Evaluation of Anti-Glucosaminidase Monoclonal Antibodies as a Passive Vaccination for Implant-Associated *Staphylococcus aureus* Osteomyelitis

By

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This dissertation is dedicated to: my wife, Amanda; my daughters, Arianna and Isabella; my father and mother, Robert and Verna Varrone; my father-in-law and mother-in-law, Richard and Laura Paddon; and all of my family and friends who have all supported me throughout this entire journey.
Biographical Sketch

John J.P. Varrone was born in New Haven, Connecticut. He received his Bachelor of Science degree in Biology (Vertebrate Physiology) from the Pennsylvania State University in 2002, and his Master of Science degree in Biology from the Southern Connecticut State University in 2009. John began his doctoral studies in Pathology at the University of Rochester School of Medicine and Dentistry in July 2009. He was awarded a Ruth L. Kirschstein NRSA T32 training grant from the Department of Orthopaedics Center for Musculoskeletal Research in May 2010, and received his Master of Science degree in Pathology from the University of Rochester School of Medicine and Dentistry in November 2011 (formally awarded May 2012). John was an invited student representative for the Department of Pathology and Laboratory Medicine Admissions Committee (2014), student liaison to the University of Rochester Board of Trustees Student Affairs Committee (2011), and is a past President, Vice President, and Social Chair of the Department of Pathology and Laboratory Medicine Graduate Student Council. He is also an active member of the American Society for Microbiology and volunteer firefighter in the Town of Chili, Rochester, New York. He pursued his research in *Staphylococcus aureus* vaccine development under the direction of Dr. Edward M. Schwarz, Ph.D., and has presented his work at the Orthopaedic Research Society (2011, 2012, 2013, and 2014) and Staphyloccocal Diseases Gordon Research Conference (2013) national meetings.
The following publications were a result of work conducted or published during doctoral study:


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Abstract

Toward the development of a passive immunization to prevent implant-associated *Staphylococcus aureus* osteomyelitis in patients undergoing total joint replacement surgery, here we evaluated the effects of a candidate anti-glucosaminidase (Gmd) mAb (1C11) in vitro and in vivo. Using our highly reproducible murine model of implant-associated osteomyelitis, we observed a reduced severity of infection in mice that received 1C11 one day prior to challenge with the bacterial inoculum, and demonstrated that tibiae explanted from these protected mice at day 14 post-infection were void of a necrotic sequestrum and had an overall reduced osteolytic lesion at the site of pin insertion. We also established the presence of defective biofilms on the surface of explanted pins from the 1C11 immunized and ΔGmd challenged mice, presumably from inhibited colonization of the implant surfaces. In vitro, 1C11 had no direct effects on proliferation, but electron microscopy demonstrated that anti-Gmd mAb phenocopies ΔGmd defects in binary fission. Additionally, 1C11 treated samples exhibited a rough-textured cell wall surface morphology studded with small knob-like structures, similar to bacteria treated with subinhibitory concentrations of vancomycin. In vitro analyses to understand the mechanism of action demonstrated that prolonged treatment (4 hours) with 1C11 promotes the formation of large clusters (“megaclusters”), of which increased their own degree of opsonophagocytosis by mouse macrophages. Collectively, these data suggest that anti-Gmd mAb may enhance the host innate immune response to *S. aureus* infection, therefore reducing bacterial spread and the overall severity of infection.
Contributors and Funding Sources

This work was supervised by a thesis committee consisting of Dr. Edward M. Schwarz (Advisor), Dr. Paul M. Dunman, Dr. Craig N. Morrell, and Dr. Lianping Xing, and Chaired by Dr. Steven R. Gill. All work described herein was performed by the author independently, with the following exceptions: scanning electron microscopy (SEM), transmission electron microscopy (TEM), 100X oil immersion light microscopy, and gold sputter coating of samples for SEM were performed by Karen L. De Mesy Bentley; sections for TEM were epoxy resin embedded, cut, and stained by Gayle Schneider; and micro-CT was performed by Michael Thullen. The *S. aureus* strains UAMS-1, UAMS-1 Δspa, and LAC USA300 (MRSA) were gifts from Dr. Paul M. Dunman. The bioluminescent LAC USA300 MRSA strain (LAC::lux) was a gift from Dr. Tammy L. Kielian of the University of Nebraska Medical Center. The *S. aureus* strain RN4220, pWedge plasmid, and phi-11 bacteriophage for development of our Gmd-deficient LAC USA300 MRSA strain (LAC ΔGmd) were gifts from Dr. Steven R. Gill. The protein A-deficient LAC USA300 MRSA strain (LAC ΔSpa) was a gift from Dr. Kohei Nishitani. Primer sets for the development of LAC ΔGmd were designed in collaboration with Dr. Gregory S. Canfield (from Dr. Steven R. Gill’s laboratory) and Dr. John M. Morrison (from Dr. Paul M. Dunman’s laboratory).

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<tr>
<td>$\alpha$T2m</td>
<td>Anti-T2 mycotoxin (irrelevant control antibody)</td>
</tr>
<tr>
<td>$\Delta$Gmd</td>
<td>Glucosaminidase-deficient mutant</td>
</tr>
<tr>
<td>$\Delta$Spa</td>
<td>Protein A-deficient mutant</td>
</tr>
<tr>
<td>1C11</td>
<td>Candidate anti-glucosaminidase monoclonal antibody</td>
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<tr>
<td>Aaa</td>
<td>Autolysin/adhesin from <em>Staphylococcus aureus</em></td>
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<tr>
<td>AGLAA</td>
<td>Alanine-Glutamine-Lysine-Alanine-Alanine peptide stem</td>
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<tr>
<td>Amd</td>
<td>Amidase</td>
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<tr>
<td>APC</td>
<td>Antigen presenting cell</td>
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<tr>
<td>Atl</td>
<td>Autolysin</td>
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<tr>
<td>BLI</td>
<td>Bioluminescent imaging</td>
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<tr>
<td>CFU</td>
<td>Colony forming unit</td>
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<tr>
<td>ClfA</td>
<td>Clumping factor A</td>
</tr>
<tr>
<td>Cy</td>
<td>Cytoplasm</td>
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<tr>
<td>DAIR</td>
<td>Debridement/antibiotics/implant retention approach</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle medium</td>
</tr>
<tr>
<td>ECM</td>
<td>Extracellular matrix</td>
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<tr>
<td>Fab</td>
<td>Fraction, antigen binding (of an antibody)</td>
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<tr>
<td>Fc</td>
<td>Fraction, crystallizable (of an antibody)</td>
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<tr>
<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FDA</td>
<td>Food and drug administration</td>
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<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>FET</td>
<td>Fisher’s exact test</td>
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<tr>
<td>Gmd</td>
<td>Glucosaminidase</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IgG1</td>
<td>Immunoglobulin G subclass 1</td>
</tr>
<tr>
<td>IgG2(a/b)</td>
<td>Immunoglobulin G subclass 2(a/b)</td>
</tr>
<tr>
<td>IgM</td>
<td>Immunoglobulin M</td>
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<tr>
<td>IVIG</td>
<td>Intravenous immunoglobulin</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>mAb(s)</td>
<td>Monoclonal antibody/antibodies</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MRSA</td>
<td>Methicillin-resistant <em>Staphylococcus aureus</em></td>
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<tr>
<td>MSCRAMMMs</td>
<td>Microbial surface components recognizing adhesive matrix molecules</td>
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<tr>
<td>NAG</td>
<td>N-acetylglucosamine</td>
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<tr>
<td>NAM</td>
<td>N-acetylmuramic acid</td>
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<tr>
<td>NETs</td>
<td>Neutrophil extracellular traps</td>
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<td>Nu</td>
<td>Nucleus</td>
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<tr>
<td>Nuc</td>
<td><em>Staphylococcus aureus</em> thermonuclease gene</td>
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<tr>
<td>O.D.</td>
<td>Optical density</td>
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<tr>
<td>PAMPs</td>
<td>Pathogen-associated molecular patterns</td>
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<tr>
<td>PBS</td>
<td>Phosphate-buffered saline</td>
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<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>qPCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
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<tr>
<td>RANKL</td>
<td>Receptor activator of nuclear factor kappa-B ligand</td>
</tr>
<tr>
<td>ROS</td>
<td>Reactive oxygen species</td>
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<tr>
<td>SCIN</td>
<td><em>Staphylococcus</em> complement inhibitor</td>
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<td>SEM</td>
<td>Scanning electron microscopy</td>
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<tr>
<td>TEM</td>
<td>Transmission electron microscopy</td>
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<tr>
<td>TJR</td>
<td>Total joint replacement</td>
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<tr>
<td>TLR-2</td>
<td>Toll-like receptor 2</td>
</tr>
<tr>
<td>TLR-9</td>
<td>Toll-like receptor 9</td>
</tr>
<tr>
<td>TMP/SMX</td>
<td>Trimethoprim/sulfamethoxazole</td>
</tr>
<tr>
<td>TSB</td>
<td>Tryptic soy broth</td>
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<tr>
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Chapter One

Introduction
1.1 Implant-associated *Staphylococcus aureus* osteomyelitis

The artificial total joint arthroplasty procedure has been marveled as one of mankind’s greatest surgical achievements,\(^1\)\(^2\) offering people with debilitating conditions of the joint, such as osteoarthritis or rheumatoid arthritis, a much higher quality of life. The overall safety and effectiveness of this procedure cannot be disputed. However, even with improvements in surgical technique and the aggressive use of prophylactic antibiotics in hospitals and clinics, one percent of total joint replacements (TJR) performed in the United States each year become infected.\(^3\) Recent reports suggest that even if the best technical skills and preventative measures could be obtained, the rate of infection, at best, would still be 0.5%.\(^4\)

Given that over 1.5 million TJR surgeries are performed annually worldwide,\(^1\) 15,000 patients each year that are developing infections as a result of their surgeries. This absolute number of infections is based on statistics from the United States and assumes that each country performing the TJR procedure has a similar one percent annual rate of infection, which we know may not be entirely accurate since hospitals and clinics around the world do not always report their annual infection rates. These implant-associated infections can be extremely debilitating to the patient, since infection of the bone (osteomyelitis) is characterized by progressive inflammatory bone destruction (osteolysis) coupled with reactive bone formation, both of which can lead to a permanent state of disrepair. Furthermore, as Americans live longer and demand a high quality of life, a steady increase in the number of procedures performed annually will likely result in an increase in the absolute number of infections.\(^5\)
Bacteria of the genus *Staphylococcus* are involved in 65-70% of all orthopaedic-associated infections,\(^6\) approximately 50% of which are clinical isolates of methicillin-resistant *S. aureus* (MRSA) acquired in both hospital and community settings.\(^7,8\) Direct inoculation via a traumatic accident, surgical incision, or accidentally-contaminated orthopaedic implant is the most common route of *S. aureus* infection, though the initial infection may occur due to hematogenous seeding of the pathogen from another site in the body.\(^4\)

Regardless of the pathogen’s origin, implant-associated *S. aureus* infections that affect the bone and surrounding tissues are often initially treated using a minimalistic and conservative method, called the debridement/antibiotics/implant retention (DAIR) approach. This approach can usually be performed when the causative pathogen is not resistant to one or more of the antibiotics currently used to treat *S. aureus* infections. However, surgeons trying to eradicate MRSA infected TJR are often required to perform an aggressive two-stage exchange arthroplasty in lieu of the DAIR approach.\(^9-12\) This two-stage procedure involves removal of the infected primary implant, thorough debridement of all necrotic bone and soft tissue surrounding the initial infection site, repeated cleansings with sterile saline, and the administration of empirical systemic and local antibiotic therapy. If the patient responds well and the infection is suppressed, then re-implantation may occur weeks or months later, or when cultures taken several days prior to the re-implantation surgery are declared culture negative.\(^5\) Unfortunately, reinfection rates for MRSA infected TJR are extremely high (15-40%), and additional surgical intervention may be required if the infection returns.\(^9-13\) If the infection cannot
be controlled with multiple rounds of surgical intervention and long-term suppressive antibiotics, then arthrodesis (joint fusion) or amputation of the affected limb may become necessary.\textsuperscript{14}

1.2 Brief history of \textit{Staphylococcus aureus}

\textit{Staphylococcus} was first identified in 1881 by the Scottish surgeon Sir Alexander Ogston, who isolated it from the skin and soft tissue wounds of patients he was treating at the Aberdeen Royal Infirmary. He named these bacteria after their ability to grow as small grape-like clusters, unlike another type of bacteria he was isolating from these wounds that grew as chains (\textit{Streptococcus}). Several years later in 1884, the German scientist Anton Rosenbach isolated single colonies of \textit{Staphylococcus} that were gold in color, to which he gave the name \textit{Staphylococcus aureus} (\textit{S. aureus}). This helped to distinguish them from other colonies of \textit{Staphylococcus} that were creamy-white in color (\textit{S. albus}; later renamed \textit{S. epidermidis}). \textit{S. aureus} was the pathogen responsible for the majority of skin and soft tissue infections found in hospitals in the late 19\textsuperscript{th} century, and, in addition to deep-seated infections of the limbs, often led to severe pneumonia and bacteremia in these patients due to a lack of treatment options at the time.

It was not until four decades later that the first antibiotics were discovered. Alexander Fleming accidentally stumbled upon penicillin in 1928 when he made an observation that the green mold \textit{Penicillium notatum} prevented his \textit{S. aureus} cultures from growing on agar plates that had become contaminated with the mold during plating. He isolated and grew the mold in pure culture, and showed that its extracts also prevented
the growth of a number of other common pathogens. However, it was not until 1941 that penicillin could be manufactured from *P. notatum* in quantities large enough to be made commercially available to the public.

The widespread use of penicillin in the 1940s successfully cured many *S. aureus* infections, but its overuse in hospitals and private clinics was resulting in the emergence of penicillin-resistant strains. In 1959, penicillin was no longer considered effective, and methicillin was introduced into the clinic as an alternative. However, in 1961 the first strain of methicillin-resistant *S. aureus* (MRSA) emerged in the United Kingdom. Vancomycin, which was introduced around the same time as methicillin and long considered the drug of last resort for the treatment of MRSA infections, eventually replaced methicillin. Though following several decades of its successful use in the clinic, the Centers for Disease Control and Prevention now reports that twelve vancomycin-resistant strains have been isolated in the United States since 2002. In fact, resistant strains of *S. aureus* have been identified for every antibiotic introduced into clinical practice.

### 1.3 *Staphylococcus aureus* and its peptidoglycan cell wall architecture

*S. aureus* is a Gram-positive, nonspore-forming coccus (spherical shaped bacterium) that is approximately one micron (\(~1 \mu m\)) in diameter. It is both coagulase- and catalase-positive, meaning that it can clot human plasma and catalyze the decomposition of the reactive oxygen species (ROS) hydrogen peroxide, respectively. As a facultative anaerobe, *S. aureus* is capable of replicating aerobically through aerobic
respiration or anaerobically through fermentation. In either case, cell division (binary fission) occurs asexually, producing two identical cells that contain the exact genomic and accessory plasmid DNA as the parent. Continuous cell division occurs in successive perpendicular planes, giving rise to small clusters of bacteria (2-8 bacteria) that define the genus (“bunch of grapes”).\textsuperscript{17,18}

Being a Gram-positive organism, \textit{S. aureus} possesses a thick peptidoglycan cell wall that immediately surrounds its plasma membrane, with very little periplasmic space separating the two structures. Compared to Gram-negative bacteria, which have a much thicker periplasmic space and are encased in a peptidoglycan cell wall that is approximately 5-10 nm thick,\textsuperscript{19} the peptidoglycan cell wall that encases \textit{S. aureus} is 20-40 nm thick.\textsuperscript{20} This thickness is required to maintain the spherical bacterium’s turgor pressure as remodeling of the cell wall occurs, and to prevent the daughter bacteria from bursting as they double in size following division.\textsuperscript{21}

The peptidoglycan cell wall is initially synthesized in the cytoplasm as a precursor disaccharide subunit that is comprised of one \textit{N}-acetylMuramic acid (NAM) and one \textit{N}-acetylglucosamine (NAG) sugar moiety, linked to one another by a single $\beta$-1,4 glycosidic bond. Next, the disaccharide subunits are stitched together on the surface of the plasma membrane by forming additional $\beta$-1,4 glycosidic bonds between them, a process that is catalytically facilitated in the cytoplasm by penicillin binding proteins (PBP). This process results in the creation of long polysaccharide (glycan) strands of alternating NAM and NAG sugar subunits, which are then further crosslinked to form the peptidoglycan superstructure.\textsuperscript{22} In the case of \textit{S. aureus}, an AGLAA (alanine-glutamine-
lysine-alanine-alanine) peptide stem branches off of every NAM subunit, and becomes crosslinked by a pentaglycine bridge that connects the lysine on one glycan strand’s NAM subunit to the second alanine on the adjacent parallel glycan strand’s NAM subunit. This finalizes the cell wall building process, and results in the highly crosslinked architecture of the peptidoglycan cell wall.

In order to separate daughter cells following binary fission, *S. aureus* must be able to hydrolyze (degrade) its own peptidoglycan cell wall in a tightly regulated and highly orchestrated manner. This degradation process requires autolytic enzymes, which are produced only on demand in order to protect the bacterium against any accidental degradation and lysis. To maintain this high level of regulation, each enzyme specifically cleaves one bond within the peptidoglycan cell wall and is only localized to that specific region of the cell wall for a brief period of time. This allows the enzyme to perform its intended function and then quickly leave the area, preventing any deleterious side effects from occurring.

The presence of cell wall associated autolytic enzymes in *S. aureus* was first recognized in the late 1950s – 1960s. The early biochemical experiments of Jack Strominger and his colleagues, which comprehensively characterized the peptidoglycan cell wall architecture of several Gram-positive bacterial species and spanned nearly two decades, paved the way for the full analysis of these autolytic enzymes and their detailed mechanism of interaction with the Staphylococci.
1.4 Interaction of *Staphylococcus aureus* with the host

Upon entry into the host, *S. aureus* is quickly recognized by cells that comprise the cellular component of the innate immune system. Pathogen-associated molecular patterns (PAMPs) on the surface of the bacterium bind to and activate Pattern Recognition Receptors (PRRs), of which results in two separate protective functions: 1) intracellular signaling and the release of regulatory molecules (i.e., cytokines) which can act directly to stimulate innate or adaptive immunity (via signaling PRRs), or 2) promotion of endocytosis through binding of the bacterium to host phagocytes (via endocytic PRRs). In the case of professional phagocytes such as the macrophage, endocytic PRRs allow the phagocyte to bind to, and then internalize, the bacterium in a process known as phagocytosis (aka, engulfment). Once engulfed, the bacterium is trapped inside a phagocytic vesicle (the phagosome), which then fuses with a lysosome thus forming the phagolysosome. The bacterium is usually destroyed once it is contained within the phagolysosome and, in the case of professional antigen presenting cells (APC) such as the macrophage, presented on major histocompatibility complex (MHC) Class II. However, regarding *S. aureus* phagocytosis, internalization does not always result in destruction of the bacterium.

Phagocytosis is also stimulated when the bacterium is opsonized, either through nonspecific binding with complement protein C3b or specific binding with antibody to an antigen on its surface, in a process called opsonophagocytosis. If pathogen-specific antibodies are present, then binding of the antibody to the specific antigen on the bacterium’s surface induces a conformational change in its Fc region. This
conformational change permits two antibody-dependent events to occur: 1) binding by complement protein C1q and activation of the complement cascade, or 2) binding to Fc receptor (FcR) on the phagocytic cell. These events then directly result in bacteriolysis or phagocytosis, respectively.

To try and avoid the host immune response, *S. aureus* must outsmart the host immune system and quickly attach itself to host tissue extracellular matrix (ECM) proteins. It binds to these ECM proteins through cell wall associated adhesins, the most common of which are called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules)\(^4\). These MSCRAMMs grant *S. aureus* the ability to bind to a wide array of host proteins, including fibrinogen, fibronectin, and collagen, and are crucial for early colonization of the host tissues.\(^{29}\) Binding of these host proteins also allows *S. aureus* to mask itself, thereby avoiding opsonization and thus preventing its recognition as a foreign pathogen.

### 1.5 *Staphylococcus aureus* immune evasion strategies

*S. aureus* has acquired, through many years of evolution and by residing on humans as a part of our normal skin and mucosal flora, a vast number of survival tools to overcome any host defenses it encounters upon entering a wound.\(^{30}\) This model also applies to MRSA.

First, *S. aureus* can evade many host defenses by: 1) expressing factors (i.e., protein A) that incorrectly orient IgG antibodies on its surface so that the bacteria are not recognized by the neutrophil or macrophage Fc receptor, 2) its ability to inactivate serum
complement with *Staphylococcus* Complement Inhibitor (SCIN), 3) its ability to form a capsule to prevent phagocytosis by neutrophils and macrophages, and 4) its resistance to lysozyme (via membrane-bound O-acetyltransferase). S. aureus can also release toxins (i.e., alpha-hemolysin) that damage the membrane of host cells, leading to leakage of intracellular components and eventual lysis, thus granting *S. aureus* cytolytic activity against leukocytes and erythrocytes.

Second, it has been shown that *S. aureus* can survive within mammalian cells, leading to additional protection from the host immune response and induction of apoptosis in professional and nonprofessional phagocytes. Research by Bayles et al. suggests that binding of *S. aureus* MSCRAMMs to the mammalian cell initiates pseudopod formation and endocytosis, allowing the bacterium to become internalized. Then, in order to release itself from within the lysosome, the bacterium can upregulate one of its cytolytic toxins (i.e., alpha-hemolysin) and induce lysis of the mammalian cell from within. Recent work by Kubica et al. confirms these findings in professional phagocytes, and states that the bacterium survives within the lysosome in a metabolically active state until the intracellular environment is perfect for escape. This would allow the bacterium to, once again, gain access to the host’s circulatory system.

Finally, *S. aureus* can survive in a dormant state referred to as a biofilm, which is impenetrable by antibodies, phagocytes, and antibiotics. The persistence of *S. aureus* infections is often attributed to biofilm formation within the necrotic bone, and recent reports suggest that these biofilms can evade TLR-2 and TLR-9 recognition in vivo. Additionally, the bacterium can upregulate factors (i.e., phenol soluble modulins) that
allow its escape from the biofilm, allowing yet another way for the bacterium to enter the host’s circulatory system and reinitiate infection. Therefore, complete removal by the host is extremely difficult, if not impossible.

1.6 Current treatment regimens for *Staphylococcus aureus* infected total joint replacements

Antibiotics are systemically (often intravenously) administered to patients undergoing a TJR procedure immediately prior to surgery as part of standard surgical prophylaxis in most hospitals and private institutions. Broad-spectrum antibiotics offer the most antimicrobial coverage, and thus are administered in order to prevent infections from a number of typical disease-causing microorganisms. However, surgical prophylaxis does not always confer protection, and recent reports suggest that “no preventative measure is likely to lower the infection rate below 0.5%.”

In the event of a MRSA infected TJR, debridement of necrotic bone and drainage of soft-tissue abscesses is necessary. Device removal is advised for early-onset and acute infections, and is strongly recommended if the infection is late-onset or chronic in nature. Parenteral (systemic and/or implanted) antibiotics are then administered for a minimum period of 8 weeks, followed by parenteral or enteral (oral) antibiotics for an additional 3-6 months in the case of osteomyelitis or an infected TJR, respectively. However, if long-term suppression is deemed necessary, the antibiotics may be continued for longer periods of time (>6 months) if implant removal was not permissible during the debridement-drainage process, or if the infection persists.
At present, combination vancomycin, daptomycin, and rifampin therapy is the recommended treatment for early-onset and acute MRSA infected TJR in adults, and is administered for a minimum of 8 weeks. Then, rifampin may be administered in combination with trimethoprim/sulfamethoxazole (TMP/SMX), a tetracycline (i.e., doxycycline), a fluoroquinolone (i.e., ciprofloxacin), or clindamycin until the infection is cleared. In the event of a late-onset or chronic MRSA infected TJR, a combination regimen involving TMP/SMX, a tetracycline, or a fluoroquinolone is given in conjunction with rifampin. In children, vancomycin alone is the recommended treatment of choice, but clindamycin, daptomycin, and/or linezolid may be used. Unfortunately, these antibiotics often come with many unwanted side effects, further substantiating the need for novel therapeutics in the clinic.

1.7 The quest for the perfect Staphylococcus aureus vaccine

Due to the overuse of antibiotics in clinics and hospitals over the past five decades, bacteria have been developing resistance at an alarming rate. History demonstrates that as fast as we are introducing new antibiotics, bacteria are developing resistance to them. Furthermore, it is projected that they will continue to do so as additional antibiotics become FDA approved and are introduced into practice. This strongly emphasizes the need for alternative or complementary therapeutics to prevent infection in patients undergoing TJR surgery. This is especially true for immunocompromised patients and the elderly who collectively account for the majority
of patients, and who are also not ideal candidates for active vaccination due to a suppressed or delayed immune response.

Despite tremendous efforts, an effective vaccine against *S. aureus* remains elusive, as several clinical trials utilizing active or passive antibody-based therapeutics have failed. These vaccine candidates may have failed in clinical trials for several reasons. First, as it is well known that *S. aureus* resides on humans as part of our normal skin and mucosal flora, much of the population already has high serum titers to this pathogen. Second, *S. aureus* is the causal organism of a very broad range of diseases, most notably sepsis, endocarditis, and osteomyelitis, thus making it extremely difficult to select a proper protective outcome. Therefore, the focus has been on trying to determine which antigen epitopes to target in order to create or enhance host immunity, and to determine which disease or disease timeframe these antigen targets are specifically involved in.

### 1.8 Glucosaminidase as a target for a *Staphylococcus aureus* passive immunotherapy

In Gram-positive bacteria, autolysins play an important role in cell separation and peptidoglycan cell wall remodeling during binary fission. The bifunctional 138-kDa *S. aureus* autolysin is transported by an unknown mechanism to the outside of the pathogen and is proteolytically processed on its cell surface to produce two extracellular peptidoglycan hydrolases, *N*-acetylmuramyl-*L*-alanine amidase (amidase, 62-kDa) and endo-β-*N*-acytethylglucosaminidase (glucosaminidase, 51-kDa), of which remain non-covalently attached to the outside of the bacterium. Cell wall binding repeat
domains, two C-terminal (R1, R2) on amidase (Amd) and one N-terminal (R3) on glucosaminidase (Gmd), are responsible for the docking of these two active enzymes to the peptidoglycan cell wall, where they bind to exert their effects before their subsequent release into the extracellular milieu.\textsuperscript{45}

Yamada et al. performed some of the first experiments identifying the cell surface localization of autolysin and its role in \textit{S. aureus} cell division.\textsuperscript{49} In 1996, this group published data demonstrating that gold-labeled antibodies generated against the autolysin gene products (Amd and Gmd) aggregated in a double-ring formation at the septum of dividing cells, giving this structure an appearance that is often referred to as an equatorial ring. They reported that as the bacteria continued to divide, approximately half of the double-ring structure stayed on each adjacent daughter cell. Based on these results, they proposed that the autolysin gene products form this double-ring structure at the site of the future septum, and then begin to digest the peptidoglycan that connects the two adjacent daughter cells. Once separated, the autolysin gene products migrate and begin to align in a ring-like formation at the next septal site, where the process is repeated.

It was two years later that Baba and Schneewind first reported that three repeat domains (R1 – R3) were necessary and sufficient for the targeting of autolysin to the Staphylococcal peptidoglycan cell wall equatorial ring.\textsuperscript{45} They demonstrated that immediately following this targeted localization of autolysin to the equatorial ring, proteolytic processing of the bifunctional protein is allowed to occur. This series of events permits the localized hydrolysis of peptidoglycan to occur, thus inducing the separation of the actively dividing bacteria. They also suggested that receptors for the
repeat binding domains must be arranged within the cell wall in a similar fashion (in an equatorial ring), and turned over frequently to prevent hydrolysis at old division sites. However, whether this docking is receptor-driven or the result of a wall teichoic acid avoidance strategy remains unknown.\textsuperscript{50}

There are many features that make Gmd a very attractive target for an anti-\textit{S. aureus} vaccine investigation. First, it is highly conserved among Staphylococci. For example, Gmd is greater than 95\% conserved across all strains of \textit{S. aureus} in the public database, and approximately 85\% conserved among other Staphylococci.\textsuperscript{51} Second, it is essential for complete separation of daughter cells following binary fission. \textit{S. aureus} bacteria deficient in Gmd still divide, but daughter cells fail to separate, leading to the generation of large clusters that fall out of suspension.\textsuperscript{52} Additionally, these clusters can be dispersed by the addition of Gmd to cultures.\textsuperscript{53} Third, Gmd is located on the extracellular surface of the bacterium, potentially focusing the immune response on a vulnerable part of the cell. In support of this, scanning electron micrographs of anti-Gmd immune complexes on the surface of \textit{S. aureus} demonstrated that the antibody binds in immediate proximity to digested cell wall.\textsuperscript{49} Fourth, Gmd appears to play a role in biofilm formation.\textsuperscript{22,54,55} For example, previous studies demonstrated that Gmd is involved in the primary attachment of \textit{Staphylococcus} bacteria to a polymer surface,\textsuperscript{55} and that the murein hydrolases are regulated by effector genes that control bacterial death and lysis in the case of biofilm formation.\textsuperscript{22,54} Finally, elevated levels of anti-Gmd antibodies were detected in serum from mice that survived a challenge with \textit{S. aureus}.\textsuperscript{56}
Collectively, this information provides a strong rationale for anti-Gmd therapy for *S. aureus* orthopaedic infections, as the antibodies have multiple potential mechanisms to achieve cytostatic and cytolytic activity. Additionally, the extremely high evolutionary conservation of the enzyme suggests that compensatory mutations to achieve antigenic variation and immune evasion may not be possible.

1.9 Thesis overview

Given that Gmd is so intimately involved in *S. aureus* cell division and the early colonization of implant surfaces, the goal of this study is to evaluate the effect of anti-Gmd mAb on *S. aureus* growth in vitro and in vivo. Using a highly reproducible murine model of *S. aureus* implant-associated osteomyelitis developed in our lab, coupled with a panel of novel assays designed and developed specifically for this thesis project, this dissertation research assessed the protective effects of one candidate neutralizing anti-Gmd mAb (1C11) by its ability to reduce the severity of infection and promote bacterial clearance by the host. We will demonstrate how 1C11 affects osteolysis of cortical bone at the site of pin insertion, and implant biofilm formation on the surface of stainless steel pins. A potential mechanism by which 1C11 protects mice from implant-associated *S. aureus* infection will also be presented. Understanding the specific early events involved in the host response to implant-associated *S. aureus* infection will aid in the development of novel antibody-based therapeutics, and may one day help eradicate these devastating infections.
Chapter Two

Materials and Methods
2.1 Preparation of monoclonal antibodies

Murine anti-Gmd IgG1 mAbs (clones 1C11, 1E12, 2D11, 3A8, and 3H6) were purified from hybridoma cell line culture supernatant as previously described. Briefly, the hybridoma cell lines were grown in DMEM media containing 10% FBS and 10 µg/ml gentamicin, and purified from the culture supernatant using Protein G sepharose (GE Healthcare, Wauwatosa, WI). Following purification of each mAb, the eluate was concentrated with Pierce Concentrators (Thermo Scientific, Rockford, IL), sterile-filtered using a 0.22 µm PES syringe filter unit (VWR, Radnor, PA), and standardized to 1 mg/ml aliquots prior to their use in our in vitro and in vivo assays. The irrelevant IgG1 control low-endotoxin/azide-free murine mAbs, anti-T2 mycotoxin (αT2m) and MOPC-21, were obtained from Southern Biotech (Birmingham, AL) and Abcam (Cambridge, MA), respectively.

2.2 Growth assessment assays

Dose-dependent effects (0.05 - 50 µg/ml) of 1C11 vs. placebo (PBS or αT2m mAb) on LAC USA300 MRSA growth was determined by longitudinal optical density (O.D.) at 490 nm in 96-well plates, tritiated-thymidine incorporation, WST-8 tetrazolium salt reduction, and quantitative colony forming unit (CFU) assays, as follows.

For the longitudinal O.D. growth assays, LAC USA300 was first grown overnight at 37°C for 18 hours in tryptic soy broth (TSB) supplemented with erythromycin (10 µg/ml). Next, 2 ml of each culture at 10^6 CFU/ml was dose-dependently treated in one of three ways: 1) sterile PBS; 2) an isotype-matched mouse IgG1 mAb (αT2m); 3) anti-
Gmd IgG1 mAb 1C11. Replicates of five were performed for each sample at a volume of 0.2 ml per well of a 96-well microtiter plate. Bacterial planktonic growth was then measured at the indicated intervals by O.D. at 490 nm, and the results were graphed as a function of O.D. vs. time.

For the tritiated-thymidine incorporation assays, LAC USA300 (1 ml per treatment group at 10^6 CFU/ml) was first added to 17X100 mm bacterial culture tubes (Fisher Scientific, Waltham, MA), and treated with placebo (volume-matched PBS) or 1C11 (50 µg/ml). Tritiated thymidine was then added to all samples to a final concentration of 10 mCi/ml per sample, placed in a 37°C static incubator for variable time points, and assayed in a Beckman Coulter LS 6500 multipurpose scintillation counter (Beckman Coulter, Miami, FL) as previously described.58

For the WST-8 proliferation assay, 10^4 CFU LAC USA300 MRSA was grown at 37°C for 3.5 hours in the presence of placebo (volume-matched PBS) or 50 µg/ml 1C11. The water-soluble tetrazolium salt, WST-8, was then added and the cultures were allowed to grow at 37°C for an additional 3.5 hours. Samples were read at 450 nm, and the average of five samples was used for the analysis.

For the CFU assay, 10^7 CFU each LAC USA300 MRSA and LAC ΔGmd were grown at 37°C for 4.5 hours in the presence of placebo (volume-matched PBS) or 75 µg/ml 1C11 (n=3 per treatment group). Serial dilutions of each sample were then plated and incubated overnight at 37°C, and the number of colonies observed was graphed as a function of log_{10} CFU for each treatment group.
2.3 Glucosaminidase inhibition assay

Gmd neutralizing activity of the mAb was determined via cell wall digestion assay. Briefly, *Micrococcus luteus* bacteria (ATCC, Manassas, VA) were grown overnight at 37°C in LB media and diluted in PBS to an optical density of 0.7 at 490 nm. Purified recombinant His-Gmd (32 µg/ml in PBS) was added to the dilutions of mAb to a volume of 0.1 ml in a 96-well microtiter plate. 0.1 ml of bacteria was then added to each well, and O.D. was measured at 0 and 30 minutes using a BioTek ELx800 plate reader (BioTek Instruments, Winooski, VT). Percent inhibition was calculated as 100*(1-(\(\Delta_{60A_{490}}\) inhibitor/\(\Delta_{60A_{490}}\) no inhibitor control)) as illustrated in Figure 2.1. For in vivo use, mAb were sterile-filtered using a 0.22 µm PES syringe filter unit (VWR, Radnor, PA) before injection.

2.4 *Staphylococcus aureus* strains

The *S. aureus* strains UAMS-1, UAMS-1 protein A-deficient (ΔSpa), and LAC USA300 (MRSA) were generous gifts from Dr. Paul M. Dunman. The *S. aureus* strain RN4220 was a generous gift from Dr. Steven R. Gill, and the protein A-deficient LAC USA300 MRSA strain (LAC ΔSpa) was a gift from Dr. Kohei Nishitani. We generated a LAC USA300 glucosaminidase-deficient isogenic mutant (LAC ΔGmd), as described below. The bioluminescent *S. aureus* strain Xen29 was purchased from Caliper Life Sciences (Hopkinton, MA). The bioluminescent strain LAC::lux was generously provided to us by Dr. Tammy L. Kielian.
2.5 Generation of glucosaminidase-deficient LAC USA300 MRSA

We generated a LAC USA300 Gmd-deficient isogenic mutant (LAC ΔGmd) strain via homologous recombination using a gene knockout shuttle vector (pWedge) provided by Dr. Steven R. Gill as follows. Upstream and downstream Gmd fragments were generated by PCR of LAC USA300 genomic DNA using the following primer sets: Gmd-Up-Fwd-eagl and Gmd-Up-Rev-sacII to amplify a 518-bp upstream portion, and Gmd-Down-Fwd-sacII and Gmd-Down-Rev-acc65i to amplify a 514-bp downstream portion (Table 2.1). The resultant PCR products were digested with SacII and ligated to form a 1032-bp fragment before purification and digestion with Eagl/Acc65I (New England Biolabs, Ipswich, MA). The digested 1032-bp fragment was then cloned into the same restriction sites in the pWedge E. coli/S. aureus shuttle vector and transfected into DH5α E. coli (Invitrogen, Carlsbad, CA) for propagation. This plasmid was then electroporated into RN4220 S. aureus to produce recombinant phi-11 bacteriophage, which was then used to transduce LAC USA300 MRSA. Genomic Gmd deletion was confirmed with PCR, and the absence of Gmd protein was confirmed by Western blotting (Fig. 2.2A) using a mix of our five anti-Gmd mAb as the primary antibody. We also confirmed that alpha-hemolysin activity (virulence) was not affected during the homologous recombination steps of the Gmd deletion process, as presented in Figure 2.2B.
2.6 Preparation of round pins for transtibial implantation

For the pathogenic challenge, Size 000 (0.25 mm diameter) round stainless steel insect pins (Austerlitz, Czech Republic) were autoclaved and stored in 70% ethanol until use. The pins were air-dried before incubation in a 5 ml overnight culture of Xen29 S. aureus for 20 minutes, and then allowed to air dry at room temperature for 5 minutes before transtibial placement. Quantification determined a bacterial load of 1.33 +/- 0.67 x10^5 CFU Xen29 per round pin, similar to a previous report.56

2.7 Preparation of flat pins for transtibial implantation

For the pathogenic challenge, 0.2 mm thick x 0.5 mm wide stainless steel ribbon (MicroDyne Technologies, Plainville, CT) was bent into a 3 mm long L-shaped (flat) pin before being autoclaved. The pins were incubated in a 5 ml overnight culture of LAC::lux MRSA for 20 minutes, and then allowed to air dry at room temperature for 5 minutes before transtibial placement. Quantification determined a bacterial load of 2.11 +/- 1.16 x10^5 CFU LAC::lux (USA300) MRSA per flat pin.

2.8 Passive immunization and assessment of implant-associated osteomyelitis in mice using round or flat pins

All in vivo experiments were performed on protocols approved by the University of Rochester Committee on Animal Resources. Forty 8-10 week old female BALB/cJ mice (The Jackson Laboratory, Bar Harbor, ME) were randomized to placebo (volume-matched PBS) or 1 mg of 1C11 mAb (40 mg/kg i.p.) one day prior to the surgery. The
mice were anesthetized with Ketamine (100 mg/kg) and Xylazine (10 mg/kg) immediately prior to surgery, then their right legs were shaved and the skin was cleansed with 70% ethanol. A 1 cm incision was made slightly medial to the tibial crest, and the skin was retracted for pin placement. In the case of the round pin, implant-associated osteomyelitis was induced in the right tibia of the mice by inserting the pin transtubially in a medial to lateral fashion, and the ends bent to prevent movement. In the case of the flat pin, a hole was first created transtubially in a medial to lateral fashion with 30G and 27G sterile needles, respectively, and then the flat pin was press-fit in the hole with the bend end of the L-shaped flat pin facing posteriorly. Finally, for either surgery, the surgical incision was closed with a single 5-0 Ethilon suture (Ethicon, Somerville, NJ).

Mice were removed from either group if they died of anesthesia following the surgery, during longitudinal Bioluminescent Imaging (BLI), or if a mouse removed its pin during the course of the 14-day experiment. BLI of all mice used in our studies was performed under Isoflurane (Butler Schein, Dublin, OH) at the indicated time points post-pin insertion using the Xenogen IVIS Spectrum imaging system (Caliper Life Sciences, Hopkinton, MA). Three minute high-sensitivity ventral images were taken at each time point to measure the total bacterial load of metabolically active bioluminescent *S. aureus* in each mouse, and then the BLI was quantified using the LivingImage 4.0 software package (Caliper Life Sciences, Hopkinton, MA). The total photon flux (in photons per second) emitted from a 1.5 cm diameter region of interest focused on the center of the pin was used for our analyses. After BLI on day 14 the mice were euthanized and their tibiae were assessed by micro-CT. The specimens were scanned at 12.5 microns isotropic
resolution using the VivaCT 40 (Scanco Medical AG, Bassersdorf, Switzerland) and 3D reconstruction of the osteolytic lesion was performed and quantified as previously described59.

2.9 Scanning electron microscopy

For explanted pin analysis by scanning electron microscopy (SEM), pins were removed from mice at sacrifice, fixed in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight at 4°C, osmicated, dehydrated, and then placed onto stainless steel stubs and sputter coated with gold for qualitative analysis.

For in vitro cluster analysis by SEM, LAC USA300 or LAC ΔGmd was grown for 12 hours in TSB media to achieve a mid-log growth suspension. For the treatment groups, bacteria were incubated with PBS, or 50 µg/ml of αT2m or 1C11 for 1 hour. Samples were then plated onto BD Biocoat poly-L-lysine coated glass coverslips (BD, Franklin Lakes, NJ), fixed, dehydrated, and sputter coated with gold for visualization. Three SEM micrographs of each sample group were randomly chosen for the analysis. Each micrograph was divided into a 3x3 grid using Adobe Photoshop CS5 (Adobe, San Jose, CA), and the number and size of clusters was counted within each grid. The total number and size of clusters was then calculated for each treatment group.

2.10 DNA purification and quantitative real-time PCR

Tibiae were decalcified identically to those used for histological analysis, in 0.5 M EDTA pH 7.5 that was changed daily and kept at 4°C throughout the entire two-week
decalcification period. Following decalcification, samples were washed three times with sterile PBS and resuspended in 360 µl buffer ATL (Qiagen, Valencia, CA) with 40 µl proteinase K (Qiagen, Valencia, CA), and then incubated at 55°C until the bone was completely digested and the solution was clear. Then, 180 µl gram positive bacteria lysis buffer (Qiagen) containing 20 mg/ml lysozyme (Sigma) and 20 µg/ml lysostaphin (Sigma) was added to lyse any gram positive bacteria present in the samples. Next, 400 µl buffer AL (Qiagen, Valencia, CA) with 5 µl proteinase K (Qiagen, Valencia, CA) was added, and the samples were placed on ice and sonicated three times, at level 15, for 10 seconds with one-minute resting intervals in between the sonications (Microson Ultrasonic Cell Disruptor; Misonex, Farmingdale, NY). After sonication, DNA was precipitated with 400 µl 100% ethanol and then isolated from the samples using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol, and the DNA was eluted with 100 µl sterile distilled water and stored at -20°C.

Quantitative real-time PCR on the S. aureus-specific nuc gene (encoding a S. aureus specific thermonuclease) was performed in order to quantify the total bacterial load in the tibiae. Primers used for amplification of the 269-bp product were as follows: 5’-GCGATTGATGGTGATACGGTT-3’ and 5’-AGCCAAGCCTTGACGAACCTAA-3’. The reactions were carried out in a final volume of 20 µl using 10 µl 2X Sso Fast EvaGreen Supermix (BioRad, Hercules, CA), 1 µM primers, 1 µl sterile distilled water, and 2 µl of the DNA template extracted from the tibiae, and run in triplicate in a Rotor-Gene Q (Qiagen, Valencia, CA). The DNA template was first denatured at 94°C for 5 minutes, followed by 40 cycles of amplification (denaturation, 94°C for 20 seconds;
annealing, 60°C for 20 seconds; extension, 72°C for 20 seconds). To calculate the absolute numbers of copies of the *S. aureus* nuc gene per tibia, we first generated a standard curve using 10-fold dilutions of a known concentration of nuc gene DNA, and ran these standards in parallel with our unknown samples. The mean of the three Ct values from each tibia sample were then compared to Ct values from the known standards, and the total number of copies of the *S. aureus* nuc gene was calculated per tibia. Since each *S. aureus* bacterium contains exactly one copy of the nuc gene, we could effectively calculate the bacterial load in each tibia using this method.

### 2.11 Phagocytosis assays

The mouse macrophage cell line RAW 264.7 (ATCC, Manassas, VA), originally isolated from adult BALB/c mice, was used in our phagocytosis assays. Stimulation was not required to induce phagocytic capability with this cell line.

For analysis by transmission electron microscopy (TEM), 1x10^8 UAMS-1 *S. aureus* in 0.4 ml of PBS was opsonized with 50 µg/ml 1C11 for 30 minutes, and then the bacteria were exposed to 0.4 ml of complement-rich mouse serum (Innovative Research, Novi, MI) and 5x10^6 RAW 264.7 mouse macrophages in 0.4 ml DMEM media (Invitrogen, Carlsbad, CA) for a period of 0, 15, 30, or 60 minutes before fixation in 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Samples were trapped in agarose, post-fixed in 1% osmium tetroxide, dehydrated, and embedded in EPON/Araldite epoxy resin. The epoxy blocks were then ultra thin sectioned (70 nm)
onto grids, stained with uranyl acetate and lead citrate, and imaged using a Hitachi 7650 TEM with attached Gatan 11 megapixel digital camera.

For the per high-power field analysis, samples were prepared according to the TEM protocol and sectioned at one micron, then stained with toluidine blue and coverslipped. These samples were then imaged at 100X under oil immersion, and then the mean number of macrophages containing internalized bacterial clusters (“megaclusters”) from three random images was calculated as the mean +/- SD.

2.12 Statistical analysis

Data are presented as mean plus or minus one standard deviation. In our 1C11 passive immunization experiments, the lowest bioluminescent value of the placebo group at each time point was used as a cut-off threshold for analysis by Fisher’s Exact Test (FET), which we used to determine significance among the treatment groups. We utilized a two-tailed unpaired student’s t-test for our in vitro experiments. Differences were considered significant at p<0.05. All data were analyzed using GraphPad Prism (version 4.0a) software (GraphPad Software, San Diego, CA).
2.13 Figures and Tables

Figure 2.1 Anti-Gmd mAb 1C11 inhibits Gmd enzymatic activity. Gmd enzymatic activity was assessed, as described in section 2.3 of this chapter. Of note is that 90% of the Gmd activity was inhibited at 50 µg/ml 1C11.
Table 2.1 Oligonucleotides used in the generation of LAC ΔGmd MRSA.
As described in section 2.5 of this chapter, primers were designed to either amplify flanking regions of Gmd or to confirm its deletion.

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gmd-Up-Fwd-eagI</td>
<td>5'-GGACCTCGGCGCGGGCTGTGAAACCAACTACA-3'</td>
</tr>
<tr>
<td>Gmd-Up-Rev-sacII</td>
<td>5'-GGACCTCGGCGGAATGCAAGCACCCTTGGTTTC-3'</td>
</tr>
<tr>
<td>Gmd-Down-Fwd-sacII</td>
<td>5'-GGACCTCGGCGGGGAAGTCGGCAATACCTTC-3'</td>
</tr>
<tr>
<td>Gmd-Down-Rev-acc65i</td>
<td>5'-GGACCTGGTACCCGATGAAATTAGCAGGGAAC-3'</td>
</tr>
<tr>
<td>pWedgeMCS-seq-FWD</td>
<td>5'-AGGCCTAATGACTGCTTTT-3'</td>
</tr>
<tr>
<td>pWedgeMCS-seq-REV</td>
<td>5'-ATTAATGCGTGCTGACG-3'</td>
</tr>
</tbody>
</table>
Figure 2.2 Generation of ΔGmd MRSA for loss of function studies.

(A) The absence of Gmd was confirmed in LAC ΔGmd by Western blotting with 1C11. Note the 58kDa Spa (*) and 53kDa Gmd (#) immunoreactive bands in the parental strain, and the absence of the Gmd band in LAC ΔGmd. The 56kDa His-Gmd protein was included as a positive control. (B) LAC ΔGmd was streaked against S. aureus RN4220 on a 5% sheep blood agar plate, and then grown overnight at 37°C. Hemolysis was observed only with the mutant, and not with RN4220 (control strain). Collectively, these data indicate that we successfully created an isogenic Gmd-deficient control for our assays, and that deletion of Gmd does not affect alpha-hemolysin activity (virulence).
Chapter Three

Passive Immunization with Anti-Glucosaminidase Monoclonal Antibody 1C11

Reduces the Severity of Infection in 50% of Mice Challenged Transtibially with

*Staphylococcus aureus*
3.1 Abstract

Toward the development of a vaccine against methicillin-resistant *Staphylococcus aureus* (MRSA) we evaluated a neutralizing anti-glucosaminidase (Gmd) monoclonal antibody (1C11) in a murine model of implant-associated osteomyelitis, and compared its effects on LAC USA300 (wild-type MRSA) vs. a Gmd-deficient isogenic strain (LAC ΔGmd). 1C11 significantly reduced the severity of Xen29 *S. aureus* infection in approximately 50% of the mice, as determined by bioluminescent imaging at day 3 post-infection (p=0.016). Interestingly, micro-CT of the osteolytic lesions revealed the absence of sequestrum and overall reduced cortical osteolysis in the protected mice, and biofilm integrity defects in 1C11 treated mice that were similar to that observed on implants from mice challenged with LAC ΔGmd. In vitro, 1C11 had no direct effects on proliferation, but electron microscopy demonstrated that anti-Gmd mAb phenocopies ΔGmd defects in binary fission. Prolonged treatment of MRSA cultures with 1C11 induced the formation of large bacterial clusters (“megaclusters”) that sedimented out of liquid culture, which was not observed in ΔGmd cultures or 1C11 treated cultures of a protein A deficient strain (LAC ΔSpa), suggesting that “megaclusters” are formed by the combined effects of Gmd inhibition and antibody-mediated agglutination. Collectively, these results suggest that passive immunization with anti-Gmd mAb 1C11 may enhance host immunity against *S. aureus* osteomyelitis, and prevent its spread in vivo.
3.2 Introduction

*Staphylococcus aureus* is the primary pathogen isolated from infected orthopaedic implants, approximately 50% of which are clinical isolates of methicillin-resistant *Staphylococcus aureus* (MRSA) acquired in both hospital and community settings. Although the number of primary infections following total joint replacement (TJR) is low (~1%), reinfection rates from MRSA are very high (15-40%) and often require a two-stage exchange arthroplasty to remedy the problem. Given that MRSA has surpassed HIV as the most deadly pathogen in the United States, accounting for 94,360 hospitalizations and 18,650 deaths each year, and the emergence of multidrug resistant *S. aureus* strains, alternatives to antibiotic therapies are now in great demand.

Despite tremendous efforts, an effective vaccine against *S. aureus* remains elusive, as several clinical trials have failed. An additional challenge in developing an effective vaccine to prevent and treat *S. aureus* TJR infection is the known immunodeficiency in this susceptible patient population due to aging, autoimmunity, obesity and diabetes. Thus, passive immunization via infusion of neutralizing antibodies against specific *S. aureus* surface proteins (i.e., ClfA) and virulence factors (i.e., alpha-toxin) is a more attractive option. Ideally, the passive immunization would comprise a dual-acting monoclonal antibody (mAb) that has direct antimicrobial effects by inhibiting a critical *S. aureus* molecule, and also have immunomodulatory effects to enhance the host response and bacterial clearance. For these reasons we have focused our efforts on the glucosaminidase (Gmd) subunit of autolysin (Atl), which several groups have identified as an immunodominant antigen. Functionally, Atl has been
shown to be essential for cell wall biosynthesis and degradation during binary fission.$^{48,49,53}$ Atl has also been shown to function as an adhesin,$^{70,71}$ a biofilm enzyme,$^{69,71}$ was identified as the molecular target of vancomycin,$^{72}$ and interferes with the production of antibodies in mice.$^{73}$

Recently, we have generated recombinant histidine-tagged Gmd protein (His-Gmd) and used it as an active vaccine to generate a panel of anti-Gmd IgG1 mAb, of which five were chosen for further analysis based on their high affinity binding to the recombinant protein. One mAb (1C11) was chosen as our leading candidate due to its ability to alter \textit{S. aureus} planktonic growth in vitro,$^{74}$ and because of its neutralizing effect on Gmd activity (as presented in Materials and Methods). We also used it to demonstrate the potential of circulating anti-Gmd antibodies as a serum biomarker of protective immunity against \textit{S. aureus} in patients with orthopaedic infections.$^{57}$ Here we formally evaluate its potential as a passive vaccine by testing the hypothesis that passive immunization protects mice from implant-associated osteomyelitis by inhibiting \textit{S. aureus} growth, binary fission, and biofilm formation.

3.3 Results

3.3.1 Anti-Gmd mAb 1C11 is identified as a leading candidate for an anti-\textit{Staphylococcus aureus} passive immunization

Thirty-three anti-Gmd mAb were commercially developed for our project by Precision Antibody (Columbia, MD), of which five were pre-chosen for the project due
to their high-affinity binding to recombinant His-tagged Gmd (data not shown). The five candidates were evaluated using an in vitro growth assay that measured the O.D. of planktonic phase Xen29 *S. aureus* cultures over time, as described in Materials and Methods. Xen29 was chosen as the model *S. aureus* strain in these early experiments because it is the bioluminescent strain that we primarily use in our in vivo assays. One mAb (1C11) had the greatest effect on Xen29 *S. aureus* growth over the course of the entire experiment (Fig. 3.1), having significantly reduced the peak O.D. at every time point following the initial bacterial lag phase of 7 hours (*p<0.05 at 7 hours growth; **p<0.01 at 9+ hours growth), and in a dose-dependent manner (Fig. 3.2). Therefore, we chose 1C11 as the leading candidate for use in our in vivo and in vitro experiments.

### 3.3.2 Anti-Gmd mAb 1C11 significantly reduces peak optical density of planktonically growing *Staphylococcus aureus* in vitro

Next, we evaluated the effect of 1C11 on planktonic phase cultures of *S. aureus* strains Xen29 (Fig. 3.3A) and UAMS-1 (Fig. 3.3B) in a parallel experiment. One hundred CFU (100 CFU) of each planktonic phase bacterial strain were grown in the presence of PBS (no treatment control), an irrelevant mAb MOPC-21 (negative control), an anti-protein A mAb (positive control), or our candidate anti-Gmd mAb 1C11, as described in Materials and Methods. The irrelevant mAb (MOPC-21) did not have a significant effect on Xen29 or UAMS-1 growth, whereas treatment with 1C11 or anti-protein A significantly reduced the peak O.D. of both strains (*p<0.05 at 7 hours growth; **p<0.01 at 8+ hours growth) (Fig. 3.3). This reduction in peak O.D. was greater in the
1C11 treated cultures, though not statistically significant when compared to the anti-protein A control. These data suggest that the binding of antigens on the surface of the bacterium directly affects bacterial growth, thus reducing the peak density (O.D.) of planktonically growing cultures over time, but that treatment with 1C11 may alter *S. aureus* growth in more than one way.

Consistent with previous data (Fig. 3.1 - 3.3) using 100 CFU Xen29 or UAMS-1, addition of 1C11 to 1,000 CFU planktonic phase LAC USA300 MRSA reduced the peak O.D. (Fig. 3.4). When compared to the data presented in Figures 3.1 and 3.3, the time at which the different treatment groups diverged was much earlier than observed with the 100 CFU cultures (4 hours vs. 7 hours growth, respectively), due to the fact that the 10-fold higher starting inoculum left its lag phase several cell division cycles sooner. We also wanted to see the effect of 1C11 on our Gmd-deficient strain, LAC ΔGmd. As expected, treatment of LAC ΔGmd with 1C11 had no effect on its growth, indicating that the presence of Gmd on the surface of the bacterium is necessary for the reduction in peak density of planktonically growing cultures in our experiments.

3.3.3 Anti-Gmd mAb 1C11 reduces the severity of infection in mice challenged transtibially with implant-associated *Staphylococcus aureus*

In order to assess the effects of 1C11 on the establishment of implant-associated osteomyelitis, we administered the mAb or placebo to mice one day prior to challenge with a bioluminescent *S. aureus* (strain Xen29) coated pin, and then monitored the infection via longitudinal BLI. Consistent with prior studies, the placebo treated mice
uniformly displayed a peak BLI on Day 3 (Fig. 3.5A top). In contrast, the 1C11 treated group displayed a bimodal response in which half of the mice displayed a similar response to the placebo mice, and the other half had a very low BLI signal (Fig. 3.5A bottom). To quantify this outcome, we performed a threshold analysis using the lowest BLI value in the placebo group as the cutoff for infection as previously described. The results demonstrated that the 1C11 passive immunization significantly (p=0.016) reduced the severity of infection in approximately 50% of the mice, as demonstrated by our quantitative analysis of the bioluminescence at day 3 post-infection (Fig. 3.5B).

We utilized micro-CT to analyze the osteolytic lesions at the site of pin insertion in tibiae removed from the infected mice at day 14 post-infection (Fig. 3.6). Interestingly, analysis by micro-CT revealed sequestrum in the tibiae with high BLI values (Fig. 3.6Aa), which was absent in the tibiae with BLI values below the infection threshold (Fig. 3.6Ab), as well as in tibiae removed from mice infected with our Gmd-deficient strain (LAC ΔGmd) (Fig. 3.6Ac). Osteolytic area was also significantly reduced in the protected mice (Fig. 3.6B). Although no formal conclusions can be made about this novel finding at this time, the results suggest that the absence of a sequestrum may be a valuable translational biomarker of protection from chronic osteomyelitis.

As determined by nuc gene quantitative real-time PCR (qPCR) on total DNA (bacterial and mouse) extracted from the tibiae at day 14 post-infection, we demonstrated that passive immunization with 1C11 had no effect on total S. aureus bacterial load (Fig. 3.7). This was not surprising, given that qPCR is capable of amplifying bacterial DNA isolated from both live and dead bacteria, and because there was no difference in
bioluminescence at the day 14 post-infection time point. Though no significant effect was observed, these data confirm that all mice received an equivalent bacterial load at the initial time of infection.

3.3.4 Anti-Gmd mAb 1C11 disrupts *Staphylococcus aureus* biofilm formation in vivo

Since Gmd has been reported to be an enzyme critically involved in *S. aureus* biofilm formation,\(^{69,75,76}\) we qualitatively assessed the biofilm on pins harvested from the infected tibiae on day 14 by SEM (Fig. 3.8). The results showed that Gmd is not required for MRSA biofilm formation in vivo, as there was clear evidence of a bacteria-embedded matrix on the pins harvested from mice passively immunized with 1C11 prior to infection with LAC USA300 MRSA, and mice challenged with LAC ΔGmd coated pins. However, further scrutiny of the SEM images revealed that in contrast to the biofilm that coats the entire surface of pins from placebo treated mice, the biofilm on the pins from 1C11 treated mice and LAC ΔGmd infected mice appeared highly fragmented morphologically. Although the cause of this fragmentation remains a topic of investigation, our interpretation of this finding is that minor defects in the WT biofilm that are generated during dehydration and other processing steps prior to SEM are exaggerated in biofilm produced in the absence of Gmd, and suggest that this enzyme may be required for biofilm integrity.
3.3.5 Anti-Gmd mAb 1C11 inhibits *Staphylococcus aureus* binary fission, forcing the bacteria to form large clusters that sediment out of liquid culture

To elucidate the mechanism by which 1C11 reduced the severity of *S. aureus* osteomyelitis in our murine model, we first evaluated the direct effects of the mAb on MRSA growth in vitro vs. untreated, control mAb treated, and isogenic LAC ΔGmd MRSA controls. Consistent with previous reports assessing the growth of Gmd- and Atl-deficient strains,71,76,77 we found that addition of 1C11 to cultures of LAC USA300 inhibited complete separation of daughter cells undergoing binary fission in a manner that appeared to phenocopy the LAC ΔGmd control (Fig. 3.9A). We previously demonstrated that when we measured the turbidity of the suspension cultures over time, 1C11 significantly reduced the peak optical density (Fig. 3.4). Coincident with this decrease in O.D. at 4 hours was the appearance of a precipitate in the bottom of 1C11 treated cultures, which SEM confirmed to be “megaclusters” of MRSA (Fig. 3.9B), with some so large that they were well over 100 bacteria in size. A quantitative analysis of MRSA clusters in suspension culture at 4 hours revealed that loss of Gmd function leads to significantly larger clusters of bacteria (Fig. 3.9C), as predicted due to defective binary fission (Fig. 3.9A). Interestingly, the control mAb also induced significantly more small clusters (7-24 bacteria) vs. the PBS control, but did not induce large clusters (>25 bacteria), suggesting limited aggregation via non-specific mAb binding to MRSA and Spa (protein A) crosslinking.

We also found that addition of 1C11 to our protein A-deficient MRSA strain (LAC ΔSpa) did not significantly change the growth pattern of suspension culture (Fig.
3.10). Thus, the observations that “megaclusters” are only found in 1C11 treated LAC USA300 MRSA cultures suggests that both inhibition of Gmd-mediated binary fission and bacterial agglutination are required for this to occur.

3.3.6 Treatment of *Staphylococcus aureus* with anti-Gmd mAb 1C11 results in a textured surface morphology marked by the presence of knob-like structures

When we analyzed SEM and TEM micrographs of LAC USA300 MRSA cultures that were treated with 1C11 for 4 hours in liquid culture, we noticed that the surface morphology was highly textured, and contained small knob-like structures (Fig. 3.11). This was very interesting because although treatment of LAC USA300 MRSA cultures phenocopied LAC ΔGmd defects in binary fission and cell separation, we did not observe this surface morphology in our LAC ΔGmd cultures.

3.3.7 Anti-Gmd mAb 1C11 does not have an effect on *Staphylococcus aureus* proliferation in vitro

To test if 1C11 inhibition of binary fission and clustering had any direct effects on MRSA proliferation, we assayed tritiated thymidine incorporation into the DNA of actively dividing log phase bacteria. No difference in proliferation rate was observed between the placebo and 1C11 treatment groups (Fig. 3.12A), which was also demonstrated with a WST-8 tetrazolium salt reduction assay (Fig. 3.13A). This finding that Gmd function is not required for normal MRSA DNA replication was confirmed in tritiated thymidine incorporation studies with LAC ΔGmd (Fig. 3.12B), and in traditional
CFU assays that failed to demonstrate significant effects of 1C11 and ΔGmd on proliferation (Fig. 3.13B).

3.4 Discussion

With the intent of developing a passive vaccine to prevent *S. aureus* and MRSA infection in patients undergoing TJR, and to treat patients with *S. aureus* and MRSA infected implants, here we evaluated the effects of a candidate anti-Gmd mAb (1C11) in vivo and in vitro. Our demonstration that 1C11 significantly reduced the severity of *S. aureus* infection in approximately 50% of mice challenged in our implant-associated osteomyelitis model is remarkable, considering the overwhelming *S. aureus* inoculum used. Challenging the mice with a lower inoculum might better mimic the conditions involved in *S. aureus* infection of humans, and thus better replicate the natural course of *S. aureus* infection. However, due to the very high number of phagocytes residing in the bone marrow of BALB/c mice and their overwhelming ability to clear implant-associated bacterial infections, it can be implied that the large presence of these phagocytes would easily destroy bacterial inocula below the 5x10^5 CFU dose we currently use.

As it is known that mice protect themselves from this infection via a Th1-driven immune response that leads to IgG2b dominated humoral immunity, we believe that this efficacy could be enhanced by conversion of the IgG1 heavy chain to enhance mAb effector function. One descriptive study from 1979 found that a heterogeneous IgG2 antibody fraction from mouse serum, but not fractions containing mouse IgM or IgG1 isotypes, possessed the capability to efficiently activate the classical and alternative
complement pathways in their in vitro assay.\textsuperscript{80} Moreover, the IgG2 fraction efficiently lysed more than 80\% of the sheep red blood cells, whereas the IgG1 fraction weakly activated the classical complement pathway, and thus only led to lysis of approximately 30\% of the sheep red blood cells. These lytic events relied on the presence of calcium, indicating that the antibody-antigen complex formation is required for complement fixation and triggering of the complement cascade, and suggests that antibodies are necessary for complement-mediated lysis. To this end, we have recently generated IgG2a and IgG2b subclasses of 1C11 that can be used in future studies to evaluate the effects of antibody heavy chain class switching in vitro and in vivo.

Our in vivo studies also produced two descriptive findings that warrant further investigation. The first is the distinctive micro-CT images of the lesions in tibiae with active vs. inactive \textit{S. aureus} infection (Fig. 3.6), which correlated with BLI in our Xen29 in vivo experiment (Fig. 3.5). The reduced presence of reactive bone on the surface of explanted tibiae at the site of pin insertion in the protected mice indicates that passive immunization with 1C11 may prevent the spread of infection to the periosteum and adjacent soft tissues. As reactive bone formation is assessed by radiography and often used as an indicator of infection in the clinical setting,\textsuperscript{81} anti-Gmd or multivalent anti-autolysin passive immunotherapies may help to control the local spread of \textit{S. aureus} and MRSA infection.

Additionally, from prior studies with anti-resorptive agents,\textsuperscript{59} we know that removal of the cortical bone adjacent to the \textit{S. aureus} coated implant is dependent on osteoclast activity that does not occur on necrotic bone. We speculate that this is due to
S. aureus-induced destruction of the RANKL gradient secreted by osteocytes and present on osteoblasts within the once healthy bone, which prevents osteoclast formation, activation, and the eventual clearance of necrotic bone. Therefore, the absence of a sequestrum may prove to be an important biomarker of protection in this murine model.

Our second observation, the biofilm laden with structural defects on pins explanted from MRSA infected mice passively immunized with 1C11, and on pins explanted from mice infected with LAC ΔGmd MRSA (Fig. 3.8), is very provocative. These data confirm the role of Gmd in early colonization of implant surfaces, and support the results obtained by Heilmann et al.\(^5\) Currently, there are no known treatments that directly target biofilm formation. Current treatments, instead, utilize antibiotic treatment to prevent host sepsis by killing planktonic bacteria that have escaped from the biofilm microenvironment, but have little to no effect on bacteria that are still contained within the biofilm.\(^5\),\(^8\) Thus, if this finding can be validated in subsequent studies, this could represent a novel approach to treating chronic S. aureus infections.

Equally important to the efficacy of a passive immunization for S. aureus and MRSA osteomyelitis is the elucidation of its mechanism of action, such that its limitations can be overcome by modifications to the mAb and co-administration of complementary treatments (i.e., mAb against other important immunodominant targets). To facilitate this for 1C11, we generated a Gmd-deficient isogenic mutant of LAC USA300 MRSA (LAC ΔGmd) and used it as a positive control in our studies. Although we could not generate a bioluminescent version of LAC ΔGmd for longitudinal BLI in the time frame of this study, and because the presence of antibiotic resistance genes used
for selection were already present in LAC::lux,\textsuperscript{38} we were able to compare the effects of 1C11 administration vs. genetic deletion of Gmd on MRSA-mediated osteolysis (sequestrum and involucrum formation) and biofilm formation in our murine model (Figs. 3.6 and 3.8, resp.). We also used this immunologic vs. genetic approach to assess the phenotype of Gmd loss of function in vitro (Figs. 3.9 and 3.12). Remarkably, with the exception of “megacluster” formation (Fig. 3.9C), 1C11 treatment appeared to phenocopy LAC ΔGmd in every experiment. Collectively, these data provide complementary proof that Gmd is required for \textit{S. aureus} binary fission, but does not have a direct effect on proliferation. Deductively, our failure to observe “megacluster” formation in: 1) MRSA cultures treated with aT2m, 2) LAC ΔGmd cultures treated with 1C11, or 3) LAC ΔSpa cultures treated with 1C11, strongly suggests that “megaclusters” are formed by the combined effects of Gmd inhibition and antibody-mediated agglutination.

Interestingly, though 1C11 phenocopied LAC ΔGmd defects in binary fission and cell separation, only the LAC USA300 MRSA cultures treated with 1C11 exhibited a fuzzy textured morphology (visible by TEM) that was studded with knob-like structures on its surface (visible by SEM) (Fig. 3.11), whereas planktonically growing placebo (PBS) treated LAC USA300 and LAC ΔGmd cultures did not have this unique textured surface morphology. This may be due to the extracellular inhibition of Gmd once it docks on the outside surface of the bacterium, which is not immediately recognized by the bacterium and counterbalanced by other \textit{S. aureus} bacteriolytic enzymes (i.e., Aaa or Sle1).\textsuperscript{70,83} In the case of intracellular (genomic) Gmd deletion, the bacterium may
automatically turn on or upregulate expression of these redundant bacteriolytic enzymes, thus allowing the bacterium to properly turn over its cell wall. As previous studies have demonstrated a similar phenotype in *S. aureus* treated with subinhibitory concentrations of vancomycin, future studies in the Schwarz lab should focus on whether this antibody-mediated and highly altered surface morphology correlates with bacterial vulnerability, and thus increased susceptibility to antibiotics.

Taken together, these results suggest that the primary mechanism of anti-Gmd humoral immunity against MRSA osteomyelitis may be opsonophagocytosis of large bacterial clusters.
3.5 Figures and Tables

Figure 3.1 Planktonic *S. aureus* growth is reduced by anti-Gmd mAb in vitro.
Four of our five original anti-Gmd mAb depressed the growth-related increase in light scattering of Xen29 *S. aureus*. The four mAb that reduced light scattering all did so to the same degree, while the isotype-matched control (MOPC-21) and the fifth mAb (3H6) were identical to Xen29 grown in the absence of any mAb. *p<0.05, **p<0.01 vs. PBS
Figure 3.2 1C11 dose-dependently decreases *S. aureus* planktonic growth in vitro. Xen29 *S. aureus* was treated with varying concentrations of anti-Gmd mAb 1C11 diluted in TSB, and O.D. (490 nm) was determined at the indicated time points. Here, we see that concentrations of 1C11 above 1.6 µg/ml reduced the O.D. of cultures at all time points, whereas those below 1.6 µg/ml had no effect on planktonic growth. As treatment with 50 µg/ml 1C11 had the greatest effect on planktonic growth, we chose this concentration for our assays. *p<0.05, **p<0.01 vs. 0.05 µg/ml 1C11 dose.
Figure 3.3 1C11 significantly reduces peak optical density of planktonically growing Xen29 and UAMS-1 S. aureus in vitro.

One hundred CFU (100 CFU) of each planktonic phase Xen29 (A) or UAMS-1 (B) were grown in the presence of PBS (no treatment control), an irrelevant mAb MOPC-21 (negative control), an anti-protein A mAb (positive control), or our candidate anti-Gmd mAb 1C11, as described in Materials and Methods. The irrelevant mAb (MOPC-21) did not have a significant effect on Xen29 or UAMS-1 growth, whereas treatment with 1C11 or anti-protein A significantly reduced the peak O.D. of both strains. *p<0.05, **p<0.01 vs. PBS
Figure 3.4 1C11 significantly reduces peak optical density of planktonically growing LAC USA300 MRSA in vitro.

O.D. of parallel cultures was measured at the indicated intervals. While no differences were observed among the PBS, αT2m, or ΔGmd cultures at any time point, the O.D. of the 1C11 treated cultures was significantly lower after 4 hours. **p<0.01 vs. PBS
Figure 3.5 Passive immunization with 1C11 reduces the severity of *S. aureus* implant-associated osteomyelitis.

(A) Mice were immunized and challenged as described in Materials and Methods. All placebo-treated mice displayed a robust BLI signal (top), while half of the 1C11-treated mice had a dramatically reduced BLI signal (bottom). (B) The Day 3 BLI data and mean for each group (black line) are presented. The lowest BLI value in the placebo group was used as the threshold value for infection (dashed red line), which revealed a significant protective effect of 1C11 as determined by Fisher’s Exact Test (p=0.016).
Figure 3.6 Passive immunization with 1C11 promotes removal of bacteria-laden necrotic cortical bone.

(A) Representative micro-CT renderings of the placebo group (a), 1C11 treated with a BLI below the threshold (b), and in mice infected with the ΔGmd strain (c) are shown. Of note is the presence of cortical bone immediately adjacent to the infected pin (sequestrum) in (a), which was removed by the host in (b) and (c). (B) Reduced osteolytic lesions were observed in the 1C11 protected and ΔGmd infected mice. *p<0.05 vs. PBS
Figure 3.7 1C11 does not decrease total *S. aureus* bacterial load at day 14 post-infection. Quantitative real-time PCR on the *S. aureus*-specific nuc gene was performed in order to quantify the total bacterial load in each tibia extracted from the mice at day 14 post-infection. Here, we demonstrated that passive immunization with 1C11 had no effect on total *S. aureus* bacterial load in any of the treatment or infection groups.
Figure 3.8 Gmd is required for normal biofilm formation on stainless steel pins in vivo.
Representative micrographs of explanted pins from placebo, 1C11 treated, and LAC ΔGmd infected mice are shown to illustrate the uniformly coated pin with minor defects in the biofilm matrix (black arrow) in the placebo group, vs. the extensively disrupted matrix in the LAC ΔGmd and 1C11 treated groups. Top: 250-300X (bar = 20 µm); Bottom: 2,000X (bar = 3 µm)
Figure 3.9 1C11 induces MRSA megaccluster formation in vitro by combined inhibition of binary fission and agglutination.

(A) Representative micrographs of LAC USA300 cultures grown for 4 hours in the presence of αT2m, 1C11, or PBS, and of LAC ΔGmd. Of note is that the defective binary fission observed with ΔGmd is phenocopied by 1C11 addition to WT MRSA. (B) O.D. of parallel cultures was demonstrated in Figure 3.3. The decreased O.D. of the 1C11 treated cultures was coincident with a visible precipitate, which revealed megacclusters of MRSA as seen by SEM. (C) Quantification of the clusters was performed and presented as the mean +/- SD (*p<0.05 vs. PBS; #p<0.05 vs. ΔGmd). A: 15,000X (bar = 0.5 µm); B: 3,000X (bar = 2 µm)
Figure 3.10 1C11 cannot induce LAC ΔSpa MRSA megaclusters.
LAC and LAC ΔSpa USA300 MRSA cultures were grown and treated with 1C11 or PBS as described in Materials and Methods. O.D. was determined at the indicated time points. Of note is that in contrast to 1C11-treated LAC cultures, which precipitated out of liquid culture causing a decrease in optical density, 1C11-treated LAC ΔSpa cultures grew similarly to PBS-treated LAC Δspa and exhibited no difference in optical density over the time course. Collectively, these data indicate that although binary fission is inhibited in 1C11-treated LAC cultures, O.D. decreases only when megaclusters form via antibody-mediated crosslinking occurs, which is heavily dependent on protein A expression and does not form in Spa-deficient mutants. **p<0.01
Figure 3.11 Treatment of *S. aureus* with 1C11 results in a textured surface morphology marked by the presence of knob-like structures.

Representative SEM (A) and TEM (B) micrographs of LAC USA300 MRSA cultures grown for 4 hours in the presence of 1C11 reveal a textured appearance that contains small knob-like structures (red arrowheads), that was not observed by SEM or TEM in the placebo (PBS) treated cultures (C – D, resp.). This textured cell wall was not observed in LAC ΔGmd cultures (not shown). A – D - 50,000X (A, C bars = 100 nm; B, D bars = 200 nm)
Figure 3.12 Gmd is not required for LAC USA300 MRSA proliferation in vitro.
(A) LAC USA300 suspension cultures, treated with PBS or 1C11, were grown in the presence of tritiated thymidine, and the radioactive incorporation (counts per minute; cpm) into bacterial DNA was determined over time. (B) The same assay was performed using LAC and LAC ΔGmd cultures. No significant difference between the groups was detected at any time point.
Figure 3.13 1C11 does not inhibit LAC USA300 or LAC ΔGmd MRSA proliferation in vitro.

(A) LAC USA300 MRSA was grown in the presence of PBS or 50 µg/ml 1C11, then the tetrazolium salt, WST-8, was added and the cultures were allowed to grow for 3.5 hours. Samples were read at 450 nm, and the average of five samples revealed no effect of 1C11 on proliferation. (B) LAC and LAC ΔGmd were grown in the presence of PBS or 75 µg/ml 1C11. Serial dilutions were plated and incubated overnight, and the number of colonies observed was graphed as a function of $\log_{10}$ CFU for each treatment group, which revealed no effect of either treatment on LAC or LAC ΔGmd proliferation.
Chapter Four

Passive Immunization with Anti-glucosaminidase Monoclonal Antibody 1C11 Acts

by Increasing *Staphylococcus aureus* Opsonophagcytosis
4.1 Abstract

We previously demonstrated that anti-Gmd mAb 1C11 alters S. aureus bacterial growth using a combined Gmd inhibition (neutralization) and a unique antibody-mediated bridging effect to agglutinate S. aureus and MRSA, thus inducing the formation of large clusters (“megaclusters”) that sediment out of liquid culture. Bacteria treated with 1C11 also exhibited a rough-textured surface morphology, of which was studded with small knob-like structures visible by both SEM and TEM. Here we demonstrate that the formation of these “megaclusters” significantly increases their degree of opsonophagocytosis by RAW 264.7 mouse macrophages in vitro. Both internalized and extracellular “megaclusters” were embedded in a thick matrix, of which resembles undigested peptidoglycan, thus confirming the intimate role of Gmd in S. aureus cell wall turnover and peptidoglycan degradation. We also demonstrated that although the rate of phagocytosis was not increased by the prolonged treatment (4 hours) of S. aureus with 1C11, more macrophages contained internalized bacteria in the 1C11 treated samples. Collectively, these data suggest that anti-Gmd mAb 1C11 may enhance the host innate immune response to S. aureus infection by promoting opsonophagocytosis of agglutinated bacteria (“megaclusters”) by resident macrophages, and thus reducing the overall severity of infection.
4.2 Introduction

Critical to the eradication of *S. aureus* infection by a passive immunization is the antibody-mediated killing of the invading bacteria. Although much work has been done on the mechanisms by which leukocytes kill *S. aureus*, including NETosis in which the bacteria are captured and destroyed by neutrophil extracellular traps (NETs), the role of B cell immunity for the prevention of *S. aureus* infection remains controversial.87,88

Despite tremendous efforts, an effective immunotherapy against *S. aureus* based on the production or passive administration of antibodies remains elusive, as several late stage clinical trials have failed.41-44 However, though the results from these clinical trials were not overwhelming, the data do suggest that a reduction in the number of *S. aureus* bacteria by immunization could be achieved.89-91 As pointed out in a recent review on the current state of *S. aureus* vaccines, it is possible that the correct antigen or combination of antigens has not yet been discovered, or that the antibodies were fully capable of opsonizing the bacteria but could not induce their lysis or phagocytosis, and eventual death.87 Therefore, the recent push has been towards the stimulation of (via active vaccination) or passive administration of antibodies that promote humoral immunity and the overall innate immune response.

The goal of passive immunization for *S. aureus* infections is to provide immediate protection to the patient by enhancing the innate immune response via addition or supplementation of protective antibodies. Direct administration of protective antibodies also circumvents the 10 – 14 day window required for active vaccination. Antibodies used for passive vaccination can be generated in a lab (i.e., mAb from hybridoma cell
lines) or isolated from donors that have high serum antibody titers against a particular antigen of interest (i.e., intravenous immunoglobulin, or IVIG). Based upon the antigen target of interest (i.e., enzyme vs. binding factor) and/or its location on the bacterium (i.e., cell surface associated vs. secreted), systemic administration of these antibodies can promote the destruction and clearance of bacteria by one or more of four mechanisms: 1) complement fixation; 2) opsonization; 3) neutralization; and 4) agglutination.

First, they can directly lyse and kill invading microorganisms (bacteriolysis) through a highly complex complement-dependent process. They do this by specifically recognizing and binding to the exposed antigen on the surface of the pathogen, which induces a conformational change in the Fc (fraction, crystallizable) region of the antibody. This conformational change allows complement protein C1q to bind (aka, complement fixation), thus permitting a highly-orchestrated series of downstream events to occur. This series of events ultimately leads to the formation of the membrane attack complex, which punches holes through the membrane of the bacterium and renders it dead. Second, antibodies can act by directly inhibiting or blocking a key process required by the bacterium for its growth inside the host. This process, called neutralization, works by either blocking a key function required by the bacterium (i.e., host cell binding) or inhibiting an enzymatic process required by the bacterium for its survival. Third, antibodies can opsonize bacteria, making them more susceptible to phagocytosis. Initially, this process acts similarly to complement fixation. The antibody specifically recognizes and binds the exposed antigen on the surface of the pathogen, which then induces a conformational change in the Fc region of the antibody. This conformational
change permits binding of the antibody Fc region to the phagocyte Fc receptor (FcR), thus allowing engulfment and phagocytosis to occur. Fourth, antibodies may agglutinate bacteria by binding one bacterium with each of its antigen binding (Fab) arms, a common mechanism of secretory IgA that induces the formation of small clusters for phagocytosis and clearance.

As presented and discussed in Chapter 3, our leading candidate mAb 1C11 alters *S. aureus* bacterial growth using a combined inhibition (neutralization) and a unique antibody-mediated bridging effect to agglutinate the bacteria. The in vitro results we presented earlier demonstrate that 1C11 acts primarily by inhibiting *S. aureus* binary fission and preventing complete cell separation of daughter bacteria following the cell division process. When this inhibitory mechanism is combined with antibody-mediated agglutination to an adjacent bacterium via Fc to spa (protein A), the bacteria are forced to grow as large clusters (“megaclusters”) that sediment out of liquid culture. Unlike the clusters formed by our Gmd-deficient *S. aureus* mutant, which were phenotypically similar but not as large as 1C11-induced “megaclusters”, these 1C11-induced “megaclusters” are opsonized with the mAb. At present, the role of bacterial agglutination and its effect on host-mediated clearance is still far from being understood, but once solved will open the door for studies involving opsonized bacterial clusters and their clearance in vivo.

Here we evaluate the formation of 1C11-opsonized *S. aureus* “megaclusters” and their effect on phagocytosis by mouse macrophages in vitro. We hypothesize that formation of these “megaclusters” promotes opsonophagocytosis, which may formally
explain the reduced severity of infection in passively immunized mice that we observed in our in vivo experiments.

4.3 Results

4.3.1 Anti-Gmd mAb 1C11 promotes opsonophagocytosis of *Staphylococcus aureus* by RAW 264.7 mouse macrophages in vitro in a time-dependent manner

Previous reports indicate that the immortalized mouse macrophage cell line RAW 264.7 is fully capable of phagocytosing and destroying bacteria in vitro.\textsuperscript{95,96} In fact, Trivedi et al. demonstrate that this macrophage cell line was able to bind and internalize IgG-opsonized beads at a higher rate than isolated mouse primary peritoneal macrophages (1.5-fold increase), as characterized by fusion of the phagosome with the lysosome, which was completed by 15 minutes.\textsuperscript{96} Based on these prior results and the fact that the RAW 264.7 macrophages do not require any differentiation to become activated, we decided to pursue this cell line for use in our in vitro opsonophagocytosis assays.

To determine the mechanism by which 1C11 reduced the severity of infection in mice passively immunized with the mAb, we first asked the question whether 1C11 could promote opsonophagocytosis by the RAW 264.7 macrophages in vitro. Before doing so, we had to first determine if this macrophage cell line was capable of internalizing (aka, phagocytosing) 1C11-opsonized *S. aureus*. This was also an important question because
we had to identify the exact amount of time required for this macrophage cell line to internalize co-cultured opsonized bacteria.

To this end, we demonstrated that naïve RAW 264.7 macrophages (Fig. 4.1A) were fully capable of efficiently internalizing antibody-opsonized *S. aureus* in a time dependent manner (Fig. 4.1B-E and 4.2, resp.). Representative TEM micrographs of the 0-minute (B), 15-minute (C), 30-minute (D), and 60-minute (E) time points show that the number of bacteria internalized per macrophage increased as a function of time, and demonstrates the kinetics of antibody-mediated macrophage phagocytosis in vitro. Representative TEM micrographs for each time point illustrate 1C11-mediated phagocytosis (Fig. 4.1B-E), in which the extracellular bacteria (black arrows) are captured by pseudopods (red arrow) and internalized by the macrophages into vacuoles (blue arrows) (Nu = nucleus; Cy = cytoplasm). A longitudinal quantitative analysis of the number of *S. aureus* bacteria per RAW 264.7 macrophage also confirmed the time-dependent significant increase in 1C11-mediated phagocytosis (Fig. 4.2). Collectively, these data established that RAW 264.7 macrophages can indeed phagocytose antibody-opsonized bacteria, and that 30 minutes is the ideal time point for our in vitro phagocytosis assays.
4.3.2 Prolonged treatment of *Staphylococcus aureus* with anti-Gmd mAb 1C11 forces the internalized bacteria to remain in the cytoplasm of RAW 264.7 mouse macrophages as large clusters held together by a matrix

Having established an ideal phagocytosis time of 30 minutes for our RAW 264.7 mouse macrophages in vitro, we next wanted to determine the effects of prolonged treatment with 1C11 on *S. aureus* phagocytosis in vitro. To accomplish this, we treated UAMS-1 *S. aureus* bacteria with 1C11 or αT2m for 4 hours before exposing them to mouse serum and RAW 264.7 macrophages for 30 minutes. Interestingly, high power TEM at 6,000X revealed that the bacteria treated with 1C11 were mostly retained in the cytoplasm as large clusters held together by a matrix (Fig. 4.3A), whereas internalized *S. aureus* in the αT2m treated cultures resided in the cytoplasm of RAW 264.7 macrophages mostly as individual bacterium (Fig. 4.3B). Higher power 24,000X magnification of these 1C11-induced internalized clusters suggest that this matrix is composed of undigested peptidoglycan (Fig. 4.3C).

4.3.3 Extracellular anti-Gmd mAb 1C11 treated *Staphylococcus aureus* “megaclusters” are also tethered in a thick matrix

Our in vitro opsonophagocytosis assays also revealed the rare presence of 1C11-induced *S. aureus* “megaclusters” extracellular to the RAW 264.7 macrophages (Fig. 4.4), of which appeared to be held together by a matrix of similar composition. These “megaclusters” were qualitatively larger than those found within the RAW 264.7 macrophages, suggesting that the 1C11-induced “megaclusters” may have to be picked
apart by macrophages in order to allow their successful engulfment and eventual destruction.

### 4.3.4 Prolonged treatment of *Staphylococcus aureus* with anti-Gmd mAb 1C11 increases its degree of phagocytosis by RAW 264.7 mouse macrophages in vitro

We predicted that the 1C11-induced formation of “megaclusters” we observed in vitro would quantitatively increase their degree of phagocytosis by the RAW 264.7 macrophages. To test this, UAMS-1 *S. aureus* bacterial cultures were treated with 1C11 for 4 hours to allow the formation of “megaclusters”, and then incubated with mouse serum and RAW 264.7 macrophages for 30 minutes to permit phagocytosis (internalization) to proceed. To quantify the effect of 1C11 clustering on *S. aureus* phagocytosis in vitro, we first determined the number of RAW 264.7 macrophages containing internalized bacteria in the αT2m treated controls using per high-power field light microscopy analysis (Fig. 4.5A). We then compared these results to parallel-run cultures of RAW 264.7 macrophages containing 1C11-induced “megaclusters” (Fig. 4.5B). The results confirmed 1C11-mediated internalization of *S. aureus* “megaclusters”, some of which were so large that they were associated with aggregated macrophages (Fig. 4.5C). Of note is that neither bacterial clustering nor clearance of *S. aureus* by aggregated macrophages was observed in the αT2m treated controls (Fig. 4.5A), indicating that these traits are the direct result of prolonged treatment (4 hours) with 1C11 in vitro.
We then compared the quantitative results obtained from the per high-power field analysis of bacteria that were opsonized with 1C11 for 30 minutes vs. bacteria that were treated with 1C11 for 240 minutes (4 hours) to induce the formation of “megaclusters”. These data revealed that the formation of “megaclusters” significantly increased their degree of phagocytosis over opsonization with the 1C11 mAb alone (p<0.01), suggesting that “megacluster” formation is required for host protection via macrophage phagocytosis (Fig. 4.6).

4.3.5 Though the rate of *Staphylococcus aureus* phagocytosis is identical, the 1C11-induced formation of “megaclusters” resulted in an increased degree of phagocytosis in vitro.

Interestingly, when we quantified the exact number of bacteria, and not “megaclusters” of bacteria, per macrophage in TEM sections, we saw that there was no difference between the irrelevant control and 1C11 treated groups (Fig. 4.7A). These data suggest that the rate of phagocytosis by macrophages is not affected by the addition of *S. aureus*-specific antibody. However, further scrutiny of these data revealed a two-fold increase in the number of macrophages that contained bacteria at the 30-minute phagocytosis time point (Fig. 4.7B), and that more macrophages contained internalized bacteria as a percent total of macrophages per slide (per representative field analyzed) (Fig. 4.7C). Collectively, these data indicate that although treatment with 1C11 does not increase the absolute number of *S. aureus* per macrophage as a function of time (the rate), the overall number of macrophages containing *S. aureus* is increased.
Taken together, these data suggest that the 1C11-induced formation of *S. aureus* “megaclusters” may enhance the ability of the host immune response to better recognize *S. aureus* and promote a rapid influx of macrophages to the site of infection.

### 4.4 Discussion

To elucidate the mechanism by which anti-Gmd mAb 1C11 protects mice from implant-associated *S. aureus* osteomyelitis, here we evaluated its effect on phagocytosis of *S. aureus* bacteria by RAW 264.7 mouse macrophages in vitro. We demonstrated that prolonged treatment (4 hours) of *S. aureus* cultures with 1C11 induced the formation of large bacterial clusters (“megaclusters”) that were rapidly internalized by the RAW 264.7 macrophages in a time-dependent manner. Interestingly, we also observed that 1C11 induced the formation of extremely large bacterial clusters much greater than 100 bacteria in size that were just as easily phagocytosed using a team approach, often requiring 4-5 macrophages on average to pick apart and fully engulf a single one of these extremely large bacterial clusters. These data are very promising, given that removal of bacteria by opsonophagocytosis is a primary effector function of the innate immune system. Therefore, the development of antibody-based therapeutics to enhance these innate capabilities is both necessary and warranted.

Our in vitro phagocytosis assays also demonstrated another interesting function of our candidate mAb 1C11. The “megaclusters” produced by prolonged treatment (4 hours) with 1C11 and internalized by RAW 264.7 mouse macrophages in vitro were embedded in a thick matrix, of which we believe to be undigested peptidoglycan, thus
confirming the intimate role of Gmd in *S. aureus* binary fission, cell separation, and peptidoglycan degradation. We initially believed that this matrix was the result of internalization by the mouse macrophages. However, though very rare in the large number of micrographs evaluated for our quantitative analyses, we observed “megaclusters” embedded in a similar matrix that were extracellular to the RAW 264.7 macrophages. This led us to believe that this matrix was not necessarily the result of “megacluster” internalization, but was rather due to 1C11-mediated inhibition of Gmd enzymatic activity, which resulted in the tethering of “megaclusters” with a thick matrix that resembles undigested peptidoglycan.

Another interesting observation is that our quantitative analysis of the exact number of bacteria per macrophage revealed no difference between the 1C11 and irrelevant control treated groups. This was surprising because we believed that 1C11 would increase the rate of *S. aureus* opsonophagocytosis (the number of bacteria internalized per minute in our 30-minute experiment) by the RAW 264.7 mouse macrophages. However, though the rate was not altered by in vitro treatment with 1C11, the number of macrophages containing internalized bacteria was two-fold higher in the 1C11 treated group (Fig. 4.7B), and was almost three-fold higher as a function of the percent total macrophages per representative field analyzed (Fig. 4.7C).

Taken together, these data suggest that although the rate of opsonophagocytosis of *S. aureus* “megaclusters” by RAW 264.7 mouse macrophages was not altered by the prolonged treatment of *S. aureus* with 1C11, the mAb is capable to recruiting more macrophages to the site of infection, thus promoting removal of the 1C11 treated bacteria
by the host. This find confirms the protective role of anti-Gmd mAb 1C11 in vitro, and indicates that passive immunization with neutralizing anti-Gmd mAb may enhance the innate immune response to *S. aureus* infection.
Figure 4.1 1C11-treated *S. aureus* is readily phagocytosed by RAW 264.7 mouse macrophages in a time-dependent manner.

1C11-treated *S. aureus* was exposed to mouse serum and naïve RAW macrophages (A) for 0 (B), 15 (C), 30 (D), or 60 (E) minutes. Representative TEM micrographs are shown to illustrate 1C11-mediated phagocytosis in which the extracellular bacteria (black arrows) are captured by pseudopods (red arrow) and internalized by the macrophages into vacuoles (blue arrows) (Nu = nucleus; Cy = cytoplasm). A-E: 12,000X (bar = 1 µm)
Figure 4.2 The number of RAW 264.7 mouse macrophages containing internalized 1C11-treated *S. aureus* is increased in a time-dependent manner.  
1C11-treated *S. aureus* was exposed to mouse serum and naïve RAW macrophages for 0, 15, 30, or 60 minutes, and then the number of macrophages containing internalized bacteria was quantified. These data confirm the time-dependent significant increase in 1C11-mediated phagocytosis, as demonstrated by TEM in Figure 4.1.
Figure 4.3 *S. aureus* megaclusters are readily phagocytosed by mouse macrophages in vitro.

*S. aureus* megaclusters were formed by growing the cultures in the presence of 1C11 for 240 minutes, and then the cultures were exposed to mouse serum and RAW macrophages for 30 minutes to allow phagocytosis to proceed. Of note is that intracellular bacterial clusters (red arrowheads) were observed only in the 1C11 samples (A). These clusters were connected by a thick matrix (A, inset), whereas intracellular *S. aureus* in the αT2m treated cultures existed in the macrophages mostly as individual bacterium (B). (C) Higher magnification of a 1C11-induced cluster revealed that the matrix appears to be undigested peptidoglycan (blue arrowheads). A-B: 6,000X (Inset = 30,000X); C: 24,000X (A, B bars = 2 μm; C bar = 0.5 μm)
Figure 4.4 Extracellular *S. aureus* megaclusters are also embedded in a thick matrix in vitro.
*S. aureus* megaclusters were formed by growing the cultures in the presence of 1C11 for 240 minutes, and then the cultures were exposed to mouse serum and RAW macrophages for 30 minutes to allow phagocytosis to proceed. These clusters were connected by a thick matrix that was also observed in samples that were not yet phagocytosed by the RAW macrophages. Magnification: 24,000X (bar = 0.5 µm)
Figure 4.5 The 1C11-induced formation of megaclusters increased their degree of phagocytosis in vitro.

*S. aureus* megaclusters were incubated with mouse serum and RAW macrophages, epoxy resin embedded, and then thin-sectioned (1 µm) before staining with toluidine blue, as described in Materials and Methods. Compared to αT2m treated samples (A), 1C11-induced megaclusters were readily phagocytosed by macrophages (B, inset) that were occasionally forced to aggregate (C) to clear the entire megacluster. (A, B bars = 10 µm; C bar = 5 µm)
Figure 4.6 The 1C11-induced formation of megaclusters increased their degree of phagocytosis in vitro over opsonization alone.

1C11-opsonized UAMS-1 *S. aureus* was incubated with mouse serum and RAW 264.7 mouse macrophages as described in Materials and Methods. Compared to bacteria that were treated with 1C11 for 240 minutes (4 hours) to induce megacluster formation, we see that opsonization alone had no effect on phagocytosis by the macrophages. However, the formation of megaclusters significantly increased their degree of phagocytosis. n.s. = not significant; **p<0.01
Figure 4.7 Prolonged treatment with 1C11 increased the number of macrophages containing internalized bacteria, but does not alter the rate of phagocytosis.

UAMS-1 S. aureus was treated for 4 hours with 1C11 or αT2m (negative control) before co-incubation with mouse serum and RAW 264.7 mouse macrophages for 30 minutes as described in Materials and Methods. **p<0.01
Chapter Five

General Discussion
5.1 General Discussion

5.1.1 Overall results, and why altering *Staphylococcus aureus* growth in vivo may benefit the host

Toward the development of a passive immunization to prevent implant-associated *S. aureus* and MRSA infection in patients undergoing TJR surgery, here we evaluated the effects of a candidate anti-Gmd mAb (1C11) in vitro and in vivo. We observed a reduced severity of infection in mice that received passive vaccination with 1C11 one day prior to challenge with a large *S. aureus* or MRSA bacterial inoculum, and demonstrated that tibiae explanted from these protected mice at day 14 post-infection revealed the absence of a sequestrum and an overall reduced osteolytic lesion at the site of pin insertion. We also established the presence of defective biofilms on the surface of explanted pins from the 1C11 passively immunized and LAC ΔGmd challenged mice, presumably from inhibited colonization of the implant surfaces. In vitro analyses of 1C11 demonstrated that the mAb promotes the formation of large bacterial clusters (“megaclusters”) with a rough-textured surface morphology, of which increased their own degree of opsonophagocytosis by mouse macrophages.

Our observation that prolonged treatment (4 hours) with 1C11 forces the formation of large antibody-opsonized *S. aureus* “megaclusters” that possess the ability to be readily recognized and phagocytosed by the host immune system is very provocative, given that the goal of any passive immunization is to directly enhance humoral immunity and the overall innate immune response. The experiments performed
in this thesis dissertation revealed that 1C11 acts on *S. aureus* by a combination of mechanisms (neutralization and agglutination) that promote its phagocytosis by macrophages. By altering *S. aureus* growth in vivo and forcing the bacteria to grow in a way in which they do not want to, we are giving the host an advantage in the host-pathogen battle that occurs immediately upon *S. aureus* entry into its human host. The data presented in this thesis dissertation suggest that passive immunization with 1C11 tilts the scale in favor of the host, permitting clearance of the 1C11-induced “megaclusters” by macrophages and the removal of necrotic, bacteria-laden bone by activated osteoclasts.

Our work is not the first to address how altering bacterial growth affects disease pathogenesis, but the role of bacterial agglutination and its effect on host-mediated clearance is still far from being understood.\textsuperscript{92-94} Several research groups believe that agglutination of *S. aureus* promotes infection and invasiveness.\textsuperscript{93,94,98} For example, a recent publication suggests that agglutination of *S. aureus* by the ClfA-mediated binding to fibrinogen may promote sepsis, and that direct inhibition or disruption of agglutinated bacteria by thrombin inhibitors and anti-ClfA antibodies may be therapeutically beneficial for preventing *S. aureus* sepsis.\textsuperscript{93} However, in contrast to the data presented by these groups, Dalia et al. made the observation that increasing pathogen size by bacterial agglutination or changes to cellular morphology promotes antibody-mediated complement lysis, complement deposition, and uptake by human neutrophils.\textsuperscript{92} The opposing results presented here indicate that alteration of bacterial growth has different outcomes based on the disease model studied and bacterial or host factors involved, and
warrants additional studies to determine the exact role of bacterial agglutination and its effects on pathogenesis and clearance.

At the time of writing this dissertation, we are unaware of any research groups that have demonstrated a protective effect of a novel combined antibody-mediated inhibition and agglutination process on *S. aureus* clearance by the host immune system. Unlike the aforementioned experiments, which suggest that fibrinogen-cloaked *S. aureus* bacterial clusters may promote or enhance sepsis and invasiveness, the bacterial “megaclusters” formed by 1C11 are antibody-opsonized and thus primed for phagocytosis, not cloaked in host fibrinogen and masked from the immune system as others have reported. The data we presented in Chapter 4 confirms this, demonstrating that “megaclusters” formed by the prolonged treatment (4 hours) of planktonic *S. aureus* cultures with anti-Gmd mAb 1C11 were easily phagocytosed by RAW 264.7 mouse macrophages in vitro. Furthermore, “megaclusters” greater than 100 bacteria in size that were too large to be internalized by one macrophage were easily picked apart and engulfed by small groups of 4-5 macrophages, which aggregated together as a team to remove the extremely large antibody-opsonized megaclusters.

The formation of these extremely large megaclusters greater than 100 bacteria in size and their phagocytosis by small groups of aggregated RAW 264.7 mouse macrophages, as demonstrated by oil-immersion light microscopy and TEM, was very interesting. However, we believe that this in vitro phenomenon would never actually occur in vivo, given that a healthy host immune system would easily recognize and
phagocytose any small clusters of *S. aureus* that have formed due to the presence of the mAb in circulation well before they became the size that we observed in vitro.

Another important observation we made was that “megaclusters” formed by the addition of 1C11 to planktonically growing cultures, though significantly larger in size, phenocopied the defective binary fission and cell separation observed in the clusters formed by our Gmd-deficient mutant during planktonic growth. These data are very provocative, as 1C11 binds and neutralizes Gmd that is exposed on the surface of the bacterium before its proteolytic processing and subsequent release from the cell wall, yet results in a phenotype similar to one that is produced intracellularly via deletion of the gene encoding Gmd.

Interestingly, though 1C11 phenocopied LAC ΔGmd defects in binary fission and cell separation that we presented in Chapter 3, only the LAC USA300 MRSA cultures treated with 1C11 exhibited a fuzzy textured morphology (visible by TEM) that was studded with small knob-like structures on its surface (visible by SEM) (Fig. 3.11A-B). We did not observe this unique rough-textured surface morphology studded with small knob-like structures in our placebo (PBS) treated or ΔGmd controls (Fig. 3.11C-D). This may be due to the extracellular inhibition of Gmd by 1C11 once it docks on the outside surface of the bacterium, which is not immediately recognized by the bacterium and counterbalanced by other *S. aureus* bacteriolytic enzymes (i.e., Aaa or Sle1),70,83 as discussed later in this chapter. In the case of intracellular (genomic) Gmd deletion, the bacterium may automatically turn on or upregulate expression of these redundant bacteriolytic enzymes, allowing the bacterium to properly turn over its cell wall and thus
avoid autolysis. As a similar morphology is observed on *S. aureus* treated with subinhibitory concentrations of vancomycin,\(^8^4\) future studies should focus on whether this antibody-mediated and rough-textured surface morphology correlates with bacterial vulnerability, and thus increased susceptibility to antibiotics. This would present a novel combination approach to destroying *S. aureus* in vivo.

### 5.1.2 The controversial role of B cells in *Staphylococcus aureus* infection, and why our current mouse models may be at fault

Given that our goal is the production of a passive immunization to prevent *S. aureus* and MRSA infection in patients undergoing TJR surgery, we must address the controversial role of B cells in *S. aureus* infection and its relation to the elusive state of *S. aureus* vaccine development using our current mouse models of infection.

In a recent review regarding the current state of *S. aureus* vaccines,\(^8^7\) Richard Proctor pointed out that it is possible that the correct antigen or combination of antigens has not yet been discovered, or that the antibodies were fully capable of opsonizing the bacterium but could not induce its death. An opponent of antibody-based therapeutics, he suggests that B cells may not be involved at all, and that immunity to *S. aureus* may instead have to rely on or involve a strong Th17 cell response to promote bacterial clearance. Data presented by Schmaler et al. supports these conclusions, but also indicates that T cells may not be required for *S. aureus* immunity.\(^8^8\) The conclusions drawn from both Proctor and Schmaler are based solely on experiments performed with their respective mouse models of *S. aureus* sepsis, which indicated that activation of B
cells (and T cells in the case of Schmaler) did not cause an increase in the number of *S. aureus* bacteria eradicated by the mouse. However, as it is known that broad outcome-based mouse models of *S. aureus* infection (i.e., systemic infection to induce sepsis) are not perfect and do not necessarily resemble the natural course of infection found in humans,\(^9^9\) as they assume a “magic bullet” will prevent all *S. aureus*-related disease phenotypes, mouse models should instead focus on one *S. aureus* disease phenotype (i.e., osteomyelitis) and relevant quantitative therapeutic outcomes (i.e., reduced osteolysis) to prove vaccine efficacy.

The mixed results obtained using broad outcome-based mouse models of *S. aureus* infection raise concern over whether our current mouse models are adequate for the development a vaccine that protects humans, and question whether the results obtained with our current mouse models of *S. aureus* infection can be used as a basis for vaccine efficacy in humans. Despite the tremendous efforts using these current mouse models of sepsis and hematogenous spreading for vaccine development, several late stage and high profile clinical trials have failed.\(^4^1-^4^4\) However, though the results from these clinical trials were not overwhelming, the data do suggest that a reduction in the number of *S. aureus* bacteria by immunization could certainly be achieved.\(^8^9-^9^1\) For example, Inhibitex (acquired by Bristol-Myers Squibb Co. in 2012) demonstrated that infants who received a high dose (750 mg/kg) of Veronate®, a pooled and purified IVIG from patients with anti-MSCRAMM antibodies, had a lower incidence of *S. aureus* infection, candidiasis, and overall death. However, these results did not hold up in a high-profile Phase III clinical trial, and the project was terminated in 2006. It has since been
concluded that the pre-clinical mouse models may not have adequately represented the proposed clinical objectives.41

To address the concerns brought about from similar studies and to avoid the same problems going forward, several research groups have begun concentrating their efforts on developing more clinically relevant models of S. aureus infection,67,75,78,100-104 of which several groups are utilizing these models for vaccine development.67,75,78 These models mimic some of the most common and/or serious complications of S. aureus infection. Additionally, the recent explosive development of bioluminescent S. aureus strains and their introduction into clinical-based research has allowed researchers to incorporate quantitative measures such as longitudinal BLI to determine bacterial load, without the need to sacrifice animals mid-experiment. Coupled with quantitative end points, such as CFU to determine total bacterial load or quantification of intracellular bacteria to measure phagocytosis, these models can be powerful tools to study disease pathogenesis and evaluate candidate anti-S. aureus therapies in vivo.

Unfortunately, the mouse models available to study S. aureus bone infection still lag. In an attempt to better understand implant-associated S. aureus osteomyelitis and its complex disease pathogenesis, many investigators still rely on the use of S. aureus intramedullary pins to induce infection in mice. This intramedullary approach results in a highly variable lesion whose precise location cannot be predetermined temporally or spatially, as the bacterial inoculum is introduced directly into the bone marrow immediately before or after a stainless steel K-wire or pin is inserted into the bone marrow cavity. Another group developed what they consider to be an extremely relevant
chronic model of implant-associated osteomyelitis using a similar intramedullary pin style approach, but instead introduce the bacterial load via intravenous infection into the mice following the pin insertion. However, this hematogenous model assumes that the large bacterial inoculum delivered to each mouse ($2 \times 10^5 - 1 \times 10^6$ CFU in 150 µl PBS) homes to the pin, and does not fully take into account the potential side effects that a systemic dose of *S. aureus* may have on other organ systems within the mouse. Furthermore, it is unknown how infection of these organs may affect the bone infection, since the immune response may not be fully engaged in the bone microenvironment.

We have already addressed and integrated many of these points by developing a highly reproducible mouse model of localized implant-associated *S. aureus* osteomyelitis, in which the bacteria inoculum is pre-coated on a stainless steel pin before transcortical implantation through the tibia. This approach allows us to control the precise location of the bacterial infection without directly affecting any other organ systems within the mouse, and thus faithfully evaluate our candidate passive immunotherapies in vivo using a wide range of quantitative analyses both noninvasively and upon sacrifice. By using this model to evaluate our anti-*S. aureus* immunotherapies in vivo, we have determined that our candidate anti-*S. aureus* mAb (1C11) reduces the severity of infection, promotes removal of bacteria-laden necrotic bone (sequestrum), decreases osteolytic area at the site of pin insertion, and prevents the spread of the bacteria into the surrounding soft tissues. In vitro assays were then able to help us establish that this protective effect is due to antibody-mediated formation of large bacterial clusters ("megaclusters") that increased their own degree of phagocytosis by
macrophages, indicating that 1C11 enhanced the phagocytic arm of each protected mouse’s innate immune response. Armed with this mouse model of implant-associated osteomyelitis, future studies in the Schwarz lab should focus on determining if these effector functions can be enhanced by utilizing 1C11 as part of a multivalent passive vaccine.

5.1.3 Why a multivalent vaccine may be necessary to defeat Staphylococcus aureus

Our observation that 1C11, alone and in combination with a neutralizing anti-Amd mAb (preliminary combination data not shown), prevented local osteolysis of cortical bone in tibiae removed from mice challenged with a LAC USA300 MRSA coated flat pin, is also very provocative. Therefore, these data suggest that inhibiting Gmd or both autolysin subunits in combination (Amd and Gmd) significantly reduces the osteolytic damage that commonly results from S. aureus invasion of healthy bone and periosteum. As it is known that multivalent active vaccination can provide excellent protection against a number of pathogens, such as those developed for pertussis, influenza, or MMR, perhaps an antibody-based anti-S. aureus passive immunotherapy directed against both subunits of autolysin will provide the most protection and prove extremely successful in humans as a pre-TJR surgery prophylaxis.

Regarding our preliminary results using a neutralizing anti-autolysin passive vaccine over the results obtained using 1C11 alone (data not shown), one would think that completely inhibiting S. aureus autolysin enzymatic activity would result in destruction of the bacterium. We initially expected these results because the rough-
textured cell wall structure we observed in Chapter 3 should result in bacterial autolysis, to which we believe would be enhanced by the addition of a second neutralizing anti-autolysin mAb. However, *S. aureus* possesses may redundant hydrolytic enzymes that may partially counterbalance some of the deleterious effects of an anti-autolysin therapeutic cocktail. For example, Heilmann et al. state that a recently discovered multifunctional *S. aureus* peptidoglycan hydrolase (autolysin/adhesin from *S. aureus*, or Aaa) can counterbalance the deleterious effect of an autolysin mutant, and on its own possesses bacteriolytic capability against a *S. aureus* cell wall substrate. Biswas et al. confirm these findings but suggest that, though the lytic activity of Aaa is increased in an autolysin mutant, it is reserved for emergency situations only and cannot “cure the severe consequences of an autolysin mutation.” Another bacteriolytic enzyme (Sle1) that possesses similar properties to Aaa, as identified by Kajimura et al., can also counterbalance a genetic deletion of autolysin in *S. aureus*. These data help to support our findings that complete inhibition of Gmd or autolysin prevents binary fission and cell separation (Fig. 3.9), but has no direct effect on *S. aureus* proliferation (Figs. 3.12 and 3.13) and bacteriolysis.

Taken together, the data presented and discussed in this thesis dissertation help us to better understand how altering bacterial growth in vivo by passive immunization with anti-Gmd mAb 1C11 may prevent *S. aureus*-induced localized bone destruction and pathogen spread. Proof of vaccine efficacy in humans may one day lead to a combination therapy that helps patients undergoing TJR surgery retain their implants, should they develop an infection as the result of their surgery.
5.2 Figures and Tables

Figure 5.1 Proposed model of Gmd inhibition or deletion on *S. aureus* growth. This oversimplified schematic demonstrates the proposed effect on a neutralizing anti-Gmd mAb, or genomic deletion of Gmd, on *S. aureus* growth in vivo. Planktonic phase bacteria (1) are unable to undergo complete separation (2), forming megaclusters. These megaclusters cannot form robust biofilms (3) during early implant colonization, or are phagocytosed (4) when discovered by the host free-floating in circulation or in the soft tissues.
Chapter Six

Clinical Implications and Final Perspectives
Based on the data presented in this thesis dissertation, we envision that a multivalent passive immunization comprised in part by anti-Gmd mAb 1C11 can be used as a protective immunomodulatory therapy administered to patients immediately prior to TJR surgery. As 1C11 works by reducing the overall severity of infection, decreasing osteolysis around the site of implant placement, preventing robust biofilm formation on implant surfaces, and enhancing the phagocytic arm of the innate immune response, treatment with 1C11 as part of a multivalent passive vaccine, or its complementary use with S. aureus specific antibiotics, may help to eradicate a S. aureus infection more quickly than with traditional broad spectrum antibiotics alone.

Similar to other antibody-based passive immunotherapies that are on the market today (i.e., Palivizumab, or Synagis®), which are commonly administered intramuscularly or systemically, 1C11 would be introduced systemically or via local intramuscular injection to all patients immediately prior to TJR surgery. As 1C11 is an IgG1 mAb, we believe that our 1C11 passive immunization would confer protection in patients undergoing TJR surgery for up to 4 weeks following the procedure. This raises one concern regarding the use of mAb as a passive vaccine. Although antibody-based passive vaccination confers immediate protection for vaccine recipients, the effects are short-lived. For example, for antibodies that have not been genetically modified, the half-lives of IgG1, IgG2, and IgG3 subclasses are 29.7 days, 26.9 days, and 15.7 days, respectively, in immunocompromised patients who are receiving IVIG preparations. These half-lives can easily be modified genetically using current technologies, but we
feel that 4 weeks offers an excellent level of protection vs. not having a successful vaccination at all.

We ultimately envision that 100% of patients undergoing TJR surgery will be administered a multivalent 1C11 passive vaccine in combination with antibiotics as part of an anti-\textit{S. aureus} prophylactic cocktail. Therefore, our current dose of 40 mg/kg in mice, for which we used to induce the protective benefits we observed in vivo, would likely have to be reduced in order for us to offer an affordable vaccine into the current market. This could be commercially achieved by increasing the binding affinity of 1C11 to Gmd. Alternatively, we could identify other neutralizing anti-Gmd mAb that bind at similar domains and therefore work similarly, though have an increased affinity for Gmd when compared to 1C11.

Future studies in the Schwarz lab will address all of the concerns discussed in this thesis dissertation, should anti-Gmd mAb 1C11 make it to clinical trials alone or as part of a multivalent anti-\textit{S. aureus} prophylactic cocktail. For now, we will continue to focus and work diligently on developing novel anti-\textit{S. aureus} immunotherapies to one-day end these devastating implant-associated orthopedic infections.
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