<0.001$, and *** $p<0.0001$ compared to control.

4. Discussion

S. aureus has been traditionally considered as an extracellular pathogen; however, it has been shown to invade and survive within both non-phagocytic and phagocytic cells. By nature, the internalization and survival of *S. aureus* within non-phagocytic and phagocytic cells would be expected to be different, and may play significantly different roles in related diseases. To our knowledge, there are no reports comparing *S. aureus* infection of a non-phagocytic cell with a phagocytic cell. Therefore, the main goal of the present study was to compare the internalization behavior and related biological responses of *S. aureus* in a non-phagocytic cell (i.e. osteoblast) and a phagocytic cell (i.e. macrophage). Our findings may contribute to the understanding of the pathogenesis of many chronic and recurrent infections.

First, we showed that *S. aureus* can be internalized by both osteoblasts and macrophages. The infection of osteoblasts and macrophages was observed as early as 0.5 h at an MOI of 500:1; the corresponding live intracellular CFUs of *S. aureus* for osteoblasts and macrophages at 0.5 h were 1.4×10^3 and 7.2×10^4 CFU/(10^5 cells), respectively (Figure 2C). With increasing infection time, the intracellular CFUs of both osteoblasts and macrophages increased significantly from 0.5 h to 2 h followed by a plateau from 2 h to 8 h (Figure 2C). Since macrophages are supposed to engulf and eliminate pathogens on contact, it was not surprising to find that, at the same infection conditions (i.e. MOI of 500:1 for 2 h), significantly more (approximately two orders) *S. aureus* (live and dead) was phagocytized by macrophages compared to those internalized by osteoblasts (Figure 4A and B). Similarly, significantly more live intracellular *S. aureus* was seen in macrophages compared to osteoblasts during infection times 2-8 h

(Figure 2A and C). Macrophages had significantly lower viability at a shorter infection time period (i.e. 2 h) and significantly higher survival at a longer infection time (i.e. 8 h) compared to infected osteoblasts (Figure 2D). In addition, it is possible that the accumulation of toxins produced by *S. aureus* [35,36] and the significantly higher levels of H₂O₂ in infected osteoblasts and macrophages and O₂⁻ in infected macrophages (Figure 5A and B) affected the viability of macrophages and osteoblasts; both decreased (almost linearly) with increasing infection time (Figure 2D). It also seems that actin cytoskeleton formation plays a major role in *S. aureus* internalization into osteoblasts since cytochalasin D, an inhibitor that disrupts actin filament polymerization, could lead to up to 50% inhibition of *S. aureus* internalization (Figure 2E). This finding was consistent with other studies [21,37] where actin filament was determined to play a dominant role in *S. aureus* internalization.

Note that our data (Figure 4C) confirmed that gentamicin treatment was effective in eliminating extracellular *S. aureus* and the post-infection CFU was indeed from live intracellular *S. aureus*. Gentamicin treatment is commonly used to eliminate extracellular bacteria [19,37], but such a procedure lacks direct confirmation of live intracellular bacteria. In this study, the dual staining approach combined with confocal microscopy presented direct evidence that no live extracellular *S. aureus* was observed after the gentamicin treatment (Figure 4C).

Next, we found that *S. aureus* could survive intracellularly for up to 5 and 7 days, respectively, within macrophages and osteoblasts (Figure 3). As a phagocytic cell, macrophages were obviously more effective than osteoblasts at not only phagocytizing but also destroying the intracellular bacteria. This observation was supported by the

findings that *S. aureus* infection not only significantly increased the H_2O_2 levels in macrophages at 1 h infection but also significantly increased the O_2^- levels in macrophages at infection times of 0.5 and 1 h (Figure 5A and B). In contrast, *S. aureus* infection induced significantly higher levels of H_2O_2 in osteoblasts at 0.5 and 1 h infection but did not induce significant changes in O_2^- levels in osteoblasts (Figure 5A and B). As a result, significantly higher intracellular CFUs were found in macrophages immediately after infection (Figure 2C) while significantly less intracellular *S. aureus* survived in the following days after infection (Figure 3). Complete elimination of the intracellular bacteria by macrophages was found at an earlier time period (day 5 vs. day 7) compared to osteoblasts (Figure 3). Survival of *S. aureus* within host cells was also reported in other cell types. For instance, *S. aureus* was found to survive within human lung epithelial cells for up to 2 weeks [38], persisting in macrophage vacuoles for 3-4 days before escaping into the cytoplasm and causing macrophage lysis [6], and remaining viable for up to 5 days within HT-29 and Caco-2 enterocytes [39]. It was proposed that, once inside osteoblasts, macrophages, or other cells, *S. aureus* may undergo phenotypic switching to small colony variants (SCVs), which are associated with increased anchoring of fibronectin-binding proteins and clumping factors on the bacterial surface [40,41]. These proteins may function as substrates for bacterial enzymes needed to evade phagocytic oxidative killing [6,42] thereby contributing to the intracellular survival of *S. aureus*. Moreover, *S. aureus* produces catalase, which catalyzes the decomposition of H_2O_2 , thereby protecting itself inside host cells such as macrophages [43]. It was believed that the phenotypic switching of *S. aureus* may make the bacteria more resistant to antibiotics [16,44]. The survival of *S. aureus* within cells like macrophages and

osteoblasts and the possible phenotypic switching of *S. aureus* may explain why antibiotics have so often failed to cure *Staphylococcal* infections [2,16,41]. Future studies may focus on investigation of the possible changes that occur to *S. aureus* after internalization into osteoblasts and macrophages.

Lastly, we found that *S. aureus* infection resulted in significantly increased levels of H_2O_2 in infected osteoblasts at infection times of 0.5 and 1 h and in infected macrophages at infection time of 1 h (Figure 5A). The O_2^- levels in infected macrophages significantly increased at infection times of 0.5 and 1 h (Figure 5B). The increase in reactive oxygen species indicates that *S. aureus* internalization induced significant stress in osteoblasts and macrophages which would produce such chemicals to eliminate the invading pathogens. *S. aureus* infection also resulted in much higher phagocytosis activity of macrophages (Figure 5D) and relatively lower (although not significant) ALP activity of osteoblasts at days 4 and 7 after infection (Figure 5C). This effect could be associated with the significant increase in H_2O_2 and O_2^- levels. It is noteworthy that, besides the significant changes in reactive oxygen species, *S. aureus* internalization in osteoblasts also led to significantly higher production of IL-6 and IL-12 [19,45], macrophage chemoattractant protein 1, IL-8, IP-10, RANTES [19,45], and RANK-L and prostaglandin E_2 (two important molecules that can promote osteoclastogenesis and bone resorption) [46].

5. Conclusions

In this study, we intended to compare two infection models of osteoblast-*S. aureus* and macrophage-*S. aureus*. We found that *S. aureus* could internalize in macrophages and osteoblasts, that the internalization increased substantially with increasing infection time from 0.5 h to 2 h, and that the internalization process may have been primarily mediated by osteoblast actin cytoskeleton formation. Internalized *S. aureus* could survive within macrophages and osteoblasts for up to 5 and 7 days, respectively. Our confocal and transmission electron microscopy observations clearly confirmed the presence of live *S. aureus* within osteoblasts and macrophages and that gentamicin treatment was effective in killing extracellular *S. aureus*; no live extracellular *S. aureus* was detected after gentamicin treatment. Transmission electron microscopy also demonstrated that the internalization in osteoblasts was not uniform; some osteoblasts had several bacteria while others had none. Confocal microscopy observation showed that significantly more *S. aureus* was internalized in macrophages compared to osteoblasts. Similarly, a significantly higher (two orders) level of live intracellular *S. aureus* was detected in macrophages compared to osteoblasts at 2 h infection among different MOIs (i.e. 100:1, 500:1, and 1000:1) and at different infection times (2-8 h) at an MOI of 500:1. However, the percentage of *S. aureus* survival after infection was significantly lower in macrophages compared to osteoblasts at post-infection days 1-6. The viability of macrophages and osteoblasts both decreased with increasing infection time between 0-8 h and macrophages had relatively lower viability at shorter infection time periods (i.e. 0.5-4 h; significant at 2 h) and higher viability at longer infection time periods (i.e. 6-8 h; significant at 8 h) compared to osteoblasts.

S. aureus infection also led to noticeable changes in macrophages and osteoblasts. Significantly increased H₂O₂ production was observed in osteoblast-*S. aureus* infection at 0.5 and 1 h and in macrophage-*S. aureus* infection at 1 h. At 0.5 and 1 h infection, significantly increased superoxide anion production was found in macrophages. Infected osteoblasts had relatively lower ALP activity at post-infection days 4 and 7 and infected macrophages had higher phagocytosis activity compared to non-infected cells. Since chronic and recurrent bone infections have been frequently reported, are often associated with *S. aureus* and may be related to the intracellular persistence of *S. aureus* within host cells (e.g. osteoblasts, macrophages), the present study helps to better elucidate *S. aureus* internalization and its effects on host cells. This study may assist in evaluating the pathogenesis of chronic and recurrent infections and developing new therapeutic strategies against intracellular bacteria and diseases.

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CHAPTER 3

Intracellular *Staphylococcus aureus* Causes Bone Infection: *In vivo* Evidence

Abstract

Chronic and recurrent bone infections occur frequently but have not been fully explained. *Staphylococcus aureus* (*S. aureus*) is often found in these chronic and recurrent infections and may be responsible for such infections. One possible reason is that *S. aureus* can internalize and survive within host cells and by doing so, *S. aureus* can evade both host defense mechanisms and most conventional antibiotic treatments. However, no *in vivo* evidence in the literature has shown that *S. aureus* internalized into host cells is directly responsible for infections. In this study, we hypothesized that intra-cellular *S. aureus* could induce infections *in vivo*. Osteoblasts were infected with *S. aureus* and, after eliminating extra-cellular *S. aureus*, the osteoblasts were inoculated into an open femur fracture rat model. Bacterial cultures and radiographic observations confirmed local bone infections in animals inoculated with intra-cellular *S. aureus* within osteoblasts alone at post-operative day 21. We present the first direct *in vivo* evidence that intra-cellular *S. aureus* could be sufficient to induce bone infection in animals; we found that intra-cellular *S. aureus* inoculation of as low as 10^2 colony forming units could induce severe bone infections. Our data may suggest that intra-cellular *S. aureus* can “hide” in host cells during symptom-free periods and, under certain conditions, they may escape and lead to infection recurrence. Intra-cellular *S. aureus* therefore could play an important role in the pathogenesis of *S. aureus* infections, especially those chronic and

recurrent infections in which disease episodes may be separated by weeks, months, or even years.

Keywords: Chronic infection, infection recurrence, intra-cellular pathogen, *Staphylococcus aureus*, *in vivo*, co-culture

Introduction

Bone infection or osteomyelitis is a significant clinical problem which can lead to prolonged hospitalization, sepsis, poor functional outcome, and even death [1-4]. Patients with traumatic open fractures have a high risk of infection (5-33%) due to microbial contamination and soft tissue damage [5], and such infections are often hard to eradicate, long-lasting (i.e. chronic), and recurrent [6]. For instance, over a million Americans are hospitalized every year for bone fractures [7]; such injuries are increasingly common because of increased survivability of high energy trauma in civilian settings as well as in combat activities. Treatment of bone infections, particularly chronic and recurrent infections, frequently requires long-term hospitalization for repeated debridement and prolonged parenteral antibiotic therapy. Bacteria including *Staphylococcus aureus* (*S. aureus*), however, are often surprisingly successful in overcoming both host defense mechanisms and antibiotic treatments resulting in chronic and recurrent bone infections; indeed, a high rate of recurrence or relapse of bone infections was reported where ~17% of infections related to traumatic extremity injuries recur or relapse [8, 9]. It is thought that bacteria may elude host defenses and antibiotics and can “hide” themselves intracellularly within host cells [10]. Unfortunately, evidence to support the hypothesis that intracellular bacteria are responsible for or contribute to bone infections in animals or humans is sparse or does not exist.

A wide range of bacterial species have been isolated related to bone infections. The pathogen *S. aureus* is the most common causative agent of bone infections, contributing to approximately 80% of all human disease cases [11]. *S. aureus* is a normal flora that colonizes the skin, skin glands, and mucous membranes, specifically in the nose

of healthy individuals. *S. aureus* expresses a large number of cell surface proteins (i.e. adhesins) of the microbial surface components recognizing adhesive matrix molecules or MSCRAMMs [12, 13] through which it can colonize the bone matrix and adhere to bone cell surfaces. *S. aureus*, while generally considered an extracellular pathogen, is one such bacterium that has the ability to invade and live within different cell types, including phagocytic and non-phagocytic cells (reviewed in reference 14). By “hiding” inside the host cells, *S. aureus* may escape from host defenses and most antibiotic treatments and may be responsible for chronic and recurrent bone infections.

To our knowledge, there is no definitive *in vivo* evidence showing that *S. aureus* “hiding” intracellularly is directly responsible for or contributes to infections. The objective of this study was to determine whether intracellular *S. aureus* can induce bone infections in a rat model. By pre-infecting osteoblasts *in vitro* with *S. aureus*, killing extracellular bacteria with gentamicin, and inoculating infected osteoblasts into an open femur fracture rat model, we examined the bone infections in rats induced by intracellular *S. aureus*.

Materials and Methods

Reagents

Dulbecco's modified Eagle's medium-F 12 (DMEM-F 12) and phosphate buffered saline (PBS) were purchased from Lonza (Walkersville, MD). Tryptic soy broth (TSB) and tryptic soy agar (TSA w/5% sheep blood) plates were from Fisher Scientific (Pittsburgh, PA). Fetal bovine serum (FBS), 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), and 100 U/mL penicillin/100 mg/mL streptomycin were obtained from Invitrogen (Carlsbad, CA). Triton X-100 was from Sigma (St. Louis, MO). *S. aureus* (American Type Culture Collection or ATCC 25923) and rat osteoblast cells (UMR-106) were purchased from ATCC (Manassas, VA).

Preparation of osteoblasts with intracellular S. aureus via co-culturing osteoblast-S. aureus

Rat osteoblast cells were seeded at a density of 5×10^5 osteoblasts/mL and cultured in 6-well plates overnight to form a monolayer in DMEM-F12 supplemented with 10% FBS and 1% penicillin/streptomycin solution. Fresh *S. aureus* inoculum was prepared by suspending 5 colonies of *S. aureus* into 5 mL TSB, and incubated at 37°C for 18 h. The bacterial suspension was centrifuged (3750 rpm) at 4°C for 15 min, washed once with PBS, and its optical density was then adjusted to have *S. aureus* of 5×10^8 colony forming units/mL (CFU/mL). Rat osteoblasts and *S. aureus* were then infected as follows (Fig. 1): (i) Osteoblast monolayer was first washed twice with PBS. *S. aureus* was pelleted, re-suspended in DMEM-F12 (without FBS and antibiotics), and then added to the osteoblast monolayer at a multiplicity of infection (MOI) of 500:1 *S. aureus* to osteoblasts. Osteoblasts and *S. aureus* were next infected at 37°C in 5% CO₂ humidified incubator for

2 h. **(ii)** The osteoblast monolayer was then washed twice with PBS to remove any non-adherent bacteria, and cell culture media supplemented with gentamicin (100 µg/mL) was added and kept for 2 h to kill remaining extracellular bacteria; gentamicin is an antibiotic that is unable to penetrate mammalian cell membranes within short time periods [2, 15, 16]. Infected osteoblasts were immediately used or kept on ice before being used in the rat animal studies. **(iii)** Samples of infected osteoblasts were lysed using Triton X-100. **(iv)** Cell lysates were cultured on blood agar plates to determine the CFU which represents the viable colonies of intracellular *S. aureus* within osteoblasts.

Figure 1. A schematic diagram describes the process of infecting osteoblasts with intracellular *S. aureus* and confirming that the osteoblasts are infected. **(i)** Rat osteoblasts were infected with *S. aureus* for 2h. **(ii)** Extracellular *S. aureus* were killed with gentamicin and washed. **(iii)** Osteoblasts were lysed using a lysing buffer. **(iv)** Cell lysates were cultured on blood agar plates to count live intracellular *S. aureus* colonies.

Animal groups and open fracture infection procedure

Sprague-Dawley rats (400-500 g) were obtained from Hilltop Lab Animals (Scottsdale, PA). All studies were approved by the West Virginia University Institutional Animal Care and Use Committee. Our open femur fracture rat model [17, 18] was modified and the surgical procedure was described in brief as follows (Fig. 2): After sedation of the animals with intraperitoneal anesthetic, the hind leg was shaved prior to fracture. A midshaft femur fracture was produced using a custom-made device (Fig. 2A), an incision was made to expose the fracture (Fig. 2B), and then the fracture was fixed using an intramedullary stainless steel Kirschner wire or K-wire (Fig. 2C). Next, 100 μ L of treatment solution was gently pipetted into the fracture site without contacting the surgical instruments (Fig. 2D). Based on the treatment solutions, rats were randomly assigned into the following six groups (six rats per group):

Group	Treatment solution (100 μL)
Group I	PBS
Group II	10^6 osteoblasts
Group III	10^6 osteoblasts combined with 20 CFU of extracellular <i>S. aureus</i>
Group IV	10^6 osteoblasts with $*10^2$ CFU intracellular <i>S. aureus</i>
Group V	10^6 osteoblasts (infected) with 10^6 CFU intracellular <i>S. aureus</i>
Group VI	10^6 osteoblasts (infected) with 10^6 CFU intracellular <i>S. aureus</i> combined with 20 CFU of extracellular <i>S. aureus</i>

* This was obtained by diluting the 10^6 CFU of intracellular *S. aureus* with non-infected osteoblasts.

The incision was subsequently closed (Fig. 2E) and post-operative radiographs were taken to verify the fracture fixation. All animals were monitored for the study duration of three weeks and euthanized on post-operative day 21.

Figure 2. The rat femur was fractured, fixed with a K-wire, and inoculated with 0.1 mL solution of PBS, osteoblasts, mixture of osteoblasts and extracellular *S. aureus* (20 CFU), infected osteoblasts with intracellular *S. aureus*, or mixture of infected osteoblasts and extracellular *S. aureus* (20 CFU). Rats were euthanized on post-operative day 21. **(A)** Creation of a femur fracture using a custom-designed setup. **(B)** Exposure of the fracture. **(C)** Intramedullary fixation of the fracture using a K-wire. **(D)** Injection of 0.1 mL solution using a pipette. **(E)** Closure of incision.

Microbiological and radiographic evaluations

On the day of euthanasia, bone and muscle tissues were collected in 15 mL centrifuge tubes with 5 mL PBS and homogenized. Samples of bone and muscle homogenates were diluted and plated on blood agar plates, and *S. aureus* colonies were counted after incubating the plates, at 37°C for 24h. In addition, K-wires that were used to fix the fractured femurs were collected at euthanasia, cut into 2cm-long pieces, rolled on blood agar plates and cultured at 37°C for 24 h. Radiographs of fractured femurs were taken on post-operative days 0, 7, and 21.

Body weight and complete blood count

The body weights of animals were measured on the day of surgery and immediately before euthanasia and the net weight gain/loss was calculated. Blood samples were collected immediately prior to euthanasia from the heart and analyzed for complete blood counts by Antech Diagnostics, Inc. (Southaven, MS).

Statistical analysis

Values of CFU, body weight gain/loss, and blood cell counts were expressed as the mean $\pm$ standard deviation. Differences in body weight gain and blood cell counts were analyzed using one-way ANOVA. P value ≤ 0.05 was considered statistically significant. JMP (V9) software was used (SAS Institute, Inc., Cary, NC).

Results

Intracellular S. aureus causes high bacterial burden in bone and muscle tissues

Rats (Groups IV, V, and VI) inoculated with infected osteoblasts (10^2 or 10^6 CFU of intracellular *S. aureus*) led to high CFU counts (i.e. $\sim 10^6$ CFU/mL) on post-operative day 21 in the bone and muscle samples (Fig. 3A). There were no significant differences in CFUs among the animal groups (i.e. Groups IV, V, and VI) inoculated with infected osteoblasts on post-operative day 21, although relatively higher CFU counts were found in the bone samples compared to the muscle samples of the same animal group. By contrast, no bacteria were observed on post-operative day 21 in the rats (i.e. Groups I, II, and III) inoculated with PBS, osteoblasts, and osteoblasts combined with 20 CFU of extracellular *S. aureus* (Fig. 3A). Correspondingly, heavy bacterial growth was seen in the blood agar plates rolled with the K-wires from animals inoculated with infected osteoblasts (10^2 or 10^6 CFU) while no bacterial colonies were found in rolling K-wires extracted from animals inoculated with PBS, osteoblasts, and osteoblasts combined with 20 CFU of extracellular *S. aureus* (Fig. 3B). Gross observation before euthanasia showed pus pockets at the fracture sites in rats inoculated with infected osteoblasts (i.e. Groups IV, V, and VI). No *S. aureus* growth (data not shown) was observed on post-operative day 21 in the blood samples collected from all the animal groups studied.

Figure 3 (A)

Figure 3 (B)

Figure 3. (A) Bone (femur) and muscle tissue homogenates reported high *S. aureus* CFUs in the animal groups inoculated with infected osteoblasts (i.e. Groups IV, V, and VI); data were from six rats. No CFU was found in the animal groups inoculated with PBS, osteoblasts, and a mixture of osteoblasts and extracellular *S. aureus* (20 CFU) (i.e. Groups I, II, and III). **(B)** K-wire rolling experiments showed high *S. aureus* growth in the animal groups inoculated with infected osteoblasts; no bacterial growth was observed in the animal groups inoculated with PBS, osteoblasts, and a mixture of osteoblasts and extracellular *S. aureus* (20 CFU).

Radiographic assessment of bone infection

No signs of infection were observed on post-operative day 21 in the radiographs of rats (i.e. Groups I, II, and III) inoculated with PBS, osteoblasts, and osteoblasts combined with 20 CFU of extracellular *S. aureus* (Fig. 4A); the X-ray images of these animals were characterized by the formation of normal bridging calluses at the fracture sites. By contrast, animal groups (i.e. Groups IV, V, and VI) inoculated with infected osteoblasts (10^2 or 10^6 CFU) exhibited severe osteolysis on post-operative day 21. Meanwhile, no severe osteolysis was observed on post-operative day 7 in the animals inoculated with infected osteoblasts (Fig. 4B).

Figure 4 (A)

Figure 4 (B)

Figure. 4. (A) Radiographs of fractured femurs on post-operative day 21 presented significant osteolysis in the animal groups inoculated with infected osteoblasts (i.e. Groups IV, V, and VI) while normal bone healing was seen in the animal groups inoculated with PBS, osteoblasts, and a mixture of osteoblasts and extracellular *S. aureus* (20 CFU) (i.e. Groups I, II, and III). (B) Radiographs of fractured femurs on post-operative day 0, 7, and 21 did not show severe osteolysis in the rats inoculated with infected osteoblasts (e.g. Group VI) on post-operative day 7.

Body weight and complete blood counts

A significant loss in body weight was observed in rat groups (i.e. Groups IV, V, and VI) inoculated with infected osteoblasts (10^2 or 10^6 CFU of intracellular *S. aureus*) compared to the animal groups (i.e. Groups I, II, and III) inoculated with PBS, osteoblasts, and osteoblasts combined with 20 CFU of extracellular *S. aureus* (Fig. 5). Interestingly, relatively more, although not significant, weight gain was seen on post-

operative day 21 in the animal groups (i.e. Groups II and III) inoculated with osteoblasts compared to the group (i.e. Group I) inoculated with PBS (Fig. 5).

Figure 5. Net weight loss of the rats on post-operative day 21 was significantly higher in the animal groups inoculated with infected osteoblasts (i.e. Groups V, and VI) compared to those in the animal groups inoculated with PBS, osteoblasts, and a mixture of osteoblasts and extracellular *S. aureus* (20 CFU) (i.e. Groups I, II, and III). Data were an average of six rats in each group.

The neutrophils in the blood of rat groups (i.e. Groups IV, V, and VI) inoculated with infected osteoblasts (10^2 or 10^6 CFU) were significantly elevated on post-operative day 21 compared to the animal groups (i.e. Groups I, II, and III) inoculated with PBS, osteoblasts, and osteoblasts combined with 20 CFU of extracellular *S. aureus* (Fig. 6). No significant differences (data not shown) were seen on post-operative day 21 in the numbers of lymphocytes, monocytes, and eosinophils among all the animal groups studied.

Figure 6. Complete blood counts on post-operative day 21 showed that neutrophil numbers were significantly higher in the animal groups inoculated with infected osteoblasts (i.e. Groups IV, V, and VI) compared to those animal groups inoculated with osteoblasts and a mixture of osteoblasts and extracellular *S. aureus* (20 CFU) (i.e. Groups II and III).

Discussion

S. aureus is a major human pathogen causing significant morbidity and mortality in both community- and hospital-acquired infections. Surprisingly, *S. aureus* is frequently successful in overcoming both host defense mechanisms and most antibiotic treatments, was found among chronic and recurrent infections, and could be responsible for such infections. This bacterium is believed to have developed several means to combat conventional antibiotic therapies, among which are its ability to survive for long periods of time inside host cells [14]. The purpose of the present study was to examine whether intracellular *S. aureus* contributes to infection *in vivo*. Effort was taken to completely eradicate extracellular *S. aureus* in the inoculums by treating them with gentamicin; the complete killing of extracellular *S. aureus* was verified in our studies as well as by others [15, 16, 19, 20]. Also, gentamicin remaining in the inoculum samples was expected to eliminate any extracellular *S. aureus* that leaked out within the first few hours after infection.

We investigated whether intracellular *S. aureus* within osteoblasts could induce infection in our open femur fracture rat model. An open femur fracture model was used because open fractures have high rates of infection and infection recurrence, and a high rate of recurrence or relapse of infections was reported in traumatic extremity injuries (8, 9). We found that animals inoculated with *S. aureus* infected osteoblasts with intracellular *S. aureus* CFU of 10^2 (i.e. Group IV) and 10^6 (i.e. Groups V and VI) all led to severe infections. The results were confirmed by culturing bone and muscle specimens from the fracture sites and explanted K-wires (Fig. 3) and visualizing via radiography of the fractured bones (Fig. 4). Inoculation of intracellular *S. aureus* of 10^2 CFU led to the

same degree of infection as 10^6 CFU inoculum, evidenced by approximately the same CFU of bone or muscle specimens on post-operative day 21 (Fig. 3). Animals inoculated with no bacteria (i.e. Groups I and II) or with 20 CFU of extracellular *S. aureus* (i.e. Group III) did not exhibit infection on post-operative day 21. No systemic infection was observed in any of the animal groups investigated because no CFU was found in blood samples. These suggest that intracellular *S. aureus* of as low as 10^2 CFU within osteoblasts induced bone infections in our rat open femur fracture model; the infection was localized.

It was interesting that severe bone infections could be induced by intracellular *S. aureus* within osteoblasts at a low rate of 10^2 CFU, which was the same quantity of inoculum as our previous rat studies where 10^2 CFU of extracellular *S. aureus* was sufficient to induce bone infections in our open fracture rat model [17, 18, 21]. This suggested that not only extracellular but also intracellular *S. aureus* of as low as 10^2 CFU could induce severe bone infections in open fractures. Note that inoculation of uninfected osteoblasts did not have an effect on infection; no CFU was observed in corresponding bone, muscle, and K-wire samples (Fig. 3). However, although not significant, increased weight gain was observed with injection of uninfected osteoblasts compared to injection of PBS (Fig. 5); the reason was unknown.

Meanwhile, neutrophils were significantly increased on post-operative day 21 in the animal groups inoculated with infected osteoblasts. This finding might suggest that neutrophils have played a deleterious role in infections induced by *S. aureus* infected osteoblasts. In the literature, neutrophils may have a protective or a deleterious role in *S. aureus* infection. On one hand, neutrophils have long been believed to provide significant

host defense against *S. aureus* infection. This is mainly because patients who are neutropenic or who have defects in neutrophil functions are more susceptible to *S. aureus* infection, and total ablation of neutrophils in an experimental mouse model of *S. aureus*-induced septic arthritis led to decreased survival of the animals and increased bacteremia [22]. On the other hand, increased neutrophils were also found to correlate with decreased survival of mice and increased bacterial burden at the infection site [23, 24].

It is important to note that both *in vitro* and *in vivo* studies have shown that *S. aureus* can internalize and survive within both phagocytic and non-phagocytic cells. *In vitro*, *S. aureus* can invade and survive within polymorphonuclear neutrophils and monocytes [25-27] and osteoblasts [28, 29]. Infected human osteoblasts were shown to release viable *S. aureus*, which were able to re-infect new osteoblasts [28]. *S. aureus* can also internalize and perhaps even replicate within bovine mammary epithelial cells, both in cell lines and in primary culture [30, 31]. *In vivo*, intracellular *S. aureus* was found, in limited cases, in animals and patients. In animal studies, *S. aureus* was found to be able to internalize and survive in osteoblasts in chick embryos [32] and in bovine alveolar cells isolated from milk of infected cows [33]. In patient studies, intracellular *S. aureus* was found inside epithelium, glandular, and myofibroblastic cells in intranasal biopsy specimens obtained, during infection symptom-free periods, in three patients with recurrent rhinosinusitis [34]. Molecular typing proved the clonal relationship of the patient-specific *Staphylococci*. Intracellular *S. aureus* was also found in surgical bone specimens from a patient who had chronic and recurrent bone infection [35]. The intracellular *S. aureus* appeared to be sequestered and protected from the host immune system within the osteoblast or osteocyte cytoplasm for years [35]. In another case,

intracellular *S. aureus* was cultured from five patients with persistent and recurrent infections [36]. Alarming, some of those intracellular *S. aureus* were small-colony variants which were non-hemolytic and non-pigmented, grew very slowly on routine culture media, and were resistant to aminoglycosides under routine conditions.

Therefore, there is some *in vitro* and *in vivo* evidence supporting the potential role of intracellular *S. aureus* in *Staphylococci* pathogenesis. However, there is no definitive *in vivo* proof before this study showing that intracellular *S. aureus* is directly responsible for or contributes to infections (e.g. chronic and recurrent infection). The results presented here are important because they have demonstrated, for the first time, that intracellular *S. aureus* could induce infection *in vivo*. Bacteria (e.g. *S. aureus*) could internalize and survive within host cells (e.g. osteoblast) and, after “hiding” for some time period (could be up to years [35]), these quiescent bacteria could escape from the infected host cells into the surrounding tissue and could be sufficient to initiate a recurrence of symptomatic infection.

Taken together, the findings reported here present the first *in vivo* direct evidence that intracellular *S. aureus* can induce bone infections and may be responsible for chronic and recurrent infections. These findings also suggested that as low as 10^2 CFU of intracellular *S. aureus* may induce severe bone infections in open fractures. These findings may greatly advance our understanding of the pathogenesis of bone infections, especially chronic and recurrent infections. The induction of bone infections by intracellular *S. aureus* was studied in this work; however, the findings are expected to be applicable to other infections/diseases and intracellular pathogens.

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CHAPTER 4

Effect of Interleukin 12 on the Killing of Intracellular *Staphylococcus aureus*

Abstract

The proinflammatory cytokine IL-12 may play a critical role in host defense against intracellular pathogens, including bacteria, viruses, and parasites. We have previously demonstrated the ability of intracellular *S. aureus* to live inside macrophages, which may explain the chronic and recurrent nature of *Staphylococcal* infections. The aim of this study was to determine the effect of IL-12 on killing intracellular *S. aureus* by macrophages. Splenic macrophages were isolated from rat spleens, infected, and treated with IL-12 or IL-12 neutralized with anti-IL-12 antibody or without IL-12. IL-12 treatment was found to exhibit a dose-dependent relationship with intracellular *S. aureus*, and resulted in significant reduction of intracellular *S. aureus* at IL-12 dosages of 250 and 2500 pg/mL. The enhancement of macrophage killing activity was most effective and significant at post-infection 24 - 36 h. Meanwhile, significantly higher levels of IFN- γ and IL-12 were released from infected macrophages treated with IL-12 compared to untreated cells. No significant changes in reactive oxygen species production were detected between treated and untreated macrophages at post-infection time 0 and 24 h. These findings demonstrate that IL-12 can stimulate macrophages to be more potent in killing intracellular *S. aureus* and could be an effective immunotherapeutic agent for preventing and treating intracellular *Staphylococcal* infections.

Keywords: Intracellular pathogens, Interleukin 12, *S. aureus*, macrophages, cytokine therapy.

Introduction

Intracellular pathogens are known to be a strong inducer of type-1 immune responses, which are characterized by the robust production of interleukin 12 (IL-12) from innate phagocytes and interferon gamma (IFN- γ) from natural killer (NK) cells and T-cells. Studies have shown that macrophages failed to be activated to kill intracellular bacteria in mice treated with IL-12 or IFN- γ neutralizing antibodies [1-6].

IL-12 is produced mainly by antigen-presenting cells and acts to connect the innate and acquired immunities by inducing differentiation of antigen-specific T-cells of Th1 phenotype and the release of IFN- γ from activated T-cells and NK cells [7-10]. IFN- γ is produced by T-cells and considered to play an important role in activating macrophages, the ultimate effector cells in host defense against intracellular infection [11, 12]. Studies have reported that macrophages can be competent cells regarding the ability to respond to IL-12, which has led to the notion that this cytokine can induce macrophage activation through an autocrine pathway [15, 16]. It has been shown that macrophages express $\beta 1$ and $\beta 2$ chains from the IL-12 receptor (IL-12R) [16]. IL-12 has also been involved in programming the macrophage response to lipopolysaccharide (LPS) by up-regulating the production of TNF- α [17].

In this study, we investigated the effects of IL-12 on the bacterial killing capacity of macrophages with intracellular *S. aureus*, and presented evidence to support the antibacterial activity of IL-12 against intracellular *S. aureus*. The findings will extend our understanding of the effects of IL-12 on macrophages and may offer insights into an immunotherapeutic approach to control chronic and recurrent *S. aureus* infection.

Materials and Methods

Reagents

Tryptic soy agar (TSA, w/5% sheep blood) plates, tryptic soy broth (TSB), phosphate buffered saline (PBS), fetal bovine serum (FBS), 45% glucose solution, 7.5% sodium bicarbonate, sodium pyruvate, and Tris-buffer were all obtained from Fisher Scientific (Pittsburgh, PA). RPMI-1640 media were purchased from LONZA (Walkersville, MD). 100 U/mL penicillin/100 mg/mL streptomycin solution, rat IL-12 p70 ELISA kit, rat IFN- γ ELISA kit, and recombinant rat IL-12 (IL-12) were from Invitrogen (Carlsbad, CA). Gentamicin, Triton X-100, lysostaphin, and ammonium chloride were obtained from Sigma (St. Louis, MO). Dichlorofluorescein diacetate (H₂DCF-DA) was from Life Technologies (Grand Island, NY). Rat anti-IL-12 antibody was purchased from R & D Systems (Minneapolis, MN).

IL-12 bioactivity

The bioactivity of recombinant rat IL-12 used in this study was assessed based on the ability of IL-12 to induce secretion of IFN- γ by macrophages. In brief, splenic macrophages of $(0.5-1) \times 10^7$ cells/mL were cultured in a 96-well plate (Fisher Scientific, Pittsburgh, PA) in RPMI-1640 supplemented with different doses (0, 1, 10, 100, and 1000 pg/mL) of recombinant rat IL-12 at 37°C in a 5% CO₂ incubator. After 48 h of culturing, the supernatant was collected for IFN- γ analysis using enzyme-linked immunosorbent assay (ELISA) by measuring the optical density at 450 nm following the manufacturer's guidelines. IFN- γ levels were determined by comparison with a standard curve. The results were expressed as mean $\pm$ SE and are presented in Figure 1, which shows that the recombinant rat IL-12 studied was bioactive.

Bacterial culture

S. aureus (ATCC 25923) obtained from the American Type Culture Collection (ATCC, Manassas, VA). A fresh inoculum was prepared by suspending 5 colonies of *S. aureus*, grown on a blood agar plate, in 5 mL TSB and incubating at 37°C for 18 h. After incubation, the *S. aureus* inoculum was centrifuged at 3750 rpm for 15 min at 4°C and washed once with 10 mL PBS to obtain $(6-8) \times 10^8$ colony forming units/mL or CFU/mL. The bacteria pellet was then re-suspended in RPMI-1640 medium free from streptomycin/penicillin and FBS.

Splenic macrophage isolation and culture

Splenic macrophages were obtained as described in (Figure 2). Spleens were obtained from healthy adult Sprague Dawley rats, and cut into small pieces in a petri-dish, and minced between the frosted ends of microscopic slides. The splenocyte suspension was filtered through a 70 µm nylon cell strainer (Fisher Scientific), and centrifuged at 2500 rpm at 4°C for 5 min. The supernatant was subsequently aspirated and the red blood cells were lysed with a 5 mL lysis buffer containing 0.17 M tris-buffer and 0.16 M ammonium chloride for 10 min. The lysis process was stopped by adding RPMI-1640 media supplemented with penicillin/streptomycin, FBS, glucose, sodium bicarbonate, and sodium pyruvate (this will be referred to as complete media). The splenocyte suspension was then centrifuged again at 2500 rpm at 4°C for 5 min followed by aspiration of the supernatant. The splenocyte pellet was washed once with complete RPMI-1640 media by centrifugation then re-suspended in complete RPMI-1640 media to obtain $(0.5-1) \times 10^7$ cells/mL. Cell viability was determined by trypan-blue exclusion assay. Splenocytes were

seeded in 48-well plate (Fisher Scientific) for 4 h and non-adherent cells (mainly B and T-cells) were washed twice with PBS and adherent cells (primarily macrophages, and referred to as splenic macrophages here) were further cultured in complete RPMI-1640 media and incubated in a 5% CO₂ incubator at 37°C for 48 h.

Figure 2. Steps for isolating splenic macrophages. **(I)** Rat spleens were collected in RPMI-1640 complete media. **(II)** Spleens were cut into small pieces in petri-dish and minced between the frosted ends of microscope glass slides to make a single cell suspension. **(III)** and **(IV)** The cell suspension was filtered through a 70 µm cell strainer. **(V)** Cells were collected by centrifugation at 2500 rpm and 4°C for 5 min. **(VI)** Cell pellet was washed once with RPMI-1640 complete media and the pellet was obtained by centrifugation. **(VII)** Red blood cells in the cell pellet were lysed with Tris-ammonium chloride for 10 min and the lysis step was stopped by adding RPMI-1640 complete media. **(VIII)** Cells were re-suspended in RPMI-1640 complete media for further culture.

Splenic macrophage infection and IL-12 treatment

Splenic macrophages of $(0.5-1) \times 10^7$ cells/mL were washed 3 times with PBS, cultured with *S. aureus* at a multiplicity of infection (MOI) of 10:1 for 2 h, washed, and incubated with gentamicin (100 µg/mL) for 2 h. The infected splenic macrophages were first treated with different doses of recombinant rat IL-12 (0, 0.25, 2.5, 25, 250, and 2500

pg/mL) in RPMI-1640 media supplemented with 5 µg/mL lysostaphin and 5% FBS for 24 h. The splenic macrophages were washed and lysed to determine the number of live intracellular *S. aureus* by plating the lysates on blood agar plates. Next, the infected splenic macrophages were treated with 250 pg/mL of IL-12 for different time periods (0, 12, 24, 36, and 48 h). The cell culture supernatants were collected for IFN-γ and IL-12 quantification using ELISA kits following the manufacturer's guidelines; duplicates were collected for each sample. The splenic macrophages were washed and lysed to determine the number of live intracellular *S. aureus*. Data were presented as mean ± SE from at least 3 different trials.

Figure 1. Quantitative measurement of bioactivity of IL-12 studied.

IL-12 action specificity

In order to further confirm the role of IL-12 treatment on infected splenic macrophages, IL-12 was pre-treated with anti-IL-12 antibody and then added to infected

splenic macrophages. As described in Chart 1, splenic macrophages of $(0.5-1) \times 10^7$ cells/mL were cultured, infected at an MOI of 10:1 for 2 h, incubated with gentamicin, and washed. IL-12 of 250 pg/mL was neutralized with 0.1 μ g/mL rat anti-IL-12 antibody for 1 h at 37°C, and subsequently added to infected splenic macrophages and incubated for 24 h. For comparison, infected splenic macrophages were also treated with or without IL-12 of 250 pg/mL for 24 h. The cell culture supernatants were collected to determine IFN- γ levels using an ELISA kit; duplicates were collected for each sample. The infected splenic macrophages were washed with PBS and lysed to determine the number of intracellular *S. aureus*.

Chart 1. A flowchart for testing IL-12 specificity by adding anti-IL-12 antibody (0.1 μ g/mL) to neutralize IL-12 (250 pg/mL) biological activity for 1 h at 37°C.

Reactive oxygen species detection

Infected or non-infected splenic macrophages of $(0.5-1) \times 10^7$ cells/mL were treated either with or without recombinant rat IL-12. At post-infection time 0 and 24 h, cells were washed once with PBS and incubated with H₂DCF-DA at 37°C for 1 h in the dark. The fluorescence intensity was measured using a fluorescent microplate reader (BioTek Instrument, Inc., Winooski, VT) at 492 nm/520 nm for 2',7'-dichlorofluorescein (DCF), converted intracellularly from H₂DCF-DA. The H₂DCF-DA fluorescent probe is

commonly used to detect reactive oxygen species. Data were average of six samples per treatment.

Statistical analysis

All data were presented as means $\pm$ SE. Statistical significance was set at $p < 0.05$. Statistical analysis was performed using GraphPad InStat Version 3.0 (GraphPad Software Inco, La Jolla, CA).

Results

Effect of IL-12 treatment on the survival of S. aureus within infected splenic macrophages

The *S. aureus* CFU within infected splenic macrophages decreased with increasing IL-12 concentration in the range of 0-250 pg/mL, and was significantly lower at IL-12 of 250 and 2500 pg/mL compared to the control (i.e. without IL-12 treatment) (Figure 3A). The intracellular *S. aureus* CFU within infected splenic macrophages that were treated with 250 and 2500 pg/mL of IL-12 was 18- and 14- fold lower than that of the control, respectively. Meanwhile, the *S. aureus* CFU splenic macrophages also decreased with increasing treatment time. The *S. aureus* CFU within splenic macrophages that were treated with 250 pg/mL of IL-12 was significantly lower at 24 and 36 h compared to the corresponding controls without IL-12 treatment (Figure 3B).

Figure 3 (A). Intracellular *S. aureus* of infected splenic macrophages that were treated with recombinant rat IL-12 at different doses (0, 0.25, 2.5, 25, 250, and 2500 pg/mL) for 24 h.

After blocking the activity of IL-12 using anti-IL-12, no significant differences were observed in intracellular *S. aureus* CFU between infected splenic macrophages without IL-12 treatment and splenic macrophages treated with antibody pre-treated IL-12 (Figure 3C).

Figure 3 (B). Intracellular *S. aureus* of infected splenic macrophages that were treated with or without recombinant rat IL-12 of 250 pg/mL for different time periods (0, 12, 24, and 36 h).

Effect of IL-12 treatment on cytokine production in infected splenic macrophages

Compared to cells without IL-12 treatment, infected splenic macrophages treated with IL-12 had significantly higher IL-12 production at the post-infection times (i.e. 12, 24, 36, and 48 h) studied (Figure 4A). Similarly, a significant increase in IFN- γ production was observed at the time periods investigated in IL-12 treated infected splenic macrophages compared to the controls without IL-12 treatment (Figure 4B). At post-

infection 24 h, the IL-12 levels in the IL-12 treated cells and the controls were approximately 427 and 324 pg/mL, respectively, and the IFN- γ levels in the IL-12 treated cells and the controls were approximately 810 and 130 pg/mL, respectively.

Figure 3 (C). Comparison of intracellular *S. aureus* among infected splenic macrophages treated with IL-12 (250 pg/mL), IL-12 (250 pg/mL) that was pre-treated with the anti-IL-12 antibody, and without IL-12 for 24 h.

After blocking the activity of IL-12 using the anti-IL-12 antibody, no significant differences were observed in IFN- γ levels between infected splenic macrophages without IL-12 treatment and splenic macrophages treated with anti-IL-12 antibody pre-treated IL-12 (Figure 4C).

Figure 4 (A). IL-12 levels of infected splenic macrophages treated with or without IL-12 of 250 pg/mL for different time periods

Figure 4 (B). IFN- γ levels of infected splenic macrophages treated with or without IL-12 of 250 pg/mL for different time periods.

Figure 4 (C). Comparison of IFN- γ levels among infected splenic macrophages treated with IL-12 (250 pg/mL), IL-12 (250 pg/mL) that was pre-treated with the anti-IL-12 antibody, and without IL-12 for 24 h.

Effect of IL-12 treatment on reactive oxygen species production in splenic macrophages

No significant differences in DCF levels were detected between IL-12 treated and un-treated splenic macrophages at post-infection time 0 and 24 h (Figure 5).

Effect of IL-12 treatment on ROS production

Figure 5. DCF levels of infected and non-infected splenic macrophages that were treated with or without IL-12 of 250 pg/mL for 24 h.

Discussion

Killing of intracellular *S. aureus* may be critical for effective therapies to treat and prevent chronic and recurrent *Staphylococcal* infections. *In vivo*, the control of intracellular pathogens depends on the activation of Th1 immune responses, suggesting a key role for macrophages. The importance of IL-12 production and subsequent IFN- γ secretion to control intracellular bacteria has been demonstrated in multiple studies [4, 6, 19, 20]. However, few studies to date have examined the activity of IL-12 on macrophage killing activity *in vitro*. Herein, we used an *in vitro* infection model of intracellular *S. aureus* to examine the effect of IL-12 on the killing activity of splenic macrophages against intracellular *S. aureus*.

We found that the intracellular killing activity of macrophages was enhanced with IL-12 treatment, since the intracellular *S. aureus* CFU decreased with increasing IL-12 doses. Compared to the control without IL-12 treatment, significant differences were observed at 250 and 2500 pg/mL (Figure 3A); and the best effective time span was found to be between post-infection 24-36 h (Figure 3B). The enhancement of macrophage killing activity by IL-12 treatment was further confirmed by neutralizing IL-12 with an antibody specific to rat IL-12 where no significant differences were observed between macrophages treated with and without IL-12 neutralized with anti-IL-12 antibody (Figure 3C). This finding was consistent with the observation that animals challenged with bacteria and subsequently receiving treatment with anti-IL-12 antibody presented more severe disease symptoms [4, 18]. Similarly, neutralizing IL-12 using the anti-IL-12 antibody increased morbidity in virus-infected mice compared with un-treated animals [24]. By contrast, exogenous IL-12 was able to induce the activities of dendritic cells and

cytotoxic T-cells against influenza virus infection when patients' own antiviral immune responses were poor [27, 26]. Exogenous IL-12 also rescued mice that were challenged with a lethal dose of encephalomyocarditis virus through IFN- γ -mediated responses [25]. As a result, Orange and Coutelier suggested that low doses of IL-12 combined with dendritic cells may be used as a potential immunotherapeutic approach against viral infections [28, 29].

We further found that the enhancement of macrophage killing activity was associated with the production of IL-12 and IFN- γ . Significantly higher levels of IL-12 and IFN- γ were detected in infected macrophages treated with IL-12 compared to controls without IL-12 treatment (Figures 4A and 4B) while no significant differences were observed in IFN- γ levels between macrophages treated with and without IL-12 that was neutralized with anti-IL-12 antibody (Figure 4C). It was clear that exogenous IL-12 treatment resulted in significantly higher production of IFN- γ and IL-12 within infected macrophages, which may reflect the notion that IL-12 could induce macrophage activation through an autocrine pathway [15, 16]. Both IL-12 and IFN- γ were previously found to be required for activating immune cells like macrophages during parasitic infections, such as *Leishmania major* [30], by inducing Th1 differentiation and activating macrophages to produce nitrogen oxide [31-33]. IL-12 also plays a critical role during the early stages of viral infection by inducing NK cell cytotoxicity and IFN- γ production, which leads to improved antimicrobial activity of phagocytic cells [27, 34-36].

Oxidative burst is one of the defense mechanisms used against pathogens, and macrophages activated by IFN- γ were found to have increased release of nitric oxide (NO) and reactive oxygen species (ROS) and enhanced bactericidal activities [13, 14].

However, some bacteria such as *Brucella*, *Salmonella*, and *Shigella* can avoid inducing oxidative burst when they encounter host cells (21, 22), and some bacteria like *S. aureus* may express enzymes that can scavenge ROS species including O_2^- (23). In this study, no significant differences in ROS levels were observed between macrophages treated with and without IL-12 at post-infection time 0 and 24 h (Figure 5). This observation may suggest an alternative killing activity than oxidative burst.

Conclusions

In this study, we examined the effect of IL-12 treatment on macrophage killing activity *in vitro* by assessing the viable intracellular bacteria and cytokine as well as reactive oxygen species production in infected splenic macrophages. We found that IL-12 treatment could significantly enhance the *in vitro* killing activity of macrophages and the enhancement was dose and time dependent. Moreover, IL-12 treatment led to a significant increase in IFN- γ and IL-12 production with no significant changes in ROS production in infected macrophages compared to cells without IL-12 treatment. The enhancement of macrophage killing activity by IL-12 was abolished when IL-12 was pre-treated with the anti-IL-12 antibody.

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CHAPTER 5

General Discussion

Although *S. aureus* has been primarily considered an extracellular pathogen, it is now clear that it can be internalized by both phagocytes and non-phagocytic cells. Intracellular *S. aureus* is considered to play an important role in their persistence (host defenses evasion) and recurrence (antibiotic failure) of infection.

The goal of my dissertation was to understand the invasion of *S. aureus* in macrophages and osteoblasts and to explore potential prophylactic and therapeutic interventions. The *in vitro* infection models demonstrate direct evidence for the ability of *S. aureus* to be internalized by both phagocytic and non-phagocytic cells. Osteoblasts are the most important cells for bone formation; therefore, any damage occurring to these cells may result in disrupted bone homeostasis. Macrophages, on the other hand, are important immune cells against invading pathogens and need to be fully efficient in clearing bacteria in a short period of time. Our data have shown that *S. aureus* could survive for several days within osteoblasts and macrophages, in the presence of antibiotics. This time may be sufficient to induce phenotypic and genotypic changes in the intracellular *S. aureus* which may make it harder to clear.

The success of the *in vitro* infection models has led us to examine the potential that intracellular *S. aureus* alone could induce infection in an animal model. By inoculating *S. aureus* infected osteoblasts, we found that intracellular *S. aureus* inoculation of as low as 10^2 CFUs could induce severe bone infections in an open femur fracture rat model.

The role of IL-12 in intracellular pathogens' clearance can be multifaceted. It is well established that IL-12 stimulates IFN- γ in T-cells and NK cells. Also, IL-12 induces CD4 Th cell differentiation toward a Th1 subset, and could have indirect, but more widespread, effects within bone tissue. Our data have shown that IL-12 treatment has led to a significant upgrade of IFN- γ production by infected macrophages and resulted in significant reduction of intracellular *S. aureus*. As a result, IL-12 may be an innovative therapeutic agent for eliminating intracellular *S. aureus* surviving within host cells.

Future Studies

According to the publication report from Web of Knowledge, there are very few publications studying the pathogenesis of *S. aureus* infection in an *in vitro* co-culture system which involves both osteoblasts and macrophages. Our data from **Chapter 4** gives a promising future for IL-12 treatment in the presence of antibiotics to eliminate intracellular *S. aureus* infection; therefore, studying the effect of IL-12 in a system where both cell types (osteoblasts and macrophages) are present will provide a better model of the actual situation.

Although our *in vivo* model is the first proof to directly link of the contribution of intracellular *S. aureus* to cause bone infection; however, antibiotics were dismissed from this model. In any clinical case of implant-associated infection, patients are treated with antibiotics for at least 6 weeks following the surgery. A better model should be designed to introduce antibiotics during the animal infection time to eliminate extracellular *S. aureus* that leaks out from osteoblasts in the first several days.

CURRICULUM VITA

Therwa Hamza

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Department of Orthopaedics

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Education

West Virginia University, School of Medicine, Morgantown, WV December 2012

PhD Candidate, Pharmaceutical & Pharmacological Sciences

- Dissertation Project: Infection models of intracellular *Staphylococcus aureus* and the impact of Interleukin 12 on intracellular *Staphylococcus aureus*

Committee Members:

- Bingyun Li, PhD (Chair)	Pharmaceutical & Pharmacological Sciences
- Yon Rojanasakul, PhD	Pharmaceutical & Pharmacological Sciences
- Patrick Callery, PhD	Pharmaceutical & Pharmacological Sciences
- Peter Gannett, PhD	Pharmaceutical & Pharmacological Sciences
- Kathleen Brundage, PhD	Microbiology, Immunology & Cell Biology

University of Jordan, Amman, Jordan 2006

- BS, Medical Analysis

Honors

Van Liere Research Day (First Place) 2012

This award was granted for Poster Presentation: "Growth of *S. aureus* and osteosarcoma cells in a co-culture environment on common orthopaedic biomaterials". McConda D, **Hamza T.**, Tidwell J., Lindsey B., Morgantown, West Virginia

WVU Doctoral Student Travel Award 2012

This award was granted for Poster Presentation: "*Staphylococcus aureus* Internalization and Associated Osteoblast Responses". **Hamza T.**, and Li B., at the Orthopaedic Research Society (ORS) Annual Meeting, San Francisco, California

Van Liere Research Day (Second Place) 2011

This award was granted for Poster Presentation: “Biological responses to intracellular *Staphylococcus aureus* by macrophages in development of a co-culture model”. Amoroso J., **Hamza T.**, and Li B., Morgantown, West Virginia

WVU Doctoral Student Travel Award 2011

This award was granted for Poster Presentation: “Intracellular *Staphylococcus aureus* infection: *In vivo* evidence for chronic osteomyelitis disease”, **Hamza T.**, Pham, D., Dietz M., Tidwell J., Jones A., Clovis N., Smith S., and Li B., at the Orthopaedic Research Society (ORS) Annual Meeting, Long Beach, California

Van Liere Research Day (Second Place) 2010

This award was granted for Poster Presentation: “Intracellular *Staphylococcus aureus* and its link with chronic osteomyelitis”, Pham, D., **Hamza T.**, Dietz M., Tidwell J., Jones A., Clovis N., Smith S., and Li B., Morgantown, West Virginia

WV Nano Research Symposium (First Place) 2009

This award was granted for Poster Presentation: “Cefazolin-embedded polypeptide nanofilms promising for infection prevention: a preliminary study on cell responses”. **Hamza T.**, Li H., Ogle H., and Li B., Morgantown, West Virginia

Laboratory Expertise

Cell Culture: Isolating and culturing mammalian cells (primary and cell lines), and culturing Gram + and Gram - bacterial strains

Immunology: Enzyme-linked immunosorbent assay (ELISA), flow cytometry, immunostaining, and confocal microscopy

Molecular Biology: Western blot, MTT viability assay, DNA proliferation assay, polymerase chain reaction (PCR), and protein purification

Publications

1. **Hamza T.**, Li B. *Staphylococcus aureus* internalization and associated osteoblast and macrophage responses. ***Microbes and Infection*** (submitted, October 2012)
2. Li H, **Hamza T.**, Li B. *In vitro* and *in vivo* antimicrobial properties of platelet-rich plasma. ***Acta Biomaterialia*** (in revision)
3. **Hamza T.**, Dietz M., Pham, D., Clovis N., Smith, S., Li B. Intracellular *Staphylococcus aureus* infection: In vivo evidence for chronic osteomyelitis disease. ***Journal of Infectious Diseases*** (submitted, August 2012)
4. McConda D, **Hamza T.**, Lindsey B. Tissue cell adherence and biofilm formation on common orthopaedic biomaterials in a co-culture environment. ***Journal of Orthopaedic Research*** (to be submitted in December 2012)

5. **Hamza T**, Barnett J, Li B. Interleukin 12 a key immunoregulatory cytokine in infection applications. *International Journal of Molecular Science* 11;789-806, 2010

Positions

- **Department of Microbiology, Immunology & Cell Biology**, Lab rotation
Dr. John Thomas. Role of biofilm in chronic wound infections
- **Department of Neurobiology & Anatomy-Eye Institute**, Lab rotation
Dr. Maxim Sokolov. Characterization of proteins involved in retinal neurodegenerative diseases
- **Department of Orthopaedics**, Lab rotation
Dr. Bingyun Li. Antibiotic-eluting nanotechnology coatings for potential prevention of device-associated infections
- **Department of Microbiology, Immunology & Cell Biology**, Lab rotation
Dr. Slawomir Lukomski. Investigate the role of Scl proteins in Group A *Streptococcus* pathogenesis
- **Mon General Hospital**, Volunteer: Pre-Admission Testing and Human Resources, Morgantown, WV
- **Al-Essra Hospital Laboratories**, Volunteer: Practical experience was gained in sample testing: Hematology, Immunology, Endocrinology, Biochemistry, Microbiology, and Parasitology, Amman, Jordan
- **The University of Jordan Hospital Laboratories**: A training program in: Out Patient Department (OPD), Blood Bank, Haematology, Histology, Biochemistry, Immunology, Endocrinology, Bacteriology, and Parasitology. Amman, Jordan

Teaching Experience

- Lecturer: (382) Biomaterials, Chemical Engineering, WVU
- Mentoring: I have mentored many students including undergraduate, graduate, and medical students. My training involved teaching to perform experiments and work independently. Also, I have helped students to write review articles, give seminars, and prepare posters to be presented at national conferences.
- Assisting Orthopaedics and Prosthodontics Residents: I have helped resident fellow to conduct their basic science research related to pathological chronic diseases such as implant-associated biofilm infections and periodontal diseases.

Workshops & Internships

USA (West Virginia)

June 2012

- Participant: Drug Discovery & Therapeutics Retreat. The aim of this workshop was to meet external and internal speakers to learn about drug discovery.

- USA (West Virginia)** October 2011
- Participant: Science: Becoming The Messenger-Division of Science & Research: This workshop was sponsored by the National Science Foundation and its main goal was to build communication skills between researchers in science and the public audience.
- USA (West Virginia)** June 2010
- Participant: Write Winning Grants: The aim of this workshop was to train faculty, post-doctoral fellows, and graduate students how to develop an irresistible fundable idea.
- France (Dijon)** June 2010
- Participant: The workshop's aim was to introduce the field of materials bioencapsulation to early-stage researchers. The training program included lectures and lab demonstrations (spray coating & drying, release profile testing, emulsification, dripping, and extrusion).
- China (Xi'an, Shanghai, Suzhou, and Beijing)** August 2008
- Presenter: The tour was funded by a grant from the National Science Foundation (NSF). The objective of this visit was to strengthen and facilitate an ongoing international collaboration in research and education in advanced biomedical devices that was initiated in 2006.
- Jordan (Amman)** April 2006
- Participant: Al-Farah Hospital Assisted Reproduction and In Vitro Fertilization (IVF) Laboratories: technical experience was gained in semen assessment, sperm preparation, oocyte collection, embryo culture and embryo transfer, and intracytoplasmic sperm injection (ICSI).

Presentations

- **Hamza T.** Li B. *Podium Presentation*. Intracellular *Staphylococcus aureus*: Challenges and Strategies. Sixteenth Annual Resident Research Symposium. Morgantown, West Virginia, May 2012.
- **Hamza T.** Li B. *Poster Presentation*. *Staphylococcus aureus* Internalization and Associated Osteoblast Responses. Orthopaedic Research Society (ORS) Annual Meeting. San Francisco, CA, February 2012.
- **Hamza T.** *Podium Presentation*. Interleukin 12: Biological Functions and Potential Clinical Applications. School of Pharmacy Research Day 2011. Morgantown, WV, October 2011.
- **Hamza T.** Li B. *Poster Presentation*. Osteoimmunology: The Inflammatory Response of Osteoblasts upon *Staphylococcus aureus* Internalization. Health Sciences Van Lier Memorial Convocation and Research Day, Morgantown, WV, March 2011.
- **Hamza T.** Pham D, Dietz M, Tidwell J, Jones A, Clovis N, Smith S, Li B. *Poster Presentation*. Intracellular *Staphylococcus aureus* infection: *In vivo* evidence for

- chronic osteomyelitis disease. Orthopaedic Research Society (ORS) Annual Meeting, Long Beach, CA, January 2011.
- **Hamza T**, Jiang B, Li B. *Poster Presentation*. A potential strategy of eradicating intracellular bacteria using antibiotic-delivery nanoparticles. WVNano Initiative Research Symposium, Morgantown, WV, April 2010.
 - Regal S, Li H, **Hamza T**, Knittle S, Li B. *Poster Presentation*. Could platelet-rich plasma be an effective treatment for bone infections? Health Sciences Van Lier Memorial Convocation and Research Day, Morgantown, WV, April 2010. (1st place award)
 - Li H, **Hamza T**, Li B. *Poster Presentation*. *In vitro* antibacterial effects of platelet-rich plasma on bacterial strains related to bone infection. Orthopaedic Research Society (ORS) Annual Meeting, New Orleans, LA, March 2010.
 - **Hamza T**, Li H, Jiang B, Knittle S, Li B. *Poster Presentation*. Prevention of bone-related infections using drug-loaded nanoparticles. The 21st EPSCoR National Conference (NSF), Washington, DC, October 2009.
 - **Hamza T**, Li H, Ogle H, Li B. *Poster Presentation*. Cefazolin embedded polypeptide nanofilms is promising for infection prevention: A study on cell responses. WVNano Research Symposium, Morgantown, WV, May 2009.
 - **Hamza T**, Li B. *Poster presentation*. Self-assembly multifunctional nanocoatings for biomedical applications. Multifunctional Nanomaterials International Symposium, Roanoke, WV, April 2008. (1st place award)
 - Ogle H, **Hamza T**, Li B. *Poster Presentation*. Antibiotic-eluting nanotechnology coatings for potential prevention of device-associated infections. WV Undergraduate Research Day at the Capitol, Charleston, WV, January 2008.

Professional Experience

- Organizing School of Pharmacy Research Day..... 2010
- Teaching Arabic As A Second Language.....2007-2010

Membership in Professional Organizations

- American Association of Pharmaceutical Scientists
- Orthopaedic Research Society
- The Association of Medical Laboratory Immunologists (AMLI)
- Microbiology Group Members

Personal Strengths and Skills

Achiever, Inducer, Positive, Restorative, and Social