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Redirection of the immune response to *Staphylococcus aureus* biofilm infection

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REDIRECTION OF THE IMMUNE RESPONSE TO
STAPHYLOCOCCUS AUREUS BIOFILM INFECTION

by

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A THESIS

Presented to the Faculty of

The University of Nebraska Graduate College

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Master of Science

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Under the supervision of Professor Tammy Kielian

University of Nebraska Medical Center
Omaha, Nebraska

June, 2017

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ABBREVIATIONS

Ab	Antibody
Ag	Antigen
AIP	Auto-inducing peptide
ANOVA	Analysis of variance
APC	Allophycocyanin
Arg-1	Arginase-1
BCA	Bicinchoninic acid
BHI	Brain-heart infusion
BMDM Φ	Bone marrow-derived macrophage
C5	Complement component 5
CA-MRSA	Community-acquired Methicillin-resistant <i>Staphylococcus aureus</i>
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CFU	Colony forming units
CHIPS	Chemotaxis inhibitory protein
COX2	Cyclooxygenase 2
CXCL	C-X-C chemokine ligand
CXCR	C-X-C chemokine receptor
DNA	Deoxyribonucleic acid
ECM	Extracellular matrix
eDNA	extracellular DNA
Erm	Erythromycin
EtOH	Ethanol
FACS	Fluorescence-activated cell sorting
FBS	Fetal bovine serum
Fc	Fragment, crystallizable
FITC	Fluorescein isothiocyanate
G-CSF	Granulocyte colony stimulating factor
GM-CSF	Granulocyte-macrophage colony-stimulating factor
G-MDSC	Granulocytic myeloid-derived suppressor cell
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

Hla	α -hemolysin
HLA	Human leukocyte antigen
H ₂ O ₂	Hydrogen peroxide
IACUC	Institutional animal care and use committee
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IP	IFN- γ induced protein
i.p.	Intraperitoneal
IRB	Institutional Review Board
KO	Knockout
K-wire	Kirschner wire
LAC	Los Angeles County
L-glut	L-glutamine
LPS	Lipopolysaccharide
LTA	Lipoteichoic acid
M Φ	Macrophage
MACS	Magnetic-activated cell sorting
M-CSF	Macrophage colony-stimulating factor
MCP-1	Monocyte chemoattractant protein-1
MDSC	Myeloid-derived suppressor cell
MIG	Monokine induced by gamma interferon
MIP	Macrophage inflammatory protein
M-MDSC	Monocytic myeloid-derived suppressor cell
MRSA	Methicillin-resistant <i>Staphylococcus aureus</i>
MET	Macrophage extracellular trap
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate
NET	Neutrophil extracellular trap
ND	Not detected
NOD2	Nucleotide-binding oligomerization domain-containing protein 2
NOS	Nitric oxide synthase
OD	Optical density
PAMP	Pathogen associated molecular pattern

PBS	Phosphate-buffered saline
PE	Phycoerythrin
PerCP	Peridinin-chlorophyll-protein complex
PGN	Peptidoglycan
PIA	Polysaccharide intercellular adhesion
PJI	Prosthetic joint infection
PMN	Polymorphonuclear cell
PRR	Pattern recognition receptor
RANTES	Regulated on activation normal T cell expressed and secreted
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
<i>S. aureus</i>	<i>Staphylococcus aureus</i>
s.c.	Subcutaneous
<i>S. epidermidis</i>	<i>Staphylococcus epidermidis</i>
TCR	T cell receptor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
WT	Wild-type

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Abstract

Staphylococcus aureus (*S. aureus*) is a leading cause of community- and healthcare-associated infections and has a propensity to form biofilms. Biofilm infections are recalcitrant to host immune-mediated clearance as well as antibiotics, making them exceptionally difficult to eradicate. The biofilm environment has been shown to skew the host immune response towards an anti-inflammatory phenotype, characterized by alternatively activated macrophages, recruitment of myeloid-derived suppressor cells (MDSCs), and minimal neutrophil and T cell infiltrates. Our laboratory has attempted to redirect the host immune response towards one that would favor bacterial clearance by employing strategies to augment pro-inflammatory mechanisms. One such approach was to utilize lipopolysaccharide (LPS), which was expected to promote pro-inflammatory activation of peripheral immune cells infiltrating the biofilm and subsequent clearance of infection. This theory was partially correct, as pro-inflammatory cytokines in the serum were significantly increased, and peripheral immune cells in the blood were more effective at killing *S. aureus ex vivo* following LPS treatment; however biofilm infection was exacerbated. Specifically, bacterial titers increased nearly 2-log with administration of LPS, and although infiltration of Ly6G⁺Ly6C⁺ MDSCs was decreased, a new population of Ly6G^{int}Ly6C⁺ cells appeared. Additionally, both Ly6G⁺Ly6C⁺ and Ly6G^{int}Ly6C⁺ populations were more suppressive with LPS treatment, partially explaining the expansion of *S. aureus* biofilm burdens. This study highlights the resilient nature of *S. aureus* biofilm infections to influence the immune response, particularly through MDSCs, even in the face of a strong pro-inflammatory stimulus. Gaining a better understanding of the mechanisms that cause this ineffective host immune response to staphylococcal biofilms is a necessary step towards eradicating these debilitating infections.

Chapter 1: Introduction

***Staphylococcus aureus* and biofilm infection**

***Staphylococcus aureus* infections and clinical significance**

Staphylococcus aureus (*S. aureus*) is a gram-positive bacterium that has remained a versatile and dangerous pathogen ever since its discovery in the 1880's [1]. The skin and nasal mucosa of approximately 30% of the world population are colonized with methicillin-sensitive *S. aureus* (MSSA), and approximately 1-2% are colonized with methicillin-resistant strains, known as MRSA, while prevalence among healthcare workers is closer to 5% [2-6]. According to a 2014 report from the CDC, over 80,000 invasive MRSA infections and more than 10,000 related deaths occur every year in the United States alone. The pathogen is responsible for a wide range of diseases, including superficial skin and soft tissue infections, respiratory infections, food poisoning, bacterial pneumonia, and sepsis [5]. The microorganism can colonize an individual for a long period with no apparent repercussions, until a breach of the skin or mucosal barrier introduces the possibility of infection. It is not well understood what determines whether an infection is contained or disseminates, and more research is needed to understand the complex interplay between *S. aureus* virulence determinants and host defense mechanisms.

***S. aureus* biofilms and prosthetic joint infections**

An important virulence determinant of *S. aureus* is its ability to form a biofilm on biological and artificial surfaces [7, 8]. A biofilm is a community of surface-associated bacteria that is enclosed in a complex matrix composed of proteins, polysaccharides, and eDNA [7-13]. Staphylococci are among the most frequent cause of biofilm-associated infections, and biofilm formation allows the bacteria to circumvent antibiotic- and immune-mediated clearance to establish persistent infections [8, 14, 15].

Biofilm development can be described as a continuous cycle with three distinct stages. The process begins by initial attachment of bacterial cells to biotic or abiotic surfaces, such as a heart valve or orthopedic device. Next, accumulation of bacteria and extracellular matrix (ECM) occurs as the biofilm matures. During the final stage, cells begin to detach from the biofilm proper, facilitating biofilm dispersal and possible reattachment at another site, continuing the cycle [8, 14, 16, 17]. Although we are beginning to understand some of the mechanisms whereby staphylococcal biofilms evade immune attack [12, 18-22], it is likely that additional pathways remain to be identified.

The biofilm mode of growth often includes dampening of protein and cell wall biosynthesis, thereby allowing the pathogen to avoid eradication by antibiotics that target actively growing cells [23-25]. Combined with its capacity for immune evasion and antibiotic resistance, staphylococcal biofilms also produce numerous virulence factors that target immune cells and damage host tissues. Only a small number of bacteria are needed to establish medical device-related infections, and their recalcitrance to antibiotics makes biofilms difficult to treat [13, 26]. In most cases, the contaminated device must be removed with an ensuing lengthy antibiotic regimen until the site is considered sterile, whereupon a new device is placed. This sequence of events prolongs recovery time and is an economic burden for the patient [27, 28]. Indeed, approximately \$1.8 billion is spent annually in the US for the treatment and clinical management of orthopedic implant-related infections [29, 30]. The cost will continue to rise, as it is projected that from 2005 to 2030, the number of total hip arthroplasty procedures will increase 174%, and total knee arthroplasties by 673% [31, 32]. When considering the increasing number of device-related procedures [32-34], nosocomial infections that can accompany these procedures in the hospital setting, and the continued emergence of

community-acquired *S. aureus* infections [35], a better understanding of the mechanisms that staphylococcal biofilms utilize to evade host immunity is one necessary step towards eradicating these chronic and debilitating infections.

Mouse model of orthopedic implant-associated biofilm infection

Our laboratory has utilized a mouse model of orthopedic implant-associated infection for the study of *S. aureus* biofilm infections and host-pathogen interactions [12, 36-39]. The proximity of the chronic biofilm infection and bone marrow make this model uniquely suited for the study of the host immune response to the invading pathogen. This model is typified by an early pro-inflammatory response resulting in the upregulation of IL-12p70, TNF- α , IL-1 β , IL-6, and IL-17 [9], but is closely followed by a chronic anti-inflammatory response. MDSCs are recruited to the site indirectly by IL-12, most likely by inducing the expression of a chemokine(s) with actions on MDSCs, which have yet to be identified [40]. These cells also increase anti-inflammatory signaling via IL-10 inhibiting immune-mediated clearance, and the biofilm persists [38, 41]. This model has allowed for a clearer interpretation of the mechanisms governing the innate immune response to *S. aureus* biofilms, but much more information remains to be elucidated in order to successfully treat these devastating infections.

Innate immune recognition and evasion of staphylococci

Innate immune cells recognize conserved pathogen-associated molecular patterns (PAMPs) expressed by microorganisms via pattern recognition receptors (PRRs) [42]. Toll-like receptors (TLRs) are a subset of PRRs that participate in innate immunity by recognizing common bacterial motifs and primarily trigger nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) activation [43-45]. These receptors are among the best-described PRRs in mammals, of which thirteen have been identified in humans, and ten in mice [42, 46]. TLR signaling, with the exception of TLR3,

recruits the adaptor molecule MyD88 to the intracellular TIR domain of the receptor and elicits a signaling cascade that induces NF- κ B activation, leading to the transcription of various cytokines, chemokines, co-stimulatory molecules, and antimicrobial peptides involved in defense responses [47].

NF- κ B regulates the expression of several genes associated with proliferation, differentiation, and cell death, as well as innate and adaptive immune responses [48], and is often targeted by microbes to subvert immune-mediated clearance [49]. As a demonstration of its importance during biofilm-mediated *S. aureus* infections, MyD88 knockout (KO) mice displayed significant increases in bacterial burdens and dissemination, fibrosis, and decreased expression of several pro-inflammatory mediators [19]. This prevented the establishment of a robust immune response needed to clear the infection, and created an environment disadvantageous to the host, as evidenced by the presence of macrophages that were polarized to an anti-inflammatory phenotype in a model of catheter-associated biofilm infection compared to wild type (WT) animals [19]. Other receptors utilize MyD88, including IL-1R and IL-18R [50], although it remains unclear whether one receptor is dominant, or if multiple pathways interact synergistically. In agreement with a role for MyD88-dependent signaling in controlling early *S. aureus* biofilm growth, a recent study demonstrated similar increases in bacterial burdens in IL-1R KO mice in a *S. aureus* orthopedic implant infection model [51]. Additionally, IL-1 β has been revealed to play a role in controlling early bacterial burdens during biofilm infections. IL-1 β KO mice displayed enhanced biofilm formation and decreased neutrophil recruitment [38]. Collectively, these studies reveal a role for innate immune mechanisms in early biofilm containment; however, it is clear that this response is not sufficient to clear biofilm infections due to their persistence in both animal models and humans. Indeed, both the MyD88 and IL-1R KO biofilm models only revealed enhanced

bacterial burdens during the early stages of infection, which dissipated over time. Upon colonization, bacteria maintain a planktonic lifestyle that elicits more traditional pro-inflammatory responses until biofilm formation has ensued, whereupon the host immune response transitions to an anti-inflammatory milieu dominated by the recruitment of myeloid-derived suppressor cells (MDSCs) and anti-inflammatory macrophages, which will be described in more detail below.

In terms of planktonic staphylococci, TLR2-mediated recognition of lipoproteins [52-55], polysaccharide intercellular adhesion (PIA), and phenol-soluble modulins (PSMs) [56, 57] leads to the production of numerous pro-inflammatory cytokines, including TNF α and IL-1 β , important regulators of the immune response. Although there has been some controversy regarding peptidoglycan (PGN) as a TLR2 ligand due to concerns of reagent purity, recent studies using ultra-pure preparations have confirmed its ability to engage the receptor [58]. Other staphylococcal PAMPs, such as lipoteichoic acid (LTA), are not inherently pro-inflammatory; however, they can augment immune activation in the presence of other PAMPs [59]. TLR9 is an intracellular PRR that recognizes unmethylated CpG DNA motifs, which occur more frequently in the bacterial genome compared to mammalian DNA [60]. Despite harboring several potent TLR ligands, staphylococcal biofilms have been reported to evade TLR recognition [12, 38] (Fig. 1.1.). Remarkably, TLR9 evasion is a hallmark of *S. aureus* biofilms [12] despite extracellular DNA (eDNA) representing a major biofilm component [61], which may be explained by leukocyte inaccessibility to eDNA when shielded by the matrix. Staphylococcal biofilms are known to evade TLR2 recognition, as evidenced by patients with mutations inactivating TLR2 have no increased incidence of post-arthroplasty *S. aureus* infection [62]. It has been shown that TLR2 activation is inhibited by staphylococcal superantigen-like protein 3 (SSL3), preventing neutrophil and monocyte

activation, as well as IL-8 production [63, 64]. Although TLR2 and TLR9 are essential for *S. aureus* recognition during planktonic infection [12], the biofilm form of growth successfully evades these extracellular sensing mechanisms.

Besides surface-associated PRRs, phagocytes are equipped with nucleotide-binding oligomerization domain (NOD) receptors that detect intracellular microorganisms, providing a second line of defense to ensure immune activation [65-68]. NOD1 is triggered by meso-diaminopimelic acid [69, 70] while NOD2 is activated by muramyl dipeptide (MDP) [65]. Both NOD1 and NOD2 recognize PGN degradation products; however, staphylococcal PGN does not contain meso-diaminopimelic acid, but it does contain MDP, making immune cells rely solely on NOD2 for sensing. MDP must reach the host cytosol to stimulate NOD2 and eventual NF- κ B activation, which may not occur frequently in the context of a biofilm infection, due to the complex matrix preventing immune cell infiltration [71, 72]. Aside from the molecules produced by staphylococci to actively block immune recognition, TLR2 and TLR9 evasion by biofilms may be further explained by ligand inaccessibility [11, 12]. For example, few planktonic bacteria are exposed at the outer biofilm surface, avoiding detection by PRRs [12, 73] and a *S. aureus*-produced matrix of polysaccharide polymers may prevent potential ligands from engaging with TLRs [74]. It is highly likely that staphylococcal biofilms produce additional factors that contribute to its ability to evade clearance in an immune competent host; however, these remain to be completely defined.

S. aureus is a common etiological agent of chronic debilitating infections, especially with the emergence of methicillin-resistant (MRSA) strains that have proven to be a therapeutic challenge. *S. epidermidis* is often overshadowed by MRSA because it encodes fewer virulence determinants by comparison; however, *S. epidermidis* is also a frequent cause of medical device-associated biofilm infections [2, 7, 75]. All humans are

colonized with *S. epidermidis*, while *S. aureus* is found in approximately 30% of individuals [76, 77]. Massey et al. predicted that for species with a high level of asymptomatic transmission, like *S. epidermidis*, less virulent strains out-compete virulent strains [78]. This would explain why *S. epidermidis* is equipped with determinants that promote persistence, such as immune evasion molecules, rather than toxins that actively attack the host. *S. epidermidis* generally acts as a commensal on the skin of humans, but harbors a limited subset of host defense mechanisms to persist in this environment, although a more robust defense is needed after penetration of the epithelial barrier [79]. Poly- γ -DL-glutamic acid (PGA) is secreted by *S. epidermidis* to promote growth and survival in the high-salt environment of human skin [80], and can form a capsule that shelters the bacteria from antimicrobial peptides and neutrophil phagocytosis [81, 82]. The *cap* gene locus drives PGA production and provides resistance to antibacterial peptides from human skin and phagocytosis. *Cap* mutant strains in a mouse catheter biofilm infection model were completely cleared [80], suggesting that PGA is critical for persistent *S. epidermidis* biofilm infection.

S. epidermidis biofilm formation is partially influenced by products from the *ica* operon. Polysaccharide intercellular adhesion (PIA) crosslinks *S. epidermidis* cells in a biofilm [83], protecting bacteria from IgG, AMPs, phagocytosis, and complement [22, 84]. C3b and IgG deposition was diminished on biofilms compared to planktonic cells, protecting biofilm-associated bacteria from neutrophil killing [84]. Another molecule, accumulation-associated protein (Aap) was shown to contribute to *S. epidermidis* biofilm formation under dynamic conditions. Namely, in a rat jugular catheter model of *S. epidermidis* infection, an Aap mutant displayed impaired colonization of the catheter surface compared to WT bacteria [85]. However, this required immunosuppression to maintain bacteremia and facilitate biofilm formation. Another study demonstrated that

macrophage activity was attenuated by *S. epidermidis* biofilms, in agreement with *S. aureus* biofilms [86]. IFN- γ production from lymphocytes occurs after stimulation by activated macrophages and only minimal levels of IFN- γ were produced following exposure to biofilm compared to planktonic organisms [87]. *S. epidermidis* is also capable of sensing host immune factors and enhancing defense systems in response to these insults [88]. For example, the antimicrobial peptide-sensing system (*aps*) is activated by a range of AMPs and causes the D-alanylation of teichoic acids [89] and phospholipid lysylation by the MprF enzyme [90]. MprF decreases the anionic charge at the bacterial surface, inhibiting the attraction of cationic AMPs. The effects of MprF expression in the context of biofilm immune evasion has not been investigated; however, it is reasonable to assume that modifying the charge at the bacterial surface would likely increase biofilm dissemination due to weakened attraction to positively charged exopolysaccharides (PIA) and proteins. *S. epidermidis* successfully utilizes a balanced system of surface modifications and secreted factors to remain undetected by host immune cells ensuring its success as a commensal and potential as an infectious pathogen. Further studies of staphylococcal biofilms and the host response to infection are needed in order to determine therapeutic targets of the immune system, and combat these persistent infections.

Immune effector cells and their role during staphylococcal biofilm infection

Macrophages

Macrophages represent an immediate line of defense against microbial invasion because all organs throughout the body harbor a resident macrophage population that is an important source of immune signaling molecules [91-93]. Macrophages also have a role in regulating tissue homeostasis by removing apoptotic cells and recycling nutrients by eliminating waste products from tissues [94-96]. Derived from bone marrow myeloid

precursors of the granulocytic-monocytic lineage, monocytes enter the systemic circulation and become macrophages after crossing endothelial venules and entering tissues [96]. These resident macrophages can be activated by various stimuli through their Toll-like and scavenger receptors, and defend the host against invading microbes. While known for their phagocytic abilities and production of antimicrobial reactive oxygen species (ROS) and reactive nitrogen intermediates (RNI), macrophages are also a major source of cytokines and chemokines that are critical for controlling immune cell recruitment/activation following bacterial exposure [97, 98]. Several *in vivo* models of staphylococcal biofilms have demonstrated that macrophages [8] and MDSCs [40, 41, 99] are the main leukocyte infiltrates, while neutrophil recruitment is mainly observed during acute infection and rapidly diminishes thereafter.

The macrophage inflammatory signature has been generally categorized into two distinct activation states, namely classically-activated (pro-inflammatory) and alternatively-activated (anti-inflammatory). These populations were originally described as M1 and M2, respectively, based on their *in vitro* responses to defined stimuli and three different polarization states have since been identified for M2 (i.e. M2a, M2b, and M2c) [100-102]. However, it is now apparent that this clear-cut dichotomy does not exist *in vivo* and macrophage activation states are more of a continuum with a mix of M1/M2 genes often being expressed, driven by the environment and stimuli the cell encounters [100]. Therefore, for the purposes of this thesis we will refer to these cells in descriptive terms as pro- or anti-inflammatory macrophages. Pro-inflammatory macrophages are a major source of pro-inflammatory cytokines (IL-6, TNF α , IL-1 β , IFN- γ , IFN- β) and ROS/RNI [103], whereas anti-inflammatory macrophages promote a fibrotic response and display attenuated microbicidal activity by expressing arginase-1 (Arg-1), IL-4, and IL-10. Inducible nitric oxide synthase (iNOS) competes with Arg-1 for the common

substrate arginine, which has been attributed to promoting microbicidal activity versus wound healing, respectively. *S. aureus* biofilms have developed mechanisms to alter macrophage phenotypes by attenuating iNOS while inducing high Arg-1 expression [12], which has been shown to promote collagen formation and fibrosis, hindering biofilm clearance [12] (Fig. 1.1.). In addition, further limiting host pro-inflammatory potential with the use of MyD88 KO mice resulted in exaggerated fibrosis in a model of *S. aureus* catheter-associated biofilm infection [19]. Indeed, biofilm-associated device infections in animal models and humans typically display strong fibrotic responses [99, 104, 105]. The fibrotic capsule may physically prevent immune cells from invading the biofilm, mask bacterial antigens, and limit antibiotic penetration, perhaps partially accounting for the recalcitrance of staphylococcal biofilms to these drugs. Additionally, fibrosis may promote dissemination and adhesion of bacteria via adhesion molecules (extracellular fibrinogen binding protein, fibronectin-binding proteins) expressed by staphylococci that bind proteins associated with the fibrotic response (i.e. collagen, fibronectin).

Despite the numerous PRRs expressed by host leukocytes and pro-inflammatory mediators induced by staphylococci, the host immune response is often not sufficient to clear biofilm infections. Macrophages have been shown to invade biofilm structures to some extent, however their ability to phagocytose biofilm-associated staphylococci is limited, and the majority of invading macrophages are killed *in vitro* [11, 12]. This failure is likely due to the inability of macrophages to physically engulf or opsonize the intact biofilm structure, possibly as a result of its size and complex structure. This hypothesis is in part supported by evidence of macrophages successfully phagocytosing bacteria from mechanically disrupted biofilms [12]. The death of invading macrophages can be attributed to *S. aureus* toxin action (i.e. Hla and LukAB); however, fluctuations in pH, oxygenation status, and release of other toxic byproducts from the biofilm may also play

a role [106-108]. Even if some macrophages manage to phagocytose bacteria from the biofilm structure, it is not sufficient to have a major impact on biofilm survival (Fig. 1.1.).

Phagocytes possess many bactericidal effector mechanisms such as, vacuole acidification, ROS/RNI, cationic molecules, myeloperoxidase, and lysozyme. *S. aureus* has developed resistance to many of these mechanisms. For example, antimicrobial peptides (AMPs) are cationic molecules that destroy bacterial cell membranes by targeting the lipid bilayer structure [109]. Staphylococci avoid AMP killing by charge modification of cell membranes, proteolytic degradation, and AMP binding and inactivation [89, 110]. Alanylation of teichoic acids (via the *dlt* operon) in the bacterial cell wall incorporates positively charged residues, allowing for biofilm formation [111], while also causing the electrostatic repulsion of AMPs and resistance to neutrophil killing [89]. Lysyl-phosphatidylglycerol modifications of teichoic acids via *mprF* or *lysC* can also confer AMP resistance [90, 112]. Staphylokinase is an exoprotein produced by *S. aureus* that binds plasminogen, inhibits the bactericidal effects of alpha-defensins [113], and induces bacterial detachment from mature biofilms [114]. The *S. aureus* metalloprotease aureolysin cleaves and inactivates the AMP LL-37 in the lysosome of macrophages and neutrophils, in addition to degrading many other substrates [115]. While its function in biofilms has yet to be studied, aureolysin expression is controlled by the *agr* system [116], a major regulator of biofilm formation [117, 118].

Lysozyme is another host lysosomal enzyme that damages the bacterial cell wall by catalyzing PGN hydrolysis. Lysozyme resistance in *S. aureus* is attributed to membrane bound O-acetyltransferase (*oatA*) that modifies N-acetylmuramyl residues in PGN, preventing lysozyme binding and degradation [119], and although its role from a biofilm perspective has yet to be investigated, its importance in immune evasion remains significant. *S. aureus* has been shown to escape the phagosome and survive

intracellularly in neutrophils and macrophages [120, 121]. This is thought to be due, in part, to antioxidant production. For example, *S. aureus* catalase neutralizes hydrogen peroxide (H_2O_2), which is utilized by leukocytes to kill bacteria, and secreted catalase from staphylococcal biofilms may prevent H_2O_2 from permeating the complex structure [122]. Molecules expressed on the surface of *S. aureus*, such as the surface factor promoting resistance to oxidative killing (SOK), also confer resistance to ROS [123]. Production of superoxide dismutases and methionine sulphoxide reductases allow *S. aureus* to resist oxidative stress [124, 125]. Reactive oxygen species can damage proteins by methionine oxidation, and *S. aureus* expresses a number of different enzymes to combat this destruction [126, 127]. Staphylococci also employ manganese (Mn^{2+}) homeostasis as a defense mechanism due to Mn^{2+} itself acting as a superoxide dismutase [128]. It is important to note that the expression of many of these genes responsible for ROS resistance and the stress response (described further below) are upregulated in biofilm compared to planktonic cells [129]. While the *agr* global regulator is the main driver of α -hemolysin and toxin production, the SaeRS global regulator in *S. aureus* also plays a part in regulating α -hemolysin, coagulase, and fibronectin-binding protein A [130-132]. Sae expression is activated by hydrogen peroxide and was found to reduce human neutrophil ROS production, allowing intracellular survival of the pathogen [133-138].

Phagocytosed bacteria that have evaded immune clearance may act as a reservoir for infection persistence [139]. *S. aureus* [140], *S. epidermidis* [141], and *S. lugdunensis* [142] have all demonstrated the ability to invade host cells and persist in a semi-dormant state, effectively avoiding exposure to antibiotic therapy [143]. These bacteria have numerous mechanisms to promote intracellular survival, providing a protective niche for pathogenic organisms. For example, capsule polysaccharide

synthesis was enhanced after phagocytosis [144] and could conceivably limit the success of degradative enzymes needed to penetrate the thick capsule. The host intracellular environment has also been shown to activate staphylococcal stress response genes. The stringent response of staphylococci is composed of two key components for the switch to tolerant phenotypes upon environmental stress, namely *rpoS* and the alarmone guanosine tetraphosphate (ppGpp) [145]. Upon amino acid deprivation, ppGpp synthesis potentiates the transition to a dormant state by decreasing protein synthesis capacity and increasing amino acid biosynthesis [146-149]. The stringent response is induced in the biofilm state, as well as after phagocytic uptake by neutrophils, allowing for intracellular *psm* expression, subsequent neutrophil lysis, bacterial escape, and survival, having major implications for infection dissemination [150].

In addition to the expression of molecules involved in immune evasion and intracellular survival, *S. aureus* strains also express a number of secreted toxins that kill host cells (Fig. 1.1.). Various molecules, such as γ -hemolysin (Hlg), α -hemolysin (Hla), and leukocidins, oligomerize and interact with specific receptors on the leukocyte surface, producing pores and inducing osmotic lysis [151]. Hlg was found to be upregulated after phagocytosis, suggesting it has a role in destroying neutrophils [144]. Although the role of Hlg in biofilm immune evasion has not been studied, a role of Hla has been defined. Scherr et al. [152] demonstrated cooperation between Hla and the bicomponent leukotoxin, LukAB, in inhibiting murine macrophage phagocytosis by *S. aureus* biofilms. Another leukotoxin, β -hemolysin (Hlb), degrades sphingomyelin causing lysis of human monocytes and inhibition of IL-8 production from endothelial cells, impeding neutrophil transmigration [153]. Hlb has also been shown to form covalent homodimers in the presence of eDNA, stimulating biofilm formation [154]. Additional

leukocidins of *S. aureus* (PCL, LukED, LukGH, and LukMF) have been described by Naimi *et al.* [155], Alonzo *et al.* [156], and Spaan *et al.* [157], although their specific functions in the context of biofilms have yet to be elucidated. The previously mentioned *sae* system as well as *agr* is responsible for regulating many of these secreted toxins and virulence-associated proteins, often increasing expression after leukocyte exposure, which contributes to bacterial persistence. Currently, many questions remain regarding the role of bacterial-derived factors in altering the host immune response towards one that favors biofilm persistence.

Myeloid-derived suppressor cells (MDSCs)

Immune responses elicited by staphylococcal biofilms share many similarities with tumors, in part, because both display significant infiltration of anti-inflammatory macrophages and MDSCs [12, 158, 159]. MDSCs are a heterogeneous population of myeloid progenitor cells that are arrested from fully differentiating into mature cells such as granulocytes, macrophages, or dendritic cells (DCs) [160-164]. MDSCs can interact with a variety of cell types, including T cells, DCs, macrophages, and natural killer cells to regulate anti-inflammatory activity and create an immunosuppressive milieu [162, 165]. It is thought that two signals are required for the differentiation and activation of MDSCs. First, MDSC expansion is thought to be induced during infection, tumors, or chronic stimulation, in response to cytokines and growth factors, such as G-CSF, GM-CSF, M-CSF, IL-6, and VEGF [162, 165]. The second signal activates MDSCs, causing increased Arg-1, NO, and suppressive cytokine production (i.e. IL-10), which has been attributed to pro-inflammatory molecules like IFN- γ , IL-1 β , IL-13, TLR ligands, and others [162]. In healthy individuals, if the two signals are not present, myeloid progenitor cells quickly differentiate and no expansion of the MDSC population occurs [165-168].

However, under inflammatory conditions, such as those created by bacterial infection, excessive cytokine production can influence the growth of MDSC populations [161, 167].

S. aureus infections have been associated with profound inhibition of T cell responses that are not attributed to T regulatory cells [169]. Instead, MDSCs were found to have a major role in regulating the immune response to biofilm infection. MDSCs inhibit antigen (Ag)-specific and polyclonal T cell activation by robust Arg-1 expression, depleting extracellular arginine needed for T cell responses [161, 170-172]. In particular, L-arginine regulates the expression of CD3 ζ , as well as the cell cycle regulators cyclin D3 and cyclin dependent kinase 4 in T cells [165, 173]. Production of ROS by MDSCs has also been shown to inhibit CD8 T cell responses to antigens, preventing an effective adaptive immune response to invading pathogens [174].

A significant MDSC infiltrate is associated with *S. aureus* biofilms *in vivo*, and our laboratory was the first to demonstrate that MDSCs play an important role in skewing monocytes/macrophages towards an anti-inflammatory phenotype during biofilm infection [158] (Fig. 1.1.). In addition to Arg-1, MDSCs from *S. aureus* biofilms displayed increased IL-10 expression. IL-10 production by MDSCs induced anti-inflammatory gene expression in monocytes, contributing to the persistence of *S. aureus* orthopedic biofilm infections [41] (Fig. 1.1.). IL-12 has also been found to promote MDSC recruitment and bacterial persistence [175], indicating a possible target to dampen MDSC infiltrates. Attenuating MDSC influx via Ab-mediated depletion at the infection site improved *S. aureus* clearance in a mouse model of orthopedic implant biofilm infection by promoting monocyte and macrophage pro-inflammatory activity [158]. These studies have shown that IL-12 is critical for MDSC recruitment to the site of infection and that IL-10 is one mechanism used by MDSCs to exert immunosuppressive functions that prevent biofilm clearance.

The evolution of immune responses can vary depending on the strength of the initial bacterial challenge, which was recently demonstrated for *S. aureus* biofilm formation. Specifically, our recent study [176] took advantage of IL-12 KO mice that displayed impaired MDSC recruitment and improved biofilm clearance to uncover an inoculum-dependent influence on subsequent immune responsiveness. This study was not feasible in WT animals because MDSC infiltrates and biofilm formation are too pronounced to discern differences. A low-challenge dose (10^3 CFU) of *S. aureus* in IL-12 KO mice showed reduced cytokine expression, MDSC recruitment, and improved bacterial clearance as compared to WT mice. In contrast, a higher-challenge dose (10^5 CFU) negated these differences, demonstrating the importance of bacterial inoculum on infection outcome. This is an important point, since some animal models utilize a large infectious inoculum or introduce implants that are pre-coated with bacteria [177-182]. By extension, a higher bacterial inoculum can accelerate biofilm formation and alter the immune response, making it difficult to discern underlying pathophysiological mechanisms. In a clinical setting, human PJIs result from colonization with low numbers of bacteria, which may provide a survival advantage during acute infection because of the inability to trigger a strong pro-inflammatory response [176]. As such, maintaining infectious doses as low as possible is desirable when attempting to best model human disease. In this regard, our recent study demonstrated similar immune infiltrates in human PJI tissues compared to the mouse model, revealing the fidelity of low bacterial challenge to reliably represent aspects of human disease [176]. The mechanism whereby IL-12 regulates MDSC recruitment and biofilm clearance is unknown, since the cytokine is best described for its ability to induce Th1 cells and adaptive immunity [183].

Information pertaining to adaptive immune responses against staphylococcal biofilms is sparse compared to what is known regarding innate immune mechanisms. *S.*

aureus is capable of targeting B cell survival and function through staphylococcal protein A (Spa). Spa is involved in biofilm formation [184], and can be found on the bacterial surface or secreted into the extracellular space where it can associate with Fc and Fab domains of immunoglobulins (Igs) [185, 186]. Immunoglobulins are generated against bacterial epitopes and allow recognition by macrophages and neutrophils via the Fc domain following opsonization. Spa binds IgG in the incorrect orientation for recognition, blocking the Fc domain, thereby preventing staphylococcal phagocytosis and complement activation via the classical pathway [187-189]. Binding of the Fab domain by Spa promotes B cell superantigen activity [190]. Protein A binds the Vh3 region of IgM on the surface of B lymphocytes, initiating proliferation and receptor-mediated programmed cell death [190]. Together, these mechanisms enact Spa as an effective suppressor of adaptive immune responses.

Just as with B cells, the known roles of T cell responses to biofilm infections are limited. Evaluation of human tissues recovered from orthopedic prosthetic surgery revealed that T cells were limited in PJIs, whereas tissues from subjects with aseptic prosthetic loosening displayed a noticeable T cell population [175]. Additionally, some *in vivo* studies indicate that T cells may play a role in orthopedic implant biofilm infections [177]. Inflammatory cytokines representative of Th1 and Th17 responses, as well as Th1-dependent antibodies, were found to be upregulated throughout biofilm infection [9]. However, it is important to note that these studies utilized implants that were pre-coated with bacteria, which likely elicits a distinct inflammatory cascade compared to low numbers of bacteria that establish biofilm infections in humans [176]. This could explain the disparity in T cell responses observed between laboratories, as other studies have shown the T cell infiltrate and contribution to immune response to be negligible [11]. *S. aureus* may promote T cell lysis by expressing δ -hemolysin, a PSM that is regulated by

agr-mediated quorum sensing and may be influenced by the biofilm state, since *agr* action has been associated with biofilm dispersal [117, 191, 192]. Staphylococcal superantigens activate vast numbers of T cells by their ability to crosslink MHC Class II on antigen presenting cells to the T cell receptor, followed by widespread T cell apoptosis that effectively prevents memory cell development [193]. *S. aureus* biofilms have been shown to produce superantigens that caused T cell activation and elicited a systemic inflammatory response in the absence of systemic infection [194]. This study was performed with transgenic mice expressing human MHC Class II (HLA-DR), since staphylococcal superantigens are not highly reactive with mouse MHC molecules [195]. *S. aureus* strains also produce a MHC class II analogue protein (Map) that reduces lymphocyte proliferation and shifts the immune response to Th2, suppressing Th1-dependent bacterial clearance [196]. However, the expression and functional impact of these molecules in the context of staphylococcal biofilm formation remain to be determined.

Neutrophils

Neutrophils are a primary line of defense against planktonic staphylococcal infections [5, 197, 198]. These phagocytic cells of the innate immune system are often called the “first responders” to an infection due to their rapid migration to sites of inflammation [197, 199]. However, unlike macrophages, neutrophil inflammatory cytokine and chemokine production is limited, and their short lifespan necessitates constant recruitment due to rapid cell turnover, making it difficult for these cells to combat a persistent biofilm infection. Engagement of PRRs activates pathways critical for bacterial phagocytosis and microbicidal activity, which elicit an oxidative burst within the phagosome, mediated by NADPH oxidase and iNOS that generate ROS and RNI, respectively. Bactericidal activity is augmented following phagosome-lysosome fusion,

since the lysosome is rich in proteases, cathepsins, defensins, and other antimicrobial effectors, creating an inhospitable environment for bacteria [200]. Despite the extensive array of antibacterial mechanisms neutrophils employ to combat infection, pathogens have evolved to evade these host-defense strategies through various means.

Staphylococci utilize several antioxidants to counteract ROS action and survive within the phagosome, including alkyl hydroperoxide reductase, staphyloxanthin, catalase, and SOK, among others [122-124, 201]. Neutrophils have been shown to phagocytose biofilm-associated bacteria but at a reduced level compared to planktonic bacteria or immature biofilms [12, 202]. Some of the antioxidants produced by *S. aureus* have been shown to be upregulated in biofilm-associated cells; specifically staphyloxanthin, catalase, and superoxide dismutase (SOD) [129], indicating that cells in a biofilm are better equipped to deal with stressful conditions upon phagocytosis. In addition, *S. aureus* biofilms do not dramatically alter their transcriptional profiles following neutrophil exposure [20] and although neutrophils infiltrate sites of early *S. aureus* biofilm infection [158], this occurs at a time when bacteria are still in a planktonic growth state (i.e. day 3 post-infection). Once mature biofilms form, around day 7 based on recalcitrance to antibiotic action, neutrophils are rare and are replaced by large numbers of MDSCs [158] (Fig. 1.1.).

The fact that few neutrophils are associated with *S. aureus* biofilm infections [12] could be attributed to the many virulence and immune evasion factors produced by *S. aureus*. In order to reach the biofilm and mediate bacterial clearance, neutrophils must adhere to and cross the capillary endothelium. This is accomplished through reciprocal interactions between endothelial receptors and ligands on the neutrophil surface. Neutrophil chemotaxis and extravasation is thwarted by multiple *S. aureus* secreted factors, such as SSLs, PSMs, chemotaxis inhibitory protein of *S. aureus* (CHIPS), formyl

peptide receptor-like 1 inhibitor (FLIPr), and FLIPr-like proteins [5]. Although these virulence determinants have been implicated in circumventing neutrophil recruitment during planktonic *S. aureus* infection, their role in preventing neutrophil influx into biofilms remains unknown. If similar mechanisms of action exist, this may be one explanation to account for the paucity of neutrophils associated with *S. aureus* biofilms. Nevertheless, a recent study from our laboratory demonstrated that the exogenous introduction of neutrophils at the site of *S. aureus* biofilm infection was not capable of preventing biofilm establishment [203], further supporting their ineffectiveness against biofilm growth.

Neutrophils are recruited to sites of infection by chemotactic gradients sensed by membrane bound G-protein coupled receptors (GPCRs), such as FPR1-3 and CXCRs [204-207]. PSMs interfere with these chemoattractants by binding human formyl peptide receptor 2 (FPR2) [208]. N-formyl peptides are found on the surface of *S. aureus* and induce chemotaxis, phagocytosis, and oxidative burst of neutrophils and monocytes [209]. CHIPS binds C5a and N-formyl peptide receptors on human leukocytes, effectively negating chemoattractant activity and preventing leukocyte recruitment to sites of infection [210]. The expression of extracellular adherence protein (Eap) by *S. aureus* binds and blocks ICAM-1, the endothelial receptor needed for leukocyte adhesion and diapedesis [211]. FLIPr and FLIPr-like inhibit FPR1 and FPR2, thereby evading recognition of secreted PSMs [208, 212, 213], and both FLIPr proteins inhibit neutrophil Ca^{2+} mobilization and actin polymerization [214-216]. Staphopain A, a protease secreted by *S. aureus* during biofilm growth, inhibits neutrophil migration toward CXCR2 chemokines by cleavage of their N-terminal domain [215]. It is important to note that many virulence factors produced by staphylococci, such as staphylococcal complement inhibitor (SCIN), extracellular fibrinogen binding protein (Efb), extracellular

complement binding protein (Ecb), and CHIPS, are highly species-specific with activity mainly limited to human cells, having little to no apparent effect on leukocytes in animal models [217]. Together, CHIPS, FLIPr, and SSLs, inhibit chemoattractant-mediated migration, effectively promoting planktonic infection; however, their involvement during staphylococcal biofilm formation remains to be determined.

Just as neutrophils have mechanisms to recognize and destroy invading pathogens via PRRs, *S. aureus* strains can respond and produce a plethora of virulence factors to counteract neutrophil function, including hemolysins, leukotoxins, iron scavengers, and stress response genes [218]. Phagocytosed staphylococci are capable of surviving ROS in phagosomes and causing host cell lysis [219]. Genes involved in capsule synthesis, gene regulation, oxidative stress, and virulence have also been reported to be up-regulated following neutrophil phagocytosis [144]. Physical and electrochemical cell wall properties resist secreted neutrophil defensins and lysozyme, while neutralizing enzymes (i.e. catalase) and carotenoid pigment confer resistance to ROS [5, 119, 220]. Immediately following phagocytosis, catalase, thioredoxin, thioredoxin reductase, SOD, alkyl hydroperoxide reductase, and glutathione peroxidase levels have been reported to be upregulated in *S. aureus*, corresponding with maximal neutrophil ROS generation [144]. Genes associated with virulence, including plasminogen binding protein, epidermin immunity/lantibiotic proteins, FnBPs, staphylocoagulase, clumping factors, γ -hemolysins, and exotoxin 2, can be upregulated upon phagocytosis [144]. The *agr* quorum sensing system has been implicated in intracellular staphylococcal survival in neutrophils and is responsible for the induction of many of the aforementioned virulence factors. The concentration of auto-inducing peptide (AIP) required for *agr* activation can reach critical levels within host cells, augmenting PSM expression and ultimately neutrophil lysis [221]. Because neutrophils

are targeted by staphylococcal virulence factors rather than undergoing programmed cell death, staphylococcal-mediated neutrophil lysis has been associated with necrosis [222-224]. The potent antimicrobial molecules released from neutrophils into the extracellular space can also cause local bystander tissue damage, further impairing bacterial clearance [225]. Although many of the neutrophil evasion tactics of staphylococci described above have yet to be studied in the context of biofilm infections, they are important to discuss in order to have a comprehensive view of staphylococcal interactions with the immune system.

In addition to phagocytosis, neutrophils employ extracellular traps to contain and destroy bacteria. Neutrophil extracellular traps (NETs), produced in response to *S. aureus* and other bacterial pathogens, are comprised of extruded DNA, histones, and microbicidal effectors [226]. Previously, it was thought that neutrophils underwent cell lysis to deploy NETs; however, a novel mechanism of NET formation that does not require neutrophil lysis has been reported by Pilszczek *et al.* [227]. In this model, neutrophils actively release intact vesicles filled with nuclear DNA into the extracellular space where they rupture and release chromatin. Entrapped bacteria are subject to peptidoglycan recognition protein S (PGRPS) and proteases, such as elastase [228]. These molecules ensnare bacteria to facilitate neutrophil phagocytosis and subsequent killing [229]. However, *S. aureus* harbors additional virulence mechanisms to subvert NETs, including nuclease and adenosine synthase that degrade and convert NET DNA to deoxyadenosine, allowing for pathogen escape [230]. Adenosine is a potent immunosuppressive molecule normally formed by cells after severe damage, such as hypoxic stress, ROS exposure, or cell lysis [231, 232]. Additionally, adenosine decreases MHC Class II expression in macrophages and dendritic cells and dampens IL-12 production [183]. By extension, staphylococcal enhancement of adenosine

production may interfere with T cell effector mechanisms and adaptive immune responses in infected hosts [233]. Adenosine triggers anti-inflammatory signaling cascades that inhibit neutrophil oxidative burst and degranulation, IL-1 production, and increase IL-10 production [234, 235]. Thus, AdsA-mediated synthesis of adenosine promotes *S. aureus* survival within neutrophils, presumably by inhibiting the superoxide burst and/or degranulation [233]. Deoxyadenosine (dAdo) triggers the caspase-3-mediated death of immune cells and macrophage exclusion from abscesses [236]. Treatment of human cells with dAdo causes intracellular dATP accumulation, which stalls DNA synthesis and triggers monocyte and macrophage apoptosis surrounding abscesses [237]. Interestingly, a global transcriptome analysis of *S. aureus* biofilm genes that were altered following macrophage exposure found that thermonuclease (Nuc) was one of the most strongly downregulated genes following 1 h of biofilm-macrophage co-culture [238]. Nuc downregulation is surprising due to the potential anti-inflammatory advantage of adenosine production by the biofilm and the predicted role of Nuc to degrade NETs. This is yet another example that much is still unknown with regards to biofilm-immune crosstalk during staphylococcal biofilm infections.

Osteocytes

S. aureus is a leading cause of bone and joint infections, such as osteomyelitis and PJIs, which can result in approximately 10-20% of bone loss near the infectious focus [239]. Implanted biomaterials are susceptible to microbial colonization and biofilm formation, favoring the onset of infection [240]. *S. aureus* has been found to affect at least two different cell types found in bone, namely osteoblasts and osteoclasts. Osteoclasts are bone matrix-degrading cells generated from the fusion of monocyte precursors and share conserved signaling pathways with monocytes and macrophages [241], while osteoblasts are derived from mesenchymal stem cells and are responsible

for bone formation. To date, staphylococcal interactions with osteoclasts and osteoblasts have been investigated using a murine *S. aureus* osteomyelitis model [242, 243]. Protein A, secreted by staphylococci, can bind pre-osteoblastic cells via tumor necrosis factor receptor 1 (TNFR1), resulting in osteoblast apoptosis [242-244]. Aside from directly preventing bone formation by destroying osteoblasts, *S. aureus* is also capable of altering osteoblast differentiation. Osteoblasts internalize staphylococci by $\alpha_v\beta_1$ integrin interacting with fibronectin binding protein on the bacterial surface. However, after internalization, bacteria persist and either induce host cell death or promote the secretion of osteoclastic cytokines such as RANK-L, enhancing osteoclastogenesis [245]. Osteoclasts are bone-degrading cells, and *S. aureus* infection of bone marrow-derived osteoclast precursors induced their differentiation into activated macrophages that secrete pro-inflammatory cytokines, enhancing the bone resorption capacity of other osteoclasts [246]. Furthermore, infection of mature osteoclasts directly enhanced their ability to resorb bone by promoting cellular fusion [246]. The hypoxic nature of healthy bone is exacerbated during infection and may explain the incredible persistence of *S. aureus* joint infections. Hypoxic growth of *S. aureus* resulted in a profound increase in quorum sensing-dependent toxin production and cytotoxicity [247].

Pro-inflammatory cytokines and biofilm persistence

A primary research objective of our laboratory is to understand how the host innate immune response is altered during biofilm infections with the goal of redirecting this response to facilitate bacterial clearance. It is clear that staphylococci evade and disrupt many facets of the innate immune response, although biofilm infections still elicit the production of a number of pro-inflammatory mediators. Compared to aseptic controls, several pro-inflammatory cytokines are elevated in mouse models of *S. aureus* orthopedic implant infection, including IL-12p40, IL-1 β , TNF- α , and G-CSF, as well as

the chemokines CXCL2, CCL2, CCL3, and CCL5 [40]. Despite the production of pro-inflammatory cytokines and chemokines, infected individuals are still unable to clear the biofilm infection. Instead, the pro-inflammatory response is likely responsible for promoting MDSC recruitment and activation, which is supported by our recent study where MDSC infiltrates were significantly reduced in IL-12 KO mice, which translated into improved biofilm clearance [40]. Recruitment of MDSCs and alternatively activated macrophages contribute to the chronicity of the infection [158]. The anti-inflammatory cytokine, IL-10, has been shown to have a role in MDSC recruitment [41] and interestingly so has the typically pro-inflammatory cytokine IL-12 [40]. Other pro-inflammatory mediators, such as IL-6, ROS, and cyclooxygenase-2 (COX2), drive MDSC activation, inducing the expression of Arg-1 and anti-inflammatory cytokines that drive the environment to one that favors bacterial persistence rather than clearance [248]. Theoretically, one could overcome the anti-inflammatory environment by eliciting a robust pro-inflammatory response that could facilitate bacterial killing. Our approach has employed lipopolysaccharide (LPS) treatments administered systemically and locally at the site of infection to augment pro-inflammatory activity.

The response to systemic LPS has been well characterized, and causes the production of several pro-inflammatory mediators through activation of the NF- κ B pathway [249]. Our hypothesis was that LPS activation of peripheral innate immune cells would promote their pro-inflammatory properties, which upon invasion would create a more hostile environment for the biofilm and promote bacterial clearance. The best laid plans of mice and scientists often go awry. Although as expected, systemic LPS treatment led to significant increases in systemic cytokine production and enhanced leukocyte killing of *S. aureus ex vivo*, biofilm burdens were increased nearly 2-log compared to vehicle treated mice. In addition, preliminary evidence has shown

increased expansion of MDSCs in the spleen of LPS treated mice. Collectively, these results suggest that systemic LPS elicits a pro-inflammatory cytokine network, which subsequently expands and activates MDSCs in the periphery to promote anti-inflammatory responses. In turn, this hinders innate immune cell recruitment, not only preventing biofilm clearance, but actually exacerbating it. Local LPS administration at the site of biofilm infection did not display the same phenotype as systemic treatment. Specifically, bacterial burdens were similar in local LPS treated and the vehicle control group, although the dose of LPS was nearly 10-fold lower than that given systemically, so further investigation is necessary. However, if the trends observed to date continue, the potential implications for this research are intriguing. Peripheral immune activation and subsequent MDSC activation and expansion, may tip the balance towards an increasingly anti-inflammatory immune response and dangerously worsen the infection.

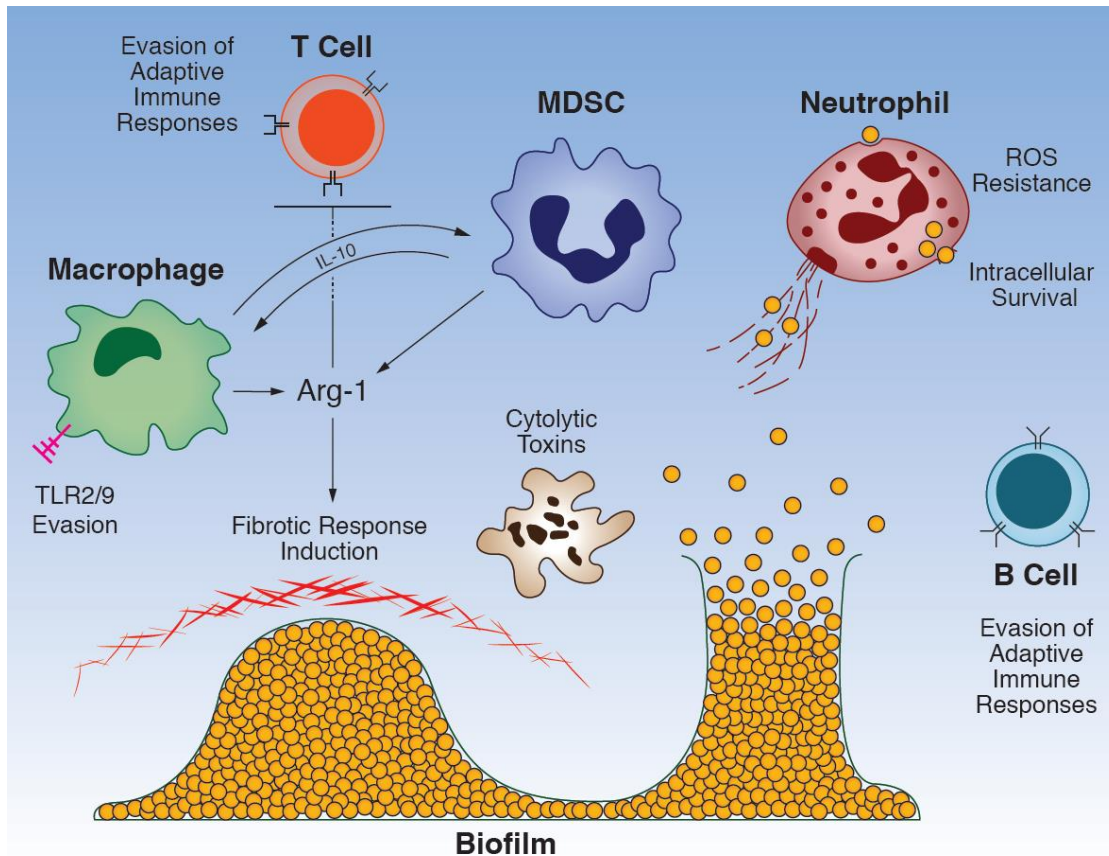


Figure 1.1. Mechanisms of staphylococcal biofilm immune evasion. Staphylococcal biofilms are adept at evading immune recognition and clearance compared to planktonic organisms. These biofilms circumvent Toll-like receptor 2 (TLR2) and TLR9, and the leukocyte response is dominated by anti-inflammatory macrophages and myeloid-derived suppressor cells (MDSCs), partially mediated by IL-10 production. Biofilms also augment arginase-1 (Arg-1) expression in macrophages and MDSCs, stimulating fibrosis and depleting extracellular arginine needed for T cell activation. *S. aureus* degrades NETs via nuclease and adenosine synthase. Phagocytosed *S. aureus* are resistant to ROS via production of several antioxidants, and are capable of intracellular survival and host cell lysis. Evasion of B cell adaptive immune responses has been attributed to Spa production by the staphylococcal biofilm.

Table 1.1. Immune-related molecules pertinent to staphylococcal biofilm infection

Biofilm Component	Abbreviation	Immune Evasion Tactic	Staphylococcal Species	References
Clumping factors	ClfA, Clfb	Fibrinogen binding proteins that shield bacteria from opsonophagocytosis and promote biofilm formation	<i>S. aureus</i> , <i>S. epidermidis</i>	[5, 250, 251]
Extracellular complement binding protein	Ecb	Binds complement and fibrinogen to disrupt phagocytosis and facilitate biofilm formation	<i>S. aureus</i>	[252]
Extracellular fibrinogen binding protein	Efb	Binds fibrinogen to evade complement-mediated clearance and promotes biofilm formation	<i>S. aureus</i> , <i>S. epidermidis</i>	[253]
Fibronectin binding proteins	FnBPA, FnBPB	Promotes cell surface adhesion and internalization	<i>S. aureus</i>	[254-256]
α -toxin	Hla	Inhibits macrophage phagocytosis and promotes cytotoxicity	<i>S. aureus</i>	[152]
Leukocidin AB	LukAB	Inhibits macrophage phagocytosis and promotes cytotoxicity	<i>S. aureus</i>	[152]
Nuclease and adenosine synthase	Nuc, AdsA	Converts NETs to deoxyadenosine, induces leukocyte death, Nuc downregulation associated with biofilm dispersal	<i>S. aureus</i>	[236]
Protein A	Spa	Induces osteoblast apoptosis, binds Ig Fc domain blocking recognition by phagocytes and complement activation	<i>S. aureus</i>	[184, 188-190, 242]
Phenol soluble modulins (PSMs)	PSM α 1-4, PSM β 1-2	Promotes biofilm dispersal, forms pores and lyses leukocytes	<i>S. aureus</i> , <i>S. epidermidis</i>	[208, 257]
SaeR/S		Regulates factors that reduce neutrophil ROS production, expression is critical for biofilm formation	<i>S. aureus</i>	[134-138]
Staphylococcal superantigen-like protein	SSL 1	Broad range MMP inhibitor, limits neutrophil chemotaxis and migration	<i>S. aureus</i>	[258-260]
Staphylocoagulase		Binds prothrombin, facilitates biofilm formation, stimulates fibrosis	<i>S. aureus</i>	[261, 262]
Staphylokinase	Sak	Neutralizes neutrophil α -defensins, induces biofilm detachment by plasminogen activation	<i>S. aureus</i>	[113, 179]
Staphopain A	ScpA	Inhibits neutrophil migration toward CXCR2 chemokines by cleavage of their N-terminal domain, induces biofilm detachment	<i>S. aureus</i>	[215, 263]
Superoxide dismutase	SodA	Neutralizes superoxide production, increased expression in biofilms	<i>S. aureus</i>	[129, 264]

Chapter 2. Materials and Methods

1) Bacterial strains and microbiological techniques

Bacterial strain

S. aureus USA300 LAC is a community-associated methicillin-resistant (CA-MRSA) strain isolated from a Los Angeles county (LAC) jail inmate with a SSTI and was also responsible for the CA-MRSA outbreak of 2002 [265-268]. We received the isolate from Dr. Frank DeLeo (National Institute of Allergy and Infectious Diseases Rocky Mountain Laboratories, Hamilton, MT) and cured it of its 27 kb LAC-p03 plasmid encoding erythromycin (Erm) resistance [269] by screening for spontaneous erythromycin sensitivity as previously described and was designated as USA300 LAC 13C. For the purposes of this thesis, this wild type strain will be referred to as USA300 LAC.

Bacterial storage and preparation

Bacterial strains were stored as glycerol stocks at -80°C, prepared by growing bacteria to exponential phase in brain-heart infusion broth (BHI; Fisher Scientific, Pittsburgh, PA) followed by centrifugation at 2,400 rpm for 10 min, 4°C. The bacterial pellet was resuspended in 1 ml of cold 1X PBS and washed by centrifuging again at 2,400 rpm for 10 min, 4°C. After discarding the supernatant, the pellet was resuspended in 20% glycerol in 1X PBS, and aliquoted into cryovials and stored at -80°C. From the freezer stock, a fresh streak plate was prepared for each experiment to avoid mutation of bacteria by prolonged storage at 4°C.

Preparation of bacteria for in vivo experiments

Overnight cultures were grown by selecting a single bacterial colony from the streak plate using a sterile loop and inoculating 25 ml of autoclaved BHI broth in a 250 ml baffled flask and incubating at 37°C overnight for 16 h with constant shaking at 250 rpm. Aliquots of 1 ml were transferred from the overnight culture into 1.5 ml Eppendorf

microcentrifuge tubes and centrifuged at 14,000 rpm, 4°C for 5 min to pellet the bacteria. The supernatant was discarded and the pellet was resuspended in 1ml PBS and subsequently washed two more times by centrifuging at 14,000 rpm for 5 min, 4°C. The washed bacteria were diluted 1:10 in PBS and the number of planktonic bacteria present was estimated by measuring the OD (BioMate 3S Spectrophotometer, Thermo Scientific, Waltham, MA) at 620 nm. To prepare inoculum for injection, the washed culture was diluted in sterile PBS after estimating the CFU/ml of the overnight culture. For example, if the overnight culture was estimated at 5.2×10^9 CFU/ml, three subsequent 1:10 dilutions would follow:

$$\begin{aligned} 1:10 \text{ dilution} &= 5.2 \times 10^8 \text{ CFU/ml} \\ 1:10 \text{ dilution} &= 5.2 \times 10^7 \text{ CFU/ml} \\ 1:10 \text{ dilution} &= 5.2 \times 10^6 \text{ CFU/ml} \end{aligned}$$

The following equation was then used to determine the amount of diluted culture needed to inject 1×10^3 CFU in 2 μ l (5×10^5 CFU/ml):

$$\begin{aligned} (5.2 \times 10^6 \text{ CFU/ml}) * x &= 5 \times 10^5 \text{ CFU/ml} * 1 \text{ml} \\ x &= 0.962 \text{ ml diluted culture} + 0.038 \text{ ml PBS} \\ &\text{or} \\ x &= 962 \mu\text{l diluted culture} + 38 \mu\text{l PBS} \end{aligned}$$

The exact concentration of cells/ml in the overnight culture was determined following preparation of bacteria for infection by serial diluting the washed culture in triplicate as follows:

10^{-2}	10 μ l of the 1ml washed overnight culture into 90 μ l PBS
10^{-3}	10 μ l of 10^{-2} dilution into 90 μ l PBS
10^{-4}	10 μ l of 10^{-3} dilution into 90 μ l PBS
10^{-5}	10 μ l of 10^{-4} dilution into 90 μ l PBS
10^{-6}	10 μ l of 10^{-5} dilution into 90 μ l PBS

Bacterial concentration of the inoculum was determined by plating 10 μ l of each dilution onto blood agar plates, incubating at 37°C overnight. The following day, the number of

bacteria was enumerated and plate counts were averaged to identify the actual CFU used for infection.

2) Mouse strains

C57BL/6 mice (8-10 weeks old) were obtained from Charles River Laboratories (Frederick, MD), from which a breeding colony was established. Mice were housed in restricted-access rooms equipped with ventilated microisolator cages and maintained at 21°C under a 12-h light/12-h dark cycle with ad libitum access to water (Hydropac; Lab Products, Seaford, DE) and Teklad rodent chow (Harlan, Indianapolis, IN). These studies were conducted in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the U.S. National Institutes of Health. The animal use protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

3) Cell culture techniques

Primary mouse bone marrow-derived MDSC culture

Adult C57BL/6 WT mice were euthanized with an overdose of inhaled isoflurane (Isothesia, VetUS, Dublin, OH) using a euthanasia chamber and cervical dislocation as the secondary method of euthanasia. The abdominal surface of each mouse was washed with an excess of 70% EtOH to minimize contamination and a subcutaneous incision was made near the midline of the abdomen. Skin was separated from the peritoneum until the hind limbs were exposed. Both hind limbs were removed at the hip joint and submerged in 1X PBS on ice until excess tissue and muscle were removed with Kimwipes. The clean bones were then placed in fresh 1X PBS on ice. The following steps were performed under aseptic conditions in a biological safety cabinet with sterile autoclaved instruments. Both ends of the bones were cut with scissors and bone marrow

was flushed with sterile, cold RPMI using a 26-gauge needle into a 50ml conical tube. After all bones were flushed, cells were pipetted to disrupt aggregates, filtered through a 70 µm cell strainer, and centrifuged at 1,200 rpm for 5 min at 4°C. The supernatant was aspirated and red blood cells lysed by the addition of 900µl sterile water for 5 s, followed by the immediate addition of 100µl 10X PBS to prevent the MDSC precursors from lysing. Finally, cells were washed with medium, centrifuged, and counted using trypan blue (Lonza, Walkersville, Germany) on a hemocytometer. Cells were plated in 175mm² tissue culture dishes at a density of 10⁷ cells/plate in 25ml of RPMI-1640 medium supplemented with 10% v/v HI FBS, 1% v/v HEPES, 1% v/v L-glutamine, 0.1% v/v antibiotic-antimycotic solution, 40 ng/ml G-CSF and 40 ng/ml GM-CSF (both from BioLegend). Cells were then incubated for 4 days at 37°C, 5% CO₂. The Ly6G⁺Ly6C⁺ MDSC population was purified from the mixed cell population by FACS and verified to possess T cell inhibitory activity. For some experiments, LPS (LPS-EB Ultrapure, Invivogen, San Diego, CA) was added at time of cell plating, or 24 h prior to MDSC harvest, at final concentrations of 1 ng/ml, 10 ng/ml, or 100 ng/ml.

4) Mouse model of *S. aureus* biofilm infection and LPS treatment

Mouse model of S. aureus orthopedic implant biofilm infection

To model infectious complications in patients following surgical device placement, a mouse model of *S. aureus* orthopedic implant biofilm infection was used. Age and sex-matched mice (8-10 weeks old) were anesthetized with a ketamine/xylazine cocktail at 100 mg/kg and 5 mg/kg, respectively (Hospira, Lake Forest, IL, USA, and Akorn, Decatur, IL, USA), and a medial parapatellar arthrotomy was performed with lateral displacement of the quadriceps-patella to access the distal femur. A 26-gauge needle was used to create a burr hole in the femoral intercondylar notch extending into the intramedullary canal, whereupon a pre-cut 0.8 cm long, orthopedic-grade Kirschner wire

(0.6 mm diameter, Nitinol (nickel-titanium); Custom Wire Technologies, Port Washington, WI, USA) was inserted, leaving approximately 1 mm protruding into the joint space. A total of 10^3 CFU of *S. aureus* USA300 LAC was inoculated at the exposed tip of the titanium implant. In some experiments, control mice received sterile implants using an identical procedure. The quadriceps-patellar complex was reduced to the midline and the fascia was sutured with 6-0 metric absorbable sutures before the skin of the surgical site was closed with 6-0 metric nylon sutures (both from Covidien, Mansfield, MA). Immediately following the surgical procedure, animals received Buprenex (0.1 mg/kg s.c.; Reckitt Benckiser Health Care, Hull, North Humberside, United Kingdom) for pain relief, and were returned to cages under a heat lamp to ensure maintenance of core body temperature until fully recovered from anesthesia. Cages were labeled with biohazard cards and monitored daily. A second dose of Buprenex was administered 24 h after surgery, and after this interval, all mice exhibited normal ambulation and no discernable pain behaviors.

Systemic and local LPS treatments

Infected mice were treated with 200 μ l doses of 12.5 μ g LPS systemically via intraperitoneal (i.p.) injection, or 5 μ g LPS subcutaneously at the site of infection (right knee). The appropriate LPS concentrations were prepared by diluting 5mg/ml LPS-EB Ultrapure (Invivogen, CA) in 1X PBS immediately prior to injection. Control (vehicle) treatment groups received 200 μ l of PBS via i.p. injection. All treatment groups were dosed at day 5 and day 6 post-infection. Animals were sacrificed at day 7 post-infection for quantification of bacterial burdens, as well as Milliplex analysis described below.

5) Recovery of biofilm infection-associated tissues

Recovery of orthopedic implant and surrounding tissues for *S. aureus* enumeration

For some experiments, prior to isoflurane exposure, approximately 250 μ l of whole blood was collected from each mouse and immediately placed in lithium heparin tubes (Terumo, Elkton, MD) to prevent coagulation. Animals were sacrificed by overdose of inhaled isoflurane, followed by cervical dislocation. For collecting inflamed soft tissue surrounding the infected knee joint, the flank and right leg were flooded with 70% EtOH and an incision was made in the flank so the skin could be removed to expose the infection site and leg. Next, the subcutaneous tissue dorsal to the patellar tendon was excised, weighed, and placed in 0.5 ml 1X PBS + 2% FBS on ice. Muscle and tendon tissues were excluded from the analysis. The tissue was dissociated with the blunt end of a plunger from a 30cc syringe and passed through a 35 μ m filter (BD Falcon, Bedford, MA). An aliquot of 150 μ l was removed for quantitation of bacterial burdens and Milliplex analysis of the supernatant. The remaining filtrate was then processed for flow cytometry as described below. After removal of the right leg at the hip joint, the muscle was removed from the knee joint and femur. The knee joint was separated from the femur allowing for removal of the implant, which was extracted from the femur and vortexed for 5 min at 1200 rpm in 100 μ l PBS to dislodge adherent bacteria. Both the knee joint and femur were weighed and placed in 500 μ l homogenization buffer before homogenization. These tissues were homogenized using two sequential procedures owing to their resilient nature: initially a 30 second dispersal using a hand-held homogenizer, followed by disruption in a Bullet Blender (Next Advance, Averill Park, NY) for 10 minutes, using 100-mm stainless steel beads (0.9–2.0 mm stainless steel blend). To determine bacterial colonization, serial 10-fold dilutions of tissue, knee, and femur homogenates as well as implant solutions were plated on trypticase soy agar with 5% sheep blood (Remel Products, Lenexa, KS). Titers are expressed as CFU per gram of tissue or per milliliter for titanium implants. Remaining homogenates were centrifuged (20,000 x g, 20 min) and frozen at -80°C for further analysis by Milliplex bead arrays as

described below. For some experiments, the spleen, and right kidney were collected to determine the degree of splenomegaly or bacterial dissemination as described above.

Whole blood S. aureus killing assay

Whole blood killing of *S. aureus* was used to determine systemic immunocompetence of infected mice. Overnight cultures were grown by selecting a single bacterial colony from the streak plate using a sterile loop and inoculating 3 ml of autoclaved BHI broth in a 14 ml round bottom tube (Corning, Reynosa, Mexico) and incubating at 37°C overnight for 16 h with constant shaking at 250 rpm. An aliquot of 1 ml was transferred from the overnight culture into 1.5 ml Eppendorf microcentrifuge tube and centrifuged at 14,000 rpm, 4°C for 5 min to pellet the bacteria. The supernatant was discarded and the pellet was resuspended in 1ml PBS and subsequently washed two more times by centrifuging at 14,000 rpm for 5 min, 4°C. The washed bacteria were diluted 1:10 in PBS and the number of planktonic bacteria present was estimated by measuring the OD (BioMate 3S Spectrophotometer, Thermo Scientific, Waltham, MA) at 620 nm. Washed bacterial were diluted to an estimated concentration of 5×10^7 CFU/ml. After collection, 150 μ l of whole blood was removed from each heparin tube and placed into individual wells of a 96-well non-tissue culture-treated plate, at which point 3 μ l washed bacteria (1.5×10^5 CFU) was added to each well, such that the final concentration of bacteria was estimated to be 10^6 CFU/ml. At 30 min, 1 h, and 2 h post-inoculation, one 30 μ l aliquot was removed from each well for bacterial enumeration. After removal of the aliquot, the 96-well plate was immediately returned to the incubator at 37°C until the next time point occurred. To determine bacterial survival, serial 10-fold dilutions (in 1X PBS) of each sample were plated onto blood agar plates, counted the following day, and averaged among treatment groups for each time point.

6) Flow cytometry

Flow cytometry was used to characterize leukocyte infiltrates in inflamed soft tissues surrounding orthopedic implants during *S. aureus* biofilm infection. Animals were sacrificed with an overdose of inhaled isoflurane; tissues were excised as previously described and placed in 500 μ l FACS buffer (2% FBS in PBS) on ice. Tissues were dissociated using the rubber end of a plunger from a 30cc syringe, and passed through a 35 μ m filter (BD Falcon, Bedford, MA). Following removal of an aliquot for bacterial quantitation and Milliplex analysis, the filtrate was washed with 1X PBS and the cells were centrifuged at 1200 rpm for 5 min, at 4°C. After discarding supernatant, the cells were resuspended and RBCs were lysed using BD Pharm Lyse (BD Biosciences, San Diego, CA) per manufacturer instructions. After lysis, cells were washed and resuspended in 500 μ l PBS followed by incubation in mouse Fc Block (2 μ l/sample, BD Biosciences, San Diego, CA) for 20 min at 4°C, to minimize nonspecific antibody (Ab) binding. Aliquots of 100 μ l were removed from each sample, pooled, and subsequently divided equally into compensation and isotype control tubes to identify gating thresholds and assess the degree of nonspecific staining, respectively. The remaining 400 μ l of each sample was split into two tubes and diluted to 500 μ l with 1X PBS. Cells were then stained with directly-conjugated antibodies for multicolor flow cytometry analysis, which included two separate panels to identify innate immune populations or T cells. Antibodies in the innate immune cell panel included CD45-APC, Ly6G-PE, Ly6C-PerCP-Cy5.5, and F4/80-PE-Cy7. Antibodies in the T cell panel included CD3 ϵ APC, CD4 Pacific Blue, CD8a FITC, Ly6C PerCP-Cy5.5, and TCR γ/δ PE. All fluorochrome conjugated antibodies were purchased from either BD Biosciences or eBioscience. To exclude dead cells from analysis, a Live/Dead Fixable Stain Kit (Life Technologies, Eugene, OR) was also used, following the manufacturer's instructions. Analysis was

performed using BD FACSDiva software with cells gated on the live CD45⁺ leukocyte population. From this population, MDSCs were gated on the Ly6G⁺Ly6C⁺ cell population, non-MDSCs were then designated as monocytes (Ly6C⁺F4/80⁻), F4/80⁺ monocytes (Ly6C⁺F4/80⁺), or macrophages (Ly6C⁻F4/80⁺).

7) Recovery of biofilm-associated leukocytes and *in vitro* assays

Cells were collected from the soft tissue surrounding infected knee joints as described above, and leukocyte populations were purified by FACS using CD45-APC, Ly6G-PE, and Ly6C-PerCP-Cy5.5. CD45⁺Ly6C⁺Ly6G^{high}, CD45⁺Ly6C⁺Ly6G^{intermediate}, and CD45⁺Ly6C⁺Ly6G⁻ cells were classified as MDSC, intermediate, and monocyte populations, respectively. The purity of MDSC cell populations was not examined after sorting owing to limited cell numbers. However, cytopins and gene expression analysis on sorted populations revealed that sorted MDSCs were highly enriched, as they displayed nuclear morphologies and characteristic markers consistent with those reported for MDSCs in the literature.

Polyclonal CD4⁺ proliferation assays

Naïve CD57BL/6 WT mice were euthanized with an overdose of inhaled isoflurane, and their flanks were flooded with 70% EtOH and spleens were isolated from the peritoneal cavity and placed into PBS + 10% FBS on ice. Spleens were dissociated using the blunt end of a 30cc syringe, and pressed through a 70 µm filter to generate a single cell suspension, centrifuged at 1200 rpm for 10 min, 4°C and RBCs were lysed using BD Pharm Lyse (BD Biosciences, San Diego, CA) per the manufacturer's instructions. After RBC lysis, the remaining cells were washed in 1X PBS, and T cells were isolated by autoMACS using a CD4⁺ T cell isolation kit (Miltenyi Biotec, Germany). CD4⁺ T cells collected by autoMACS were immediately labeled with eFluor 670 cell proliferation dye (eBioscience) according to the manufacturer's instructions. For establishing the

functional activity of MDSCs and other leukocytes associated with *S. aureus* orthopedic biofilm infections, T cell proliferation assays were performed. Briefly, eFluor 670–labeled CD4⁺ T cells were plated at 1.5×10^5 cells/well in a 96-well round bottom plate in RPMI 1640 with 10% FBS, supplemented with 100 ng/ml recombinant mouse IL-2 (Invitrogen, Frederick, MD). FACS-purified CD45⁺Ly6C⁺Ly6G^{high}, CD45⁺Ly6C⁺Ly6G^{intermediate}, and CD45⁺Ly6C⁺Ly6G⁻ cells were added at 1:1 or 1:5 ratios to CD4⁺ T cells subjected to polyclonal stimulation with 4µl/well CD3/CD28 Dynabeads (Life Technologies, Oslo, Norway). Controls of labeled T cells only or labeled T cells incubated with Dynabeads were also included. Cell co-cultures were incubated at 37°C for 72 h, whereupon the extent of T cell proliferation was determined by flow cytometry and supernatants were saved for cytokine evaluation by Milliplex analysis.

8) MILLIPLEX multi-analyte bead array

To evaluate a panel of cytokines/chemokines in the milieu of orthopedic implant-associated infected tissue, knee joint and femur, a custom-designed mouse microbead array was used according to the manufacturer's instructions (MILLIPLEX; Millipore, Billerica, MA). This array allows for the simultaneous detection of 19 different inflammatory mediators in a single homogenate, and includes: G-CSF (granulocyte colony-stimulating factor), GM-CSF (granulocyte macrophage colony-stimulating factor), IL-1 α , IL-1 β , IL-6, IL-7, IL-9, IL-10, IL-12p40, IL-13, IL-17, CCL2 (monocyte chemoattractant protein 1, MCP-1), CCL3 (macrophage inflammatory protein 1 α , MIP-1 α), CCL5 (regulated upon activated T cell expressed and secreted, RANTES), CXCL2 (MIP-2), CXCL9 (monokine induced by IFN- γ , MIG), CXCL10 (IFN-induced protein 10, IP-10), TNF- α , and VEGF (vascular endothelial growth factor). Results were analyzed using a Bio-Plex workstation (Bio-Rad, Hercules, CA) and normalized to the amount of

total protein recovered, determined by a bicinchoninic acid assay (BCA, Bio-Rad), to correct for differences in tissue sampling size.

9) Statistical analysis

Significant differences between experimental groups were determined using an unpaired two-tailed Student *t* test or a one-way ANOVA with Bonferroni's multiple comparison post hoc analysis in GraphPad Prism 4 (La Jolla, CA). For all analyses, a *p* value <0.05 was considered statistically significant.

Chapter 3: Systemic pro-inflammatory signaling augments

***S. aureus* biofilm development**

Abstract

S. aureus is a leading cause of nosocomial and community-associated infections, and has a propensity to form biofilms on native tissue and artificial surfaces. These infections are difficult to treat with antibiotics and evade immune recognition and clearance, placing a significant economic burden on the patient and healthcare system. The ineffective immune response has been attributed to the skewing of macrophages towards an anti-inflammatory phenotype and the recruitment of MDSCs. This promotes fibrosis rather than bacterial clearance, and biofilm persistence. In an effort to overcome this immune evasion, we have used a strong pro-inflammatory stimulus (LPS) to promote the infiltration of bactericidal cells into the biofilm. Two modes of LPS delivery were examined; namely systemic administration to activate peripheral immune cells and promote their recruitment to the biofilm, as well as local LPS treatment in an attempt to revert the anti-inflammatory state of resident leukocytes to a pro-inflammatory state. Several pro-inflammatory mediators were elevated in the serum following systemic LPS administration, which correlated with improved *S. aureus* killing *ex vivo*; however bacterial titers at the site of biofilm infection were significantly increased by 2-log. This was attributed to enhanced suppressive activity of infiltrating MDSCs, and the introduction of a Ly6G^{int}Ly6C⁺ population that was also capable of immune suppression. These results demonstrate the importance of a pro-inflammatory milieu for promoting MDSC expansion and activation, which exacerbates biofilm establishment. Therefore, methods to augment *S. aureus* biofilm clearance should proceed with caution to avoid inadvertent promotion of biofilm growth.

Introduction

The host immune response to *S. aureus* biofilms is largely categorized as anti-inflammatory, with macrophages and MDSCs playing a major role in infection outcome. Although an early pro-inflammatory response is evident at the site of infection, leukocytes are incapable of effectively clearing the biofilm [40]. Many factors are known to contribute to this impaired immune response to biofilms, including *S. aureus* secretion of leukocidins, chemotaxis inhibitors, superantigens, and other proteins and molecules that interfere with antimicrobial immunity [5, 12, 270]. Aside from staphylococcal-produced factors, the immune cell infiltrate consists largely of MDSCs, which dampen macrophage pro-inflammatory activity and transform the infection site into an anti-inflammatory environment [40, 158]. MDSCs produce factors that are capable of inhibiting T cell responses, as well as several anti-inflammatory cytokines that play a role in macrophage polarization [165]. Macrophages can exhibit different activation states, depending on the local microenvironment and the signals they receive. For example, a pro-inflammatory macrophage has an increased capacity to eliminate bacteria by producing cytokines and chemokines to regulate other immune cells as well as ROS and RNI production [271, 272]. However, in the case of *S. aureus* biofilm infections, infiltrating macrophages and the overall immune response, are biased towards an anti-inflammatory state. It is the goal of our laboratory to reprogram this response towards one that favors bacterial clearance.

Lipopolysaccharide is a potent stimulator of innate immunity in a wide variety of species. Only 1 to 2 μg is enough to cause a lethal reaction in humans, whereas rabbits and mice can survive doses of up to 10 μg and 50 μg , respectively [273-275]. A larger LPS dose induces septic shock through the actions of excessive pro-inflammatory cytokines, such as TNF- α and IL-1 β ; however at lower doses, LPS can trigger numerous

physiological immunostimulatory effects [276]. Recognition of LPS through TLR4 leads to the activation of several NF- κ B-mediated factors and the production of pro-inflammatory mediators [277, 278]. Therefore, we hypothesized that LPS treatment would be an attractive approach to reprogram the immune response to an established biofilm infection to promote bacterial clearance.

Patients undergoing joint replacement surgery are at an increased risk of developing a biofilm infection, with *S. aureus* being the most frequent etiological agent [279-281]. Our laboratory utilizes a mouse model of post-arthroplasty joint infection that mimics PJI and displays evidence of biofilm formation on the infected implant [39, 158, 282, 283]. Contrary to our prediction, systemic LPS treatment of infected mice promoted biofilm growth rather than clearance, which was associated with significant increases in numerous pro-inflammatory cytokines, including CCL2, CXCL9, CCL3, CXCL10. Likewise, G-CSF, IL-6, and IL-10 expression was also elevated; factors that are known to induce MDSC expansion and activation [284-288]. Indeed, MDSC expansion in the blood and spleen of LPS treated mice was evident, yet traditional Ly6G⁺Ly6C⁺ MDSC populations were decreased at the site of biofilm infection, whereas a novel population of Ly6^{int}Ly6C⁺ cells was increased. Additionally, the MDSCs recovered from LPS treated mice were found to be more suppressive than MDSCs from vehicle treated animals. In contrast to what was observed at the site of biofilm infection, bacterial growth in whole blood was inhibited with LPS treatment, indicating that the systemic immune response remained bactericidal. These results indicate that an attempt to bolster the immune response to biofilm infection can lead to opposite effects locally and systemically. Specifically, the immune response at the site of infection appears to become more anti-inflammatory, while the systemic response becomes hyper-inflammatory. Collectively, these studies remind us of the complicated interplay dictating the inflammatory response

to *S. aureus* biofilms, and the need for more research to understand and ultimately thwart these dangerous infections.

Results

LPS treatment enhances intrinsic pro-inflammatory cytokine production during *S. aureus* biofilm infection. LPS is the major component of the outer membrane of gram-negative bacteria and is recognized by the immune system via TLR4 as an indicator of bacterial infection, causing a rapid inflammatory response [289-291]. Here we utilized LPS administration in a mouse model of *S. aureus* orthopedic implant-associated biofilm infection in an attempt to revert the immune response from an anti-inflammatory to a pro-inflammatory state. To assess the effects of LPS treatment, we first examined inflammatory mediator production in the serum and soft tissue surrounding the joint in the mouse model. Aseptic implants elicited transient inflammatory mediator production, most likely originating from the trauma generated during the surgical procedure (Fig. 3.1). In the serum, several pro-inflammatory mediators, including G-CSF, IL-6, CXCL10 (IP-10), CCL2 (MCP-1), and CXCL9 (MIG) were significantly increased in LPS compared to vehicle treated mice (Fig. 3.1). Other cytokines in the serum were also increased following LPS treatment, including, GM-CSF, IL-9, CCL3 (MIP-1 α), and CXCL2 (MIP-2 α), although these did not reach statistical significance (Fig. 3.1). Unexpectedly, levels of the anti-inflammatory cytokine, IL-10, were also increased with LPS treatment, along with decreased production of IL-1 α and CCL5 (RANTES) (Fig. 3.1). Cytokine production was also measured in the tissue surround the implanted device, but was difficult to interpret due to a large variation among samples (Fig. 3.2). Collectively, these data demonstrate that LPS administration actively augments production of several inflammatory mediators.

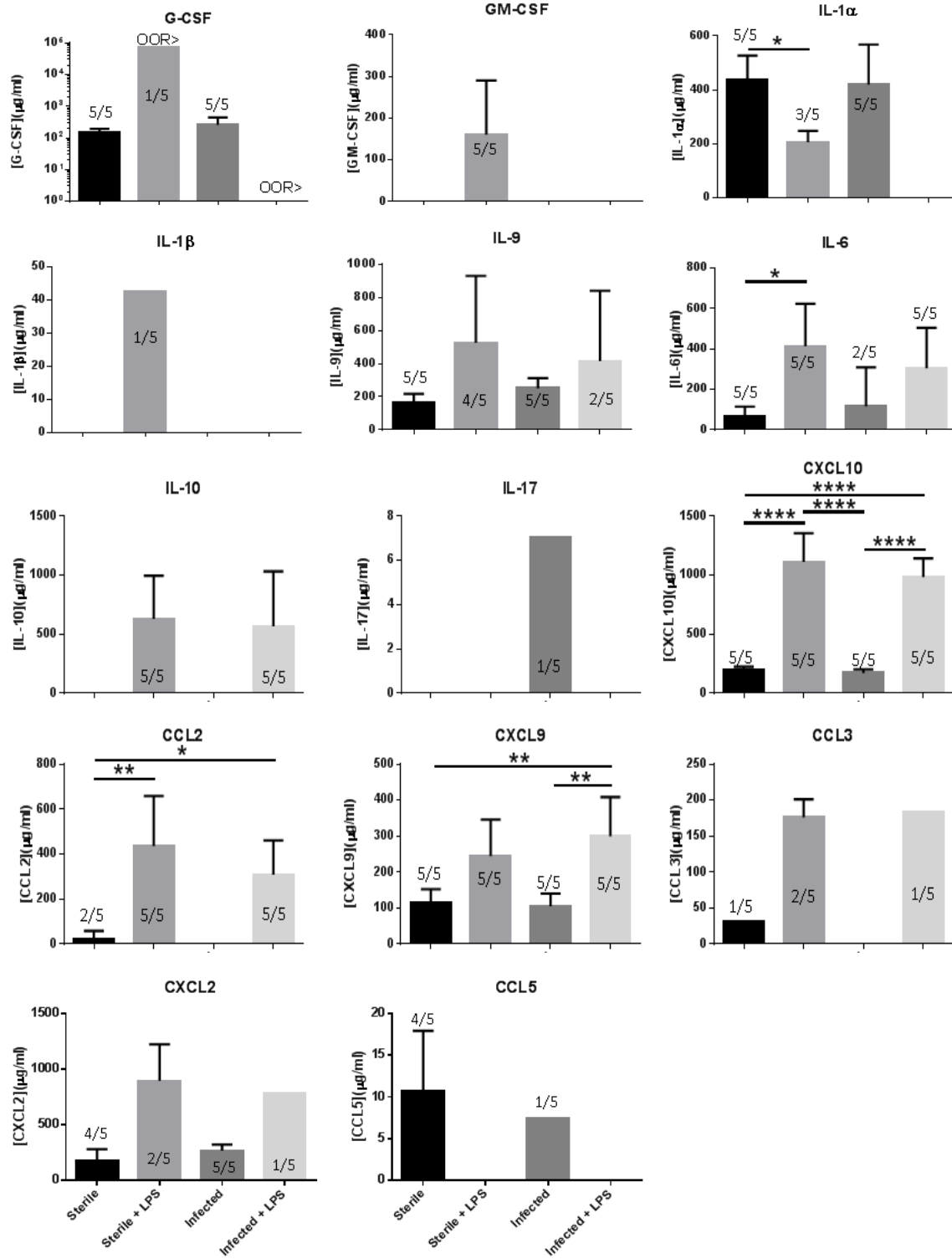


Figure 3.1. LPS treatment alters inflammatory mediator production in serum during *S. aureus* orthopedic biofilm infection. Serum collected at day 7 post-infection from sterile and infected mice that were treated with vehicle or 12.5 μg LPS via i.p. injection at days 5 and 6, whereupon expression of G-CSF, GM-CSF, IL-1 α , IL-1 β , IL-9, IL-6, IL-10, IL-17, CXCL10, CCL2, CXCL9, CCL3, CXCL2, and CCL5 was quantitated by Milliplex. Results were normalized to the amount of total protein recovered to correct for alterations in tissue sampling size. Results are representative of 5 mice per group, with the number of measurable samples labeled as "X/5". Significant differences are denoted by asterisks (* $p < 0.05$, ** $p < 0.01$, **** $p < .0001$; one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis).

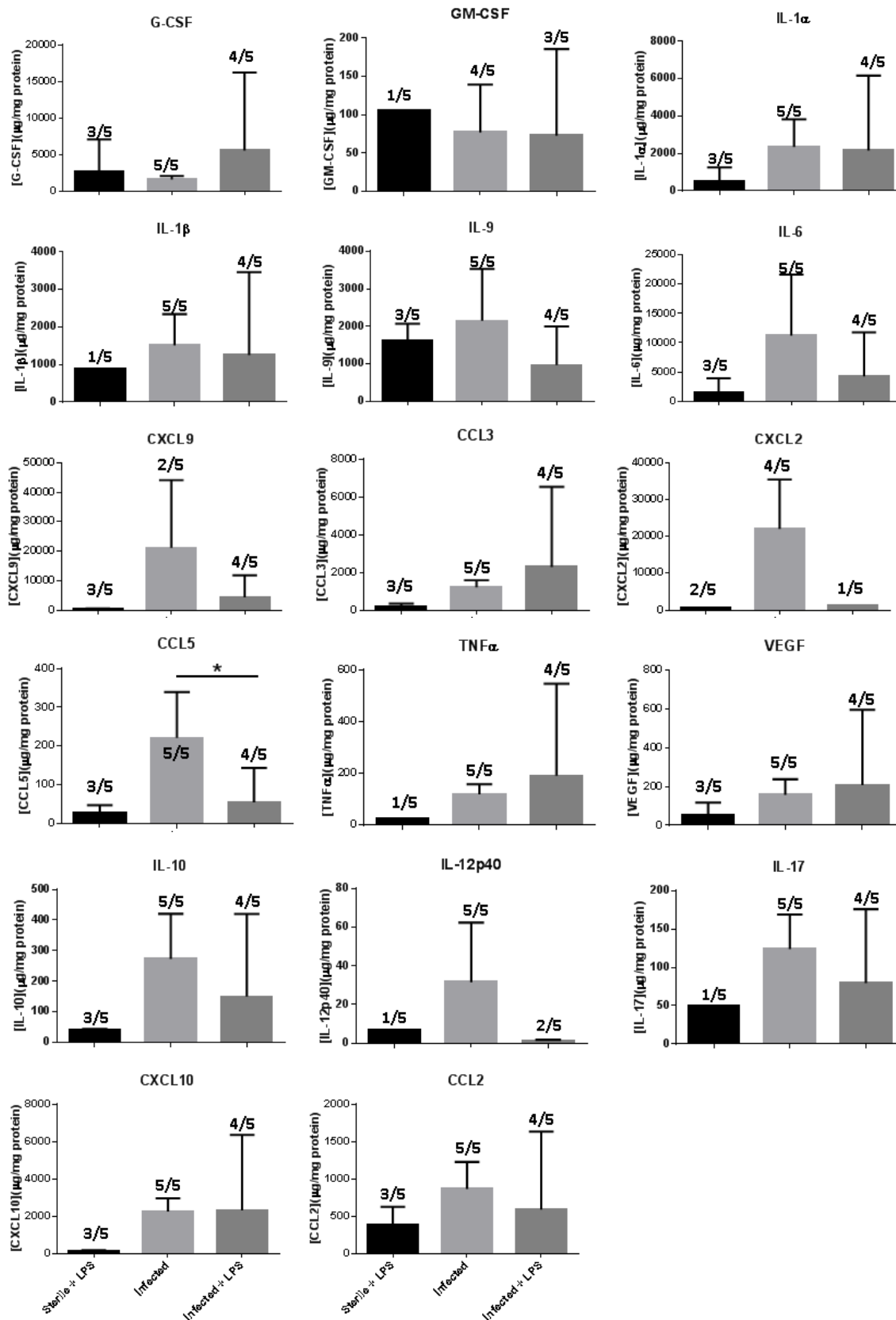


Figure 3.2. LPS treatment alters inflammatory mediator production in tissue during *S. aureus* orthopedic biofilm infection. Tissue homogenates surrounding orthopedic implants were prepared at day 7 post-infection from sterile and infected mice that were treated with vehicle or 12.5 µg LPS via i.p. injection at days 5 and 6, whereupon expression of G-CSF, GM-CSF, IL-1α, IL-1β, IL-9, IL-6, IL-10, IL-12p40, IL-17, CXCL10, CCL2, CXCL9, CCL3, CXCL2, CCL5, TNF-α, and VEGF was quantitated by Milliplex. Results were normalized to the amount of total protein recovered to correct for alterations in tissue sampling size. Results are representative of 5 mice per group, with the number of measurable samples labeled as “X/5”. Significant differences are denoted by asterisks (* $p < 0.05$; one-way ANOVA with Bonferroni’s multiple comparison post-hoc analysis).

LPS treatment enhances *S. aureus* killing by peripheral blood leukocytes *ex vivo*.

To determine if LPS treatment promoted the antimicrobial activity of peripheral blood leukocytes compared to *S. aureus* biofilm infected mice alone, whole blood was collected and cultured with planktonic *S. aureus ex vivo* to assess bactericidal activity. Using this approach we were able to distinguish differences in the ability of whole blood from different treatment groups to kill *S. aureus*. Blood from all treatment groups resulted in reduced bacterial counts after a 30 min incubation (Fig 3.3). In accordance with increased cytokine production, the blood of LPS treated mice was more effective at killing *S. aureus* than the vehicle treated animals (all groups harbored *S. aureus* orthopedic implant infection). After 30 and 60 min, only systemic LPS treatment displayed improved killing compared to vehicle treated mice; however, after 2 h of incubation, blood from both systemic and local LPS administration groups displayed enhanced killing ability (Fig 3.3B). These results indicate that LPS treatment creates a hostile systemic environment for *S. aureus* and prevents bacterial persistence in the blood, which is likely due to increased inflammatory mediator production.

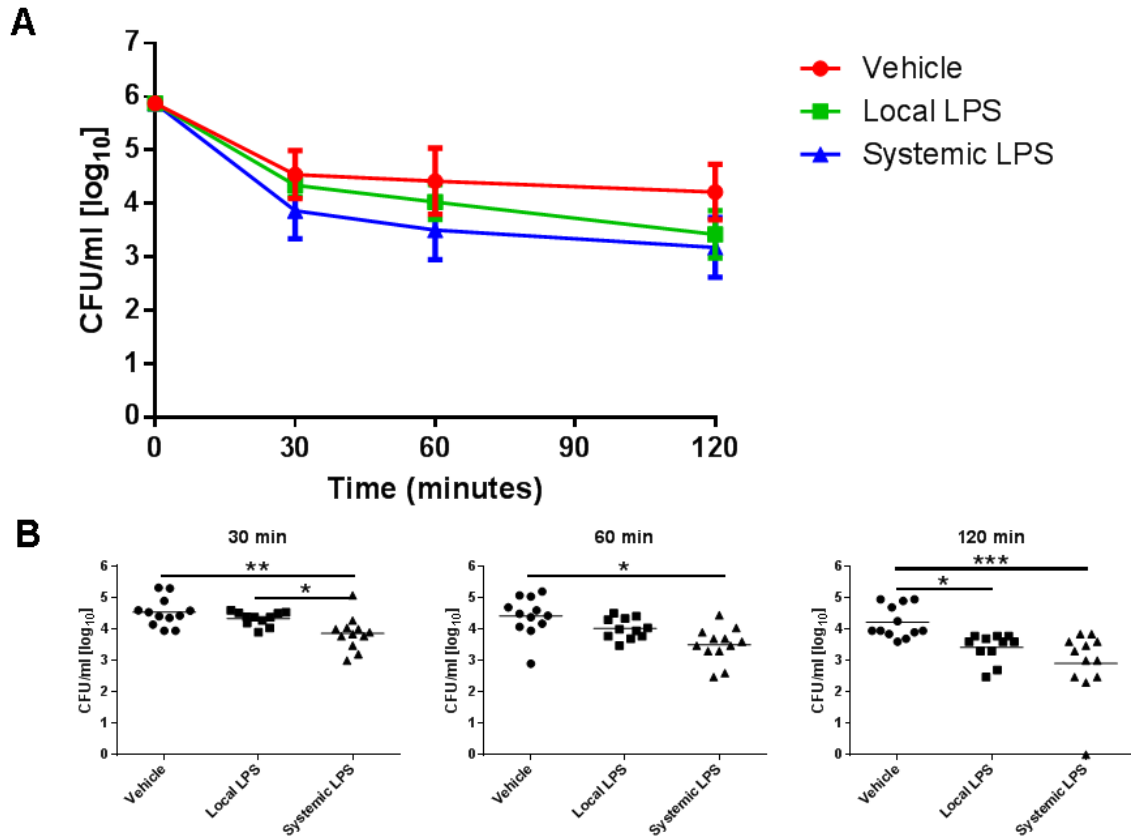


Figure 3.3. LPS treatment enhances *S. aureus* killing in whole blood. (A) Time-course of microbicidal ability of whole blood recovered from mice with *S. aureus* orthopedic biofilm infection that received vehicle, local, or systemic LPS treatments (5 and 12.5 μ g, respectively). (B) Bacterial survival in whole blood at 30 min, 1 h, and 2 h. All groups were inoculated at a concentration of 10^6 CFU per mL. Results are expressed as Log₁₀ CFU per mL and are representative of 12 mice per group from two independent experiments. Significant differences between treatment groups are denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$; one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis.

LPS treatment increases *S. aureus* growth during orthopedic implant biofilm

infection. Previous work from our laboratory has demonstrated that augmenting pro-inflammatory activity, specifically in macrophages, is critical for biofilm clearance *in vivo* [203]. Along the same lines, LPS treatment increased production of multiple pro-inflammatory cytokines, leading to improved clearance of bacteria in the blood *ex vivo* (Fig. 3.1 and 3.3). Despite the elevated pro-inflammatory immune response systemically, LPS treatment did not facilitate bacterial clearance at the site of infection, but rather promoted biofilm growth (Fig. 3.4). Systemic LPS treatment caused nearly a 2-log increase in bacterial burdens in the surrounding tissue after 7 days of infection, while local LPS treatment displayed a similar increase in bacterial titers, but to a lesser degree (Fig. 3.4). Differences in bacterial burdens among groups in the femur, joint, and implant were not significant; however, it was interesting to note that more mice in the LPS-treated groups had bacterial burdens that were below the limit of detection at these sites compared to vehicle-treated mice (Fig. 3.4). Collectively, these results suggest that immune activation via LPS treatment can improve antibacterial activity in the peripheral blood, but dramatically worsens localized infection in tissues surrounding the infected joint.

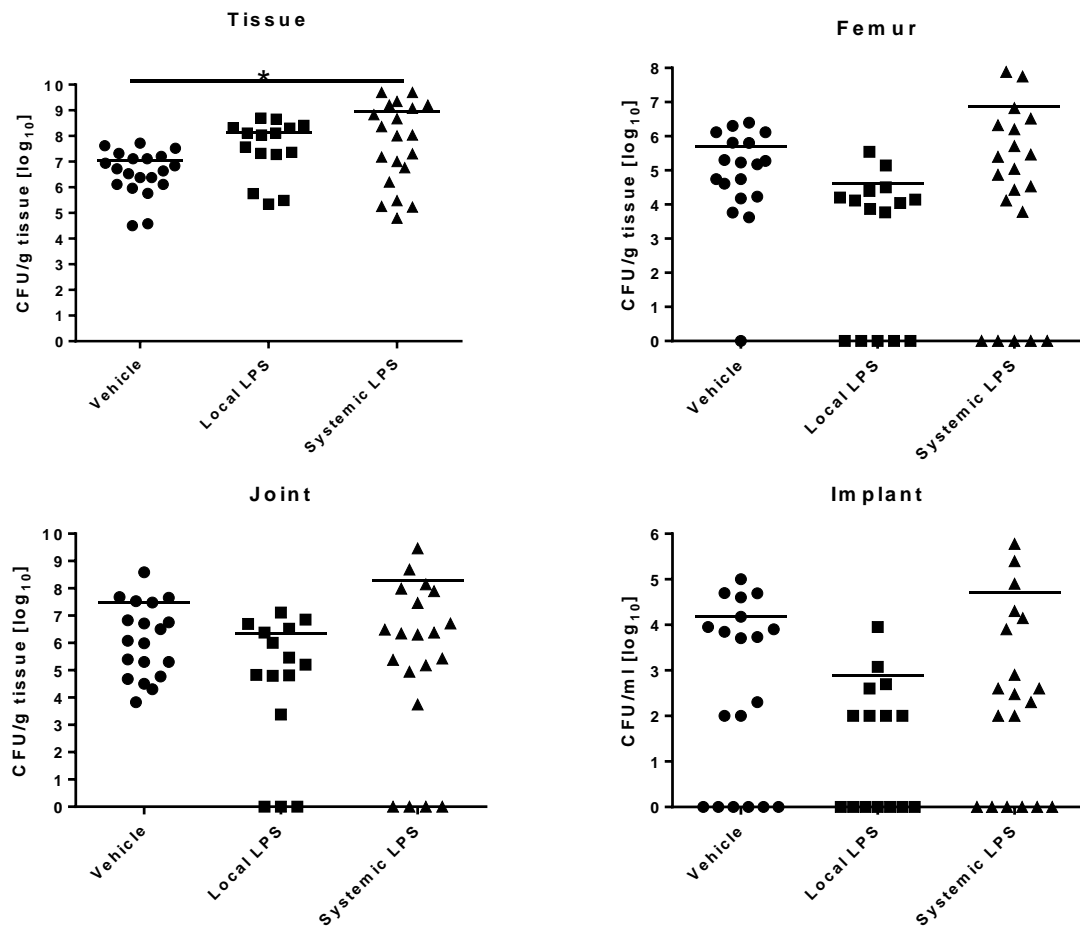


Figure 3.4. LPS treatment alters *S. aureus* biofilm burdens. Bacterial burdens associated with the knee joint, surrounding soft tissue, femur, and orthopedic implant at day 7 post-infection following local or systemic LPS (5 and 12.5 μg , respectively) or vehicle treatment. Results are expressed as CFU per mL for orthopedic implants or CFU per gram of tissue to correct for differences in tissue sampling size. Results are representative of 5-10 mice per group from three independent experiments, for a total of 20 mice per group. Significant difference between treatment groups are denoted as $*p < 0.05$; one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis.

LPS treatment alters immune cell populations in the blood, spleen, and at the site of *S. aureus* biofilm infection. Pro-inflammatory cytokines have been reported to recruit and activate MDSCs [162, 285], as well as regulate macrophage and T cell activation [40, 292, 293], which could partially explain the exacerbation of infection in LPS treated mice. MDSCs are significantly elevated in the *S. aureus* orthopedic implant infection model compared to aseptic implants, and have been shown to be critical factors in attenuating innate immune cell influx and biofilm clearance [40, 41, 158]. Based on the unexpected disconnect between pro-inflammatory cytokine production and infection outcome, we examined differences in immune cell infiltrates of LPS and vehicle treated mice.

Examination of leukocyte recruitment revealed that LPS only induced significant differences in innate immune cell populations in mice with concurrent *S. aureus* biofilm infection and not with aseptic implants. Specifically, LPS treatment had no significant effect on MDSC, monocyte, F4/80⁺ monocyte, or macrophage populations (Fig. 3.5). In agreement with previous reports from our laboratory, *S. aureus* biofilm infection increased MDSC recruitment concomitant with reduced monocyte and macrophage populations at the site of infection, compared to sterile implants [40, 41, 158] (Fig. 3.5). However, LPS treatment reduced Ly6G⁺Ly6C⁺ infiltrates that our laboratory has demonstrated to be immune suppressive MDSCs [158] (Fig. 3.5A). Specifically, local LPS administration resulted in approximately a 20% reduction of MDSCs, while systemic LPS treatment reduced the MDSC infiltrate more drastically, by nearly 60% (Fig. 3.5A). In contrast, monocyte populations increased with both LPS paradigms, whereas only systemic LPS treatment caused a significant increase in macrophage infiltrates (Fig. 3.5B and D). F4/80⁺ monocytes were not significantly different across any treatment

group (Fig. 3.5C). Distributions of these Ly6G, Ly6C, and F4/80⁺ cell populations are presented in Figure 3.6.

Alterations in leukocyte frequencies in the blood and spleen were also examined to elucidate the peripheral immune response to *S. aureus* and effects of LPS. Despite the improved killing ability of whole blood following LPS treatment, MDSCs in the blood were actually increased (Fig. 3.7A and 3.8). However, the frequency of other pro-inflammatory leukocyte populations, including monocytes, F4/80⁺ monocytes, and Ly6C⁻F4/80⁺ cells were also enhanced with LPS treatment (Fig. 3.7B-D and 3.8), perhaps compensating for the increase of MDSCs and enhanced *S. aureus* bactericidal activity. Examination of splenic leukocytes revealed an expansion of MDSCs with LPS treatment in infected mice (Fig. 3.9A and 3.10), while all other cell populations remained the same (Fig. 3.9B-C and 3.10). Previous studies have shown that under proper conditions, such as polymicrobial sepsis or in combination with IFN- γ , LPS administration can lead to the expansion and activation of MDSCs in the spleen [164, 294]. This expansion could account for increased MDSCs in the blood following LPS treatment (Fig. 3.7 and 3.8). At face value, these data appear to be contradictory. Namely, MDSC infiltrates are reduced in the joint of LPS treated mice, yet bacterial burdens are increased (Fig. 3.5 and 3.4). In contrast, MDSCs are increased in the blood of LPS treated mice, but peripheral blood leukocytes display improved killing of planktonic *S. aureus* (Fig. 3.7 and 3.3). Further explanation is needed to reconcile these seemingly contradictory results.

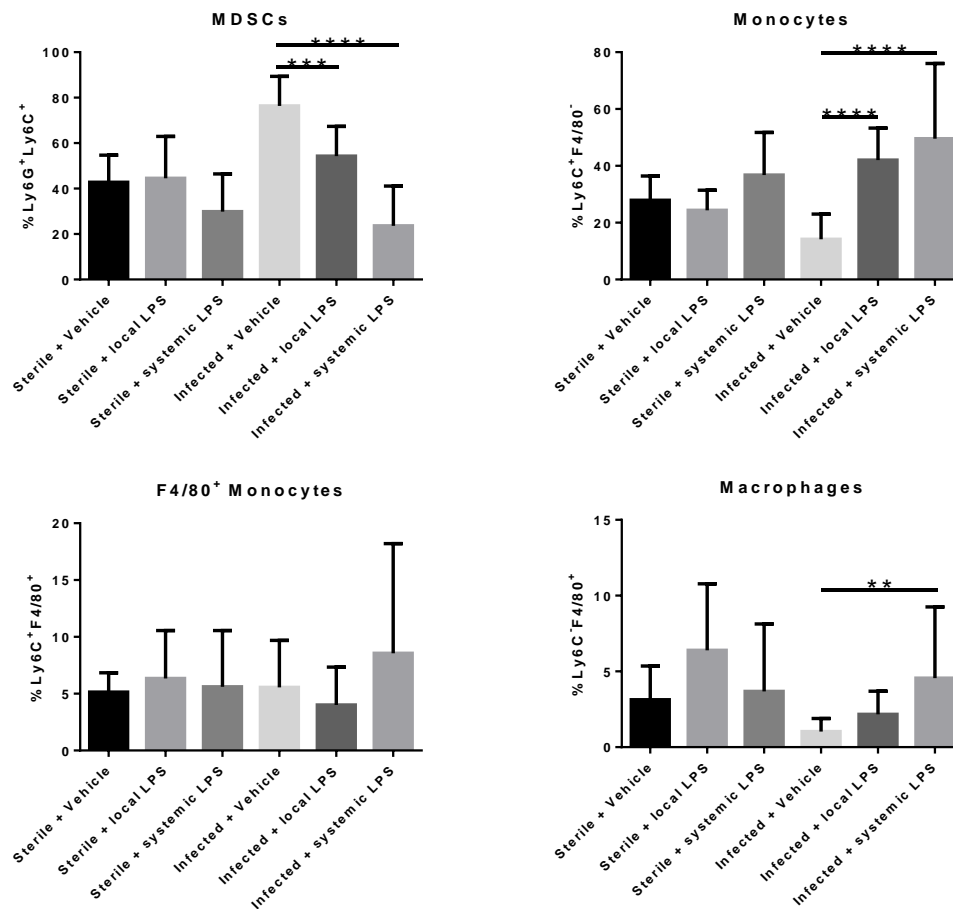


Figure 3.5. LPS administration alters leukocyte infiltrates during *S. aureus*

orthopedic biofilm infection. Implant-associated tissues from sterile and infected mice treated with local or systemic LPS (5 and 12.5 μg , respectively) or vehicle were collected 7 days after implantation of the orthopedic device, and analyzed by flow cytometry. Each bar graph represents quantitation of MDSCs (Ly6G⁺Ly6C⁺), inflammatory monocytes (Ly6G⁻Ly6C⁺), F4/80⁺ monocytes (Ly6C⁺F4/80⁺), and macrophages (Ly6C⁻F4/80⁺) in each treatment group. Results are expressed as a percentage of the total CD45⁺ leukocyte population. Results are representative of 5 mice per group from three independent experiments. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis, among sterile or infected groups separately.

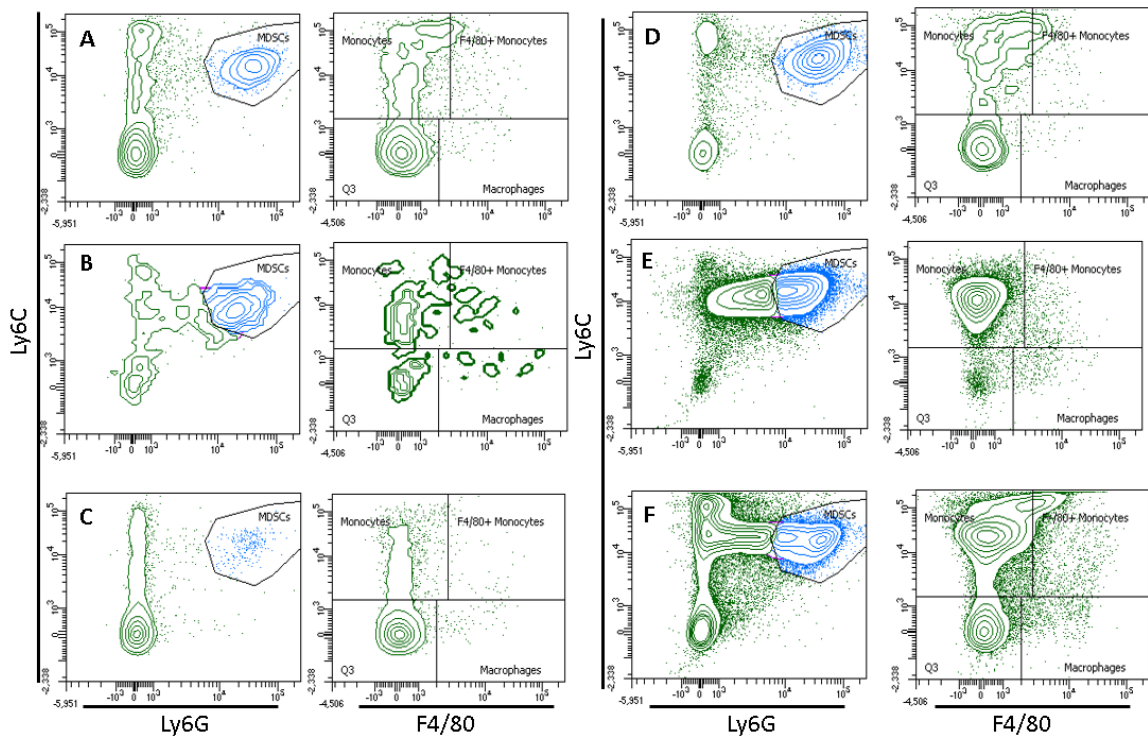


Figure 3.6. LPS administration alters leukocyte infiltrates during *S. aureus*

orthopedic biofilm infection. Implant-associated tissues from sterile and infected mice treated with local or systemic LPS (5 and 12.5 μg , respectively) or vehicle were collected 7 days after implantation of the orthopedic device, and analyzed by flow cytometry. Representative contour plots of MDSCs ($\text{Ly6G}^+\text{Ly6C}^+$), inflammatory monocytes ($\text{Ly6G}^-\text{Ly6C}^+$), F4/80^+ monocytes ($\text{Ly6C}^+\text{F4/80}^+$), and macrophages ($\text{Ly6C}^-\text{F4/80}^+$) present in each treatment group. **(A)** Sterile implant, vehicle treatment, **(B)** sterile implant, local LPS treatment, **(C)** sterile implant, systemic LPS treatment, **(D)** infected implant, vehicle treatment, **(E)** infected implant, local LPS treatment, and **(F)** infected implant, systemic LPS treatment.

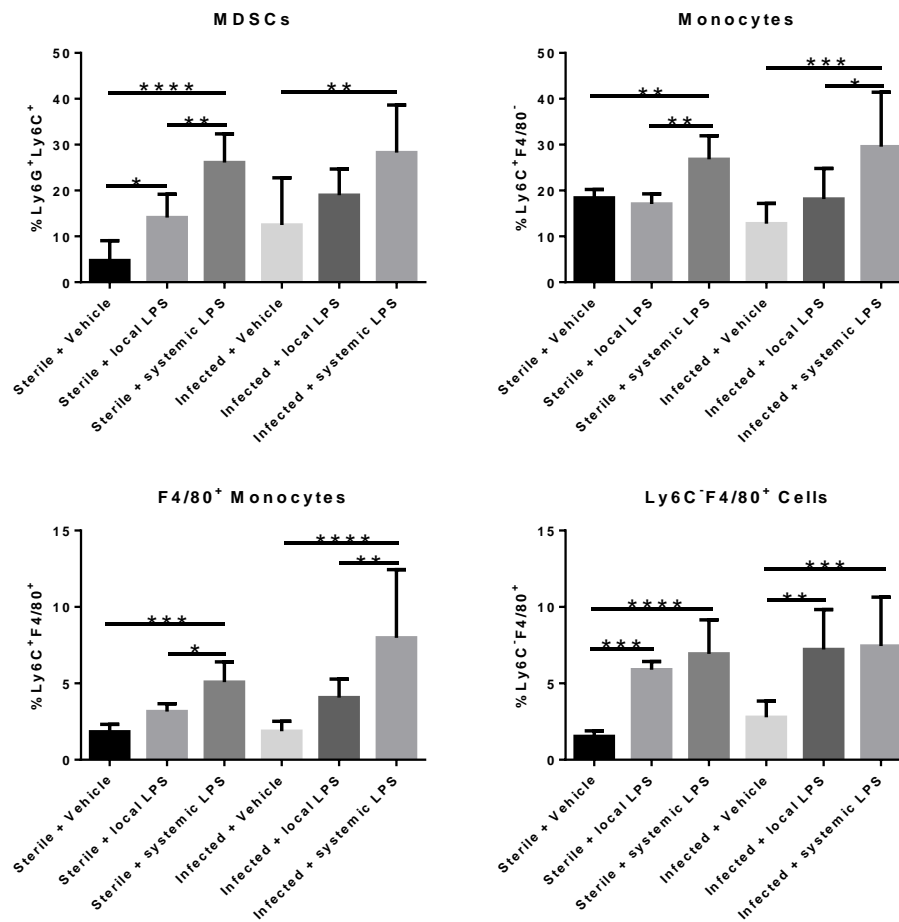


Figure 3.7. LPS administration alters peripheral blood leukocyte populations

during *S. aureus* orthopedic biofilm infection. Blood from sterile and infected mice

treated with local or systemic LPS (5 and 12.5 μg , respectively) or vehicle were collected 7 days after implantation of the orthopedic device, and analyzed by flow cytometry. Each

bar graph represents quantitation of MDSCs (Ly6G⁺Ly6C⁺), monocytes (Ly6G⁻Ly6C⁺), F4/80⁺ monocytes (Ly6C⁺F4/80⁺), and Ly6C⁻F4/80⁺ cells present in each treatment

group. Results are expressed as a percentage of the total CD45⁺ leukocyte population.

Results are representative of 5 mice per group from two independent experiments. * $p < 0.05$,

** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Bonferroni's multiple

comparison post-hoc analysis, among sterile or infected groups separately.

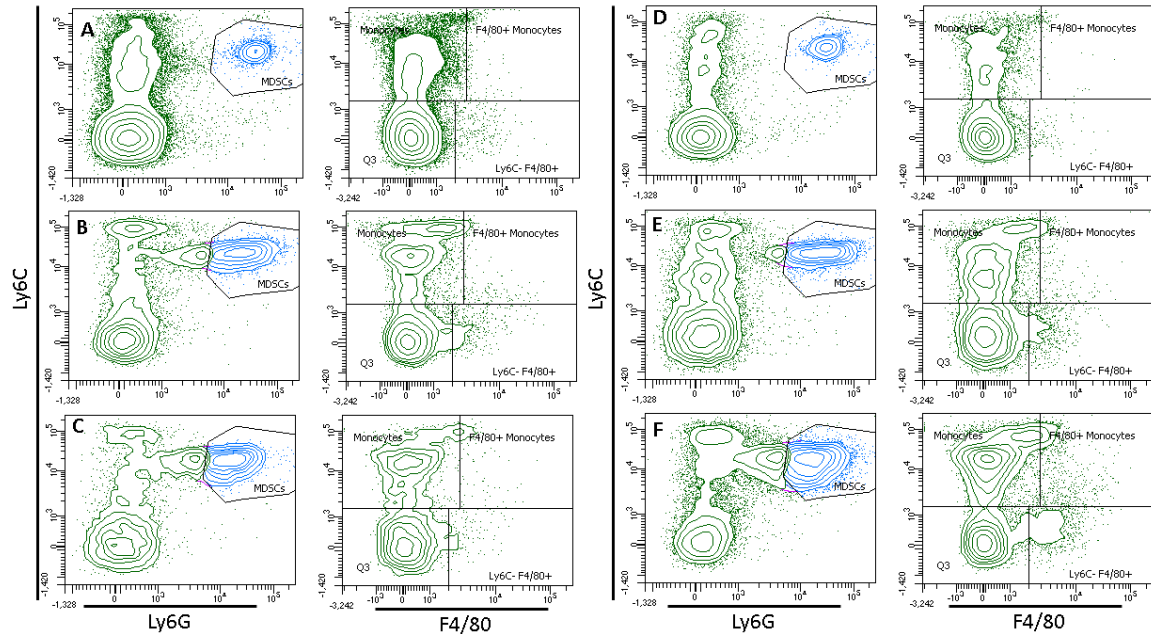


Figure 3.8. LPS administration alters blood leukocyte populations during *S.*

***aureus* orthopedic biofilm infection.** Blood from sterile and infected mice treated with local or systemic LPS (5 and 12.5 μg , respectively) or vehicle were collected 7 days after implantation of the orthopedic device, and analyzed by flow cytometry. Representative contour plots of MDSCs ($\text{Ly6G}^+\text{Ly6C}^+$), monocytes ($\text{Ly6G}^-\text{Ly6C}^+$), F4/80^+ monocytes ($\text{Ly6C}^+\text{F4/80}^+$), and $\text{Ly6C}^-\text{F4/80}^+$ cells present in each treatment group. **(A)** Sterile implant, vehicle treatment, **(B)** sterile implant, local LPS treatment, **(C)** sterile implant, systemic LPS treatment, **(D)** infected implant, vehicle treatment, **(E)** infected implant, local LPS treatment, and **(F)** infected implant, systemic LPS treatment.

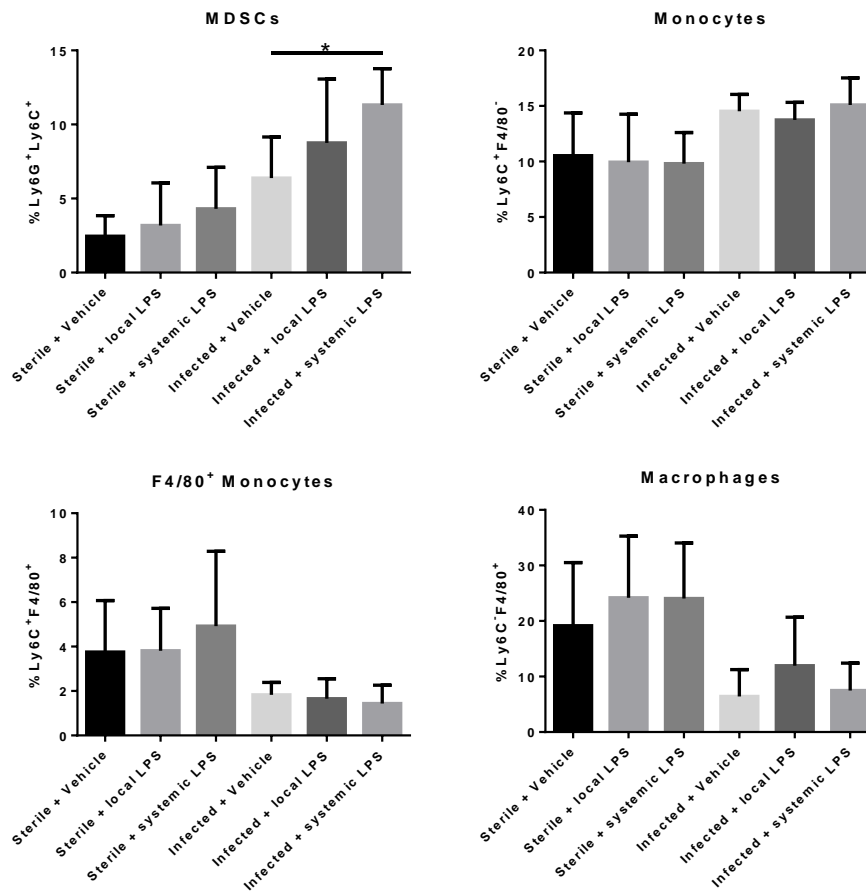


Figure 3.9. LPS administration alters splenic leukocyte populations during *S.*

aureus* orthopedic biofilm infection.** Spleens from sterile and infected mice treated with local or systemic LPS (5 and 12.5 μg , respectively) or vehicle were collected 7 days after implantation of the orthopedic device, and analyzed by flow cytometry. Each bar graph represents quantitation of MDSCs (Ly6G⁺Ly6C⁺), monocytes (Ly6G⁻Ly6C⁺), F4/80⁺ monocytes (Ly6C⁺F4/80⁺), and Ly6C⁻F4/80⁺ cells present in each treatment group. Results are expressed as a percentage of the total CD45⁺ leukocyte population. Results are representative of 5 mice per group from two independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, * $p < 0.0001$; one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis, among sterile or infected groups separately.

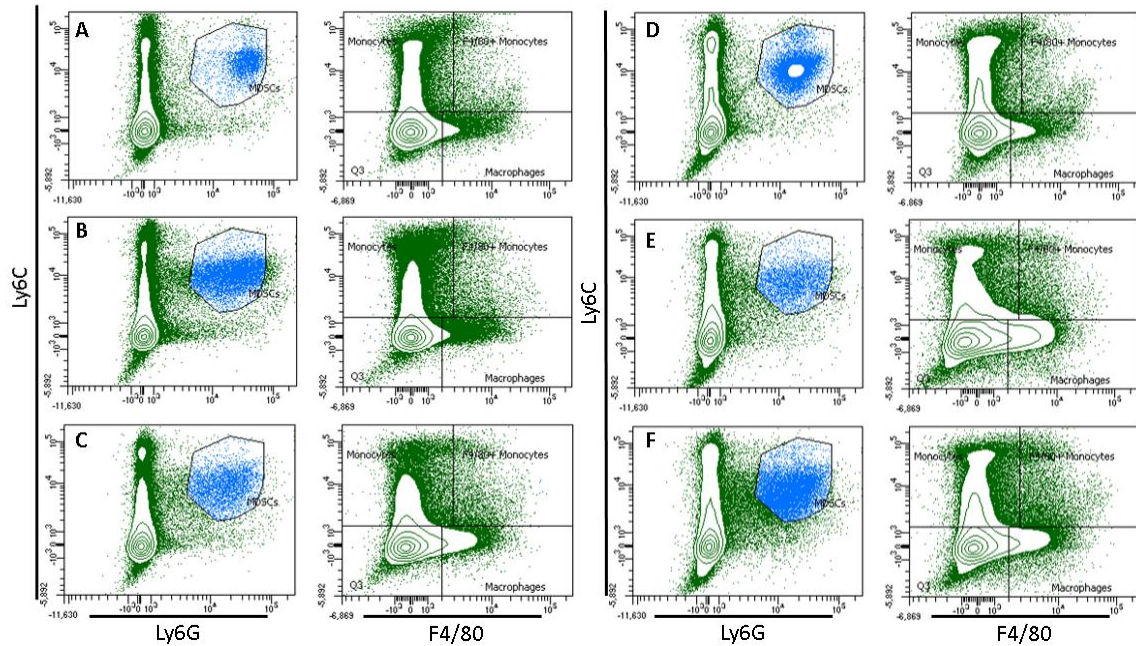


Figure 3.10. LPS administration alters splenic leukocyte populations during *S. aureus* orthopedic biofilm infection. Splens from sterile and infected mice treated with local or systemic LPS (5 and 12.5 μg , respectively) or vehicle were collected 7 days after implantation of the orthopedic device and analyzed by flow cytometry. Representative contour plots of MDSCs ($\text{Ly6G}^+\text{Ly6C}^+$), inflammatory monocytes ($\text{Ly6G}^-\text{Ly6C}^+$), F4/80^+ monocytes ($\text{Ly6C}^+\text{F4/80}^+$), and macrophages ($\text{Ly6C}^-\text{F4/80}^+$) present in sterile and infected animals of each treatment group. (A) Sterile implant, vehicle treatment, (B) sterile implant, local LPS treatment, (C) sterile implant, systemic LPS treatment, (D) infected implant, vehicle treatment, (E) infected implant, local LPS treatment, and (F) infected implant, systemic LPS treatment.

LPS treatment augments MDSC inhibitory activity. Our results have demonstrated that LPS treatment alters MDSC infiltrates into *S. aureus* biofilm infections, namely causing a shift from a predominant Ly6G⁺Ly6C⁺ towards a Ly6G^{low}Ly6C⁺ population, which introduced a Ly6G^{intermediate}Ly6C⁺ population (Fig. 3.6, 3.8, and 3.10). We hypothesized that Ly6G expression was downregulated in traditional Ly6G⁺Ly6C⁺ MDSCs following LPS treatment, nevertheless, these cells remained suppressive, accounting for the increased biofilm burdens. To determine the immunosuppressive nature of each population, Ly6G⁻Ly6C⁺ monocytes, Ly6G^{int}Ly6C⁺ Ly6G intermediates, and Ly6G⁺Ly6C⁺ MDSCs were purified by FACS and co-cultured with CD4⁺ T cells to determine which cell types could suppress T cell proliferation (Fig. 3.11). As expected, monocytes from all treatment groups did not inhibit T cell expansion (Fig. 3.12A). Ly6G intermediate cells from mice receiving systemic LPS appeared to possess some suppressive activity; however, this did not reach statistical significance (Fig. 3.12B). In general, MDSCs from LPS treated animals were more suppressive compared to MDSCs from the vehicle treatment mice (Fig. 3.12C). Although these differences were relatively modest, it is important to note that differences in total numbers of suppressive cells can amplify inhibitory activity *in vivo*. It is possible that by promoting MDSC activation and expansion, LPS treatment enhances anti-inflammatory activity at the site of infection, inhibiting immune-mediated clearance, inadvertently augmenting biofilm growth.

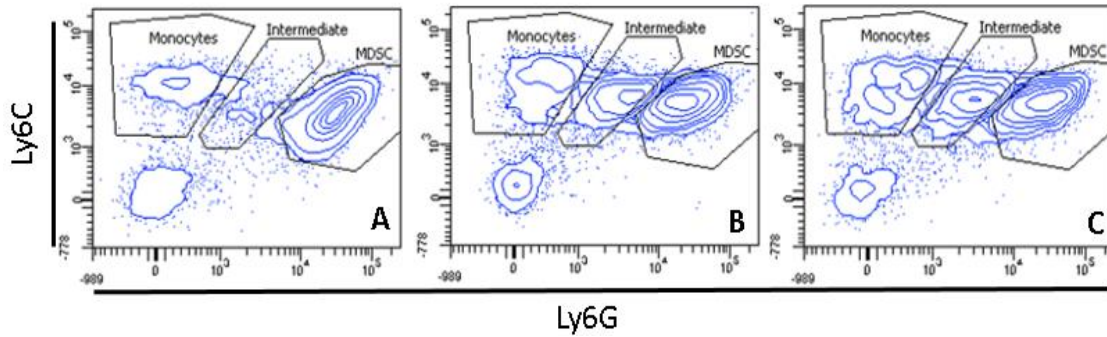


Figure 3.11. FACS purification of different leukocyte populations after *S. aureus* orthopedic biofilm infection. Leukocyte infiltrates associated with *S. aureus*-infected joints were collected by FACS at day 7 based on Ly6G expression. Contour plots of sorted monocytes (Ly6G^{hi}Ly6C⁺), Ly6G intermediates (Ly6G^{int}Ly6C⁺), and MDSCs (Ly6G^{lo}Ly6C⁺) in each treatment group: vehicle (**A**), local LPS (**B**), systemic LPS (**C**). All cell populations displayed are CD45⁺.

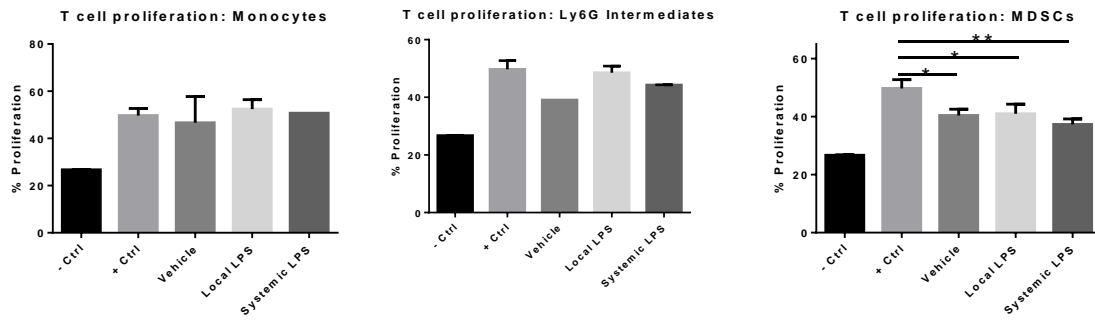


Figure 3.12. Effects of LPS treatment on MDSC T cell suppressive activity.

Leukocyte infiltrates associated with *S. aureus*-infected joints were collected by FACS at day 7 based on Ly6G expression. Analysis of *ex vivo* polyclonal CD4⁺ T cell proliferation following a 1:1 co-culture with (A) monocytes (Ly6G⁻Ly6C⁺), (B) Ly6G intermediates (Ly6G^{int}.Ly6C⁺), and (C) MDSCs (Ly6G⁺Ly6C⁺) for 72 h. Results are representative of one to three replicates. * $p < 0.05$, ** $p < 0.01$; one-way ANOVA with Bonferroni's multiple comparison post hoc analysis. (- Ctrl) T cells only; (+ Ctrl) T cells incubated with CD3/CD28 Dynabeads.

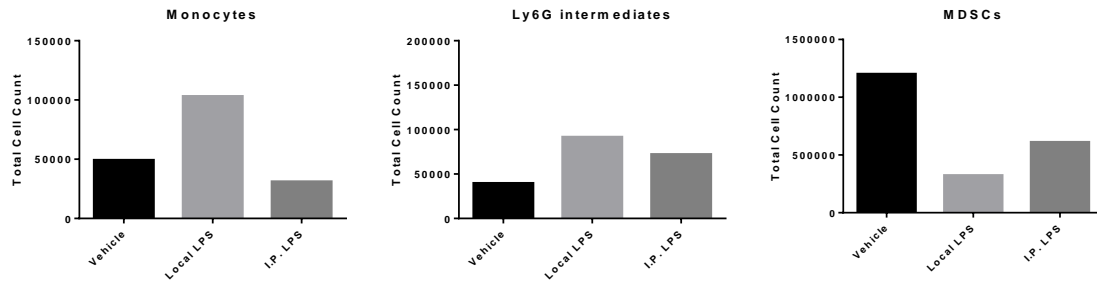


Figure 3.13. Impact of LPS on leukocyte abundance during *S. aureus* orthopedic biofilm infection. Abundance of the FACS purified Ly6G populations that were examined for T cell suppressive activity in Figure 3.XX above, including (A) monocytes (Ly6G⁻Ly6C⁺), (B) Ly6G intermediates (Ly6G^{int}-Ly6C⁺), and (C) MDSCs (Ly6G⁺Ly6C⁺). Results represent cells collected from eight animals per treatment group.

Transient but not sustained exposure to LPS causes an expansion of bone-marrow derived MDSCs *in vitro*. LPS is known to activate mature MDSCs in the presence of pro-inflammatory cytokines [294]; however, based on our findings we wanted to determine if LPS alone was capable of triggering MDSC expansion. To investigate this possibility, bone marrow cells were cultured for 4 days and treated with LPS either at the time of plating or the last 24 h prior to cell harvest. During this time, media was not changed. Sustained LPS treatment did not significantly affect MDSC expansion, even at the highest concentration of LPS examined (Fig. 3.14). Monocyte differentiation decreased approximately 20% with the highest LPS concentration; however macrophages and F4/80⁺ monocytes were not significantly altered (Fig. 3.14, 3.15).

In contrast, a dose-dependent increase in MDSCs was observed when cells were exposed to LPS for the last 24 h, with 100 ng/ml resulting in the greatest expansion (Fig. 3.16). It is likely that this increase in MDSCs may be attributed, in part, to reduced monocytes with increasing LPS concentrations, which are presumably shifting more towards an MDSC phenotype, as macrophages and F4/80⁺ monocytes remain unchanged following LPS treatment (Fig. 3.16, 3.17).

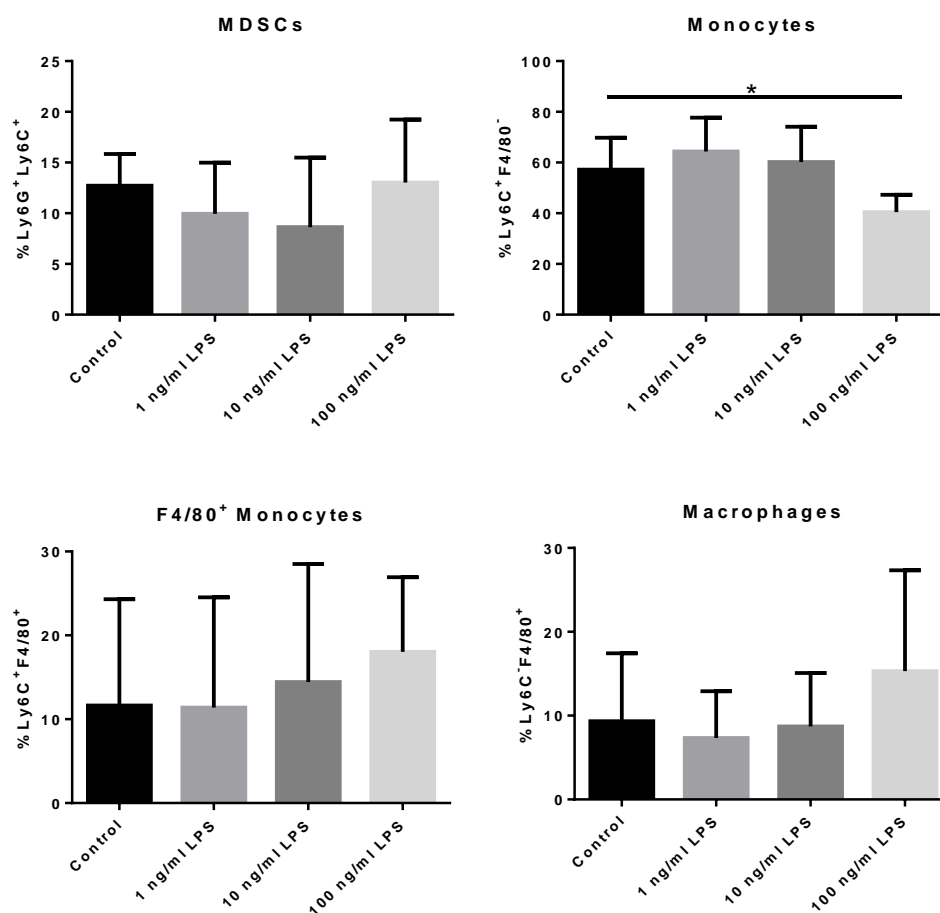


Figure 3.14. LPS treatment for 4 days does not alter bone marrow-derived leukocyte populations *in vitro*. Bone marrow cells were seeded in non-treated tissue culture plates with 1, 10, or 100 ng/ml LPS, or PBS as a control. After 3 days, cells were treated with 40 ng/ml IL-6, incubated for another 24 h, and stained for flow cytometry. Each bar graph represents quantitation of MDSCs (Ly6G⁺Ly6C⁺), monocytes (Ly6G⁻Ly6C⁺), F4/80⁺ monocytes (Ly6C⁺F4/80⁺), and macrophages (Ly6C⁻F4/80⁺). Results are expressed as a percentage of the total CD45⁺ leukocyte population. Results are representative of 9 replicates from three independent experiments. * $p < 0.05$, one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis.

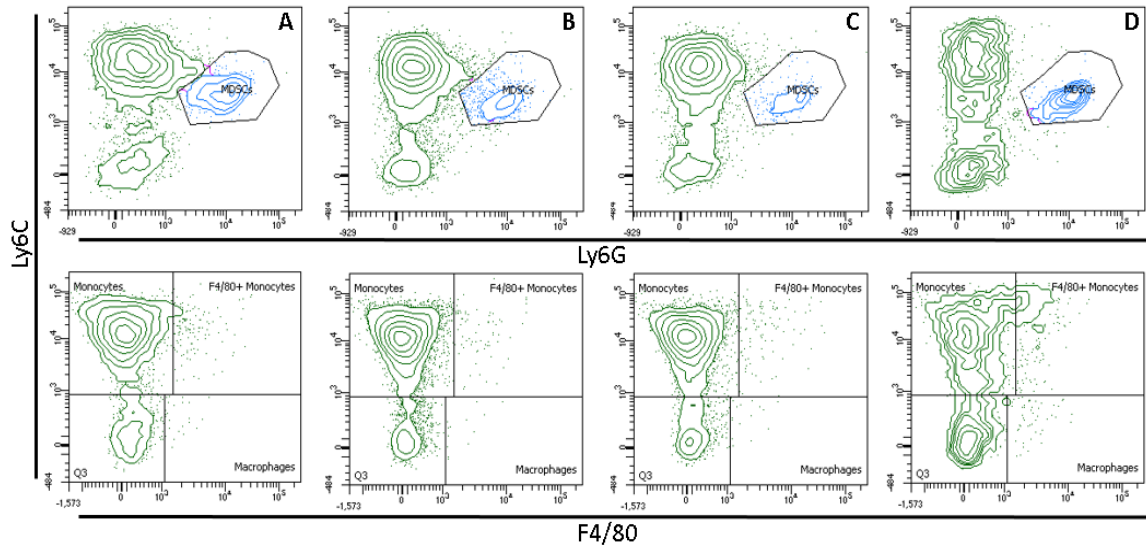


Figure 3.15. LPS treatment for 4 days does not alter bone marrow-derived leukocyte populations *in vitro*. Bone marrow cells were seeded in non-treated tissue culture plates with 1 (B), 10 (C), or 100 (D) ng/ml LPS, or PBS (A) as a control. After 3 days, cells were treated with 40 ng/ml IL-6, incubated for another 24 h, and stained for flow cytometry. Representative contour plots of MDSCs (Ly6G⁺Ly6C⁺), monocytes (Ly6G⁻Ly6C⁺), F4/80⁺ monocytes (Ly6C⁺F4/80⁺), and macrophages (Ly6C⁻F4/80⁺) are shown.

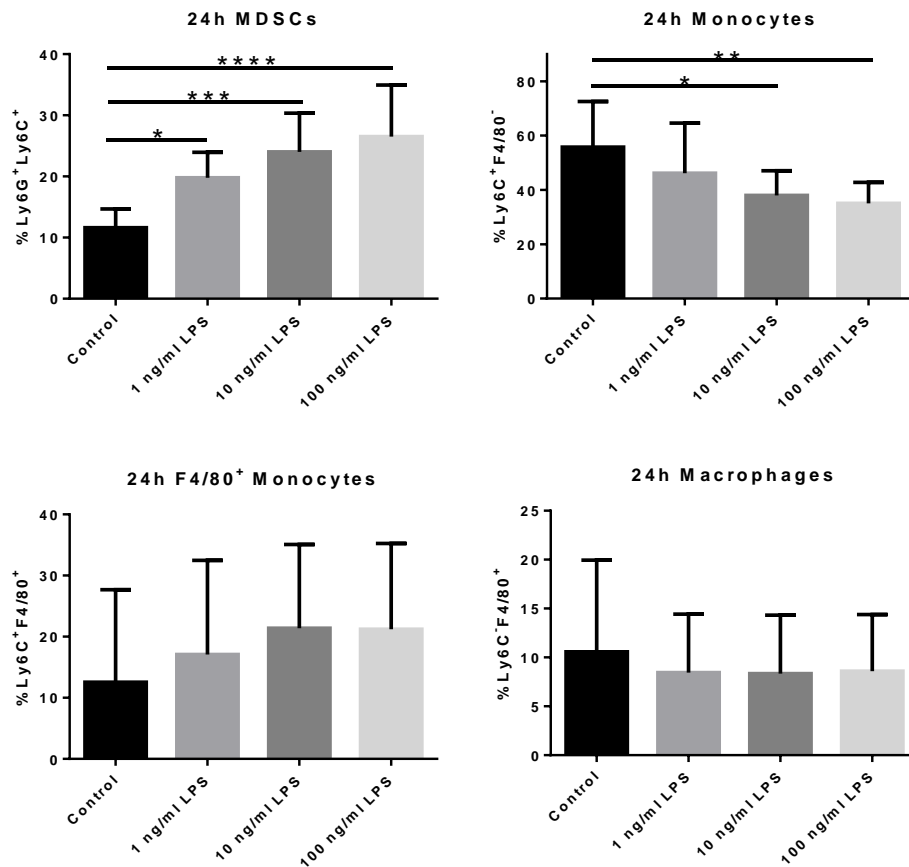


Figure 3.16. LPS treatment for 24 h expands bone marrow-derived MDSCs *in vitro*.

Bone marrow cells were seeded in non-treated tissue culture plates and incubated for 3 days, whereupon cells were treated with 40 ng/ml IL-6 \pm 1, 10, or 100 ng/ml LPS, or PBS as a control. After incubating for another 24 h, cells were collected and stained for flow cytometry. Each bar graph represents quantitation of MDSCs (Ly6G⁺Ly6C⁺), monocytes (Ly6G⁺Ly6C⁻), F4/80⁺ monocytes (Ly6G⁺F4/80⁺), and macrophages (Ly6G⁻F4/80⁺) in each treatment group. Results are expressed as a percentage of the total CD45⁺ leukocyte population. Results are representative of 9 replicates from three independent experiments. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; one-way ANOVA with Bonferroni's multiple comparison post-hoc analysis.

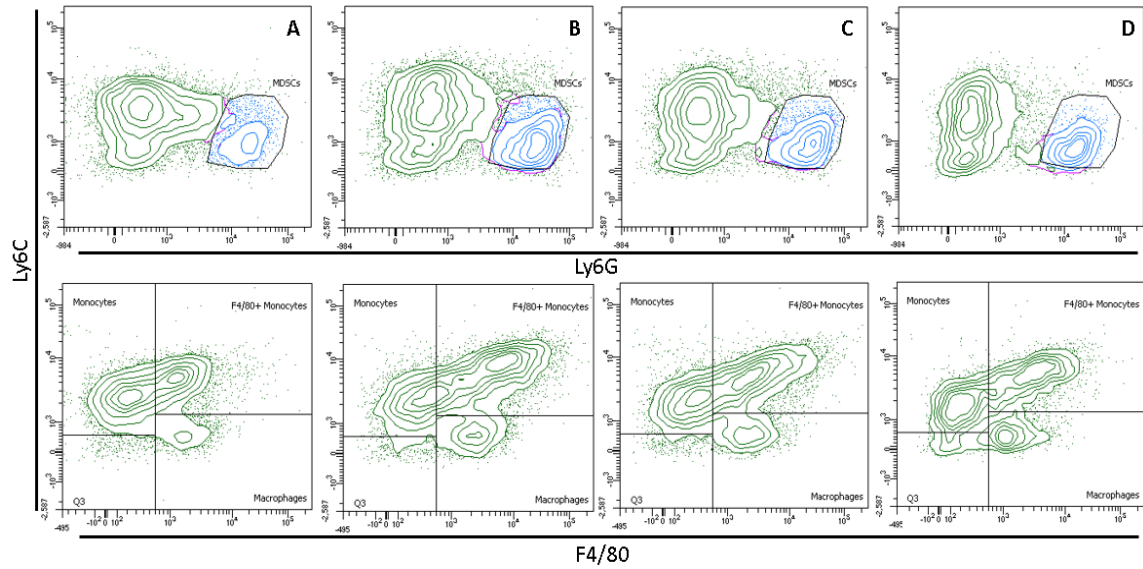


Figure 3.17. LPS treatment for 24 h expands bone marrow-derived MDSCs *in vitro*. Bone marrow cells were seeded in non-treated tissue culture plates and incubated for 3 days, whereupon cells were treated with 40 ng/ml IL-6 \pm 1 (B), 10 (C), or 100 (D) ng/ml LPS, or PBS (A) as a control. After incubating for another 24 h, cells were collected and stained for flow cytometry. Representative contour plots of MDSCs (Ly6G⁺Ly6C⁺), monocytes (Ly6G⁻Ly6C⁺), F4/80⁺ monocytes (Ly6C⁺F4/80⁺), and macrophages (Ly6C⁻F4/80⁺) in each treatment group.

Chapter 4. Discussion and Future Directions

Discussion

The immune response to *S. aureus* biofilms is largely ineffective, leading to chronic infections that require a significant amount of time, money, and antibiotics to treat. *S. aureus* evades and manipulates the host immune response through numerous mechanisms, including interference with antibody-mediated opsonization and complement activation, resistance to cationic antimicrobial peptides and ROS, impairment of phagocyte recruitment, and production of several leukocidins and toxins [5, 295]. Biofilm formation further protects the bacteria from antibiotics and host-mediated killing, making these infections particularly dangerous and difficult to eradicate. A major goal of our laboratory is to redirect the immune response to the biofilm to promote the recruitment and activation of bactericidal cells, rather than immune suppressive cells.

Prior studies have shown that *S. aureus* biofilms polarize macrophages toward an anti-inflammatory state and facilitate the preferential recruitment of MDSCs [12, 158]. MDSCs are notable for their expression of several immune-suppressive factors, such as Arg-1 and IL-10. Arg-1 activity depletes extracellular arginine, causing T cell dysfunction, and reduces its availability for iNOS function by macrophages [296]. We predicted that the introduction of a potent pro-inflammatory stimulus, specifically LPS, would induce the recruitment of leukocytes that were already programmed for bactericidal activity, which would aid in *S. aureus* biofilm clearance. Although LPS treatment did augment systemic pro-inflammatory mediator production, including G-CSF, IL-6, CXCL10, CCL2, and CXCL9, effects on bacterial clearance was mixed. For example, bacterial burdens were increased in the tissue surrounding the infected joint following LPS administration; however, more mice cleared the infection from sites distal to the primary infection site, such as the femur and deeper joint capsule. These findings suggest that LPS-dependent

activation could be limiting bacterial dissemination from the initial site of infection while simultaneously promoting a local microenvironment that augments biofilm growth. As further evidence suggesting that LPS treatment may discourage *S. aureus* dissemination, whole blood collected from LPS treated mice displayed improved killing of planktonic *S. aureus*. Collectively, these results suggest that leukocytes that have yet to have direct contact with the biofilm are effectively stimulated by LPS; however, the same cannot be said for immune cells located at the site of biofilm infection.

Although Ly6G⁺Ly6C⁺ MDSCs were decreased following LPS treatment, biofilm burdens were increased. This appears to be counterintuitive, as previous studies from our laboratory have partially ascribed biofilm immune evasion to MDSC activity, since Ab-mediated depletion of MDSCs promotes monocyte/macrophage pro-inflammatory activity and biofilm clearance [158]. However, it is important to consider that MDSCs are a heterogeneous population of immature monocytes and granulocytes that can be divided into different subsets based on their differential expression of Ly6C and Ly6G [161, 163, 297]. Namely, polymorphonuclear (PMN) and monocytic (M)-MDSCs are defined as CD11b⁺Ly6G⁺Ly6C^{lo} and CD11b⁺Ly6G⁻Ly6C^{hi}, respectively, and in our model of *S. aureus* orthopedic biofilm infection, the most suppressive cell population was found to be Ly6G⁺Ly6C⁺, highlighting the variable phenotype of these cells depending on environmental context [158, 285, 298]. Due to this variability of phenotypic markers, a functional assay, specifically the inhibition of T cells, is the gold standard used to define MDSCs [299]. Since LPS administration drastically changed the systemic immune environment by increasing pro-inflammatory mediator production, it was reasonable to predict that the phenotype and function of MDSCs may be significantly altered from what our laboratory has defined previously [40, 158, 276].

Further examination of Ly6G⁺Ly6C⁺ infiltrates revealed that although this population decreases in number with LPS treatment, it becomes more suppressive than in vehicle treated control animals. Additionally, the Ly6G^{int}Ly6C⁺ population is expanded by LPS administration, and displays some suppressive activity, although not significantly different from the vehicle control. Direct comparison of these cells is difficult due the low numbers that can be collected from vehicle treated mice; however, if the suppressive potential of Ly6G^{int}Ly6C⁺ cells can be confirmed in future studies their impact could be significant considering their abundance in the tissue of LPS treated mice. The expansion of this Ly6G-intermediate population combined with the increased suppressive capability of the classical MDSC population at the site of infection likely account, in part, for the increased biofilm growth in LPS-treated mice.

To explain the altered leukocyte infiltrates with LPS treatment, we examined cytokine/chemokine profiles in the different treatment groups. Overall, LPS administration appears to elicit a generalized upregulation of numerous pro-inflammatory factors, with a few exceptions. Specifically, G-CSF, IL-6, CXCL10, CCL2, and CXCL9 were significantly increased in the serum of LPS-treated mice, while only IL-1 α and CCL5 were reduced. Although typically considered pro-inflammatory, these cytokines can execute multiple functions, where the environmental context dictates the outcome. G-CSF and IL-6 are known to be important for MDSC expansion and activation, while CCL2 has been linked to MDSC migration [300-302]. Additionally, IL-10 levels were increased with LPS treatment; a pleiotropic cytokine that possesses numerous functions. In particular, IL-10 skews macrophages towards an anti-inflammatory phenotype and is a known product of activated MDSCs [303-305]. Our laboratory has shown that IL-10 contributes to *S. aureus* biofilm infection persistence, since IL-10 KO mice displayed reduced MDSC influx, increased monocyte and macrophage infiltrates, and improved

biofilm clearance compared to WT animals [41]. Overall, this cytokine profile represents an environment that is highly favorable for MDSC recruitment and proliferation, via CCL2, IL-6, and G-CSF, as well as heightened MDSC activity through increased IL-10 production.

With increasing distance from the primary site of biofilm infection, another conundrum emerges. Namely, although MDSC infiltrates increase following systemic LPS administration, whole blood from this treatment group displayed improved bacterial killing compared to the PBS-treated control group. This result may be explained by the increased recruitment of bactericidal effectors, such as monocytes and Ly6C⁺F4/80⁺ cells. We propose that these cells remain pro-inflammatory and are minimally inhibited by surrounding MDSCs, perhaps because the peripherally expanded MDSC population is not fully activated, and may not become activated until coming into contact with unknown factors in the biofilm milieu. Meanwhile in the blood, phagocytes are hyperactivated by LPS treatment, promoting phagocytosis and bactericidal activity, which discourages dissemination. As stated earlier, this is further supported by the increased incidence of bacterial clearance in the femur and joint of LPS treated mice. These results suggest that LPS alone is not enough to activate MDSCs. Our *in vitro* studies revealed that sustained LPS treatment did expand MDSCs from the bone marrow. Previously, it has been reported that LPS in combination with IFN- γ could lead to enhanced MDSC functions, such as NO release and T cell suppression; however, these effects were not observed with single treatments *in vitro* [294]. Pertinent to the experiments described in this report, IL-6 acts as the second signal rather than IFN- γ , and concomitant treatment with LPS and IL-6 leads to an increased expansion of MDSCs rather than treatment with IL-6 alone. As we continuously discover in immunology, timing is everything. Simultaneous exposure of an immune stimulant and

MDSC activators leads to a significant effect on cell populations, while staggered treatment has little to no effect.

Collectively, these studies demonstrate that the immune response and function of effector cells can be highly variable depending on the environment in the context of *S. aureus* biofilm infections. Attempts to redirect the anti-inflammatory immune response to biofilms towards a more pro-inflammatory response had unexpected consequences. Namely, hyperactivation of the immune response through LPS treatment may limit bacterial dissemination, but worsens localized biofilm infections. The host immune system-biofilm interaction is multi-faceted and complex, and there are still many areas that remain to be examined. Identifying the factors that control the immune switch from a pro-inflammatory to anti-inflammatory state at the site of biofilm infection is an ongoing effort in our laboratory.

Future Directions

Determine the suppressive capability of peripheral MDSCs

Although the results and conclusions proposed by this study are intriguing, further examination of certain aspects is needed to confirm preliminary observations. In particular, MDSCs in the blood of LPS-treated mice are suspected to be inactive, due to the bactericidal nature of the other peripheral blood leukocyte populations; however, this has yet to be confirmed. The activation state of peripheral blood MDSCs can be confirmed by analysis of iNOS and IL-10 expression, as well as their ability to inhibit T cell proliferation. MDSCs from the blood and biofilm-infected tissues can be collected by FACS, and their suppressive capability compared to confirm that peripheral blood MDSCs are not fully activated, and require additional signals to promote an anti-inflammatory environment. Additionally, MDSC expansion was observed in the spleen of LPS treated mice but these cells are expected to be non-suppressive, however this remains to be determined.

Utilization of CXCR2 KO mice to examine the effect of LPS on *S. aureus* dissemination

Bacterial dissemination to systemic organs is typically not observed in wild type mice during *S. aureus* orthopedic biofilm infection; therefore, it is difficult to determine if LPS treatments truly prevent bacterial dissemination with the current data. Utilization of CXCR2 KO mice could be an acceptable alternative to address this question. CXCR2 is responsible for the recognition of multiple chemokines including CXCL1, CXCL3, CXCL5, and CXCL7 in mice [306-309], which likely regulate MDSC influx into *S. aureus*-infected tissues. Recognizing that there are confounding factors with regards to

differences in leukocyte infiltrates, CXCR2 KO mice display significant bacterial dissemination to systemic organs due to unchecked bacterial expansion, and may be a good model to determine if systemic LPS treatment could thwart biofilm dissemination by augmenting the bactericidal activity of peripheral blood leukocytes.

Transcriptional analysis of biofilm-associated MDSCs

From the unexpected results of this study, and many others, it is clear that there is much left to be learned about the immune response to *S. aureus* biofilm infections. Our laboratory has initiated steps towards gathering more information through the transcriptional profiling of biofilm-associated MDSCs and macrophages via RNA sequencing (RNA-Seq). Differential expression analysis revealed thousands of genes that were up- or down-regulated in both immune cell types when comparing responses to planktonic *S. aureus* vs. biofilms. This data set has confirmed previous results published by our laboratory [12, 41, 158], including increased IL-10 production by biofilm-associated MDSCs, and decreased expression of several pro-inflammatory cytokines from biofilm-associated macrophages. Further mining of this data set will likely identify other potential processes regulating immune responses to biofilm infections. The use of bioinformatics to perform pathway analysis and gene ontology enrichment analysis will highlight interesting genetic targets to explore, and will provide plenty of future directions in our laboratory for years to come. Understanding the role of immune cells during *S. aureus* biofilm infections, including the signals required to expand and activate MDSCs, as well as the signals that inhibit macrophages and other pro-inflammatory cells, may allow for the development of novel therapeutic targets that will improve the immune response and allow the host to clear these infections with minimal antibiotic intervention.

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