

CHARACTERIZATION OF HOST-PATHOGEN INTERACTIONS DURING EARLY
STAPHYLOCOCCUS AUREUS BIOFILM FORMATION ON SURFACES

by

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DEDICATION

To my wife and best friend Cameron. My dissertation would not have been possible without your tireless support and sacrifice. I could not have asked for a better partner in this endeavor- you have been by my side every step of the way, even when you were on the other side of the country.

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ABSTRACT

Implanted biomaterials such as orthopedic screws, prosthetic joints, pacemakers, and catheters are essential components of modern medicine. Unfortunately, implanted foreign bodies are susceptible to biofilm infection, leading to a persistent and difficult to treat disease state. Biofilm infections readily tolerate clearance from the immune system, however much of our understanding of the mechanisms governing persistence are formulated around the biofilm state during advanced infection. By comparison we have a poor understanding of the early stages of infection. Specifically, how contaminating organisms initially evade host immune defenses and establish a robust infection remains ill-defined. In this work, we interrogated interactions between *Staphylococcus aureus* (*S. aureus*), a frequent culprit in biomaterial infections, and early contributors to host immunity. Using *in vitro* time-lapse microscopy, we observed that human neutrophils readily phagocytose and kill single cells or small clusters of *S. aureus* cells that are attached to a surface. *S. aureus* cells that go undiscovered during the initial stages of neutrophil surveillance form biofilm aggregates that rapidly gain tolerance to neutrophil killing. *In vivo* models of implant infection demonstrated that surface adherent bacteria can evade discovery due to delayed or heterogeneous neutrophil recruitment to the surface. Biofilm aggregate formation was impaired in a strain deficient in the two-component gene regulatory system SaeR/S and the resulting cells were highly susceptible to neutrophil killing. Inhibition of aggregation was dependent on serum complement proteins C3 and factor B, suggesting that SaeR/S regulated factors actively inhibit host complement to facilitate aggregation. Taken together, these data suggest that the formation of immune-tolerant biofilm aggregates may contribute to chronic device related infections by protecting bacteria from phagocyte killing. These studies provide vital insight into the host pathogen interactions on contaminated biomaterial surfaces and highlight early events that may determine infection outcome.

CHAPTER ONE

BIOFILM INFECTIONS OF IMPLANTED BIOMATERIALS

Implant-Associated Infections

One of the earliest studies to explicitly link biofilm as the causative agent of an implant associated infection presents in its case report a description of the hallmark progression of a biofilm infection (Marrie et al., 1982). In brief, a man was admitted with *Staphylococcus aureus* (*S. aureus*) septicemia and treated with intravenous antibiotic treatment. His condition improved, however the infection recurred one week following discharge. Following a second treatment of antibiotics, he was discharged, only for the infection to return a third time. At this point, a transvenous pacemaker implanted six years prior was removed and examined by scanning electron microscopy, revealing a significant *S. aureus* biofilm on the intracardiac portion of the pacemaker leads. Replacement of the pacemaker with a new epicardial pacemaker appeared to resolve the infection at the time of the study's submission 10 months later. Indeed, this case study traces a common course for implant associated infections as these infections are commonly antibiotic tolerant, recurring, and due to the biofilm state of bacteria or fungi (Costerton et al., 1999; Mah and O'Toole, 2001; Stewart, 2015; Stewart and Costerton, 2001). The lack of success generally seen by antibiotic therapy alone often necessitates the removal and/or replacement of the implanted device for successful clearance of the bacteria. In cases such as a pacemaker or prosthetic joint, this constitutes major surgery with significant risk to the patient and additional economic burden (Kurtz et al., 2012).

Implanted medical devices are common solutions to a wide range of health problems and improve the lives of many thousands of people each year. Indwelling medical devices can range in complexity, size, and location from simple orthopedic screws or catheters to total joint

replacements and cardiac pacemakers. The risk of infection is a consistent potential complication with any implanted biomaterial and unfortunately many decades of research into clever antimicrobial surface strategies have not yet produced an actionable solution (Busscher et al., 2012; Campoccia et al., 2013). We believe that this is due in part to insufficient focus on the early host immune response and a fundamental lack of understanding as to how contaminating bacteria evade killing by innate immune defenses. It was observed many decades ago that the presence of a foreign body, in this case a suture, reduced the infectious dose of *S. pyogenes* by nearly 10,000 fold compared to an intradermal injection (Elek and Conen, 1957). Later observations demonstrated that guinea pigs readily cleared up to 10^8 CFUs *S. aureus* injected subcutaneously, whereas 10^2 - 10^3 CFUs could cause an infection to a subcutaneously implanted “tissue cages” (perforated polymethacrylate or polytetrafluoroethylene tubes). Importantly, polymorphonuclear leukocytes (PMNs) recovered from the tissue cage demonstrated reduced bactericidal capabilities, suggesting immune function is impaired near the foreign body (Zimmerli et al., 1982, 1984).

Despite significant research into the immune response to “sterile” implanted biomaterials, often referred to as the foreign body response (Anderson et al., 2008), there is still little consensus on how host immune defenses break down in the presence of a foreign body and fail to eliminate contaminating pathogens. Indeed, this problem is somewhat difficult to study as most healthy individuals or laboratory animals do not develop infections associated with implanted biomaterials. Thus, many models of infection require artificially high doses of bacteria to consistently cause infection. It is highly unlikely that an immune response to an implant with tens of thousands to millions of bacteria on the surface would be identical to the response to an implant with hundreds of bacteria. Indeed, the latter is much more likely to reflect true levels of contamination that occur during surgery, which have been estimated to be on the order of 10^2 bacteria/cm² per hour of surgery (Busscher et al., 2012; Fitzgerald, 1979). Rather,

there are likely comorbidities that contribute to impaired immunity and insufficient clearance of bacteria from the implant surface (Nguyen et al., 2013; Stewart and Bjarnsholt, 2020). An improved description of the immune response in a realistic context is essential to uncover how and why immunity fails and contaminating bacteria are able to survive and form a biofilm. This specifically necessitates an understanding of the behavior of both the host and the pathogen during these early time points following implantation.

Biofilm Tolerance to Antimicrobials

Bacterial biofilms can exist as surface associated or otherwise aggregated communities of bacteria or fungi that produce a protective matrix and develop distinct structure and physiology. As a biofilm community matures, tolerance to antimicrobial chemotherapy tends to manifest, creating a significant challenge for killing and removal. Typically, steps to disrupt biofilm greatly aid in clearance (e.g. brushing of teeth or wound debridement), however these approaches are not practical for indwelling medical devices. The development of tolerance has been shown to be associated with both the areal cell density of microorganisms within a biofilm and the relative age or maturation state of biofilms (Stewart, 2015). The reduced effectiveness of antimicrobials against biofilm can be attributed to several factors. First, there can be decreased penetration of an antimicrobial, depending on the agent and organism (de Beer et al., 1994; Costerton et al., 1999; Stewart et al., 2001). Neutralization of the antimicrobial by organisms towards the top of the biofilm in concert with slower diffusion in cell aggregates compared to water may limit antimicrobial penetration and exposure of organisms at the substratum or aggregate center (Stewart, 2015). Second, there are localized alterations in cell physiology and gene expression, including an upregulation of stress responses, within a biofilm compared to planktonic cells. Biofilms are inherently heterogeneous communities due to chemical gradients and differences in local microenvironments within the biofilm (Stewart and

Franklin, 2008). Consumption of oxygen and nutrients, as well as reduced diffusion into the biofilm, create chemical gradients that alter bacterial activity at different points in the biofilm (Rani et al., 2007; Xu et al., 1998). The changing availability of nutrients as well as buildup of bacterial waste products throughout a biofilm results in differential gene expression within the biofilm (Lenz et al., 2008; Williamson et al., 2012) and altered cells states, such as stress-adapted, dormant, or non-growing states, that contribute to antimicrobial tolerance (Stewart, 2015). Indeed, many antibiotic classes require active bacterial growth to be effective (Taber et al., 1987; Tuomanen et al., 1986), and dormant cells have been shown to be more effective at surviving antibiotic treatment (Williamson et al., 2012). Nutrient limitation in biofilm can also lead to upregulation of stress responses related to hypoxia, growth arrest, and metal limitation that help to protect cells (Hentzer et al., 2005; Stewart et al., 2019). Finally, the active production of antimicrobial-inhibiting proteins such as catalase (Elkins et al., 1999), β -lactamase (Anderl et al., 2000; Bagge et al., 2004) or efflux pumps (Høiby et al., 2010) can directly aid in the protection of biofilm cells from specific antimicrobials. Together, the complex chemistry of the biofilm microenvironment combined with active stress responses can greatly aid in the tolerance of biofilm to antimicrobial therapy. In the context of an implant associated infection, this can result in recurrent infections due to insufficient biofilm killing by antibiotics and failure to clear the bacteria by host immune responses.

Immune Response to Implanted Biomaterials

The mechanisms by which biofilms tolerate host immunity are not as well described as antimicrobial tolerance but are an area of expanding research. Immediately following implantation, host proteins such as complement, albumin, fibrinogen, fibronectin, and vitronectin within interstitial fluid or blood will adsorb to the biomaterial surface and initiate an inflammatory response (Anderson et al., 2008). The immune response to a biomaterial surface will vary

based on many factors, such as the implant location, material type, and surface properties and is an area of heavy research focus, specifically geared towards concerns of biocompatibility. Importantly, host proteins that bind to the implant surface such as fibronectin and fibrinogen can help mediate bacterial adhesion via specific adhesins (Arciola et al., 2018). Adsorbed fibrinogen has been demonstrated to be proinflammatory, helping to mediate phagocyte adherence to implant surfaces (Hu et al., 2001; Kourtzelis et al., 2013; Tang and Eaton, 1993; Tang et al., 1996). Additionally, complement deposition and activation occurs on the biomaterial surface (Tang et al., 1998; Zimmerli and Sendi, 2011). Together, these proteins and other molecular signals such as damage-associated molecular patterns (DAMPs) can help to initiate acute inflammation and phagocyte recruitment to the implant surface. The exact timing of this acute recruitment, especially with respect to neutrophils to the implant surface, remains poorly described during the early minutes to hours following implantation.

Neutrophils exit the blood stream and are recruited to the site of infection where they eliminate pathogens through the production of reactive oxygen species (ROS), release of antimicrobial peptides, and Neutrophil Extracellular Trap (NET) formation (Kolaczowska and Kubes, 2013). Pathogens opsonized by C3b and IgG are phagocytosed and exposed to superoxide, hydroxyl radicals, hydrogen peroxide, and through interaction with the myeloperoxidase enzyme (MPO), hypochlorous acid within the phagosome (Amulic et al., 2012; El-Benna et al., 2016). Degranulation leads to the release of several bactericidal proteins such as α -defensins, lysozyme, proteases, and chelators into the phagolysosome or extracellular environment (Amulic et al., 2012). Antimicrobial NETs that contain histones and antimicrobial proteins immobilize and can directly kill extracellular pathogens (Kolaczowska and Kubes, 2013). In the presence of a foreign body, an object too large to phagocytose, neutrophils and other granulocytes are known to undergo “frustrated phagocytosis”. Granulocytes in this state demonstrate partial degranulation and release of ROS resulting in localized inflammation and

reduced immune cell function (Anderson et al., 2008; Wright and Gallin, 1979; Zimmerli et al., 1984). Indeed, the impairment in immune cell function near the implant surface observed in Werner Zimmerli's tissue cage model has been proposed as a contributing factor to the increased susceptibility to infection (Zimmerli et al., 1982, 1984).

Biofilm Tolerance to Host Immunity

Independent of any immune dysfunction caused by the biomaterial surface itself, biofilms resist killing by host immune cells through several mechanisms. 1.) Biofilms can secrete virulence factors or toxins in large local concentrations to directly interfere with or lyse host cells. 2.) Biofilms experience decreased penetration, phagocytosis, and killing by leukocytes. 3.) The infectious microenvironment surrounding the biofilm can alter immune cell efficacy and function.

Virulence Factors

Pseudomonas aeruginosa biofilms have a well-documented mechanism for evading neutrophil killing through the production of rhamnolipids, a class of biosurfactants. Rhamnolipid production is dependent on quorum sensing (QS) that commonly occurs in biofilms and results in the direct lysis of neutrophils (Jensen et al., 2007). Elimination of QS signaling decreased phagocytosis, PMN infiltration into the biofilm, and improved clearance in *in vivo* models of biomaterial infection (van Gennip et al., 2009, 2012). Phenol-soluble modulins (PSMs) are surfactant-like peptides that are produced by both *S. aureus* and *S. epidermidis*. Directly regulated by the Agr QS system, PSMs can lyse eukaryotic cells, including neutrophils, to evade host immunity (Cheung et al., 2014; Peschel and Otto, 2013; Surewaard et al., 2013). Lysis due to PSMs is more commonly observed in *S. aureus*. While *S. epidermidis* encodes the highly cytolytic PSM δ , it is expressed at low levels (Cheung et al., 2010). Additionally, in a subcutaneous implant model, the PSMs produced by *S. aureus* were demonstrated to

contribute to biofilm structuring, detachment, and dissemination of cells to lymph nodes (Periasamy et al., 2012). *S. aureus* biofilms secrete five bicomponent leukocidins (LukAB, LukED, PVL, HlgAB, HlgCB) that can lyse neutrophils and induce premature NET formation (Bhattacharya et al., 2018). Deletion of these toxins resulted in significantly decreased survival of bacteria in a chronic porcine burn wound model, demonstrating a significant role for these released toxins in biofilm survival. LukAB specifically was subsequently shown to be essential for evasion of neutrophils that were in direct contact with and actively phagocytosing *S. aureus* biofilm (Bhattacharya et al., 2020). Similarly, LukAB and α -hemolysin have been implicated in macrophage lysis and protection of biofilm on a femoral implant from immune clearance (Scherr et al., 2015). *S. aureus* biofilms grown in mammalian cell culture medium will produce staphylococcal complement inhibitor (SCIN) and chemotaxis inhibitory protein of staphylococci (CHIPS) during the first few hours following attachment, demonstrating an early strategy aimed at impairing immune recognition and phagocyte recruitment (Sultan et al., 2018).

Protection from phagocytosis

The biofilm extracellular matrix is composed of extracellular polymeric substance (EPS) that can include polysaccharides, proteins, extracellular DNA (eDNA) and lipids. EPS aids in cellular adhesion and cohesion and provides protection to cells within the matrix. There is a growing body of evidence for the benefit of EPS in protection from immune cell killing is mediated by its ability to physically shield biofilm cells from harm. While bacterially derived eDNA has long been recognized an important component of the biofilm matrix (Montanaro et al., 2011; Qin et al., 2007; Whitchurch et al., 2002), the presence of host-derived DNA in biofilm infections is more commonly being recognized. Neutrophil derived DNA was found to contribute to biofilm formation in CF sputum (Walker et al., 2005) and a more recent study found host-derived DNA surrounding *P. aeruginosa* biofilms in a murine implant model (Alhede et al.,

2020a). This neutrophil derived DNA is most likely due to NETosis or necrotic cell death. The demonstration that secreted *S. aureus* leukocidins can induce NETosis without physical contact suggests an active strategy to both impair neutrophil function and establish a barrier of host-derived eDNA between the bacterial cells and recruited leukocytes (Bhattacharya et al., 2018). *S. aureus* can subsequently produce nuclease to degrade surrounding NETs and allow dispersal of cells (Berends et al., 2010; Bhattacharya et al., 2020; Sultan et al., 2019). NETosis has also been demonstrated as a strategy to contain *P. aeruginosa* biofilm keratitis to prevent dissemination to the brain, at the cost of increased antibiotic tolerance, highlighting the role of NETs in containing pathogens (Thanabalasuriar et al., 2019). Additionally, *S. aureus* has been demonstrated to co-opt host fibrinogen through formation of protective fibrin barriers in a coagulase and von Willebrand factor binding protein dependent manner (Guggenberger et al., 2012). Cells encased in fibrin barriers were physically shielded from neutrophil phagocytosis and killing.

Biofilm architecture has been implicated in leukocyte resistance. Investigation of QS dysfunction in *S. aureus* demonstrated a clear fitness advantage to QS cheaters in biofilms through changes in biofilm structure (He et al., 2019). As previously discussed, QS in *S. aureus* induces expression of PSMs that remodel biofilm and promote detachment (Periasamy et al., 2012). QS deficient mutants (Δagr) developed dense biofilms that were resistant to leukocyte penetration and killing, resulting in increased survival in an *in vivo* catheter infection model (He et al., 2019). *S. aureus* biofilms show increased tolerance to neutrophil penetration and phagocytosis as they mature, with “younger” biofilms being more susceptible to phagocytic killing (Günther et al., 2009). Similarly, differential penetration by peripheral blood leukocytes was observed depending on the growth conditions (static vs. shear conditions) and the age of the biofilm, however impaired phagocytosis was observed in both conditions (Leid et al., 2002). Human neutrophils remain phagocytically active but display reduced motility and penetration

into *P. aeruginosa* biofilm, undergo partial degranulation and produce a reduced oxidative burst without effectively clearing biofilm bacteria (Jensen et al., 1990; Jesaitis et al., 2003).

Further evidence for the protective role of EPS can be found in studies that disrupt the biofilm matrix to improve susceptibility to killing. *P. aeruginosa* lacking genes for the production of the exopolysaccharide alginate is more susceptible to killing by human leukocytes (Leid et al., 2005). *S. epidermidis* deficient in the major EPS component poly-*N*-acetylglucosamine (PNAG) was more susceptible to neutrophil killing *in vitro* and in a catheter infection model (Kristian et al., 2008). The Bakaletz lab has produced an extensive body of work investigating antibody mediated disruption of biofilm in otitis media. Antibodies to PNAG protected mice against *Streptococcus pyogenes*, *Streptococcus pneumoniae*, *Listeria monocytogenes*, and *Neisseria meningitidis* (Cywes-Bentley et al., 2013). Antibody targeting of DNABII proteins, proteins that bind and crosslink eDNA in the biofilm of many species, have been shown to disrupt biofilm, increasing antibiotic susceptibility and immune clearance, again demonstrating a critical role of the biofilm matrix in persistence (Devaraj et al., 2018; Martyn et al., 2020; Novotny et al., 2013, 2021). Together, there is a clear body of evidence demonstrating the role of the biofilm matrix in the protection of biofilm bacteria from host immune defenses.

The infectious microenvironment

The high density of bacterial cells within a biofilm aid in the formation of an infectious microenvironment that harbors nutrient and oxygen gradients (Bjarnsholt et al., 2022; Stewart and Franklin, 2008). This altered environment, while influencing bacterial metabolism and antibiotic tolerance as described above, also has a profound effect on the activity of host immune defenses within the environment and directly contributes to bacterial persistence. The observation that the *S. aureus* biofilm microenvironment can skew macrophage polarization towards an anti-inflammatory phenotype that was ineffective at clearing biofilm cells has

sparked an extensive line of work investigating the cellular immune response to biofilm infection and its role in biofilm survival (Thurlow et al., 2011). Pro-inflammatory macrophages primarily utilize aerobic glycolysis while anti-inflammatory macrophages utilize oxidative phosphorylation (Yamada and Kielian, 2019). Thus, the nutrients available within the microenvironment can directly influence the activity of recruited macrophages, affecting antimicrobial activity and cytokine production. Myeloid derived suppressor cells (MDSCs) are recruited to sites of biofilm infection via IL-12 (Heim et al., 2015b) and play a significant role in creating an anti-inflammatory environment through IL-10 production (Heim et al., 2015a, 2014) that allows bacterial persistence, potentially in an attempt to prevent tissue damage from sustained inflammation in response to the infection. Notably, MDSCs and elevated IL-10 levels can be isolated from tissues associated with prosthetic joint infections (Heim et al., 2018). Specifically, biofilm derived lactate was demonstrated to increase IL-10 expression by MDSCs through inhibition of specific histone deacetylases, linking biofilm metabolites to the anti-inflammatory environment observed surrounding biofilm infections (Heim et al., 2020). Importantly, macrophages that were stimulated to be pro-inflammatory were more effective at clearing *S. aureus* biofilm than non-activated macrophages or neutrophils in an adoptive transfer catheter infection model, further linking the anti-inflammatory polarization to bacterial persistence (Hanke et al., 2013). In a similar study, metabolic reprogramming of monocytes towards a pro-inflammatory state via an oxidative phosphorylation inhibitor increased clearance of biofilm (Yamada et al., 2020). Contrary to these studies, a chronic tibial pin infection has been reported to produce sustained pro-inflammatory cytokines, and the authors suggested that the inflammatory tissue damage may contribute to *S. aureus* persistence (Prabhakara et al., 2011). Of note, the two models differed in their initial inoculum, with work from the Kielian group often employing a low (10^3 CFUs) inoculum and the study by Prabhakara *et al.* inoculating with a higher (10^5 CFUs) dose. It is unclear if this distinction would significantly alter the course of the

immune response or not. In addition to the immunoregulatory changes that can occur in the biofilm microenvironment, availability of oxygen can greatly alter the efficacy of neutrophil killing. Oxygen can be depleted by the biofilm itself or by neutrophils surrounding the biofilm, resulting in diminished capability for ROS production and bactericidal activity by neutrophils (Kolpen et al., 2010; Mandell, 1974; Wu et al., 2018). Indeed, neutrophils demonstrated impaired motility and reduced killing of surface attached *S. aureus* in hypoxic (2% O₂) and anoxic (0% O₂) conditions (Ghimire et al., 2019). These studies provide direct evidence demonstrating the immune-limiting and immune-modulating properties of the infectious microenvironment that aid in biofilm survival and persistence.

Pathogenesis of Implant Infections: More to Uncover

Four decades of research have reinforced a common theme: biofilm infections are incredibly difficult to eliminate once established. The mechanisms described above provide a basic model for bacterial persistence within the host, but largely depend on the presence and development of a mature biofilm infection. Specifically, many of the mechanisms would require densities of bacteria sufficient to: 1.) Produces localized concentration gradients of nutrients or oxygen. 2.) Produce concentrations of effector molecules such as toxins or antimicrobial inhibitors sufficient to allow bacterial survival. 3.) Produce sufficient EPS to inhibit phagocytosis, immune cell killing, or antimicrobial transport. Thus, a fundamental question remains: How do small amounts of contaminating microorganisms that are introduced to the implant surface or peri-implant tissue initially evade host immune defenses and form a mature biofilm?

To answer this question, it is helpful to consider the events that might occur immediately following implantation. Two of the most plausible routes of implant contamination are through hematogenous seeding and contamination during surgery. Hematogenous seeding of implanted surfaces is a commonly proposed route of implant infection, especially if the infection occurs

several years after the initial device placement. Indeed, patients are at a significantly higher risk of periprosthetic joint infection following bouts with *Staphylococcus aureus* bacteremia (Lalani et al., 2008; Sendi et al., 2011). In the case study presented at the beginning of this chapter, the patient's transvenous pacemaker became infected following an elbow injury, six years after the procedure, suggesting a hematogenous route of infection (Marrie et al., 1982). Hematogenous seeding has also been produced in experimental models, however these models require relatively high bacterial burdens to achieve implant infection (Wang et al., 2017; Zimmerli et al., 1985). Thus, hematogenous seeding may occur predominantly in cases where there is an exceedingly high bacterial burden in the bloodstream, such as during sepsis. Conversely, the high fraction of implant-associated infections caused by microbes commonly found within the skin microbiome suggests contamination of the surgical wound during implantation. For example, *S. epidermidis* is a frequent culprit in orthopedic implants (Arciola et al., 2005, 2018; Costerton et al., 1999) and *Cutibacterium acnes*, associated with the upper back, can cause infections to shoulder implants (Fatima et al., 2021; Kolakowski et al., 2018). Bacteria can exist in the skin in large aggregates 10 – 50 μm in diameter, which may offer them a significant level of protection from phagocytosis and killing by immune cells and antimicrobials (Alhede et al., 2020b; Ring et al., 2017). Additionally, the prospect of “cryptic” infections, infections that present few symptoms and are not detected by traditional methods, may occur at rates higher than appreciated and potentially result in active infections long after the initial contamination, making it difficult to identify the origin of contamination (Moojen et al., 2010). In practice, both skin and hematogenous contamination may contribute to the problem, and likely have slightly different pathogeneses.

Once bacteria have reached the implant surface, host defense may be compromised in several ways that contribute to increased susceptibility. Importantly, implanted biomaterials are not vascularized and may cause significant trauma to the local area. Thus, there may be

decreased delivery of oxygen, nutrients, and serum factors to the implant surface. In their tissue cage model of implant infection, Zimmerli and colleagues demonstrated diminished complement C3 levels (18.6% of serum values) and decreased antibiotic concentrations compared to serum levels within tissue cage fluid (Widmer et al., 1990; Zimmerli et al., 1982). Furthermore, immune cells must be recruited from the blood stream or surrounding tissue to the implant surface, which may take a significant amount of time depending on the local geometry and degree of damage to the vasculature. Implanted biomaterials also fail to “alert” the host of contamination. While host cells and tissue may recognize foreign microbes and initiate an immune response through cytokine secretion, generating a highly directed immune response, the inert biomaterial has no mechanism to identify microbial contamination and communicate with the host. Discovery of a few contaminating bacteria may thus be predicated on the immune response to the biomaterial itself. Finally, the presence of the biomaterial itself has been shown to induce immune cell dysfunction. Leukocytes recovered from tissue cage fluid demonstrated reduced phagocytic capability, reduced superoxide production, and reduced levels of granule enzymes (Zimmerli et al., 1984). However, injection of PMNs isolated from peripheral blood directly into the tissue cage reduced infection rates, suggesting that the initial response to the biomaterial, and the subsequent “frustrated phagocytosis”, impaired the ability of early responders to clear bacteria. However, it has previously been demonstrated that neutrophil phagocytosis and killing of single surface attached *S. aureus* cells can readily occur *in vitro* (Ghimire et al., 2019). It is likely that the material type, location, conditioning film, and local immune milieu contribute to the function, or dysfunction, of immune cells on a biomaterial surface.

It is clear that we have an incomplete understanding of the early immune response to biomaterials in the context of infection and the factors that determine whether bacteria are cleared or persist. The research described in this thesis sought to characterize interactions between neutrophils and surface adherent bacteria, specifically *S. aureus*, at the earliest time

points following attachment. Our fundamental goal is to better understand the conditions that allow for successful clearance of contaminating bacteria and to identify the mechanisms by which nascent biofilm can escape immune clearance. We contend that bacteria are readily cleared from an implant surface and the surrounding peri-implant tissue if the following steps occur. 1.) Neutrophils must be recruited to the implanted surface from the bloodstream; 2.) Neutrophils must adhere to and surveil the surface to discover contaminating bacteria; 3.) Discovered bacteria must be successfully phagocytosed and killed. If these events do not occur in a timely manner, bacteria will proliferate and form immune tolerant biofilm aggregates that can evade killing and persist (Figure 1). Chapter 2 describes studies that investigate the conditions on a surface that allow for neutrophil persistence through delayed discovery and biofilm aggregate formation. The experiments in Chapter 3 identify the SaeR/S two-component regulatory system as essential for neutrophil-tolerant biofilm aggregate formation by regulating virulence factors that interfere with serum complement. Two appendix chapters are also included that explore specific *S. aureus* virulence factors involved in neutrophil evasion (Appendix A) and the role of biomaterial surface adsorbed serum proteins in facilitating neutrophil motility and discovery of bacteria (Appendix B). Together, this work thoroughly interrogates the recruitment of neutrophils and the subsequent discovery and killing of surface associated *S. aureus*. We have identified areas in which this immune response can fail, suggesting mechanisms of pathogenesis and providing targets for the development of novel prophylactic therapies.

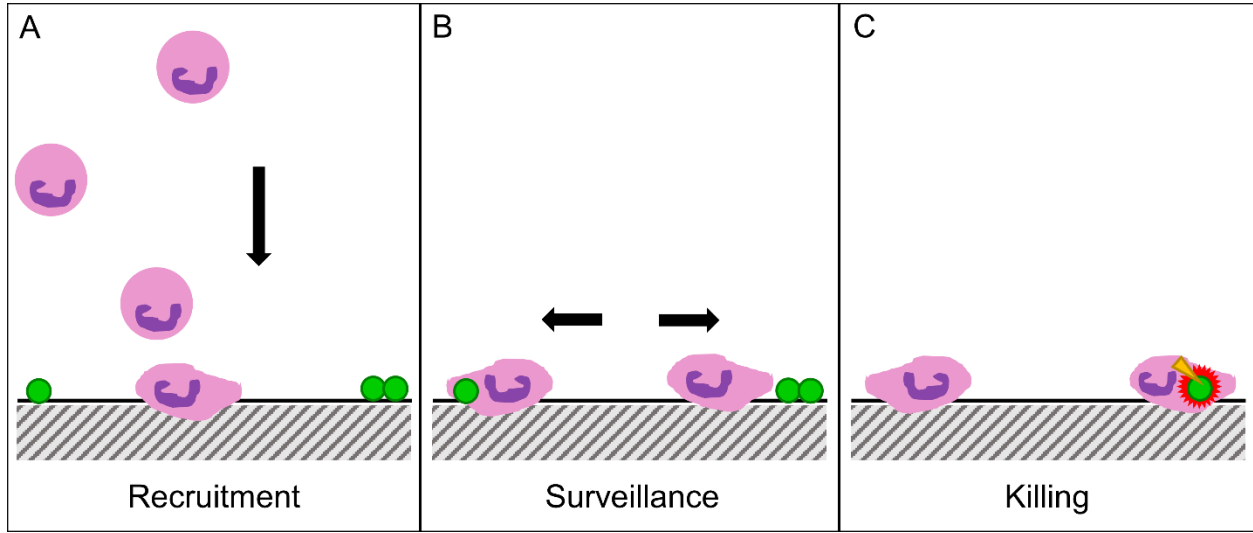


Figure 1: Proposed steps required for successful clearance of contaminating microorganisms from a biomaterial surface. (A) Neutrophils (pink) are recruited from the local vasculature to the implant surface as part of the immune response to a foreign body. (B) Neutrophils adhere to the surface and discover bacteria (green) through chemotaxis or random motility. (C) Phagocytosed bacteria are killed through production of ROS and degranulation. We hypothesize that these events must occur prior to the formation of immune-tolerant biofilm aggregates for clearance to occur.

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CHAPTER TWO

DELAYED NEUTROPHIL RECRUITMENT ALLOWS NASCENT STAPHYLOCOCCUS
AUREUS BIOFILM FORMATION AND IMMUNE EVASION

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Delayed neutrophil recruitment allows nascent *Staphylococcus aureus* biofilm formation and immune evasion

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ABSTRACT

Biofilms that form on implanted medical devices cause recalcitrant infections. The early events enabling contaminating bacteria to evade immune clearance, before a mature biofilm is established, are poorly understood. Live imaging *in vitro* demonstrated that *Staphylococcus aureus* sparsely inoculated on an abiotic surface can go undiscovered by human neutrophils, grow, and form aggregates. Small (~50 µm²) aggregates of attached bacteria resisted killing by human neutrophils, resulting in neutrophil lysis and bacterial persistence. *In vivo*, neutrophil recruitment to a peritoneal implant was spatially heterogeneous, with some bacterial aggregates remaining undiscovered by neutrophils after 24 h. Intravital imaging in mouse skin revealed that attached *S. aureus* aggregates grew and remained undiscovered by neutrophils for up to 3 h. These results suggest a model in which delayed recruitment of neutrophils to an abiotic implant presents a critical window in which bacteria establish a nascent biofilm and acquire tolerance to neutrophil killing.

1. Introduction

The bacterial or fungal biofilm state is often responsible for chronic, persistent infections on implanted surfaces and is an area of great concern to the medical community [1–4]. Biofilms that mature on implanted medical devices become recalcitrant to both antimicrobial therapies and host immune defenses [5–7]. Decades of research into novel biomaterial surfaces and antimicrobial coatings have yet to alleviate the issue. We suspect that an improved understanding of the innate immune response to an emergent biofilm is necessary to inform better strategies for preventing these serious infections.

Staphylococcus aureus readily forms biofilms on biotic and abiotic surfaces and has been a persistent threat to public health both in

hospital-associated and more recently, community-associated infections [8,9]. In addition to causing skin and soft tissue infections, sepsis, and necrotizing pneumonia, both methicillin-resistant and sensitive strains of *S. aureus* are common culprits in implant-associated infections, especially periprosthetic joint infections (PJI) and other orthopedic implant infections [9–13]. Two-stage revision, one of the most used procedures to treat PJI, is expensive, prolonged (6–12 weeks), and only has a success rate between 65 and 90% [14,15]. As is often the case in PJI, removal of an infected implant is frequently necessary to resolve biofilm infections [12,14,15]. In addition to causing serious harm to the patient, joint infections significantly increase economic costs, with studies finding total joint arthroplasty infection can triple or quadruple costs in Europe and the United States, respectively [16,17].

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In addition to forming biofilms, *S. aureus* expresses potent immunomodulatory virulence factors, making it an ideal organism to study host-pathogen interactions on a surface [18]. Mature *S. aureus* biofilms have been shown to utilize several mechanisms to modulate host immune responses such as inducing premature neutrophil extracellular trap (NET) formation [19], inhibiting complement [20], preventing phagocytosis [21,22] and attenuating the anti-bacterial activity of macrophages [23]. These and other studies have identified numerous strategies used by mature *S. aureus* biofilms to interfere with the normal host immune defenses. Nevertheless, it is unclear how, and at what stage in biofilm formation tolerance to host immune defenses manifests.

Previously, we have demonstrated that clearance of *S. aureus* aggregates attached to an abiotic surface *in vitro* depended on high densities of human neutrophils [24]. In addition, the aggregate size appears to play a role in the efficiency of phagocytosis [25]. Therefore, it is reasonable to hypothesize that clearance of a contaminated surface *in vivo* would require fast recruitment and high densities of neutrophils. Neutrophil recruitment *in vivo* is thought to occur within a few hours (2–4 h) after infection or injury [26,27] and this timeframe may serve as a critical window for bacteria to establish biofilm aggregates. To our knowledge, this time scale of recruitment has not been investigated in the context of an implanted surface. In this present study, we hypothesized that delayed neutrophil recruitment to a *S. aureus*-contaminated implant site allows for nascent biofilm formation and tolerance to host defenses *in vivo*.

For successful clearance of contaminating bacteria, we hypothesize that the following steps must occur: (i) Neutrophils must be recruited to the contaminated surface, via host- or pathogen-derived signals; (ii) Neutrophils must adhere to and patrol the surface in search of bacteria; (iii) Contaminating bacteria must be discovered by recruited neutrophils; and (iv) Neutrophils must phagocytose and successfully kill the discovered bacteria. We contend that these events must occur in a timely manner, before *S. aureus* biofilm aggregates develop tolerance to host defenses.

In this work, we investigated how small ($\sim 5\text{--}100\ \mu\text{m}^2$) *S. aureus* aggregates evade phagocytosis and killing by neutrophils by challenging adherent bacteria with human neutrophils *in vitro* during the early stages of biofilm formation. We additionally explored the dynamics of early neutrophil recruitment to a *S. aureus* contaminated surface using *in vivo* intravital imaging and neutrophil localization using a peritoneal implant model. Of note, we observed that human neutrophils readily clear lone cells or small groups of *S. aureus*. However, *S. aureus* rapidly becomes resilient through the formation of multicellular aggregates. *In vivo*, we observed that neutrophil recruitment is spatially and temporally heterogeneous and can take several hours to occur, potentially providing sufficient time for contaminating microorganisms to mount mechanical and leukotoxic defenses prior to engagement by leukocytes.

2. Materials and methods

2.1. Bacteria and neutrophil preparation

Staphylococcus aureus strain AH2547 (HG001 + pCM29, courtesy of Alex Horswill), a known biofilm-forming strain [28] and MW2-GFP (MW2 + pCM29) [29] with constitutive expression of a green fluorescent protein, was grown overnight in tryptic soy broth supplemented with 10 $\mu\text{g}/\text{ml}$ chloramphenicol. Overnight cultures were centrifuged for 5 min at 4000 rpm, rinsed, resuspended in phosphate buffered saline (PBS), and serially diluted. Cells were attached to a 4-chambered glass bottom Petri dish (Cellvis, CA, USA) to facilitate live-cell imaging. To attach cells, varied concentrations of cells were added to the surface in PBS at a volume of 10 μL . After 30 min of incubation at 37 $^{\circ}\text{C}$, unattached bacteria were gently rinsed from the surface with PBS. Each chamber of the Petri dish was filled with 1 ml of 10% fresh human serum in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} to simultaneously coat the surface with serum and opsonize bacteria and

incubated at 37 $^{\circ}\text{C}$ for 30 min. Human neutrophils were isolated from heparinized venous blood obtained from healthy donors following a standard IRB-approved protocol. All donors provided written consent to participate in the study. Neutrophils were isolated under endotoxin-free conditions ($<25\ \text{pg}\ \text{ml}^{-1}$) and purity ($<1\%$ PBMC contamination) and viability ($<2\%$ propidium iodide positivity) of neutrophil preparations were assessed by flow cytometry as previously described [30,31]. Neutrophils were kept on ice until stained with LysoBrite™ Red (AAT Bioquest, CA, USA) according to the manufacturer's instructions. Propidium iodide was added to the bulk medium just prior to neutrophil addition at a concentration of 5 $\mu\text{g}/\text{ml}$. To enumerate surviving bacteria, bacteria were removed from the surface by a sterile scraper and pipetting, vortexed in dilution tubes with PBS, and plated on tryptic soy agar in triplicate. Plates without detectable bacteria were counted as 0.5 colonies prior to log transformation.

2.2. Microscopy

A Leica SP5 inverted confocal scanning microscope was utilized for all *in vitro* imaging. GFP-tagged bacteria and neutrophils were excited with the 488 nm and 561 nm laser lines, respectively. A LiveCell (Pathology Devices, CA, USA) environmental chamber system was utilized to maintain 5% CO_2 , 20% O_2 , 50% humidity, and 37 $^{\circ}\text{C}$ for sample incubation during imaging. Image stacks with 1- μm z-slices were recorded sequentially at 1–2 min intervals over a 4-h time course using a 20 $\times$ objective lens. At least two fields of view from each chamber of the dish were generally imaged per experiment. Each well was then imaged using a 7 $\times$ 7 stitched tile scan image with a 10 $\times$ objective to quantify total amount of bacteria and count neutrophils remaining on the surface.

2.3. Image analysis

MetaMorph version 7.8.13 (Molecular Devices) image analysis software was used to measure the change in bacterial biomass by quantifying the threshold area of bacterial green fluorescence over the period of 4 h. Maximum growth rates were calculated by fitting an exponential curve to the data within log phase. Movies were prepared with Imaris version 8.0, 9.2, or 9.3 (Bitplane). To quantify neutrophil motion, the "Spots" module in Imaris was utilized to track 3D-objects over time, display their path, and analyze their motion. Neutrophils were automatically identified as "Spots" based on size and manually edited and confirmed when needed. Motion was tracked using the Brownian motion algorithm. To quantify the fraction of a field of view "patrolled" by a neutrophil over 4 h, neutrophil tracks determined by the "Spots" module were set to be cylinders 10.87 μm in diameter. The image was then imported into MetaMorph, and the percentage of the field of view covered by a neutrophil track was determined by thresholding.

For the analysis of individual neutrophil-bacteria interactions, the "Spots" feature was used to identify neutrophils and propidium iodide staining events and the "Surfaces" feature was used to dynamically track bacterial growth. From the 4 experiments, two fields of view for each condition were analyzed. 8 interactions per field of view were analyzed for control wells (8 fields of view $\times$ 8 interactions, $n = 64$) while all interactions that could reasonably be distinguished in the head start wells were analyzed (8 fields of view, $n = 70$ interactions). Measurements of the bacterial aggregate were recorded at the start and end of each interaction with a neutrophil, and propidium iodide staining or aggregate breakup events were cataloged. The percentage of aggregates inducing PI staining was calculated by dividing the number of aggregates that caused PI staining of at least one phagocytosing neutrophil by the total number of aggregates discovered by a neutrophil in each field of view.

Maximum growth rates of individual aggregates were calculated by fitting an exponential curve to volume measurements calculated using "Surfaces" in Imaris. Aggregates that were not detected for at least 88% of the frames were discarded. To determine the change in bacterial

biomass over time from *in vivo* experiments, Imaris surface output files were analyzed in MATLAB and the total GFP volume (μm^3) was calculated for each frame. Percent change in biomass was calculated by comparing the last frame to the first frame for the final continuous field of view in each position. *In vivo* growth rates were calculated by fitting an exponential curve to the data collected in a continuous field of view that appeared to be out of lag phase and free of significant signal noise (such as due to animal movement).

Leukocyte counts from SEM images were produced by manually counting all visible cells that could be distinguished as distinct leukocytes and normalizing to the field of view area.

All images and videos from obtained from intravital microscopy were analyzed using Imaris version 9.5.1. GFP *S. aureus* clusters were quantified over time using the surface function and volume and neutrophil recruitment was tracked using the spot function. For quantification of neutrophil migration within the top 20 μm of collagen, spots were filtered for any spot entering the defined area of interest, top 20 μm of collagen, in the z-plane. Discovery frequency was quantified by applying a filter to the GFP bacteria surface “intensity mean” of tdTomato channel and thresholding was automatically applied by default settings.

2.4. Peritoneal implant model

The peritoneal implant model was performed as previously described with modifications [32,33,34]. Overnight cultures of *S. aureus* strain HG001 were grown in TSB for 20 h with shaking at 150 rpm and resuspended in 5 ml TSB before dilution. Sterile silicone tubes (i.d. 4 mm, o.d. 6 mm, length 4 mm) were incubated in a diluted overnight culture of *S. aureus* in 0.9% saline ($\text{OD}_{600} = 0.1$) for 1 h at 100 rpm. Female C57Bl/6J mice were purchased from Janvier and allowed two weeks to acclimate prior to surgery. Mice were anesthetized with Hypnorm-midazolam (Hypnorm [0.315 mg fentanyl citrate/ml and 10 mg fluanisone/ml] and midazolam [5 mg/ml] and sterile water [1:1:2]) via subcutaneous injection in the groin area. 1 ml sterile PBS was injected into the mouse peritoneum 4 h prior to implantation. Implants were inserted into the peritoneal cavity via a 1 cm groin incision. The mice were given bupivacaine and Temgesic for postoperative pain and euthanized by cervical dislocation at the end of the experiment. Several implants were removed and fixed in 2% glutaraldehyde for analysis by scanning electron microscopy while the remaining implants were put in 2 ml 0.9% NaCl and kept on ice until further processing. To quantify colony forming units on implants pre-insertion, representative silicone tubes were sonicated in 0.9% saline and dilutions were plated on tryptic soy agar. To determine the number of neutrophils recruited to the peritoneum, a lavage was performed by injecting 5 ml of PBS into the peritoneal cavity and gently massaging the abdomen before withdrawing the peritoneal fluid (PF) [35]. PF was stored on ice until preparing the samples for determination of recovered neutrophils by flow cytometry. To enumerate the neutrophils in the PF a 100 μL sample was added to a TruCount Tube (340334, Becton Dickinson). Ten μL of Anti-Ly-6G-FITC, mouse (130-102-295, Miltenyi Biotec), Anti-Ly-6G-APC, mouse (130-102-342, Miltenyi Biotec), and CD45-PerCP, mouse (130-102-469, Miltenyi Biotec) were added and the samples were incubated on ice for 30 min in the dark before fixation by addition of 1 ml of 10% (v/v) FACS lysing solution (349202, Becton Dickinson). Stained and fixed samples were analyzed by flow cytometry using a FACSCanto II (Becton Dickinson). Light scatter and fluorescence parameters for $>10,000$ events were recorded after gating on forward light scatter and fluorescence for CD45 staining to exclude debris, cell aggregates and bacteria. The instrument was calibrated with CSTbeads (Becton Dickinson, Franklin Lakes, NJ, USA). All experiments were authorized by the National Animal Ethics Committee, Denmark, Permit number 2018-15-0201-01500.

2.5. Scanning electron microscopy

Specimens were fixed in 2% glutaraldehyde in 0.05 M sodium phosphate buffer, pH 7.4. Following 3 rinses in 0.15 M sodium phosphate buffer (pH 7.4) specimens were post fixed in 1% OsO₄ in 0.12 M sodium cacodylate buffer (pH 7.4) for 2 h. Following a rinse in distilled water, the specimens were dehydrated in 100% ethanol according to standard procedures and critical point dried (Balzers CPD 030) with CO₂. The specimens were subsequently mounted on stubs using double adhesive carbon tape (Ted Pella) as an adhesive and sputter coated with 6 nm gold (Leica ACE 200). Specimens were examined with a Quanta 3D SEM (FEI, Eindhoven, The Netherlands) operated at an accelerating voltage of 2 kV.

2.6. Intravital imaging in a murine skin flap model

Animal experiments were performed with male adult 7-8-wk-old mice and all experimental animal protocols were approved by the University of Calgary Animal Care Committee and followed guidelines established by the Canadian Council for Animal Care (protocol number AC19-0138). All mice were housed under specific pathogen-free conditions and received sterilized rodent chow and water *ad libitum*. CatchupTM mice in which neutrophils are tagged with a tdTomato fluorescent protein were anesthetized and kept at body temperature using a heating pad [36]. A jugular catheter was inserted to maintain anesthesia and deliver systemic treatments. The dorsal flank skin was exteriorized on the right flank as previously described [26]. The tissue was covered with a cover slip that had been seeded with GFP-tagged *S. aureus* at surface densities similar to those used in *in vitro* experiments (10^3 – 10^4 CFU/cm², pregrown in RPMI medium, rinsed with HBSS). Superfusion buffer (HBSS without calcium, magnesium, or phenol red, ThermoFisher) was then perfused across the exteriorized skin tissue to keep the skin moist at a flow rate set to 0.17 ml/min.

Mice were imaged for up to 180 min after being anesthetized using a Leica SP8 upright multiphoton microscope. Laser excitation at 940 nm was used to excite tdTomato and GFP with external detectors (HyD-RLD2 BP 585/40 for tdTomato, HyD-RLD3 BP 525/50 for GFP) and second harmonic generation (external detector HyD-RLD4 BP 450/70) to visualize skin collagen. Laser power, detector settings and acquisition settings were maintained throughout each experiment. Blood vasculature was labeled intravascularly using QtrackerTM 655 Vascular Labels (ThermoFisher) with laser excitation at 940nm (external detector HyD-RLD1 BP 675/50).

A 3D tile scan (4×4 fields of view; each field of view $350 \times 350 \times 100 \mu\text{m}^3$) was first collected to get an overview of the GFP *S. aureus* aggregates on the coverslip. From there, 3 fields of view ($350 \times 350 \times 80 \mu\text{m}^3$) were selected within the 3D tile scan to select the video positions. Images were collected every 45 s for up to 180 min.

2.7. Statistical treatment of data

Data were analyzed using GraphPad Prism version 8.4.2. Normality was assessed by the D'Agostino-Pearson or Shapiro-Wilk test. Normal data was tested with an unpaired student's *t*-test, one-way ANOVA with Tukey's post hoc test, two-way ANOVA with Bonferroni's or Tukey's multiple comparisons test or Brown-Forsythe ANOVA with Dunnett's T3 multiple comparisons test as appropriate. Otherwise, the Kruskal-Wallis test or Mann-Whitney test was applied. The test applied is described in each figure legend.

3. Data availability

The raw and processed data required to reproduce these findings are available to download from <https://datadryad.org/stash/share/WHrJ0eXSaqg3jdFH9pOPwUlablkGwL2RVvFePLu3fc4>.

4. Results

4.1. High surface densities of neutrophils are required for effective clearance of *S. aureus*

We utilized an *in vitro* confocal microscopy approach to observe interactions between adherent *S. aureus* and human neutrophils (polymorphonuclear leukocytes, PMNs). Green fluorescent protein (GFP) expressing *S. aureus* AH2547 was sparsely seeded on a glass surface, challenged with fluorescently labeled neutrophils, and continuously imaged for 4 h. In the absence of neutrophils, bacteria grew and formed dense aggregates on the surface with a collective specific growth rate of $0.80 \pm 0.16 \text{ h}^{-1}$. Interestingly, the growth rate of individual *S. aureus* aggregates was quite variable, ranging from -0.2 to 1.6 h^{-1} (Figure S1A). Clearance of bacteria was highly dependent on the density of neutrophils on the surface, with effective killing occurring under higher neutrophil conditions (Fig. 1A and B). Low neutrophil conditions resulted in killing of some attached bacterial aggregates, but survival and prolific growth of the many undiscovered aggregates. Conversely, sufficiently high neutrophil concentrations allowed rapid discovery and elimination of adherent bacteria before aggregates were able to grow significantly (Fig. 1A and B). Neutrophil surface densities greater than 10 000 neutrophils/cm² often enabled effective clearance of bacteria, resulting in 1.5–2 log reductions in bacterial burden compared to control wells without neutrophils (Fig. 1C). Clearance of the bacteria correlated well with the ability of neutrophils to “patrol” large surface areas (Fig. 1D). Net bacterial growth as measured by GFP area within a given field of view (FOV) was halted when approximately 50% of the surface was patrolled and reductions in bacterial burden greater than one log were consistently observed at approximately 80% coverage of the surface by neutrophil tracks (Fig. 1D). When densities of both neutrophils and bacteria were low, the outcome was stochastic and the result often depended on timely discovery of the bacteria, which we have previously described in a mathematical model [24]. Thus, we have observed that a high surface density of neutrophils is required to effectively discover and

eliminate *S. aureus* sparsely adherent to an abiotic surface.

4.2. Neutrophil recruitment to a peritoneal implant is heterogeneous

To observe spatial patterns of immune cell recruitment to a contaminated biomaterial *in vivo*, we utilized a previously described murine peritoneal implant model [32,33]. A silicone tube was homogeneously coated with a low density of *S. aureus* and inserted into the mouse peritoneum via groin incision. Mice were euthanized 24 h post implantation and the implants were removed for analysis. We estimated the initial bacterial burden on the implant surface to be approximately $4.1 \pm 1.7 \times 10^5$ CFUs/implant via plate count and $1.2 \pm 0.51 \times 10^6$ cells/cm² based on counts from scanning electron microscopy (SEM) micrographs ($n = 9$ FOVs from 2 implants) (Fig. 2A). After 24 h in the peritoneum, the mean log reduction of viable bacterial counts recovered from implants (referenced to the pre-insertion count) was 1.98 ± 0.73 ($n = 11$). Flow cytometry of immune cells collected by peritoneal lavage showed that neutrophils were highly prevalent in the mouse peritoneum at 24 hpi, constituting $53 \pm 6.8\%$ of host cells ($n = 8$ mice), whereas the background levels of neutrophils in animals that did not receive an implant was $0.7 \pm 0.8\%$ ($n = 8$). The concentration of neutrophils in peritoneal lavage fluid was $4.3 \pm 3.3 \times 10^3$ cells/ml in animals that did not receive an implant and rose to $4.1 \pm 0.9 \times 10^5$ cells/ml in animals harboring inoculated implants for 24 h. Interestingly, although the distribution of *S. aureus* appeared uniform across the implant surface prior to insertion, recruitment of immune cells to the implant was spatially heterogeneous. By SEM, we observed the formation of distinct patches of host material on the implant surface (Fig. 2B). Examination of these patches at higher magnification revealed aggregations of leukocytes (Fig. 2C). We estimated the local surface density of these leukocyte patches to be $6.2 \pm 5.6 \times 10^5$ leukocytes/cm² based on counts of observable cells in areas where leukocytes were present ($n = 10$ FOVs from 2 implants) (Fig. 2F). Several FOVs were devoid of leukocytes (Fig. 2F). Magnification of areas lacking these leukocyte patches revealed that *S. aureus* aggregates remained on the surface with little to

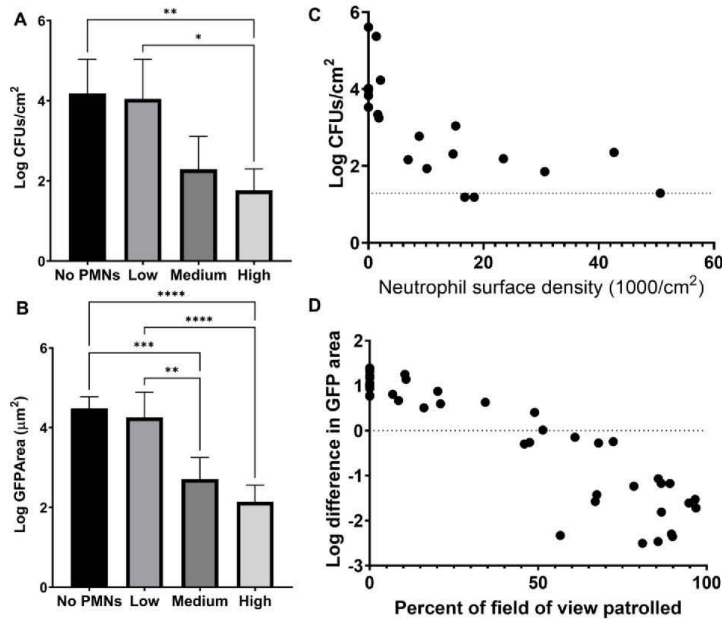


Fig. 1. High neutrophil densities are required for adequate clearance of attached *S. aureus* *in vitro*. Approximately 2×10^5 CFU/cm² *S. aureus* GFP were initially added to the surface and challenged with PMNs for 4 h. (A) *S. aureus* CFUs recovered and (B) total remaining GFP signal from wells with no, low ($1.7 \pm 0.27 \times 10^3$ PMNs/cm²), medium ($1.0 \pm 0.31 \times 10^4$ PMNs/cm²), or high ($2.8 \pm 1.3 \times 10^4$ PMNs/cm²) numbers of PMNs. Data are mean $\pm$ SD ($n = 4-7$). Kruskal-Wallis Test (panel A) and one-way ANOVA with Tukey's post hoc test (panel B) ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). (C) CFUs recovered from challenge plotted against varied neutrophil surface densities. Dashed line represents limit of detection. $n = 5$ independent experiments. (D) Log difference in GFP area per field of view as a function of the percentage of the field of view covered by neutrophil tracks. $n = 40$ FOVs from 5 independent experiments.

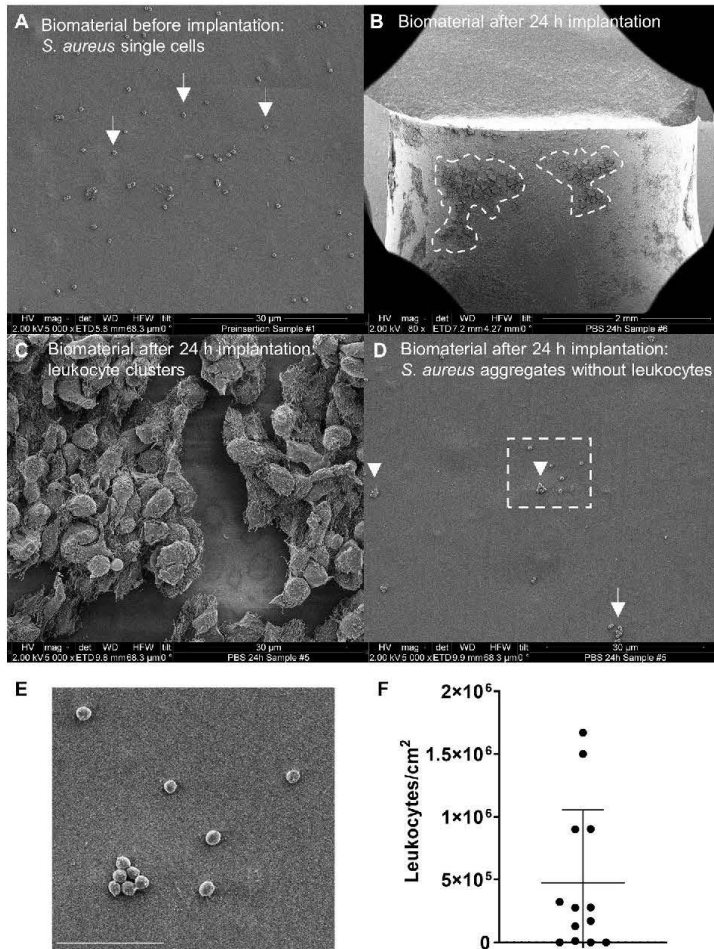


Fig. 2. Immune cell recruitment to an inoculated peritoneal implant is heterogeneous. (A) SEM micrographs showing adherent *S. aureus* cells on the surface of a silicone tube prior to implantation into the mouse peritoneum. (B–D) Representative SEM micrographs from silicone tubes removed from the mouse peritoneal cavity after 24 h. (B) At low (80×) magnification, distinct areas of significant immune cell recruitment can be observed as dark patches on the implant surface (highlighted by dashed lines). (C) Higher magnification (5000×) reveals the dark patches outlined in (B) to be leukocyte clusters. (D) Higher magnification (5000×) reveals sections of the implant devoid of leukocytes. (E) Inset of the region denoted by the white box in (D). Scale bar = 5 μm. (F) Estimated leukocyte numbers on the implant surface from SEM images, normalized to FOV area (n = 13 FOVs).

no interaction with recruited immune cells (Fig. 2D). Additionally, while the SEM images of implants pre-insertion revealed mostly single or paired groupings of *S. aureus* cells (Fig. 2A), the areas without recruited immune cells on explanted surfaces contained clusters of *S. aureus* with larger numbers of cells, suggesting growth (Fig. 2E). These observations support the hypothesis that some bacteria on a contaminated surface may escape detection due to heterogeneous recruitment of immune cells, providing these cells an opportunity to develop into more resilient aggregates.

4.3. *S. aureus* aggregates resist clearance by neutrophils *in vitro*

We have observed previously that *S. aureus* aggregates discovered by neutrophils later in an *in vitro* experiment tended to be more tolerant to neutrophil clearance [24]. Therefore, we sought to further investigate and quantify the effect of aggregate size and timing of discovery on *S. aureus* survival of neutrophil challenge. *S. aureus* was sparsely seeded on glass and then allowed to form aggregates for 4 h prior to the addition of neutrophils. In control wells without neutrophils, preformed *S. aureus* aggregates grew into larger aggregates on the surface after 4 h of

imaging (Fig. 3Ai-ii). Clusters of *S. aureus* that were given a 4 h head start prior to the addition of neutrophils resisted clearance, resulting in large aggregates remaining on the surface at the end of the experiment (Fig. 3Aiii-iv). However, CFU-matched single-cell *S. aureus* were readily cleared by the neutrophils within 4 h (Fig. 3Avii-viii). We quantified the log CFU of *S. aureus*/cm² in the presence and absence of neutrophils for both head start (4 h) and no head start (single-cell *S. aureus*) and found that in the presence of neutrophils, there was a significant reduction of *S. aureus* recovered from the surface post-imaging (Fig. 3B). Furthermore, neutrophils were significantly more effective at clearing single-cell *S. aureus* compared to preformed aggregates (Fig. 3C). This suggests that surface attachment alone is not an inherent barrier to neutrophil clearance, but within a short period of time *S. aureus* aggregates can develop tolerance to neutrophils.

We observed that some aggregates failed to decrease in size after co-localization with a neutrophil and that some neutrophils disappeared during this interaction. These observations led us to investigate whether larger *S. aureus* aggregates were lysing neutrophils. We added propidium iodide (PI), a membrane impermeable dye, to our assay to investigate whether neutrophil membrane integrity was compromised upon

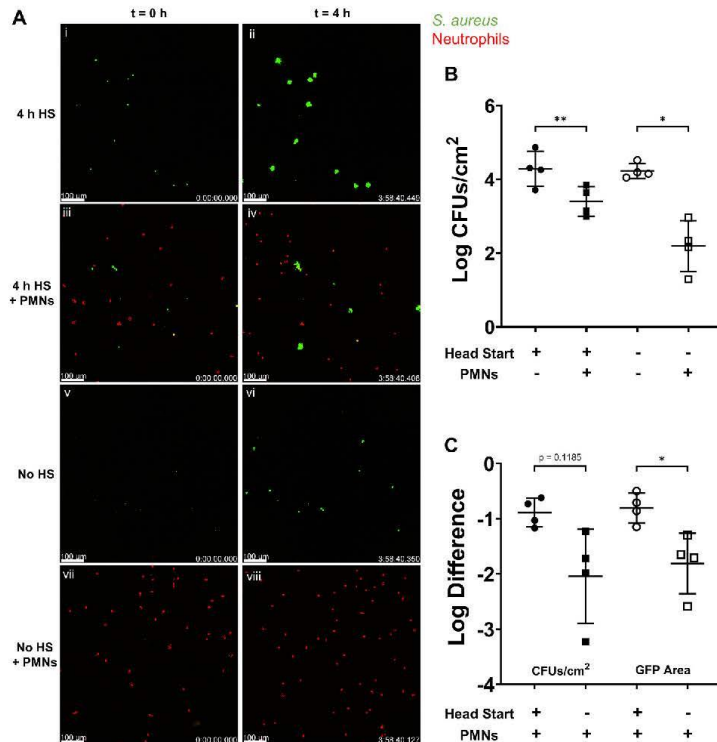


Fig. 3. *S. aureus* aggregates become resilient to neutrophil clearance after 4 h of growth *in vitro*. *S. aureus* (green) clusters were seeded at $\sim 10^2$ CFU/cm² and grown for 4 h (HS) or seeded at $\sim 10^3$ CFU/cm² and grown for 0.5 h (No HS) prior to the start of imaging, with or without neutrophil (red) addition ($\sim 2 \times 10^4$ PMNs/cm²). (A) Representative microscopy images at t = 0 h and t = 4 h. (B) Log CFUs/cm² recovered from wells post imaging. (C) Log difference in CFUs/cm² (black points) and GFP area (white points). Data are mean $\pm$ SD (n = 4 independent experiments). Paired student's t-test *p < 0.05, **p < 0.01.

interaction with large *S. aureus* aggregates. Neutrophils interacting with larger aggregates often stained extensively with PI, suggesting these aggregates were able to damage neutrophil membrane integrity (Video S1). Neutrophils became PI positive at a much higher frequency when *S. aureus* aggregates were given a head start prior to the addition of neutrophils (Fig. 4A–C). Under the head start condition, $86 \pm 19\%$ of aggregates in a given FOV induced PI staining of at least one neutrophil while we did not observe any single cells of *S. aureus* causing PI staining of neutrophils in this assay (Fig. 4C, Video S1). We analyzed the outcome for each bacterial aggregate present in a field of view and found that aggregates greater than roughly $50 \mu\text{m}^2$ caused frequent neutrophil lysis and aggregates greater than $75 \mu\text{m}^2$ caused neutrophil lysis in all of the interactions observed in these experiments (Fig. 4D). Larger aggregates tended to persist, as aggregates with initial area greater than $75 \mu\text{m}^2$ at the time of interaction with a neutrophil often failed to decrease or even grew in size from the time of discovery (Fig. 4E). We also observed on occasion that large aggregates could be broken up or fractured into smaller pieces by multiple neutrophils and that these smaller pieces were more readily cleared (Video S2). These events did not seem to depend solely on the size of the aggregate (Figure S2A). Rather, aggregates appeared to fracture when a high number of neutrophils attempted to phagocytose a portion of the aggregate over the course of the experiment (Figure S2B). Non-fractured aggregates tended to grow slightly over the 4 h observation period following discovery by the first neutrophil, however when fracture occurred, there was a slight decrease in the total volume of all fractured pieces (Figure S2C). This suggests a better outcome when neutrophils are successfully able to fragment a portion of the bacterial cluster compared to when the clusters remain intact.

Supplementary data related to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2021.120775>

4.4. Minimal neutrophil recruitment to a *S. aureus*-contaminated coverslip *in vivo*

We adapted a murine skin intravital microscopy model [26,37] to visualize *in vivo* neutrophil behavior on a coverslip inoculated with bacteria in real time using resonant-scanning multiphoton intravital microscopy. This design paralleled the *in vitro* experiments described above. Approximately 10^4 CFUs of *S. aureus* AH2547 GFP were seeded onto a glass coverslip and allowed to grow for 4 h to form aggregates (head start) and the contaminated coverslip was interfaced with the dermis. With the multiphoton system, dermal collagen was visualized via second harmonic generation (SHG), neutrophils were fluorescently labeled with tdTomato in the Catchup^{IVM-red} reporter mouse [36] and *S. aureus* was visualized with GFP. Previously, it has been shown that neutrophils are robustly recruited to a localized *S. aureus* infection in the skin within 2 h [26], so we asked whether neutrophils were recruited in a similar timeline to *S. aureus* aggregates on a coverslip. Over the course of 180 min, there was minimal neutrophil recruitment to the *S. aureus* coverslip surface (Fig. 5A–C, Video S3). In many FOVs, we observed zero or one neutrophil and the one neutrophil was often deeper in the collagen away from the *S. aureus* aggregates (Fig. 5A). In the rare occurrence where we saw neutrophil recruitment (Fig. 5B), we quantified how many neutrophils migrated towards the *S. aureus* aggregates within the top 20 μm of collagen (Fig. 5D–E). We recorded three FOVs per mouse (from n = 4 independent experiments) and observed around 10 neutrophils migrate into the top 20 μm of collagen in only two of the

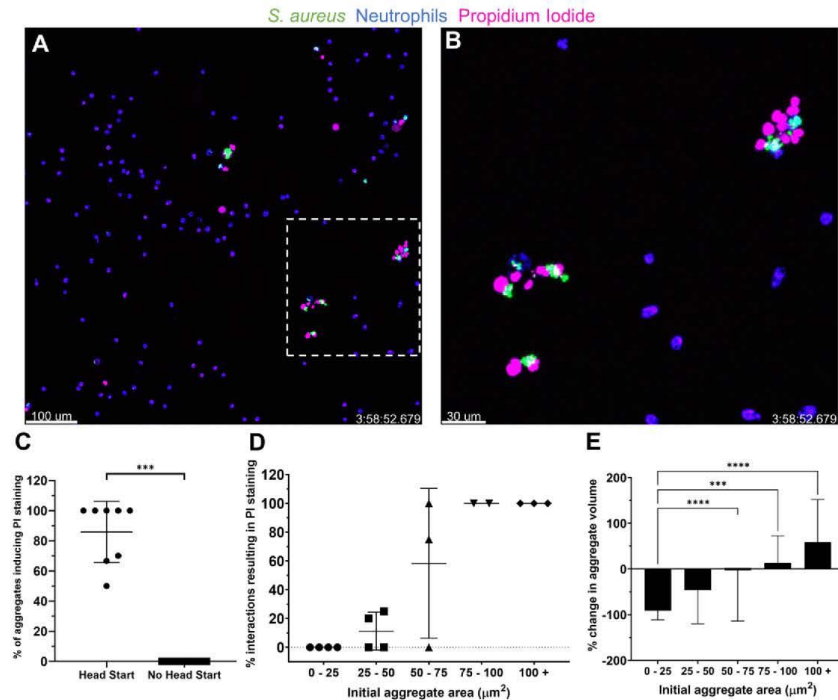


Fig. 4. Larger *S. aureus* aggregates cause neutrophil lysis and resist clearance *in vitro*. (A) *S. aureus* aggregates (green) were given 4 h to grow prior to the addition of neutrophils (blue). Representative images at $t = 4$ h show propidium iodide (PI) staining (pink) of neutrophils interacting with larger, persistent clusters of *S. aureus*. (B) Enlarged image of the inset in (A). (C) Percentage of *S. aureus* aggregates causing PI staining of at least one neutrophil. Data are mean $\pm$ SD ($n = 8$ FOVs per group from 4 independent experiments. Mann-Whitney test $***p < 0.001$). (D) Rates of PI staining of neutrophils as a function of the bacterial aggregate area at the beginning of the interaction. Each point represents the calculated average of all interactions within the size boundaries from a given experiment. The number of observed interactions per point ranged from $n = 1$ –19 collected from 16 FOVs from 4 independent experiments. (E) Change in volume of an aggregate from the first frame in which a neutrophil phagocytoses it to the last frame that it is visible. Data are mean $\pm$ SD (Bins contain 6–58 measurements from 16 FOV from 4 independent experiments. Kruskal-Wallis test $***p < 0.001$, $****p < 0.0001$).

twelve FOVs (Fig. 5D), suggesting very few neutrophils are recruited to the *S. aureus*-contaminated coverslip *in vivo* during these early time points. Of those neutrophils that were able to migrate towards the coverslip surface, we further quantified how many associated with *S. aureus* aggregates (Fig. 5F) and found again that few neutrophils were interacting with *S. aureus* (Fig. 5F and G, Video S4). To confirm these results, we repeated the experiment using a clinically relevant methicillin-resistant *S. aureus* strain MW2 and again observed minimal neutrophil recruitment (Figure S3). Using single-cell *S. aureus* attached to the coverslip for 30 min (no head start), we observed no neutrophil recruitment to the coverslip over 180 min (Fig. 5H). In many of the videos we occasionally observed one or two neutrophils, but this was transient as the neutrophils seemed to be passing by in deeper skin capillaries (data not shown). Importantly, there was no neutrophil recruitment to a sterile coverslip (Fig. 5I). At the end of the 180 min of imaging, a fluorescent vascular dye was injected to visualize dermal blood vessels and capillaries within the dermal collagen (Fig. 5J). This larger stitched image highlights that there was no neutrophil recruitment to a sterile coverslip, though we were able to detect one or two neutrophils within capillaries (Fig. 5Ki-ii) and within larger dermal vessels (Fig. 5Kiii).

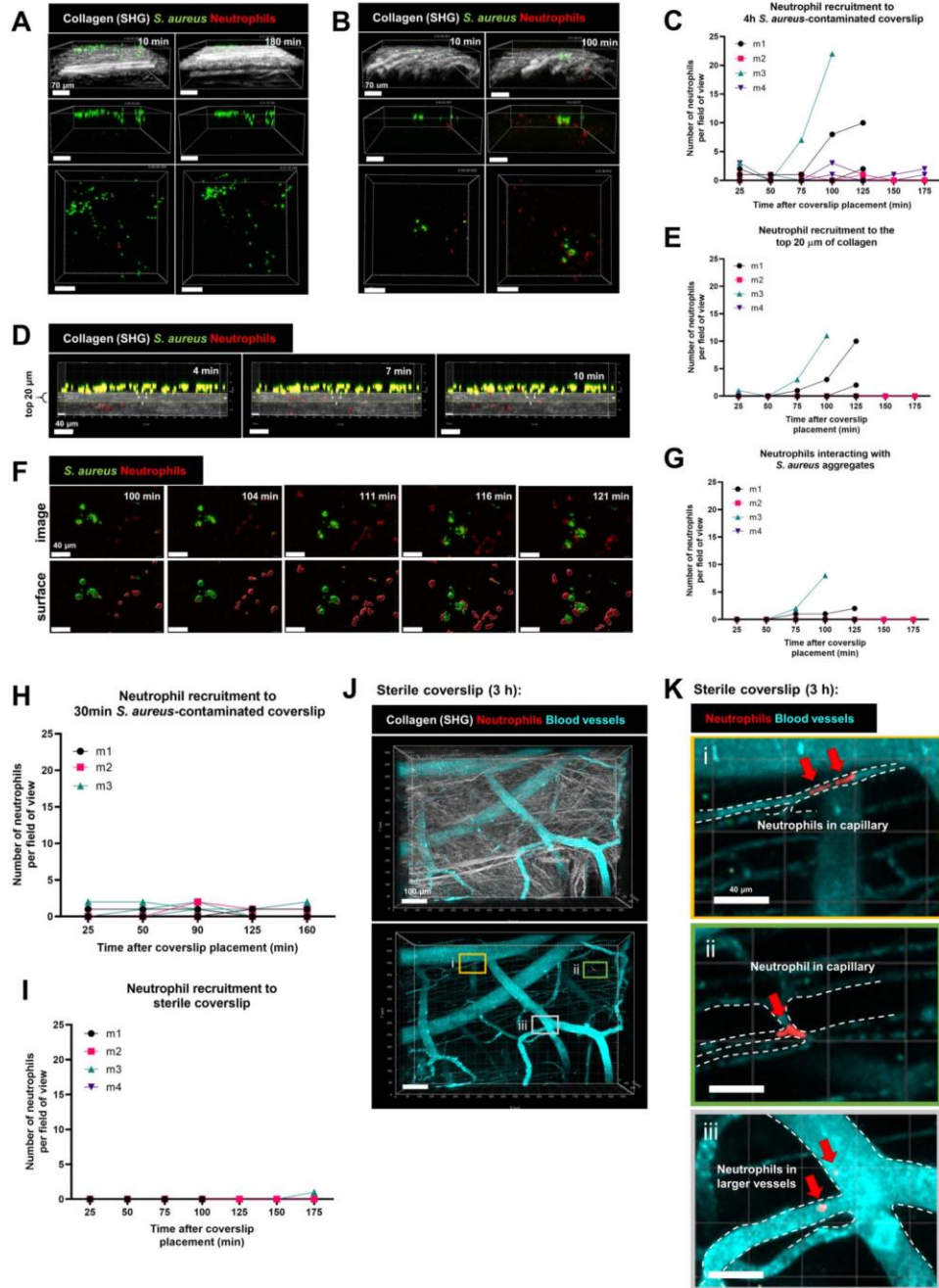
Altogether, these data show that very few neutrophils are recruited to a *S. aureus* contaminated coverslip interfaced with the dermis over the course of 180 min *in vivo*. On the rare occasion where neutrophils

migrated to the coverslip surface, they can interact with *S. aureus* aggregates, but the earliest this occurred was about 1–2 h after placing the coverslip onto the skin. In most FOVs, neutrophil recruitment was not observed over the imaging window of 180 min.

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4.5. *S. aureus* aggregates grow on the coverslip *in vivo*

To understand the dynamics of bacterial growth *in vivo*, we measured the increase in surface volume of *S. aureus* aggregates over the course of imaging. It was clear that the bacterial aggregates became larger from the time the coverslip was added to the skin (Fig. 6A) to the end of imaging at 180 min (Fig. 6B). *S. aureus* aggregates were closely associated with the collagen surface (Fig. 6C) suggesting they are in physical contact with the host dermal tissue. *In vitro*, *S. aureus* required supplementation of HBSS with serum to grow, demonstrating that host factors were required to support bacterial growth and that the superfusion buffer (HBSS) alone would not explain the observed growth (Fig. S1B and S1C). We measured the growth rate of *S. aureus* over the 3 h of imaging and found the average growth rate to be $0.46 \pm 0.45 \text{ h}^{-1}$ (median 0.25 h^{-1}) for strain AH2547, however significant heterogeneity was observed between FOVs (Fig. 6D and E). Similar results were obtained with strain MW2 (Figs. 6E, S3C). These data demonstrate that



(caption on next page)

Fig. 5. Minimal neutrophil recruitment to a *S. aureus*-contaminated coverslip *in vivo*. *S. aureus* aggregates were given 4 h to grow prior to placing the coverslip onto the dermal skin flap for intravital imaging. Representative 3D images of (A) minimal neutrophil recruitment and (B) moderate neutrophil recruitment at 10 min and 180 min after coverslip placement onto skin flap. Top: side view showing collagen (SHG) signal, middle: side view without collagen, bottom: top view without collagen. Scale bars = 70 μm . (C) Quantification of neutrophil recruitment to the 4 h *S. aureus*-contaminated coverslip. (D) Representative timelapse images of neutrophils (red spots) trafficking through the collagen into the top 20 μm . Scale bars = 40 μm . (E) Quantification of the number of neutrophils in the top 20 μm of collagen. (F) Representative timelapse images of neutrophils interacting with *S. aureus* aggregates at the coverslip surface over the course of 20 min. Scale bars = 40 μm . (G) Quantification of the number of neutrophils interacting with *S. aureus*. Quantification of (H) neutrophil recruitment to a single-cell 30-min attached *S. aureus*-contaminated coverslip and (I) sterile coverslip. (J) Representative 3D stitched image of a sterile coverslip after 180 min of imaging, showing dermal collagen (white), blood vessels (cyan) and neutrophils (red). Bottom: identical 3D image without collagen. Scale bars = 100 μm . Three boxes (i, ii, iii) are selected to show one or two neutrophils in capillaries (K i, ii) and two neutrophils in larger dermal blood vessels (K iii). Scale bars = 40 μm . For C,E,G,H,I, n = 3–4 mice from 3 to 4 independent experiments listed as m1, m2, m3, m4. Data show 3 fields of view per mouse.

once the coverslip was placed in close contact with host tissue, bacteria started to grow and form aggregates before neutrophils arrived.

4.6. Recruited neutrophils discover some aggregates *in vivo*

Next, we asked whether neutrophils pre-recruited to the skin with the chemokine macrophage inflammatory protein 2 (MIP-2) would affect bacterial aggregate discovery and clearance. Mice were treated with MIP-2 4 h before the start of imaging (Fig. 7A). In contrast to experiments without MIP-2 treatment (Fig. 5A and B), many neutrophils were present in the dermis at the outset (Fig. 7B and C). Over the course of imaging, we did not see a significant increase in the number of neutrophils indicating that no additional neutrophils were recruited into the dermis during this time (Figure 7B, S4A and S4B). Within an individual animal, neutrophil recruitment to the coverslip was also heterogeneous where some fields of view had more neutrophils than other fields of view from the same mouse (Figures S4C and S4D, Video S5). Although we did see neutrophils enter the field of view during the 180 min window (Figure S4C and S4D), this was not due to an increase in total neutrophil recruitment (Fig. 7B, S4A and S4B) but a change in neutrophil localization (Fig. 7C). At 10 min many of the neutrophils were 50–100 μm away from the *S. aureus* aggregates but by 180 min, the neutrophils migrated towards the coverslip and were much closer to the aggregates (Fig. 7C). Indeed, we observed a significant change in neutrophil localization as neutrophils migrated 50–100 μm towards the bacterial aggregates in the z-plane, which suggests that the existing neutrophils pre-recruited by MIP-2 addition were able to migrate towards *S. aureus* (Fig. 7C and D). After a 30 min single-cell attachment, many neutrophils chemotaxed towards the *S. aureus* single cells and migrated very close to the coverslip interface with a mean distance of 26 μm away from the average plane of the bacterial aggregates (Fig. 7D). We next compared this to a head start experiment where we allowed *S. aureus* aggregates to grow for 4 h to the coverslip. Although neutrophils did migrate towards these larger aggregates, there was strikingly a significant decrease in neutrophil localization and these neutrophils were much further away from the aggregates, with a mean distance of 42 μm away (Fig. 7D). The frequency of aggregate discovery by neutrophils was calculated using Imaris by applying a filter to the aggregate surface (Fig. 7E, Video S6). When bacteria were only attached for 30 min, there was a significant increase in aggregate discovery between 10 min and 180 min (Fig. 7F and G). At the 180 min time point, neutrophils more frequently discovered bacteria in the 30 min attachment condition (45–75% discovery) compared to a 4 h aggregate (25–35% discovery) (Figs 7F, G, and S4E). Mice that were not treated with MIP-2 had negligible bacteria discovery (under 10% discovery) at the 180 min time point (Figures 7F, G and S4E). To identify any relationship between bacterial load and number of neutrophils recruited, we analyzed the trends of total bacterial surface volume at the 180 min time point against total number of neutrophils (Figure S4F). Interestingly, without MIP-2 pre-treatment we did not see a positive correlation between the number of neutrophils and the bacterial surface volume, suggesting that at these bacterial burdens and time points, increased bacterial biomass does not directly lead to increased neutrophil recruitment (Figure S4F).

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Overall, pre-recruitment of neutrophils to the skin with MIP-2 led to a marked reduction in *S. aureus* aggregate volume over the course of the experiment when *S. aureus* was given a head start to form aggregates (Fig. 7H). *S. aureus* only given 30 min to form aggregates saw a slight but not statistically significant reduction in volume after MIP-2 treatment (Fig. 7H). This demonstrates that neutrophils must be present in high densities in order to have an effective response to remove the contaminating bacteria. Due to the limited imaging window of 180 min, we never observed full clearance of bacteria from the coverslip even with pre-recruitment of neutrophils, and some bacterial aggregates were left undiscovered for the entire 180 min of imaging (Fig. 7G).

5. Discussion

S. aureus is a major pathogen of implant-associated infections and serious complications can arise if the device is not surgically removed. Presently, it is unclear how small amounts of bacteria evade the host immune system and establish an infection. These early events in the interaction between host and pathogen are currently under-studied and we therefore investigated initial interactions between neutrophils and nascent *Staphylococcus aureus* biofilms *in vitro* and *in vivo*.

We hypothesized that successful clearance of contaminating bacteria from an implant surface requires that neutrophils are recruited to the surface, adhere to and patrol the surface, discover contaminating bacteria, and successfully phagocytose and kill the bacteria. We confirmed that neutrophils are able to kill surface attached *S. aureus in vitro* if a sufficient number of neutrophils are present on the surface and if *S. aureus* did not have time to form nascent biofilm aggregates. Deficiencies in neutrophil recruitment could permit biofilm formation in two ways: 1) Delayed recruitment could lead to the formation of neutrophil-tolerant biofilm aggregates prior to the appearance of a robust innate immune response, 2) An insufficient number of neutrophils “patrolling” an implanted surface could result in some of the attached *S. aureus* remaining undiscovered, again allowing aggregate formation. While we may expect *in vivo* to see directed recruitment of neutrophils to contaminating organisms due to chemotactic gradients produced by local cells that may initially discover contaminating microorganisms (macrophages, epithelial cells, etc.), we clearly observed *in vivo* that neutrophil recruitment can be highly heterogeneous. Thus, a high recruitment of neutrophils to an implanted surface is likely still important to enable full surface coverage and ensure that any contaminating microorganisms are destroyed promptly. Alternatively, during infection bacterial aggregates often exist in the peri-implant space and may not require direct neutrophil attachment to the surface for discovery. Robust recruitment to the peri-implant space to effectively discover these aggregates likely is still essential for clearance in this scenario.

We observed that *S. aureus* aggregates begin to demonstrate tolerance and the ability to lyse neutrophils at a relatively small aggregate size of approximately 50 μm^2 . Accordingly, it is plausible that *S. aureus* requires only a small amount of time, perhaps on the order of hours, prior to the arrival of host innate immune defenses in order to be able to mount a defense and tolerate neutrophil challenge. *S. aureus* aggregates

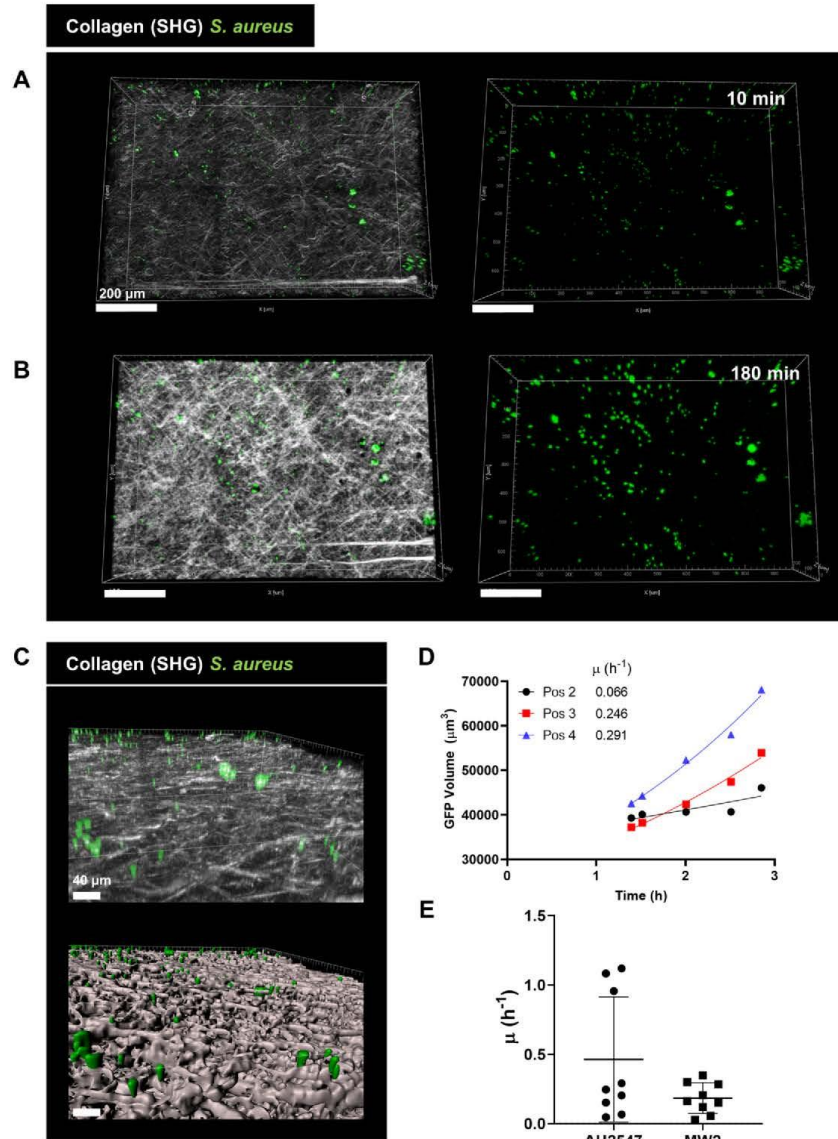


Fig. 6. *S. aureus* aggregates grow on the coverslip *in vivo*. (A) Representative intravital image of a 3D region recorded at (A) start of imaging (10 min after coverslip addition) and (B) end of imaging (180 min after coverslip addition). Left: collagen (white) and *S. aureus* (green), right: *S. aureus* signal only. Scale bars = 200 μm . (C) 3D reconstruction of the coverslip surface coverslip showing GFP *S. aureus* in close contact with collagen. Top: 3D image, bottom: surface reconstruction with Imaris. Scale bars = 40 μm . (D) Representative quantification of *S. aureus* growth *in vivo* over 180 min of imaging with an exponential curve fit to the data. Reported value μ represents the specific growth rate satisfying equation $X = X_0 e^{\mu t}$. Data shown includes representative points outside of lag phase from one mouse with three fields of view. (E) Growth rate values measured *in vivo*. Points represent value measured from a single FOV. Error bars indicate mean $\pm$ SD. (n = 3 mice/condition with 3 FOV per mouse).

appear to resist clearance by neutrophils both by the physical protection afforded by forming an aggregate and elaboration of virulence factors. Aggregates broken up by several neutrophils appeared to be slightly more susceptible to neutrophil killing, highlighting a benefit for the microorganisms of developing a structurally durable aggregate. After 4 h

of aggregate formation, *S. aureus* caused significant cytotoxic damage to interacting neutrophils *in vitro*, likely due to the production of pore-forming toxins such as γ -hemolysin or Pantone-Valentine leukocidin, resulting in persistence of the bacteria [18,19]. These behaviors are strikingly similar to examples of classical biofilm behavior that

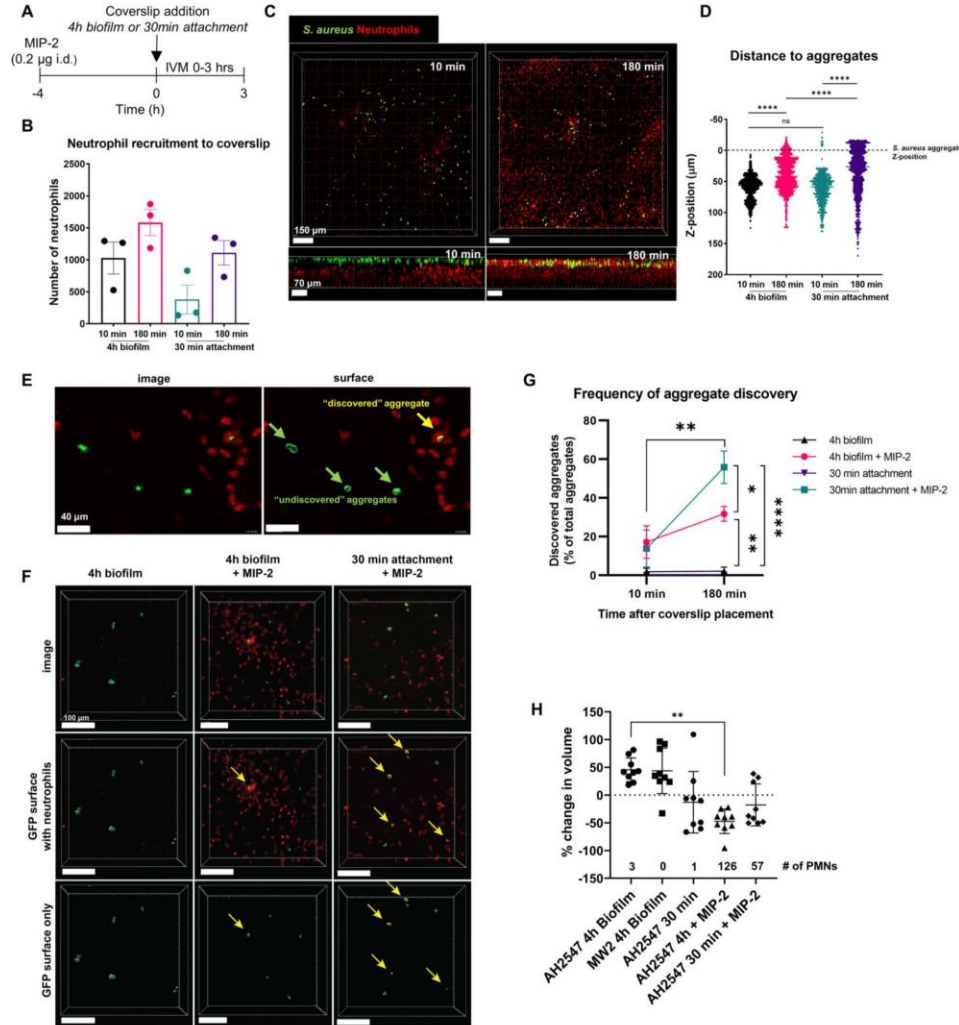


Fig. 7. Pre-recruitment of neutrophils to the skin results in *S. aureus* aggregate discovery and increased clearance. (A) Experimental timeline for MIP-2 treatment and intravital microscopy. (B) Quantification of neutrophil recruitment at 10 min and 180 min after coverslip placement. Error bars indicate the mean $\pm$ SEM (n = 3/condition, one-way ANOVA with Tukey's post-hoc test). (C) Representative 3D stitched image showing neutrophil migration through the z-plane towards *S. aureus* on the coverslip at 10 min and 180 min after coverslip placement. Top panels show XY plane viewed from the top (scale bars = 150 μ m), bottom panels show XZ plane viewed from the side (scale bars = 70 μ m). (D) Quantification of neutrophil distance to *S. aureus* aggregates. Z-position was normalized to the averaged Z-position of *S. aureus* aggregates at each time point. Dashed line indicates the average z-position of *S. aureus* aggregates. Each dot represents one neutrophil. Data are mean $\pm$ SEM (n = 3 mice/condition, one-way ANOVA $p < 0.0001$, Tukey's post hoc test **** $p < 0.0001$). (E) Representative image showing one bacterial aggregate discovered by neutrophils and three other bacterial aggregates that remain undiscovered. Left: intravital image, right: surface reconstruction with a filter applied to label the discovered bacteria aggregate (yellow) and undiscovered aggregates (green). Scale bars = 40 μ m. (F) Representative intravital images of aggregate discovery. Left: 4 h biofilm with no MIP-2 treatment. Middle: 4 h biofilm with MIP-2 treatment. Right: 30 min attachment with MIP-2 treatment. Yellow arrows point to discovered aggregates. Scale bars = 100 μ m. (G) Quantification of aggregate discovery across the three conditions: 4 h biofilm, 4 h biofilm + MIP-2, 30 min attachment + MIP-2. Data are mean $\pm$ SEM (n = 3 mice/condition, Two-way RM ANOVA $p < 0.01$, Tukey's post hoc test * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ compared between groups at the indicated time point, Bonferroni's multiple comparisons test ** $p < 0.01$ compared over time within the same group). (H) Percent change in GFP volume between the last frame and first frame for the final continuous FOV for each position. Numbers above the x-axis indicate the average number of neutrophils in the FOV at the end of imaging. Data are mean $\pm$ SD (Each data point indicates one FOV, n = 3 mice/condition, Kruskal-Wallis Test * $p < 0.05$, *** $p < 0.001$).

facilitates immune evasion [19,22,38]. However, here we have shown that tolerance to neutrophil clearance is occurring at a very early stage in biofilm development while previous work has primarily investigated immune cell interactions with mature *in vitro* biofilms. While these aggregates are quite small compared to a typical *in vitro* biofilm, aggregates of similar sizes have been reported in several types of human biofilm infections, such as implant associated infections [39]. These results suggest that contaminating *S. aureus* may be able to quickly establish a biofilm aggregate that can become relatively protected from early host immune defenses.

To investigate the dynamics of neutrophil recruitment *in vivo*, we utilized a peritoneal implant model and a dermal intravital imaging model to characterize and quantify leukocyte recruitment to contaminated surfaces. At 24 h after peritoneal implantation, we observed highly heterogeneous recruitment of immune cells to a *S. aureus* contaminated silicone implant. While immune cells were strongly recruited to certain areas of the implant, SEM of seemingly bare areas only a few millimeters away revealed persistence of *S. aureus* cells. This observation suggests that discovery of bacteria on an implant surface could take longer than 24 h in some models, allowing a window for bacteria to grow and form more resilient aggregates. This mirrors our measurements *in vitro* (Fig. 1D). We hypothesize that these leukocyte patches observed could develop in part due to the expression of chemotactic factors following neutrophil discovery and phagocytosis of bacteria or abiotic debris. This phenomenon has been described in other contexts as neutrophil swarming and is driven by LTB₄, a molecule known to be produced by neutrophils [40]. LTB₄-dependent neutrophil swarming was originally described during sterile injury, but recently neutrophil swarming was also observed during a *S. aureus* infection in the lymph node, indicating that neutrophils are capable of swarming in the presence of bacterial infection [41]. Indeed, we observed both *in vitro* and *in vivo* that on occasion once neutrophils encountered a *S. aureus* aggregate a neutrophil swarm formed (data not shown). The role of LTB₄ in the control and clearance of *S. aureus* aggregates from a contaminated biomaterial is a mechanism we would like to pursue in future studies.

This study also used intravital microscopy to track the progression of *S. aureus* aggregate growth and neutrophil recruitment to the coverslip *in vivo*. First, we have demonstrated that once a contaminated coverslip is placed onto host tissue, bacteria are able to grow well before neutrophils can be recruited from the bloodstream. Robust neutrophil recruitment was not observed over 180 min of imaging, suggesting that a longer imaging window might be necessary to capture the onset of neutrophil recruitment in most cases. Although a previous *S. aureus* gel bead model resulted in neutrophil recruitment within 2 h [26], this disparity may have been due to a difference in dosage of bacteria (10^6 CFU/bead vs. 10^4 CFU/coverslip). It seems reasonable to assume that even the bacterial density utilized in our study would exceed the number of contaminating organisms present on an implant surface following surgery, suggesting delayed recruitment of neutrophils is plausible in human patients. Furthermore, differences in models (*S. aureus* bead vs. contaminated coverslip) could give rise to differences in neutrophil recruitment *in vivo*. We have also calculated the growth rate of *S. aureus* attached to a foreign object *in vivo*, which adds to the limited knowledge of *in vivo* bacterial growth rates reported. Since bacteria were able to grow significantly over the first 180 min, this indicates that early events are critical for bacteria to establish a protective niche.

We chose to use the chemokine MIP-2 as it is a potent chemo-attractant to recruit neutrophils to sites of inflammation [27]. By pre-recruiting neutrophils, we were able to see aggregate discovery by neutrophils and a marked decrease in bacterial aggregate volume at the end of imaging, suggesting that neutrophils have the ability to clear contaminated surfaces *in vivo* if they reach the infected site quickly. However, future modification of the model will be necessary to follow up on this result, as we were unable to image for longer than 3 h. It was also interesting that more neutrophils discovered aggregates that were

only grown for 30 min compared to 4 h to form a biofilm, which highlights the importance of early bacterial discovery on contaminated implants before bacteria have the opportunity to form a biofilm.

6. Conclusion

In this work, we investigated the interactions between nascent *S. aureus* biofilms and human neutrophils *in vitro* and the recruitment dynamics of neutrophils *in vivo*. We observed a brief window of time where *S. aureus* aggregates are readily phagocytosed and killed by neutrophils if discovered, but these aggregates become more resilient once they exceed approximately $50 \mu\text{m}^2$. Leukocyte recruitment to a peritoneal implant was heterogeneous after 24 h and we observed many undiscovered bacteria remaining on the surface, suggesting that discovery of contaminating microbes can take at least 24 h. We also observed *in vivo* with intravital microscopy that minimal numbers of neutrophils were recruited to a contaminated coverslip placed onto a dermal skin flap within 3 h. Even though pre-recruitment of neutrophils with MIP-2 resulted in a decrease in bacterial aggregate volume, some aggregates remained undiscovered by neutrophils, suggesting robust recruitment is required for sterilization.

Future studies should further investigate the time scales and distribution of neutrophil recruitment times to a contaminated surface *in vivo* and the potential roles of other immune cell types during the early stages of infection. Identification of mechanisms used by *S. aureus* to evade neutrophils through toxin production and aggregation could help to better inform pathogenesis and suggest therapeutic targets. Finally, our results raise the intriguing proposition of artificially “boosting” neutrophil recruitment near an implanted surface as a prophylactic measure and we aim to explore the viability of this treatment in a variety of clinically relevant models.

Credit author statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.biomaterials.2021.120775>.

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Video S6: Frequency of aggregate discovery by neutrophils (red). Discovered aggregates are labeled yellow, undiscovered aggregates remain green.

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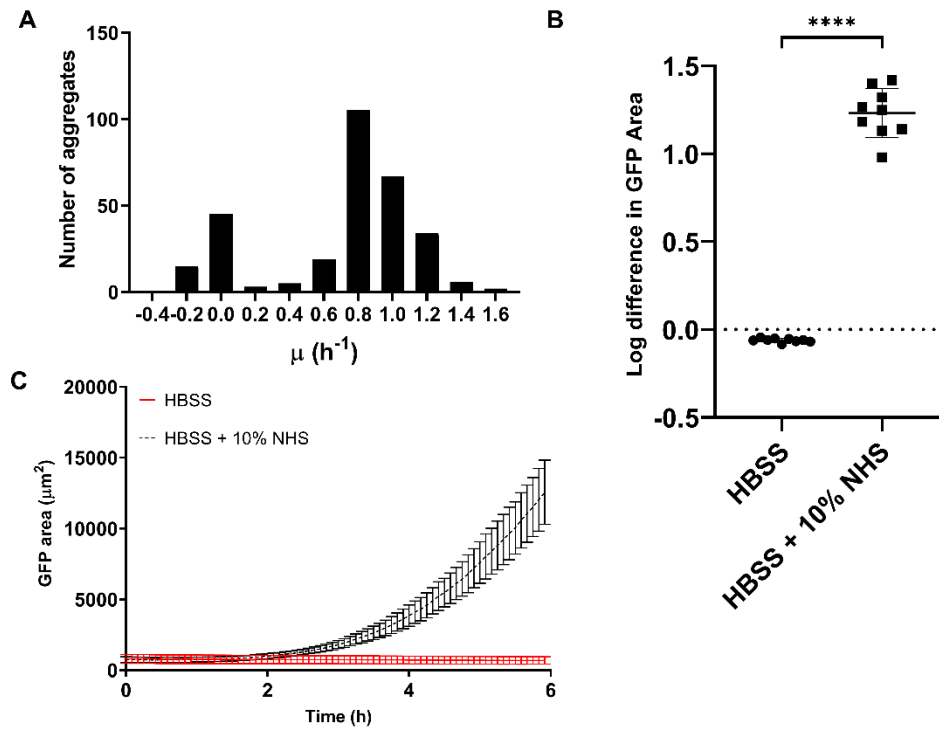


Figure S1: *In vitro* growth kinetics of *S. aureus* strain AH2547. (A) Individual *S. aureus* aggregate growth in 10% NHS is heterogeneous *in vitro*. Growth rate μ was calculated by fitting an exponential curve to volume measurements for continuously present aggregates in each field of view. (N = 302 individual aggregates measured from 8 FOVs from 4 independent experiments). (B) Log difference in GFP area for a given field of view from t = 0 to t = 6 h in Hank's Balanced Salt Solution (HBSS) or HBSS with 10% normal human serum (NHS). Error bars indicate mean $\pm$ SD (N = 9 FOV per condition from 2 independent experiments). Unpaired t-test with Welch's correction ****p<.0001). (C) FOV-averaged GFP area over time for *S. aureus* in HBSS or HBSS with 10% NHS. Error bars indicate mean $\pm$ SD (N = 9 FOVs per condition from 2 independent experiments).

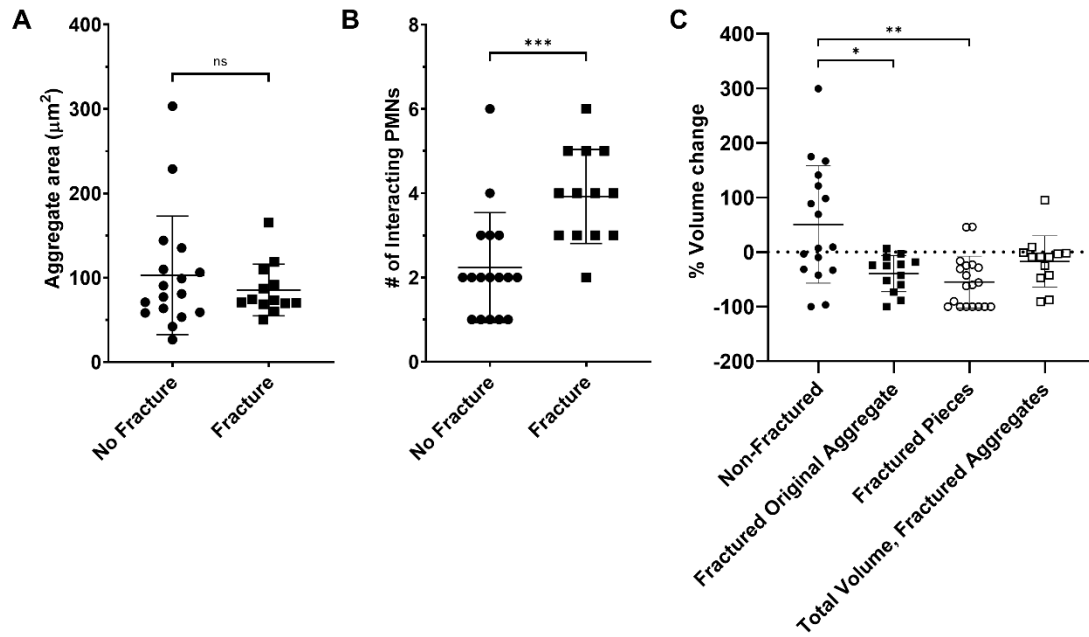


Figure S2: *S. aureus* aggregates resist clearance by neutrophils through aggregation. (A) Initial aggregate area for cases where neutrophils fractured an aggregate into smaller pieces or when it was unable to break up the aggregate. Error bars indicate mean $\pm$ SD (N = 17 no fracture cases, n = 13 fracture cases, collected from 4 independent experiments. Mann-Whitney test ns = Not Significant). (B) Number of neutrophils interacting with a given aggregate over the 4 h observation period in cases where the aggregate did or did not fracture. Error bars indicate mean $\pm$ SD (N = 17 no fracture cases, n = 13 fracture cases, collected from 4 independent experiments. Mann-Whitney test ***p < .001). (C) Percent change in volume of an aggregate from the time of initial interaction with a neutrophil to the final frame where the aggregate is present. Percent change was calculated for aggregates that do not break up, the original aggregate when fracture occurs, the individual pieces broken off of a fractured aggregate, and the sum of the original fractured aggregate and its derivative pieces. Error bars indicate mean $\pm$ SD (N = 17 no fracture cases, n = 13 fracture cases from 4 independent experiments. Brown-Forsythe ANOVA with Dunnet's T3 Multiple Comparisons test *p < .05, **p < .01).

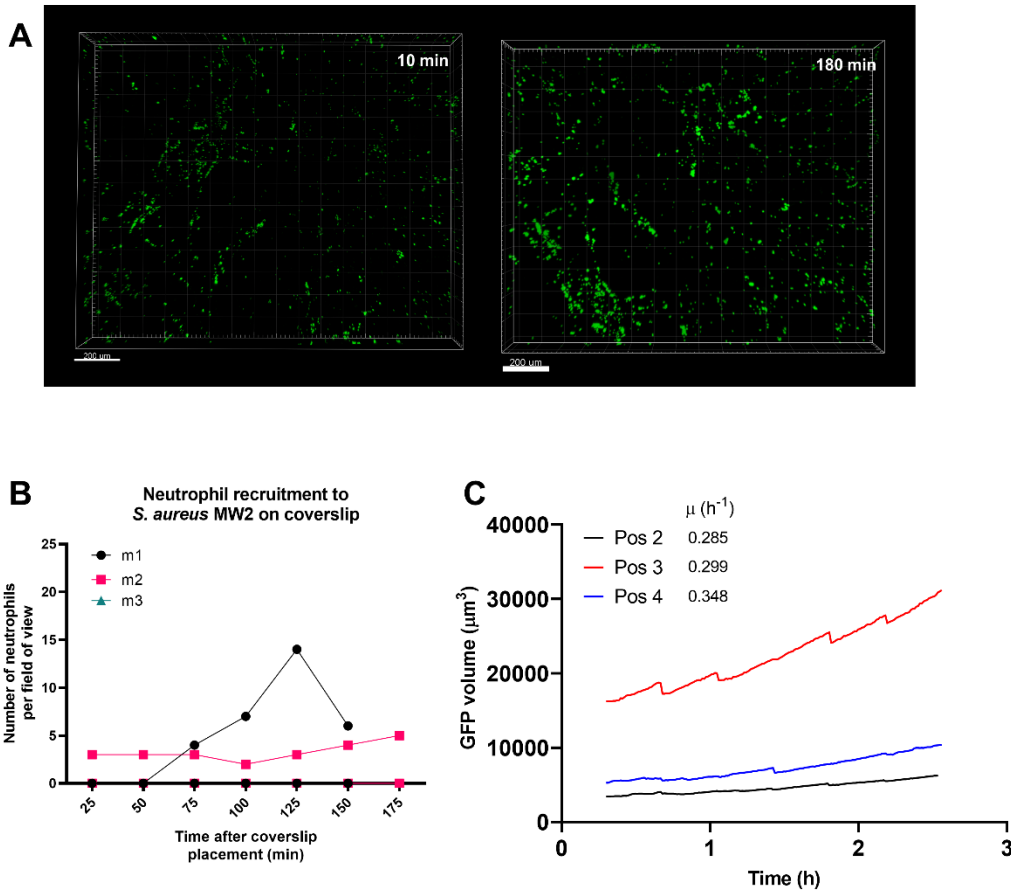


Figure S3. Minimal neutrophil recruitment to *S. aureus* MW2 on a coverslip despite bacterial growth. (A) Representative intravital image of a 3D region taken at 10 min after coverslip addition to the mouse skin flap (left) and 180 min after coverslip addition (right). Scale bars = 200 μm . (B) Quantification of neutrophil recruitment to the 4 h *S. aureus* MW2-contaminated coverslip ($n = 3$ mice from 3 independent experiments, data show 3 fields of view per mouse). (C) Quantification of *S. aureus* MW2 growth *in vivo* over 180 min of imaging. Reported value μ represents the specific growth rate satisfying the equation $X = X_0 e^{\mu t}$ using data outside of lag phase (starting at 0.75 h). Data is representative from one mouse showing three fields of view.

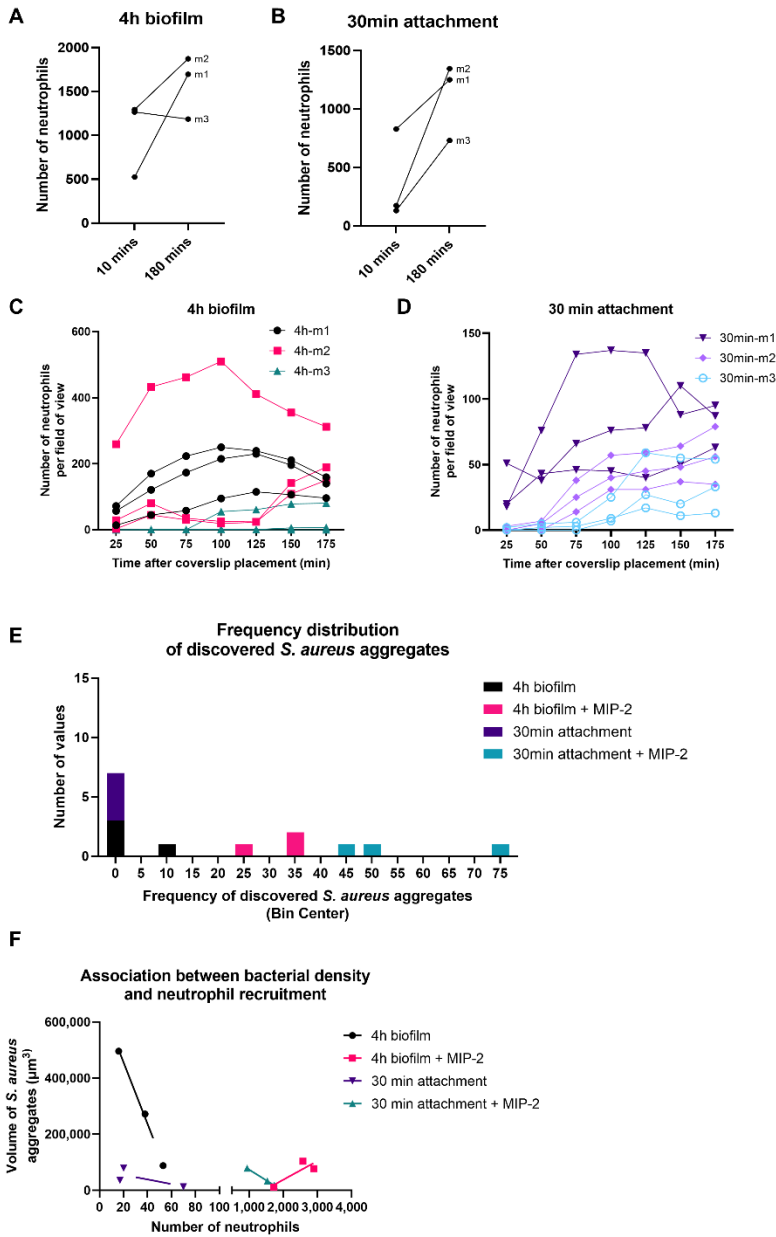


Figure S4. Dynamics of neutrophil recruitment to the *S. aureus*-contaminated coverslip after MIP-2 treatment. Quantification of total neutrophils from 3D regions with (A) 4 h biofilm on coverslip and (B) 30 min attached *S. aureus* on coverslip showing changes in individual mice ($n=3$ per group). (C-D) Heterogeneity of neutrophil recruitment to the *S. aureus* coverslip with (C) 4 h biofilm or (D) 30 min attachment. Each line shows neutrophil recruitment from one field of view (3 fields of view per mouse, $n=3$ per group). (E) Frequency distribution of discovered *S. aureus* aggregates at 180 min post coverslip addition ($n=3-4$ per group). (F) Association between bacterial density and neutrophil recruitment at 180 min post coverslip addition ($n=3-4$ per group).

CHAPTER THREE

S. AUREUS SAER/S REGULATED FACTORS OVERCOME HUMAN COMPLEMENT C3
MEDIATED INHIBITION OF AGGREGATION TO EVADE NEUTROPHIL KILLING

Contribution of Authors and Co-Authors

Manuscript in Chapter 3

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Contributions: Designed and conducted experiments, performed data analysis, drafted the manuscript.

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Contributions: Designed and conducted experiments.

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Contributions: Provided laboratory resources.

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Contributions: Designed experiments, provided laboratory resources, revised the manuscript, supervised the study, and acquired funding.

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Contributions: Designed experiments, provided laboratory resources, supervised the study, and acquired funding.

Manuscript Information

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Abstract

Infections caused by biofilm forming microorganisms are a relatively uncommon but serious complication for implanted biomaterials, such as orthopedic implants and pacemakers. *Staphylococcus aureus* is a frequent culprit in implant-associated infections and employs an impressive collection of virulence factors to escape killing by the host immune system. The specific immune evasion strategies *S. aureus* employs during early biofilm formation on a surface are still relatively unknown. Time-lapse confocal microscopy was employed to quantify interactions between *S. aureus* biofilm aggregates and human neutrophils *in vitro* and identify specific mechanisms of tolerance to neutrophil killing. The SaeR/S two-component gene regulatory system was essential for aggregate formation and subsequent neutrophil evasion. Inhibition of LAC Δ saePQRS aggregation required serum complement, as complement inhibition restored biofilm aggregation. The ability to prevent aggregation was dependent on complement proteins C3 and factor B, but not complement C4 or C5, suggesting that this activity occurs at the level of the alternative C3 convertase, C3bBb or the alternative C5 convertase, C3bBbC3b. Inhibition of LAC Δ saePQRS aggregation was also species dependent, as human, monkey, and rabbit serum strongly inhibited aggregation while mouse and guinea pig serum failed to inhibit aggregation at all. These data suggest a novel function of serum complement, the ability to inhibit *S. aureus* aggregation, is directly overcome by SaeR/S regulated factors to aid biofilm formation and immune evasion.

Introduction

Chronic biofilm infections are inherently difficult to treat due to their natural developed tolerance to both antibiotic therapies and host immune defenses (Costerton et al., 1999; Parsek and Singh, 2003). *Staphylococcus aureus* (*S. aureus*) is commonly implicated in biofilm

infections associated with orthopedic implants (Arciola et al., 2005, 2018). In addition to readily forming biofilms, *S. aureus* produces many virulence factors to interfere with host immunity (Guerra et al., 2017). To date, it remains unclear how small amounts of contaminating bacteria successfully evade the potent host innate immune defenses and subsequently develop into robust biofilm infections. We have developed a model postulating that delayed discovery of contaminating aggregates on a surface by neutrophils, key early responders to *S. aureus* infections, leads to formation of mechanically resilient and difficult to kill biofilm aggregates. Indeed, *S. aureus* aggregates on a surface that reach an approximate size of $\sim 50 \mu\text{m}^2$ resist killing by human neutrophils and cause neutrophil lysis (Pettygrove et al., 2021). The period in which bacteria are susceptible to clearance is therefore dependent on the initial size of contaminating aggregates, the growth rate of the bacteria *in vivo*, and the speed of recruitment and successful discovery of bacteria by host immune defenses. An improved understanding of the mechanisms by which *S. aureus* aggregates tolerate neutrophil killing will provide crucial insight into the events that determine whether bacteria are cleared or persist. Therefore, we sought to identify virulence factors that contribute to *S. aureus* biofilm aggregate tolerance to neutrophil clearance.

While neutrophils constitute a critical cellular component of early innate immunity, the complement system is crucial for effective recognition of pathogens and initiation of inflammation. Complement is a tightly regulated proteolytic cascade of serum proteins that can be activated through three separate pathways - the classical pathway, the lectin pathway and the alternative pathway - that begin by recognition of foreign surfaces by host proteins and converge at the point of C3. The classical and lectin pathway utilize the classical C4b2a C3 convertase to cleave C3 into C3a and C3b, while the alternative pathway relies on the C3bBb convertase. These convertases further complex with C3b to form C5 convertases that cleave C5 to release C5a and C5b. C3a and C5a are powerful anaphylatoxins that help to initiate

inflammation by recruiting immune cells and priming neutrophils (El-Benna et al., 2016; Lambris et al., 2008). Microbial surface-deposited C3b serves as a crucial opsonin to assist phagocytes in the recognition and subsequent phagocytosis and killing of foreign microbes. C5b deposited on the surface of serum sensitive microbes can lead to lysis by formation of the C5b-9 membrane attack complex (Lambris et al., 2008). This cascade thus plays a significant role in the effective recognition of microbes, recruitment of immune cells, and subsequent killing by phagocytes.

In this work, we demonstrate that the SaeR/S two component system is required for the formation of a mechanically stable biofilm aggregate and an isogenic deletion mutant of this system becomes highly susceptible to clearance by neutrophils *in vitro*. The defective aggregation phenotype observed in a $LAC\Delta saePQRS$ (hereafter referred to as $LAC\Delta sae$) mutant was dependent on serum complement proteins, specifically C3 and factor B (fB). Depletion of C3 or fB, but not C5, restored aggregation. Together, these results suggest a previously undescribed function of human complement that is strongly inhibited by SaeR/S regulated factors to evade human neutrophil evasion during early biofilm formation.

Results

Neutrophils fail to clear *S. aureus* USA300 biofilm aggregates in vitro.

We have previously demonstrated that human neutrophils readily clear single cells and very small clusters of methicillin sensitive *S. aureus* from a surface but struggle to kill larger biofilm aggregates (Ghimire et al., 2019; Pettygrove et al., 2021). We utilized the same time-lapse confocal microscopy approach to confirm these results with a clinically relevant methicillin resistant strain of USA300 *S. aureus*, LAC. LAC cells expressing green fluorescent protein (GFP) were briefly attached to a glass surface and subsequently incubated in 10% normal human serum (NHS) in Hank's Balanced Salt Solution for 1, 2, or 3 h to allow biofilm aggregates

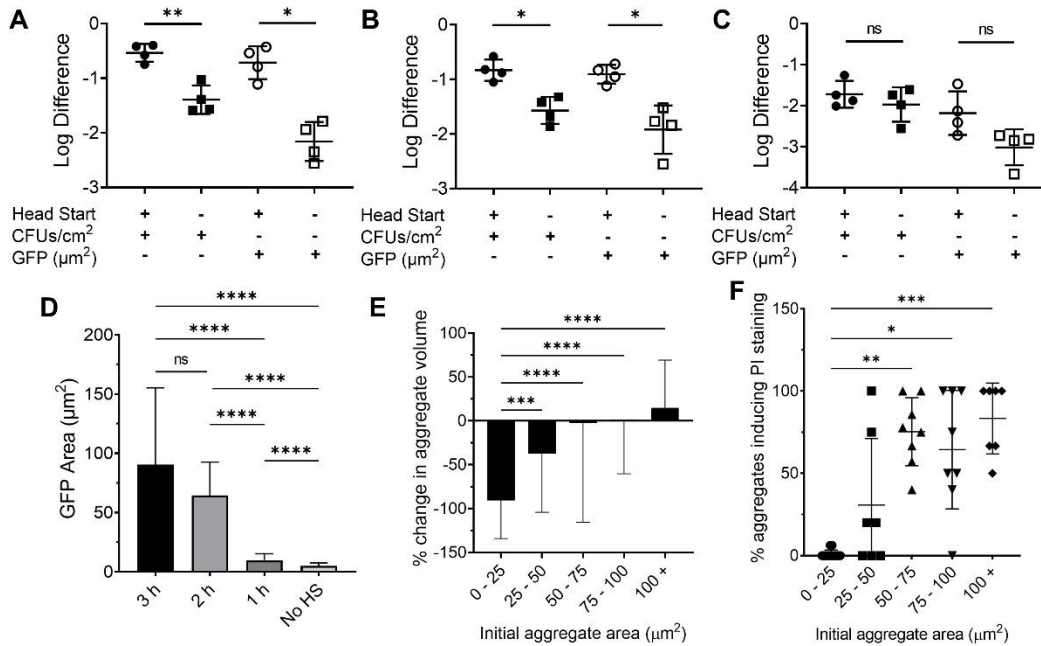


Figure 1: *S. aureus* strain LAC biofilm aggregates rapidly gain tolerance to neutrophil killing. (A) Survival of aggregates grown for 3 h prior to neutrophil addition, compared to control wells without neutrophils (N = 4 independent experiments. Paired t test * $p < 0.05$, ** $p < 0.01$). (B) Survival of aggregates grown for 2 h prior to neutrophil addition, compared to control wells without neutrophils (N = 4 independent experiments. Paired t test * $p < 0.05$). (C) Survival of aggregates grown for 1 h prior to neutrophil addition, compared to control wells without neutrophils (N = 4 independent experiments. Paired t test). (D) Average bacterial aggregate size at the start of imaging (N = 72 – 1474 aggregates per condition from 12 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons **** $p < 0.0001$). (E) Change in aggregate volume following phagocytosis by a neutrophil. (N = 15 – 92 aggregates per bin from 12 independent experiments. Kruskal-Wallis test with Dunn's multiple comparisons *** $p < 0.001$, **** $p < 0.0001$). (F) Percentage of aggregates in a FOV that induce PI staining of at least one neutrophil (N = 7 – 12 FOVs per bin. Kruskal-Wallis test with Dunn's multiple comparisons * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). Error bars indicate mean $\pm$ SD.

to develop prior to neutrophil addition. Neutrophils were added to wells and the *S. aureus*-neutrophil interactions were imaged for 4 h. Bacterial aggregates that were grown for 2 or 3 h demonstrated population level tolerance to neutrophil killing compared to CFU-matched single cell (non-aggregated cells) controls (Figure 1A, 1B). Cells that were grown for 1 hour demonstrated little additional tolerance to neutrophil clearance compared to single cell controls (Fig. 1C). After 2 h of growth, many aggregates had formed that were approximately 50 – 60

μm^2 , large enough to resist killing by neutrophils (Figure 1D, 1E). In addition to resisting neutrophil killing, aggregates of this size began to induce significant neutrophil membrane damage, with aggregates greater than $50 \mu\text{m}^2$ frequently causing propidium iodide staining of at least one interacting neutrophil (Figure 1F). Together, these data confirm previous results demonstrated in a laboratory strain of *S. aureus* and more robustly establish that relatively small *S. aureus* biofilm aggregates can tolerate human neutrophil challenge.

The SaeR/S gene regulatory system is essential for biofilm aggregate formation and tolerance to neutrophils.

We sought to explore the mechanisms by which *S. aureus* tolerates neutrophil killing during early biofilm formation by investigating two major regulators of *S. aureus* virulence, the SaeR/S two-component system and the Agr two-component system. The SaeR/S system has been demonstrated to be important for disease pathogenesis in a number of models such as skin infections (Montgomery et al., 2010; Nygaard et al., 2010), sepsis (Voyich et al., 2009), and bacterial pneumonia (Borgogna et al., 2018; Montgomery et al., 2010). The Agr system is important for biofilm maturation and detachment (Boles and Horswill, 2008; Yarwood et al., 2004), neutrophil evasion in the dermis (Matsumoto et al., 2021), phagosome escape (Münzenmayer et al., 2016; Pang et al., 2010; Surewaard et al., 2013), bacteremia (Wang et al., 2007), skin infections, and pneumonia (Montgomery et al., 2010). Together, these two systems regulate or co-regulate several important virulence factors such as secreted leukocidins, phenol soluble modulins, hemolysins, complement inhibitors, and MSCRAMMs (Liu et al., 2016; Nygaard et al., 2010). We attached $\text{LAC}\Delta\text{sae}$ or $\text{LAC}\Delta\text{agr}$ cells to a glass surface, statically incubated in 10% NHS, and observed growth over 4 h by confocal microscopy. While LAC and $\text{LAC}\Delta\text{agr}$ cells formed dense aggregates (Figure 2A, 2C), $\text{LAC}\Delta\text{sae}$ demonstrated a profound aggregation defect (Figure 2B, Video S1). Cells regularly broke away from aggregates, demonstrating impaired cell-cell adhesion and drifted across the field of view (FOV), suggesting

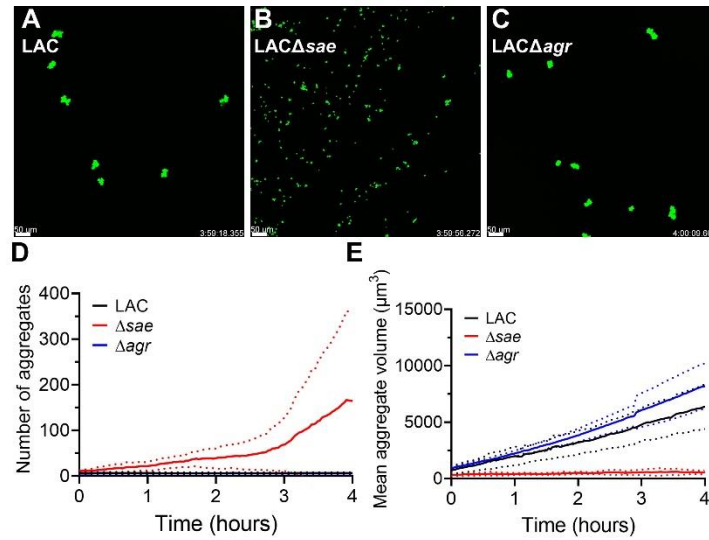


Figure 2: LAC Δ sae demonstrates an aggregation defect in human serum. Representative images LAC (A), LAC Δ sae (B) and LAC Δ agr (C) aggregation in NHS after approximately 4 h of growth. Scale bar = 50 μ m. (D) Number of bacterial objects detected in each FOV. (N = 16 (LAC) and 8 FOVs (Δ sae and Δ agr) FOVs per condition) (E) Average aggregate volume in each FOV. (N = 16 (LAC) and 8 (Δ sae and Δ agr) FOVs per condition). Solid and dotted lines indicate mean $\pm$ SD, respectively. Data collected from 8 (LAC) or 4 (Δ sae and Δ agr) independent experiments.

impaired cell-surface adhesion. Unlike the wild type (WT) strain, we observed an increase in the number of LAC Δ sae aggregates in each FOV (Figure 2D) and these aggregates remained relatively small in size (Figure 2E). Conversely, the number of LAC or LAC Δ agr aggregates remained relatively static (Figure 2D) and these aggregates grew significantly (Figure 2E). SaeP, SaeQ, and SaeS are all membrane bound proteins and SaeP has been shown to interact with biofilm matrix proteins (Kavanaugh et al., 2019). The aggregation defect that we observe does not appear to be due to direct interactions with the SaeP or SaeQ proteins as a LAC Δ saePQ mutant did not display an aggregation defect (Figure S1A). Furthermore, a LAC Δ saeR^{D51A} strain, which has an impaired ability to transcribe virulence factors due to reduced phosphorylation of the response regulator protein SaeR (Nygaard et al., 2018), still demonstrates a moderate aggregation defect (Figure S1B).

We next challenged LAC Δ sae and LAC Δ agr cells with human neutrophils to determine if either system's absence increased susceptibility to clearance. Because we began to see tolerance to neutrophil killing in the WT strain after approximately 2 h of growth (Figure 1B), we attached cells and allowed them to grow for 2 h prior to the addition of neutrophils. On average, LAC Δ sae aggregates were significantly smaller at the time of discovery and phagocytosis compared to either the LAC or the LAC Δ agr aggregates (Figure 3A). Both deletion strains demonstrated a significant decrease in the fraction of aggregates that induced neutrophil lysis, however the LAC Δ sae strain nearly eliminated all PI staining (Figure 3B). A significantly higher fraction of LAC Δ sae aggregates in each FOV imaged were completely cleared (no visible GFP signal remaining) than LAC aggregates (Figure 3C). LAC Δ agr aggregates demonstrated a slight, but not statistically significant, increase in susceptibility compared to WT (Figure 3C). These data demonstrate that at the level of individual aggregate-neutrophil interactions, LAC Δ sae is highly susceptible to neutrophil killing. We additionally saw significantly more clearance of bacteria across an entire FOV with the LAC Δ sae strain (Figure 3D), but not with the LAC Δ agr strain (Figure 3E). Thus, SaeR/S regulated factors are essential for biofilm aggregate formation in human serum and subsequent tolerance to killing by human neutrophils.

Biofilm aggregation in serum appears to be protein mediated

We next aimed to identify the SaeR/S regulated factors that facilitate aggregation in human serum. To gain hints into the mechanism of aggregation, we treated WT aggregates with varying concentrations of Proteinase K or DNase. Proteinase K treatment led to concentration dependent fracturing of biofilm aggregates into smaller groups of cells (Fig. S2A, S2B). Cells treated with Proteinase K more frequently moved across the FOV after they were released from the static aggregates (Fig. S2C). DNase treatment did not lead to fracturing of aggregates or increased cell release (Fig. S2D – F), suggesting that aggregates at this stage form through a

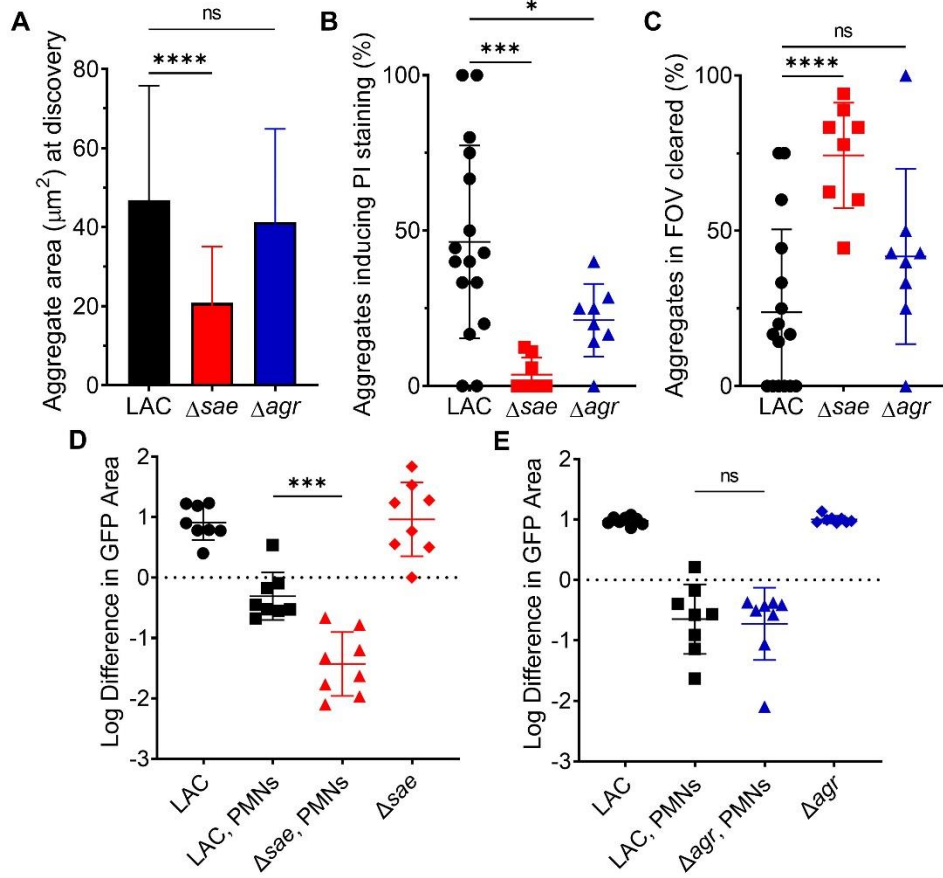


Figure 3: LAC Δsae but not LAC Δagr shows increased susceptibility to neutrophil clearance *in vitro* during early biofilm formation. (A) Biofilm aggregate size at the time of phagocytosis by a neutrophil (N = 77 (LAC), 86 (Δsae), and 40 (Δagr) aggregates. ANOVA with Dunnett's multiple comparisons test ****p < 0.0001). (B) Percentage of aggregates in a FOV that induce PI staining of at least one neutrophil (N = 16 (LAC) and 8 (Δsae and Δagr) FOVs. Brown-Forsythe with Dunnett's T3 multiple comparisons test *p < 0.05, ***p < 0.001). (C) Percentage of aggregates in a FOV that were phagocytosed and had a 100% reduction in volume. (N = 16 (LAC) and 8 (Δsae and Δagr) FOVs. Brown-Forsythe with Dunnett's T3 multiple comparisons test ****p < 0.0001). (D, E) Log difference in GFP area within a FOV over 4 h of imaging. (N = 8 FOVs per condition. Unpaired t test ***p < 0.001). Error bars indicate mean $\pm$ SD. Data collected from 8 (LAC) or 4 (Δsae and Δagr) independent experiments.

protein-dependent mechanism. Many previous reports have identified *S. aureus* proteins that facilitate biofilm formation *in vitro* in different assays and conditions (Speziale et al., 2014). We leaned on this existing literature to suggest potential SaeR/S regulated genes that might be responsible for the poor aggregation phenotype that we observe in the LAC Δsae strain. Proteins of interest that we screened included fibronectin binding proteins (LAC $\Delta FnbPs$, Fig. S1C) (Gries

et al., 2020; O'Neill et al., 2008), extracellular adherence protein (LAC Δ *eap*, Fig. S1D) (Johnson et al., 2008; Thompson et al., 2010), extracellular matrix binding protein (LAC Δ *empbp*, Fig. S1E) (Johnson et al., 2008), α -hemolysin (LAC Δ *hla*, Fig. S1F) (Caiazza and O'Toole, 2003), protein A (LAC Δ *sbi* Δ *spa*, Fig. S1G) (Merino et al., 2009). Aggregation did not appear to be dependent on coagulation of residual fibrinogen in serum, as a coagulase and von Willebrand factor binding protein knockout did not display an aggregation defect (LAC Δ *coa* Δ *vwb*, Figs. S2H-J) (Guggenberger et al., 2012) and no effect was observed in clumping factor A or B deficient strains (LAC Δ *cfa*, Fig. S2K, LAC Δ *cfb*, Fig. S2L) (McAdow et al., 2011). Although aureolysin has been demonstrated to be upregulated in a LAC Δ *sae* mutant (Cassat et al., 2013), the poor aggregation phenotype is not a product of upregulation of bacterially derived proteases, as a mutant deficient in the major *S. aureus* proteases and SaeR/S (Gries et al., 2020) still retained the poor aggregation phenotype (Fig. S2M). While these experiments only looked at a select few candidate genes, these experiments suggest a novel aggregation phenotype in serum.

Complement proteins prevent biofilm aggregation by the SaeR/S deletion mutant

To gain further clues into what *S. aureus* proteins may be involved in aggregation, we sought to determine if a serum protein, such as residual fibrinogen, was mediating aggregation in a manner similar to previous reports (Crosby et al., 2016; Knott et al., 2021; McAdow et al., 2011; Pestrak et al., 2020). To remove residual fibrinogen and other serum proteins, we ethanol-precipitated human serum overnight (Knott et al., 2021). No difference in aggregation was observed between NHS and ethanol treated serum for WT, however the LAC Δ *sae* strain was able to aggregate in ethanol treated serum (Figure S3A - D), suggesting a serum protein actively inhibited aggregation rather than facilitated it. Like ethanol precipitation of serum proteins, heat inactivation of serum complement prior to incubation with cells resulted in

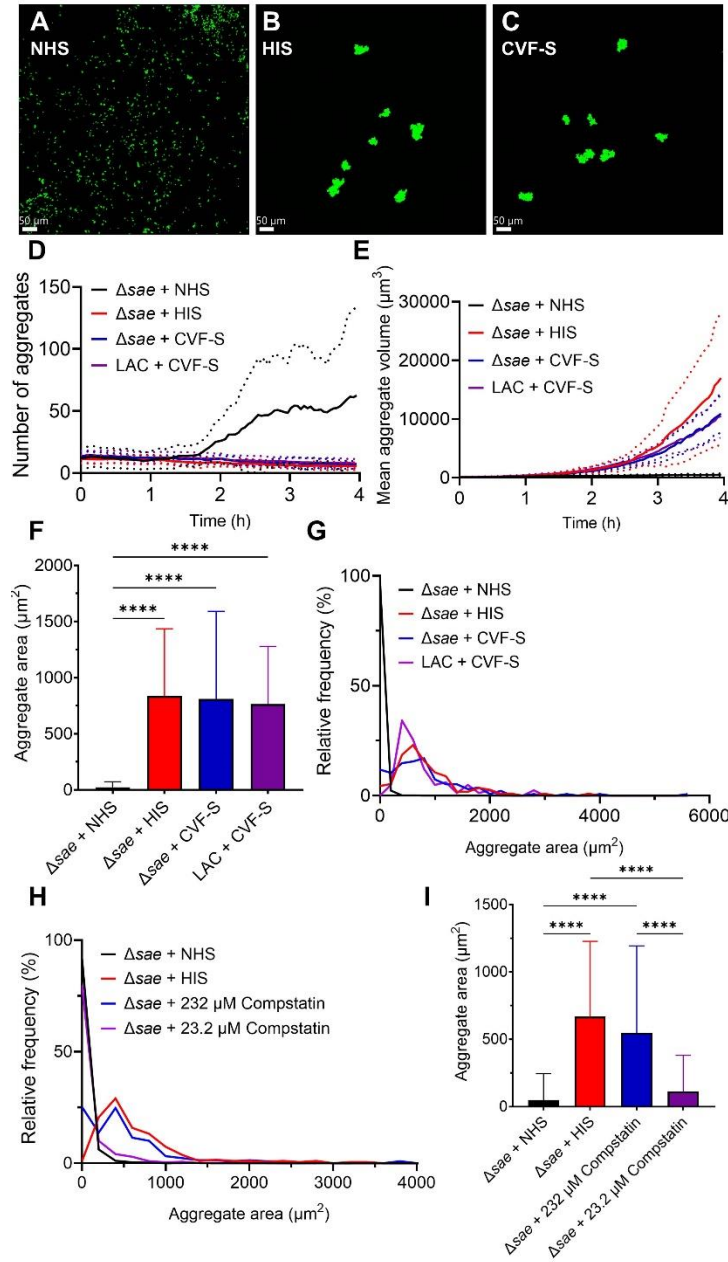


Figure 4: Complement inhibits aggregation by LAC Δ sae. Representative images of LAC Δ sae aggregation in NHS (A), HIS (B), or CVF-S (C) after approximately 4 h of growth. Scale bar = 50 μ m. (D) Number of bacterial objects detected in each FOV. (N = 6 FOVs per condition) (E) Average aggregate volume in each FOV. (N = 6 FOVs per condition). (F) Average aggregate area from representative FOVs collected after approximately 4 h of growth. (N = 7530 (Δ sae + NHS), 113 (Δ sae + HIS), 135 (Δ sae + CVF-S), and 82 (LAC + CVF-S) aggregates collected from 9 FOVs per condition. Brown-Forsythe with Dunnett's T3 multiple comparisons test. ****p<0.0001). (G) Distribution of aggregate sizes shown in panel F. Bin size = 200 μ m. (H) Distribution of aggregate sizes shown in panel I. Bin size = 200 μ m (I) Average aggregate area from representative FOVs collected after approximately 4 h of growth. (N = 3917 (Δ sae + NHS),

189 (Δ sae + HIS), 218 (Δ sae + 232 μ M compstatin), and 1161 (Δ sae + 23.2 μ M compstatin) aggregates collected from 9 FOVs per condition. Brown-Forsythe with Dunnett's T3 multiple comparisons test. **** $p < 0.0001$). Data collected from $N = 3$ independent experiments. Solid and dotted lines indicate mean $\pm$ SD, respectively.

restoration of aggregation in LAC Δ sae (Figure 4A, B, Video S2). Cobra venom factor (CVF), which binds to factor B to form a highly stable C3 convertase with C5 convertase activity (Von Zabern, 1993), was used to specifically deplete C3 and C5 complement proteins from serum. LAC Δ sae cells grown in serum treated with CVF for 1 h prior to incubation (CVF-S) showed a restoration of aggregation (Figure 4C). Growth of WT in CVF-S did not appear different from growth in NHS. The number of aggregates in each FOV for LAC Δ sae in heat inactivated serum (HIS) or CVF-S was similar to WT in CVF-S, while LAC Δ sae aggregates fractured (Figure 4D). At 4 h, LAC Δ sae aggregates in HIS or CVF-S were significantly larger than ones grown in NHS (Figure 4E, 4F). LAC Δ sae cell clusters grown in NHS were almost exclusively $< 200 \mu\text{m}^2$ ($24.7 \pm 48.6 \mu\text{m}^2$) whereas WT or LAC Δ sae cells grown in HIS or CVF-S regularly formed aggregates larger than $750 \mu\text{m}^2$ (Figure 4G). Addition of compstatin, a peptide that binds C3 and prevents cleavage by C3 convertases (Janssen 2007), produced a dose-dependent restoration of aggregation by LAC Δ sae (Figure 4H, 4I). Together these data demonstrate that complement proteins downstream of C3 cleavage by convertases can prevent aggregation of LAC Δ sae and further suggest that *S. aureus* overcomes this aggregation inhibition via SaeR/S regulated factors.

To identify more specifically the complement proteins that prevent LAC Δ sae aggregation, we investigated LAC Δ sae aggregation in human sera that had been depleted of C3 and C5 by immunoaffinity chromatography. *S. aureus* requires serum supplementation to grow in HBSS (Pettygrove et al., 2021) and we observed a severe growth defect in 10% depleted serum, suggesting that key nutrients were removed during the chromatography process. *S. aureus* will grow in RPMI 1640 medium alone, and NHS or HIS supplementation in

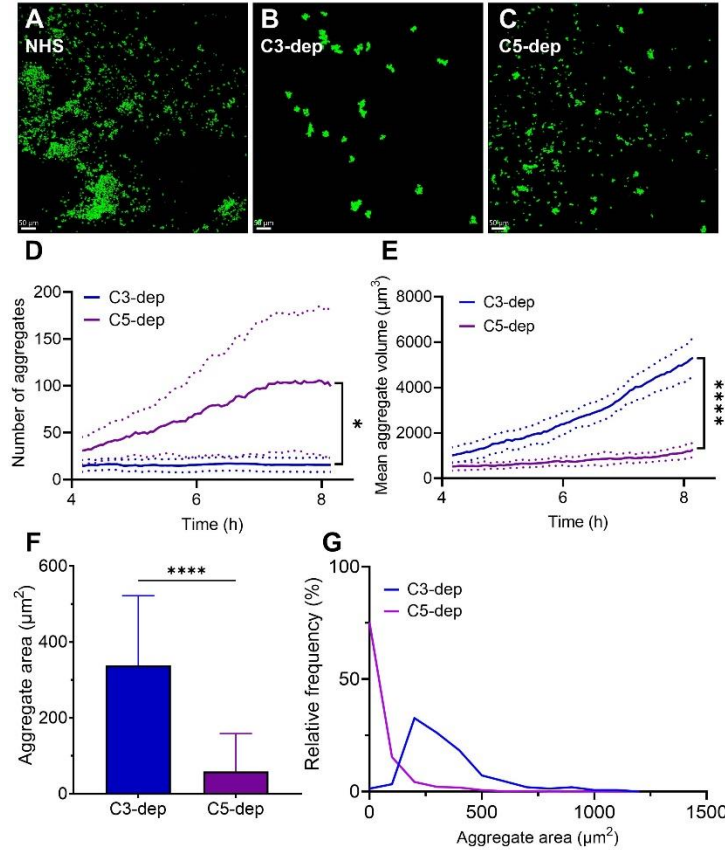


Figure 5: Complement C3 but not C5 is required for inhibition of LACΔsae aggregation. Representative images of LACΔsae aggregation in NHS (A), C3-depleted human serum (B), and C5-depleted human serum (C) after approximately 8 h of growth. Scale bar = 50 μm. (D) Number of bacterial objects detected in each FOV. (N = 6 FOVs per condition. Unpaired t test with Welch's correction * $p < 0.05$). (E) Average aggregate volume in each FOV. (N = 6 FOVs per condition. Unpaired t test with Welch's correction **** $p < 0.0001$). (F) Average aggregate area from representative FOVs collected after approximately 8 h of growth. (N = 153 (C3-dep) and 1341 (C5-dep) aggregates collected from 9 FOVs per condition. Mann Whitney test. **** $p < 0.0001$). (G) Distribution of aggregate sizes shown in panel F. Bin size = 100 μm. Data collected from N = 3 independent experiments. Solid and dotted lines indicate mean $\pm$ SD, respectively.

this medium recapitulated our previously observed aggregation phenotypes (Figure 5A, S4).

While slower, growth of LACΔsae in C3-depleted serum resembled growth in HIS and CVF-S as aggregates remained cohesive and grew significantly in size over time (Figure 5B, D, E, Video S3). Conversely, growth of LACΔsae in C5-depleted serum resembled growth in NHS in both the gradual increase in the number of cell clusters over time and the low, relatively constant,

aggregate size (Figure 5C – E, Video S4) (See Figure 4D, E for comparison). After 8 hours of growth, aggregates in C3-depleted serum were significantly larger than aggregates in C5 depleted serum, and aggregates grown in C5 depleted serum were predominantly less than 100 μm^2 in size (Fig 5F, G). These data demonstrate that complement C3, but not C5, is required to prevent aggregation of *LAC Δ sae*.

While investigating the potential role of human specific virulence factors (Rooijakkers et al., 2005) in complement inhibition and aggregation, we observed differential *LAC Δ sae* aggregation in sera from different species. We quantified growth and aggregation of *LAC Δ sae* in human, mouse, goat, horse, guinea pig, pig, rabbit and cynomolgus monkey sera and observed varying degrees of aggregation inhibition (Figure S5). Human, monkey, and rabbit sera demonstrated the strongest ability to inhibit bacterial aggregation (Figure S5A, G, H). Pig and horse sera demonstrated intermediate phenotypes (Figure S5D, F), while guinea pig, mouse, and goat sera failed to inhibit bacterial aggregation at all (Figure S5B, C, E). We observed differences in growth rate that made direct comparisons between aggregate area difficult. However, the sera from different species grouped similarly to their observed phenotypes when separated by both average aggregate area and the number of aggregates in each FOV (Figure S5I). Differences in aggregation were significant between human sera and goat, mouse, and guinea pig sera (Figure S5J). These data demonstrate that the ability to inhibit bacterial aggregation is not conserved among different complement systems.

Inhibition of aggregation is dependent on the alternative pathway

We next queried whether differences in aggregation were due to differential deposition of C3b on the bacterial cell surface, potentially blocking cell-cell adhesion in a previously undescribed mechanism. Many complement interfering proteins produced by *S. aureus* are SaeR/S regulated and we had hypothesized that we would observe significant differences in

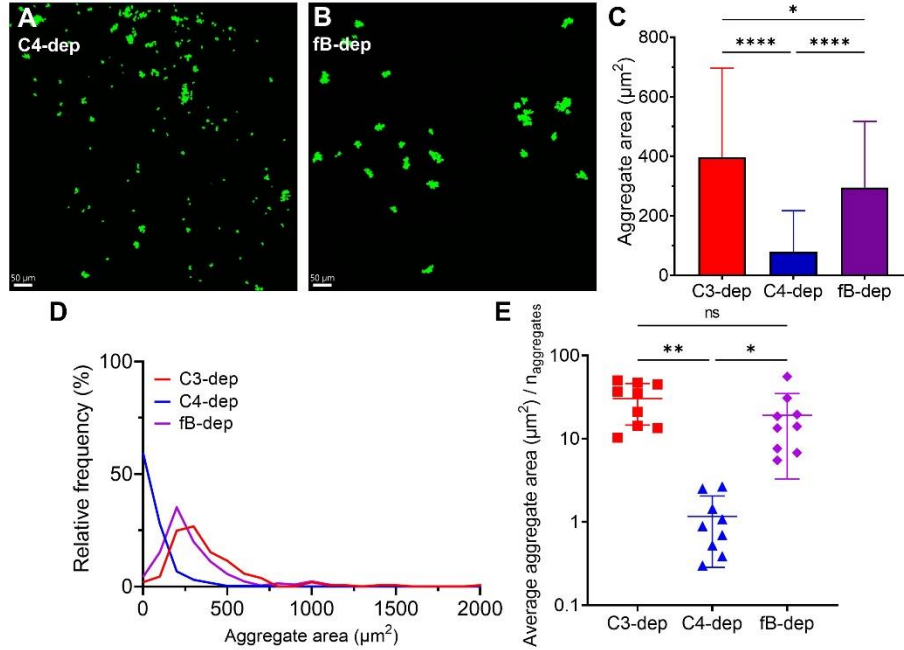


Figure 6: Inhibition of *LACΔsae* aggregation occurs via the alternative pathway. Representative images of *LACΔsae* aggregation in C4-depleted human serum (A) and factor B-depleted human serum (B) after approximately 8 h of growth. Scale bar = 50 μm. (C) Average aggregate area from representative FOVs collected after approximately 8 h of growth. (N = 157 (C3-dep), 964 (C4-dep), and 216 (fB-dep) aggregates collected from 9 FOVs per condition). (D) Distribution of aggregate sizes shown in panel C. Bin size = 100 μm. (E) Aggregation score after 8 h of growth (N = 9 FOVs collected per condition). Data collected from N = 3 independent experiments. Error bars indicate mean ± SD. Kruskal-Wallis test with Dunn's multiple comparisons. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

C3b deposition between WT and *LACΔsae* (Liu et al., 2016; Nygaard et al., 2010). Surprisingly, we observed little difference in C3b opsonization of LAC and *LACΔsae* using either confocal immunofluorescence (Figure S6A) or flow cytometry (Figure S6B). We speculate that given the low density of *S. aureus* cells and the high serum concentration used in this assay, bacterially derived complement interfering proteins were likely present in insufficient concentrations to fully inhibit complement deposition as has previously been reported (Jongorius et al., 2007; Woehl et al., 2014). Thus, it appears unlikely that simply the presence of C3b on the surface is responsible for the striking difference in aggregation.

Impaired LAC Δ sae aggregation in human, rabbit, and monkey sera is phenotypically similar to our proteinase K treatment of WT *S. aureus* cells (Figure S2). A recent study demonstrated that the alternative C3 convertase is capable of cleaving the *Neisseria meningitidis* Neisserial Heparin Binding Antigen (Di Fede et al., 2018). Together, these observations raise the possibility that inhibition of aggregation was due to direct protease activity on *S. aureus* surface proteins. Experiments with C3-depleted sera demonstrated that inhibition of aggregation is not due to the classical pathway (C4b2a) convertase, however we had not yet ruled out direct action by the alternative pathway (C3bBb) convertase. To further investigate this possibility and additionally determine if inhibition occurs through both the classical/lectin pathway and alternative pathways, we grew cells in C4 and fB depleted sera (Figure 6). Aggregation remained strongly inhibited in C4-depleted sera while LAC Δ sae cells aggregated in C3 or fB-depleted serum (Figure 5B, 6A, 6B, Video S5, Video S6). We observed significant decreases in aggregate size using C4-depleted sera compared to C3 or fB-depleted sera and a concurrent increase in aggregate numbers in the imaged FOVs (Figure 6C – 6E). There was a slight decrease in average size between aggregates grown in C3 and fB-depleted sera, however the two shared broadly similar phenotypes (Figure 5B, 6B-6E). These data demonstrate that neither the classical nor the lectin pathway play a significant role in inhibiting bacterial aggregation under the tested conditions, rather this phenomenon occurs specifically via the alternative pathway.

Discussion

S. aureus is an important pathogen in implant-associated infections as well as skin infections, pneumonia, infective endocarditis and sepsis (Arciola et al., 2005; Kobayashi and DeLeo, 2009; Lowy, 1998). Specific to biofilm infections, there is scant evidence demonstrating how contaminating bacteria evade early host immune defenses, as much of the focus has been

on how the pathogen modulates and inhibits immune function later in the infection (Bhattacharya et al., 2018; Hanke et al., 2013; Heim et al., 2020; Scherr et al., 2015). In this work, we demonstrate that the clinically relevant *S. aureus* strain LAC rapidly develops neutrophil tolerant biofilm aggregates in human serum. Importantly, the development of mechanically stable aggregates and resulting tolerance to neutrophil killing is dependent on SaeR/S regulated factors. Aggregate formation by a $LAC\Delta sae$ deletion strain was directly inhibited by serum complement in a C3 and fB dependent manner. Therefore, we propose a model wherein human complement can prevent aggregation of *S. aureus*, but this effect is overcome by SaeR/S regulated virulence factors. While a few studies have demonstrated that serum or serum proteases can affect biofilm formation (Abraham and Jefferson, 2010; Arenas et al., 2021; Di Fede et al., 2018; Ding et al., 2014; She et al., 2016), this is the first study to our knowledge to implicate complement in inhibiting bacterial cell-cell adhesion. This observation could have implications beyond implant associated infections, such as in sepsis and infective endocarditis. The data in this report demonstrate that even “small” biofilm aggregates can become recalcitrant to neutrophil killing. Thus, reduction of bacterial aggregation by complement would be a potent defense mechanism as it would greatly improve the ability of phagocytes to effectively clear bacteria.

The observation that WT *S. aureus* is not affected by complement mediated disaggregation is likely due to the numerous, redundant virulence factors known to interfere with complement function. Staphylococcal complement inhibitor (SCIN) can inhibit activity for both the classical (C4b2a) and alternative (C3bBb) C3 convertases and interfere with C3b deposition (Rooijackers and van Strijp, 2007; Rooijackers et al., 2005, 2007). The SCIN homologues SCIN-B and SCIN-C similarly interfere with C3 convertase activity (Jongerijs et al., 2007). The C3-binding domain of extracellular fibrinogen-binding protein (Efb) can bind surface bound C3b to block C3b containing convertase from cleaving its substrate (Jongerijs et al., 2007; Rooijackers

et al., 2007). Extracellular complement-binding protein (Ecb) can also inhibit C3b containing convertases on the microbe surface (Jongerijs et al., 2007) and can block C3b recognition by CR1 (Amdahl et al., 2017), thereby inhibiting phagocytosis. Additionally, Efb and staphylococcal immunoglobulin-binding protein (Sbi) have been demonstrated to bind host plasminogen, which can be converted to active plasmin by secreted staphylokinase (Okada et al., 2000), facilitating degradation of C3, C3a, and C3b near the cell surface (Koch et al., 2012). Sbi can also directly inhibit complement C3 through binding the C3a and C3dg fragments or through its IgG binding capabilities (Burman et al., 2008; Upadhyay et al., 2008). Extracellular adherence protein (Eap) inhibits the classical C3 convertase through binding to C4b and can reduce classical and lectin pathway deposition of C3b on the microbe's surface (Woehl et al., 2014). SplB can cleave and inactivate various complement proteins including C3 and C3b (Dasari et al., 2022).

Staphylococcal superantigen-like protein 7 (SSL7) was demonstrated to directly bind C5 and IgA to reduce C5a production (Bestebroer et al., 2010; Langley et al., 2005; Laursen et al., 2010). Protein A (Spa) can additionally inhibit the classical pathway through binding of IgG (Cruz et al., 2021). Other reports have identified additional evasion mechanisms by interaction between clumping factor A (Hair et al., 2008) and SdrE (Sharp et al., 2012) with complement regulatory proteins.

Based on our observation that inhibition of aggregation cannot occur without C3 or fB, interference with the alternative C3 convertase or C5 convertase is the most likely step at which *S. aureus* can restore aggregation. Efb, Ecb, and the three SCIN proteins all have demonstrated roles for interfering with the alternative convertases (Jongerijs et al., 2007), and it is additionally possible that Sbi or splB may be able to disrupt convertase function through cleavage of C3b (Dasari et al., 2022; Koch et al., 2012). Importantly, these proteins have been demonstrated to be regulated by SaeR/S by transcriptional analysis (Nygaard et al., 2010) and identification of SaeR binding sequences (Liu et al., 2016). While there certainly may be other SaeR/S regulated

genes that could impact the aggregation phenotype described above, Efb, Ecb, Sbi, SCIN, SCIN-B, SCIN-C, and SplB represent promising targets to investigate. We believe that it is highly likely that some or all these complement inhibiting factors are important to overcome the anti-aggregative effects of complement, given the redundant function of many of these virulence factors. Indeed, screens of various mutants containing deletions in up to three of these genes in various combinations have yet to recapitulate the phenotype observed with $LAC\Delta_{sae}$ (data not shown).

Complement interference is a common strategy by many pathogens such *Streptococcus*, *Serratia marcescens*, and *Porphyromonas gingivalis* due to its importance in initiating inflammation and phagocytosis (Domenech et al., 2013; Lambris et al., 2008; Rooijakkers and van Strijp, 2007). Aggregation and biofilm formation are generalizable defense strategies that are employed by many bacterial species to impair phagocytosis and increase survival. We therefore hypothesize that complement mediated inhibition of bacterial aggregation is not specific to *S. aureus* and may be overcome by other bacteria that produce tools to disrupt complement. Thus, it will be important to gain a mechanistic understanding of how complement inhibits *S. aureus* aggregation and search for this phenotype with other pathogenic organisms. Future work on this subject should seek to identify the specific mechanism by which complement C3 and fB inhibit bacterial aggregation, whether by direct action of the alternative convertases, activation of an additional serum protein, or another novel mechanism. This data could inform the design of novel therapeutics aimed at disrupting bacterial aggregation. Additionally, further identification of the specific *S. aureus* virulence factors required to overcome complement mediated aggregation inhibition could aid strategies for novel inhibitors or vaccine targets, especially in the case of secreted factors such as SCIN or Efb.

Materials and Methods

Bacteria and neutrophil preparation

All strains used in this study are listed in Table S1. Plasmid pCM29 (Pang et al., 2010) was isolated from strain AH1704 (*E. coli* BW25141) and sequentially transformed into competent *S. aureus* RN4220 and then CG100 to produce strain BP10. *S. aureus* was grown overnight in tryptic soy broth (TSB) or TSB supplemented with 10 µg/ml chloramphenicol when required for plasmid maintenance. Overnight cultures were centrifuged for 5 min at 4000 rpm, rinsed, resuspended in tryptic soy broth, and serially diluted and adjusted by optical density. Cells were attached to a 4-chambered glass bottom Petri dish (Cellvis, CA, USA, Cat no. D35C4-20-1.5-N) to facilitate live-cell imaging. To attach cells, 10 µL of diluted cell suspension was added to the surface and incubated for 30 min at 37° C. Following incubation, unattached bacteria were gently rinsed from the surface with PBS. Each chamber of the Petri dish was filled with 0.5 - 1 mL of 10% fresh human serum or modified serum in Hank's Balanced Salt Solution (HBSS) with Ca²⁺ and Mg²⁺ to condition the surface and opsonize bacteria and incubated at 37 °C for at least 30 min prior to neutrophil addition. Human neutrophils were isolated from heparinized venous blood obtained from healthy donors following a standard IRB-approved protocol. All donors provided written consent to participate in the study. Neutrophils were isolated under endotoxin-free conditions (<25 pg ml⁻¹) and purity (<1% PBMC contamination) and viability (<2% propidium iodide positivity) of neutrophil preparations were assessed by flow cytometry as previously described (Voyich et al., 2005, 2009). Neutrophils were kept on ice until stained with LysoBrite™ Red (AAT Bioquest, CA, USA, Cat no. 22645) according to the manufacturer's instructions. In neutrophil challenge experiments, neutrophils were immediately added to the surface following staining and 5 µg/mL propidium iodide was added to each well at this time. To enumerate surviving bacteria, bacteria were removed from the surface with a

sterile scraper and pipetting, vortexed in dilution tubes with PBS, and plated on tryptic soy agar in triplicate. Plates without detectable bacteria were counted as 0.5 colonies prior to log transformation.

Proteins and serum preparation

Serum was obtained from healthy donors by venous blood draw as described above. Heat inactivated serum was prepared by heating aliquots of fresh human serum to 56 °C for 20 – 30 minutes. CVF depleted serum was produced by incubating 10 µg/mL CVF (Complement Technology, Tyler, TX, USA, Cat no. A150) with serum for 1 h at 37 °C, then diluted to 10% in HBSS. C3, C4, C5, and fB-depleted sera were obtained from Complement Technology (Cat no. A314, A308, A320, and A335, respectively) and diluted to 10% in RPMI 1640. Compstatin (Selleck Chemicals, Houston, TX, USA, Cat no. S8522) was added to 10% NHS in HBSS to a final concentration of 232.2 or 23.2 µM and mixed just prior to incubation with bacteria. Ethanol precipitated serum was prepared by adding 200 proof absolute ethanol to a final concentration of 10% in serum and incubating overnight at 4°C (Knott et al., 2021). Serum was centrifuged and the resulting supernatant was diluted 1:10 in HBSS. All animal sera were thawed at 37 °C and diluted to 10% in HBSS prior to incubation with bacteria. Guinea pig and cynomolgus monkey sera were obtained from Complement Technology (Cat no. GPS and NCYS, respectively). Goat and mouse sera were obtained from MP biomedical (Irvine, CA, USA, Cat no. 092939149 and 0215228205, respectively). Horse serum was obtained from Cytiva (Marlborough, MA, USA, Cat no. SH30074.02) and pig serum was obtained from Gibco (Waltham, MA, USA, Cat no. 26250084). Proteinase K (Cat no. EO0491) and DNase I (Cat no. EN0521) were obtained from ThermoFisher Scientific.

Microscopy

A Leica SP5 inverted confocal laser scanning microscope was utilized for all imaging. GFP-tagged bacteria and propidium iodide were excited with a 488 nm laser and neutrophils were excited with a 561 nm laser. An Okolab (Ambridge, PA, USA) Uno Stage Top Incubator with stand-alone humidity controller or a LiveCell (Pathology Devices, CA, USA) environmental chamber system was utilized to maintain 5% CO₂, 20% O₂, 90% humidity, and 37 °C for sample incubation during imaging. 12 – 20 µm image stacks with 1-µm z-slices were recorded sequentially at 1–2 min intervals over a 4 h time course using a Leica 20x/0.7 NA dry objective lens. Two fields of view were imaged per chamber in each experiment. In neutrophil challenge experiments, each well was subsequently imaged using a 7 × 7 stitched tile scan with a 10x/0.3 NA dry objective to quantify total amount of bacteria remaining on the surface. For serum aggregation assays, three representative fields of view were imaged following time-lapse microscopy from each well.

Image analysis

MetaMorph version 7.8.13 (Molecular Devices, San Jose, CA, USA) image analysis software was used to measure bacterial aggregate area. Movies were prepared with Imaris version 9.8 or earlier (Oxford Instruments, Abingdon, UK). For the analysis of individual neutrophil-bacteria interactions, the Imaris Spots feature was used to identify neutrophils and propidium iodide staining events and the Surfaces feature was used to dynamically track bacterial growth over time. Discovery of bacteria by neutrophils was determined by identifying the first frame in which neutrophils co-localized with a bacterial aggregate and remained co-localized in subsequent frames. Measurements of the bacterial aggregate size were recorded at the start and end of each interaction with a neutrophil, and propidium iodide staining events were cataloged. The percentage of aggregates inducing propidium iodide staining was calculated by dividing the number of aggregates that caused propidium iodide staining of at

least one phagocytosing neutrophil by the total number of aggregates discovered by a neutrophil in each field of view.

C3b surface deposition

LAC and LAC Δ sae aggregates were grown in 10% NHS in HBSS for 4 hours prior to blocking and antibody staining. Surface associated C3b was detected with a mouse anti-human C3b antibodies (Invitrogen, Clone 6C9, Cat no. MA1-70053) and Goat anti-mouse secondary antibodies conjugated with Alexa Fluor 633 (Invitrogen, Cat no. A-21052) and imaged by confocal microscopy. C3b deposition on planktonic cells was determined by incubating cells in 10% NHS for 30 minutes prior to blocking and antibody staining. Antibody binding was quantified using a BD LSRFortessa (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and data was analyzed using FlowJo v10.

Acknowledgements

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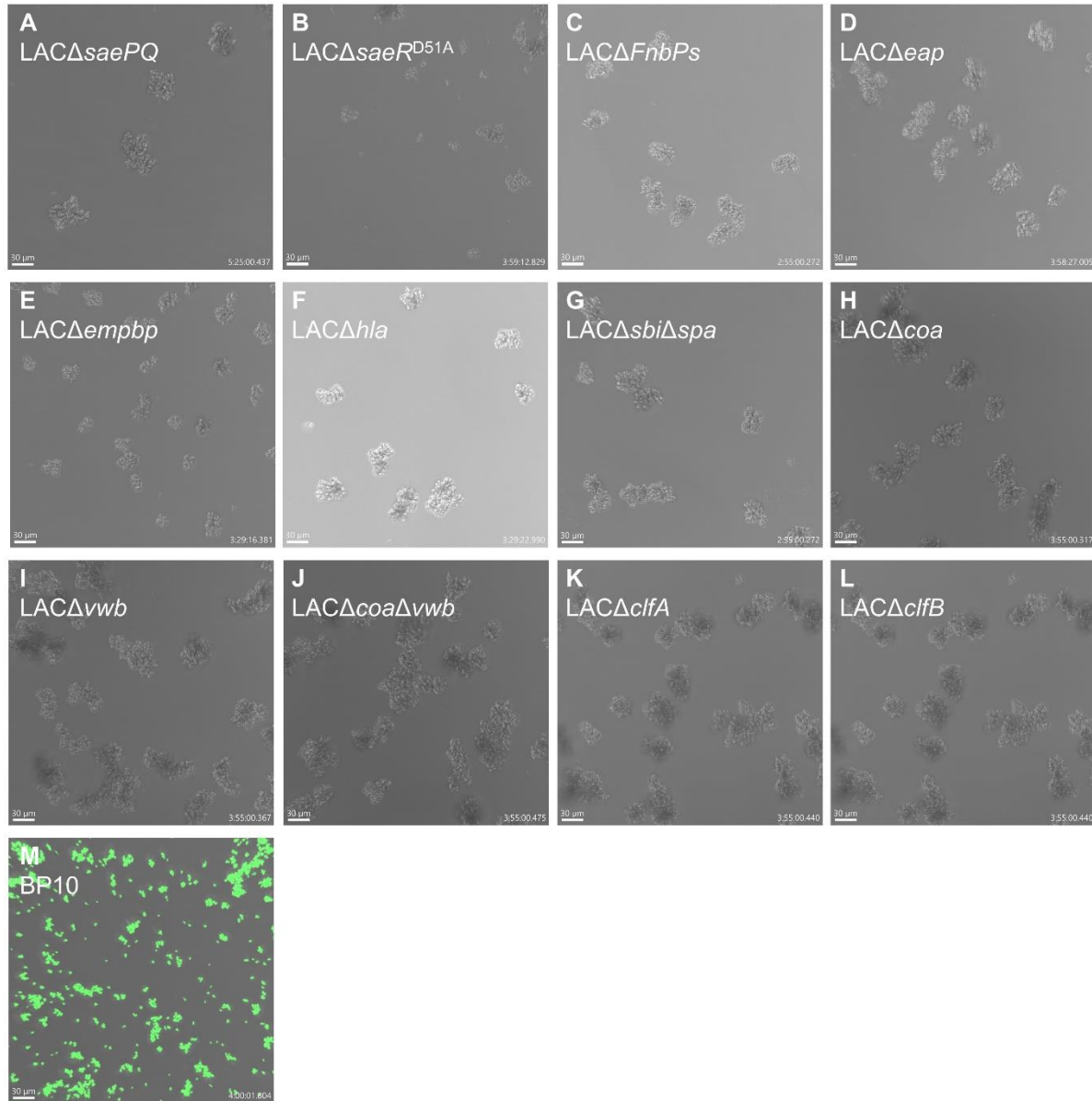
Supplementary Material

Figure S1: Aggregation phenotypes of selected *S. aureus* isogenic gene deletion mutants. Images are representative of each strain's behavior. Images taken at varying points in growth between 4 and 8 h after addition of NHS. Time stamp is indicative only of the time elapsed during imaging, not total incubation time. Scale bar = 30 μm. Strain BP10 (Panel M) is LACΔaurΔsspABΔscpAΔsplA-FΔsaePQRS + pCM29.

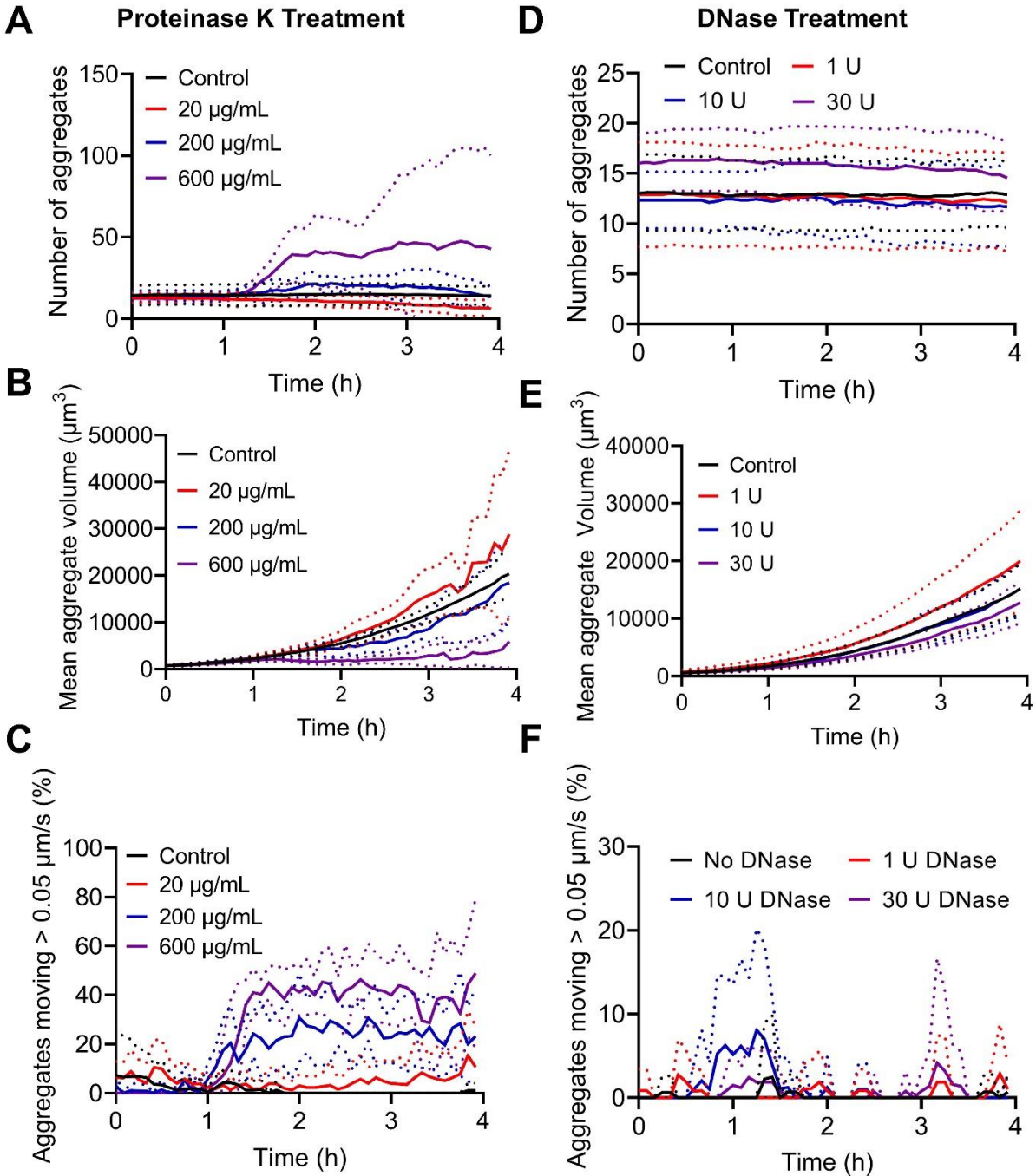


Figure S2: Proteinase K but not DNase treatment inhibits *S. aureus* LAC biofilm aggregate formation. Adherent cells were grown for two hours then treated with varying doses of Proteinase K (left column) or DNase I (right column). Number of aggregates per field of view for Proteinase K treated (A) or DNase I treated (D) *S. aureus*. Average aggregate size per field of view for Proteinase K treated (B) or DNase I treated (E) *S. aureus*. Percentage of non-static aggregates in a FOV for Proteinase K treated (C) or DNase I treated (F) *S. aureus*. Solid and

dotted lines indicate mean $\pm$ SD, respectively. Data collected from 9 FOVs per condition from 3 independent experiments per treatment.

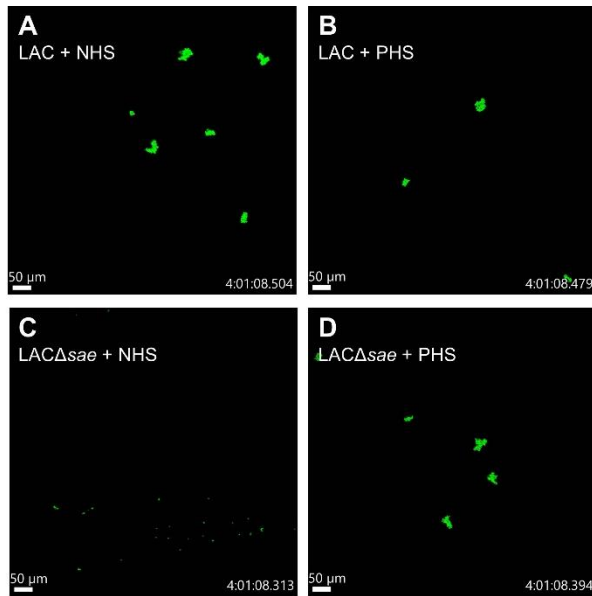


Figure S3: *S. aureus* aggregate formation in ethanol-precipitated serum. Surface adherent *S. aureus* was grown for 4 h in the presence of 10% NHS or 10% ethanol-precipitated serum (PHS). LAC *S. aureus* aggregates form aggregates in both normal human serum (A) and ethanol-depleted serum (B). LAC Δ sae fails to form aggregates in NHS (C) but is capable of aggregation in PHS (D). Scale bar = 50 μ m.

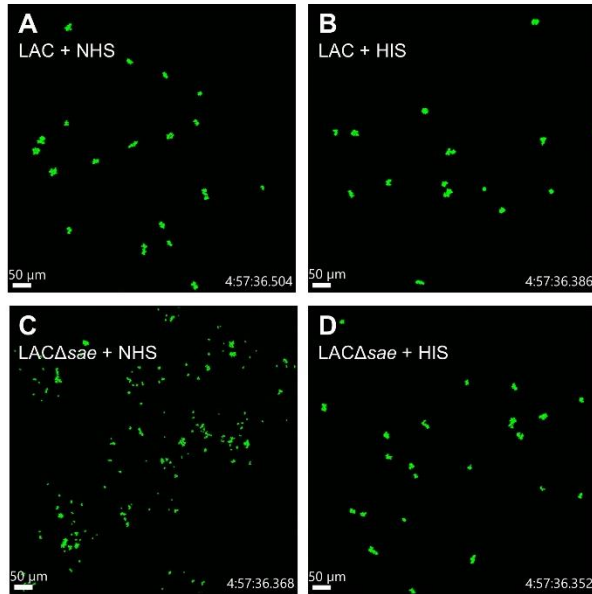


Figure S4: *S. aureus* aggregate formation in RPMI and NHS. LAC and LAC Δ sae were grown for 5 hours in RPMI supplemented with 10% NHS or HIS. LAC formed aggregates in NHS (A) and HIS (B). LAC Δ sae aggregate formation was impaired in NHS (C) but not HIS (D). Scale bar = 50 μ m.

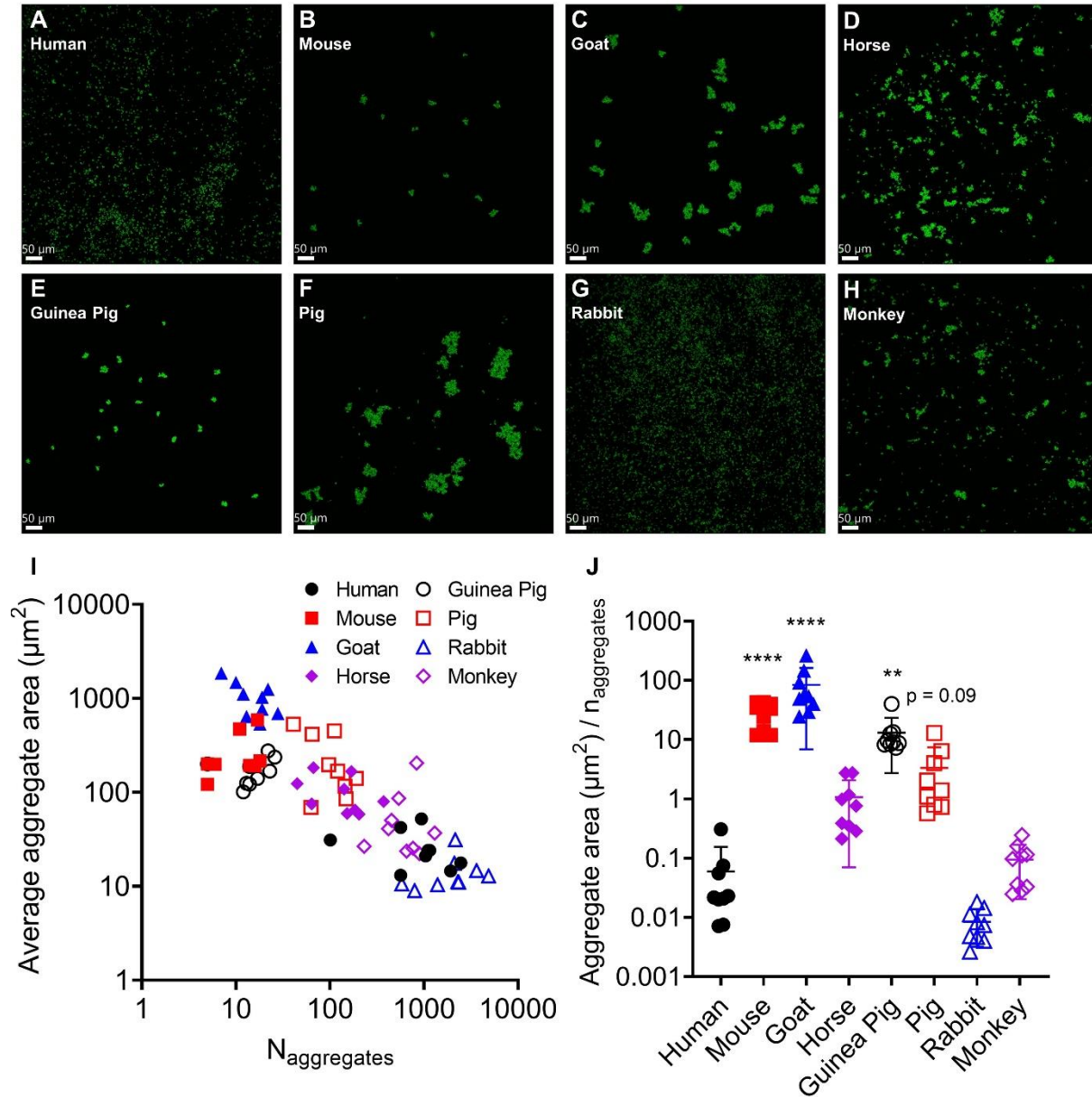


Figure S5: Inhibition of LAC Δ sae aggregation in serum is species dependent. (A-H) Representative micrographs demonstrating differential aggregation phenotypes in varying sera after 4 h. Scale bar = 50 μ m. (I) Aggregate area and number of aggregates in each FOV. (J) Aggregation score in sera from varying species (Kruskal-Wallis test with Dunn's multiple comparisons test. ** $p < 0.01$, **** $p < 0.0001$ compared to human serum). Data collected from 9 FOVs from 3 independent experiments per condition.

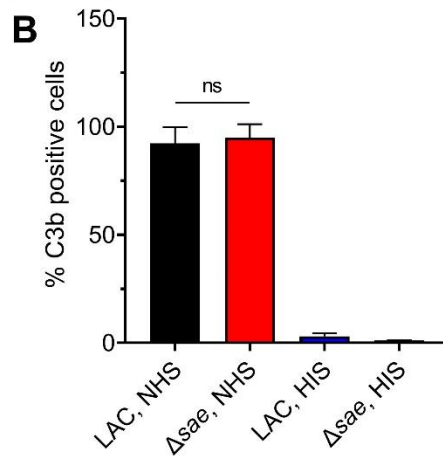
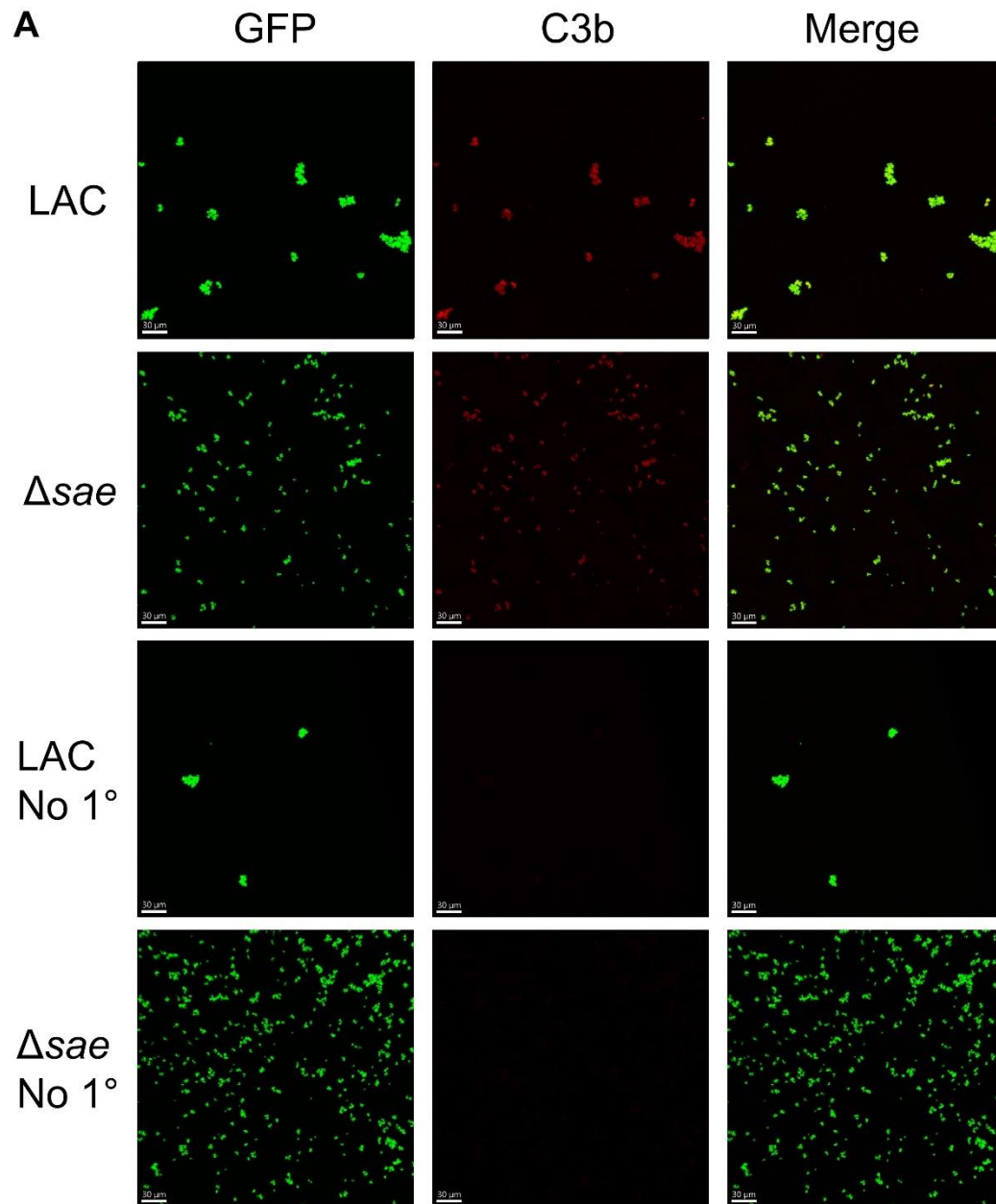


Figure S6: Surface C3b deposition does not vary between LAC and LAC Δ sae in 10% NHS. (A) Representative confocal immunofluorescence images assessing C3b deposition on biofilm aggregates. Bottom two rows contain images from wells that contained no primary antibody to C3b. Scale bar = 30 μ m. (B) C3b deposition on planktonic cells incubated in 10% NHS or 10% HIS (N = 3 independent experiments. Paired t test).

Table S1: Strains used in this study

Strain	Description	Source/Reference
<i>S. aureus</i> AH1263	LAC erms sensitive isolate	Boles 2010
<i>S. aureus</i> AH1726	LAC (AH1263) erm ^S + pCM29 (PsarA_sGFP, cmR)	Chiu 2013
<i>S. aureus</i> AH5406	LAC (AH1263) Δ saePQRS + pCM29 (PsarA_sGFP, cmR)	This Study
<i>S. aureus</i> AH2768	LAC (AH1263) agr::tet + pCM29 (PsarA_sGFP, cmR)	Paharik 2017
<i>E. coli</i> AH1704	<i>E. coli</i> BW25141 + pCM29 (PsarA_sGFP, cmR)	
<i>S. aureus</i> BP1	RN4220 + pCM29 (PsarA_sGFP, cmR)	This Study
<i>S. aureus</i> AH1919	LAC(AH1263) Δ aur Δ sspAB Δ staphopainA, spl::erm	Wörmann 2011
<i>S. aureus</i> CG100	LAC (AH1919) Δ saePQRS	Gries 2020
<i>S. aureus</i> BP10	CG100 (AH1919 Δ saePQRS) + pCM29 (PsarA_sGFP)	This Study
<i>S. aureus</i> AH4035	LAC (AH1263) Δ coa	Trivedi 2018
<i>S. aureus</i> AH4060	LAC (AH1263) Δ vWbp	Trivedi 2018
<i>S. aureus</i> AH4062	LAC (AH1263) Δ coa Δ vWbp	Trivedi 2018
<i>S. aureus</i> AH4037	LAC (AH1263) Δ clfA	Kwiecinski 2019
<i>S. aureus</i> AH2905	LAC (AH1263) Δ clfB::Tn Mariner	
<i>S. aureus</i> LAC Δ FnbPs	LAC erm ^R background	This Study
<i>S. aureus</i> LAC Δ saePQ	LAC erm ^R background	Collins 2020
<i>S. aureus</i> LAC Δ saeR ^{D51A}	LAC erm ^R background	Nygaard 2018
<i>S. aureus</i> LAC Δ eap	LAC erm ^R background	This Study
<i>S. aureus</i> LAC Δ empbp	LAC erm ^R background	This Study
<i>S. aureus</i> LAC Δ hla	LAC erm ^R background	This Study
<i>S. aureus</i> LAC Δ sbi Δ spa	LAC erm ^R background	This Study

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CHAPTER FOUR

CONCLUSIONS AND FUTURE STUDIES

Conclusions

Implanted biomaterials have become regular components of modern treatment plans for a variety of conditions and can vastly improve patient quality of life. Unfortunately, all implanted foreign bodies have significantly increased risk for developing infection and despite considerable research there are few anti-infective strategies available that have demonstrated clinical efficacy (Busscher et al., 2012). In Chapter 1, we reviewed mechanisms used by biofilms to subvert host defenses including the production of virulence factors and a protective matrix, as well as the altered infectious microenvironment. While these mechanisms of immune tolerance help to explain persistence of a biofilm, many of these phenomena require high cell densities and a “mature” biofilm infection to manifest. Thus, the research described in this thesis aimed to shift the focus to the host immune response at the very early time points following implantation and contamination. At these early time points, pathogens may be most susceptible but manage to evade the early innate immune response, constituting a potential inflection point in the contest between host and pathogen. Identifying the immune evasion strategies or fundamental deficiencies in the host immune response may provide pathways to better prophylactic treatment strategies. We hypothesized that neutrophils can readily eliminate surface adherent bacteria, but clearance is dependent on the swift recruitment of neutrophils and discovery of bacteria.

In Chapter 2, we investigated interactions between human neutrophils and nascent *Staphylococcus aureus* biofilms and the recruitment of neutrophils to a biomaterial surface in two models of implant infection. In these studies, we observed via time-lapse confocal

microscopy that surface adherent *S. aureus* will rapidly form biofilm aggregates in the presence of serum that are tolerant to neutrophil killing *in vitro*. Once aggregates reached an area of approximately 50 – 75 μm^2 , we measured diminished killing and increased neutrophil membrane damage, resulting to bacterial persistence. *In vivo*, we found that leukocyte recruitment to a peritoneal implant was highly heterogeneous, leading to survival and growth of undiscovered bacteria at 24 hours post implantation. In a subcutaneous intravital imaging model, neutrophil recruitment and subsequent discovery rarely happened within 3 hours post implantation, while bacteria continued to grow and form aggregates. Together, these experiments suggest a model wherein the recruitment of neutrophils to a biomaterial surface, and subsequent discovery of contaminating bacteria, can take several hours, allowing bacteria to grow and form neutrophil-tolerant biofilm aggregates. Furthermore, bacteria inhabiting the skin can already exist in relatively large aggregates (Alhede et al., 2020). Thus, bacteria from the skin that contaminate the surgical wound or implant surface may have a significant “head start” on biofilm formation, further supporting this hypothesized model.

Chapter 3 described studies elucidating the mechanisms of biofilm aggregate tolerance to neutrophil killing through the identification of specific relevant virulence factors. *S. aureus* mutants defective in the SaeR/S two component gene regulatory system or the Agr quorum sensing system were challenged with neutrophils and interactions were again quantified using time-lapse confocal microscopy. $\text{LAC}\Delta\text{sae}$ demonstrated a significant aggregation deficiency and cells were highly susceptible to neutrophil clearance. Inhibition of aggregation by $\text{LAC}\Delta\text{sae}$ was dependent on serum complement C3 and fB, suggesting a mechanism dependent on the alternative C3 or C5 convertase. These data highlight a novel interaction during the pathogenesis of an implant infection: serum complement can reduce *S. aureus* aggregation, rendering bacteria more susceptible to neutrophil clearance, but is inhibited by SaeR/S regulated factors to allow aggregation.

In the appendices, we detail related preliminary studies seeking to identify specific SaeR/S regulated virulence factors that contribute to neutrophil evasion and find evidence to suggest that bacterial aggregation, rather than the production of lytic toxins, provide protection from neutrophil clearance during early biofilm formation. Additionally, we performed preliminary experiments investigating the role of biomaterial surface adsorbed host proteins, namely iC3b and fibrinogen, in mediating adhesion to and motility on a surface. Together, the data presented here help to describe a new model of implant infection wherein disease can occur through a combination of delayed neutrophil recruitment and bacterial discovery and concurrent formation of neutrophil-tolerant biofilm aggregates.

Future Studies

The work presented here has opened many avenues for continued exploration. In the intravital imaging studies described in Chapter 2, we could not fully describe the time scale of neutrophil recruitment as technical limitations prevented imaging beyond three hours. We rarely observed significant recruitment or bacterial discovery within three hours post implantation. Thus, the exact timing of neutrophil recruitment and the subsequent discovery of bacteria remains an open question. Future studies should utilize both conventional mouse models and a modified intravital imaging model to measure recruitment at time points beyond three hours to determine when neutrophils arrive and consequently, the approximate window of unimpeded growth that bacteria may experience post implantation. In this work, we deliberately focused on neutrophil recruitment and interactions with *S. aureus* due to their important role as early responders and controllers of staphylococcal infections. However, the role of resident and recruited macrophages and monocytes should be determined *in vivo* during these early time points as well.

Several key questions raised in Chapter 3 remain unanswered and warrant additional investigation. While we have determined that inhibition of LAC Δ sae aggregation is dependent on C3 and fB, the exact mechanism must still be determined. One possibility is that the C3 or C5 alternative convertase is directly cleaving *S. aureus* surface proteins required for cell-cell adhesion. There is evidence of the alternative C3 convertase acting on bacterially derived proteins in a report documenting cleavage of Neisserial Heparin Binding Antigen, a virulence factor produced by *Neisseria meningitidis*, by the alternative C3 convertase (Di Fede et al., 2018), which provides a precedent for this hypothesis. Alternatively, the convertase(s) may simply activate another serum zymogen or protease that is responsible for inhibiting biofilm formation. Plasma kallikrein and plasmin were recently described to inhibit biofilm formation for *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Haemophilus influenzae*, and *Bordetella pertussis* (Arenas et al., 2021). To test these two hypotheses, experiments assaying LAC Δ sae aggregate formation in the presence of the alternative C3/C5 convertase and other serum proteases such as plasmin or kallikrein will be conducted.

On the pathogen side, it is clear that several redundant SaeR/S regulated factors inhibit complement to protect aggregation. An isogenic deletion mutant of genes known to produce proteins involved in complement interference at the C3bBb level should be constructed to attempt to recapitulate the phenotype observed in the LAC Δ sae mutant. In the event that knocking out the known complement interfering genes does not fully explain the phenotype, RNA sequencing experiments can also be conducted to compare transcriptional activity between LAC and LAC Δ sae to suggest any additional proteins that may not have known complement interfering activity. The observation that LAC Δ sae forms aggregates in complement deficient serum (C3-dep, fB-dep, CVF-dep, compstatin inhibited) does suggest that cell-cell adhesion is mediated by an SaeR/S independent factor or factors. Studies should seek to identify these protein(s) through SDS-PAGE detection of proteins cleaved in serum and mass

spectrometry, followed by validation with isogenic deletion mutants. The role of impaired aggregation must also be confirmed *in vivo* to determine its effect on biofilm establishment, persistence, and dissemination. We observed that 10% mouse serum fails to prevent $LAC\Delta sae$ aggregation, suggesting that a murine model may not recapitulate the phenotype observed *in vitro*. Thus, a rabbit model may be best suited for these studies as rabbit serum robustly inhibited aggregation. Finally, the ability of complement to inhibit aggregation of other bacterial or fungal species must be investigated to determine 1.) If this action by complement represents a universal defense mechanism and 2.) What, if any, other pathogens have adapted strategies to overcome it. Preliminary experiments growing *Staphylococcus epidermidis* in normal and heat inactivated serum suggest aggregation inhibition occurs and is not overcome by wild type bacteria (data not shown).

While specific *S. aureus* virulence factors that inhibit complement may represent interesting vaccination targets in the future, we believe that our data suggest a potential prophylactic role for immunomodulatory therapies. Several pre-clinical studies have suggested cytokine or cell-therapy based approaches to preventing or treating biofilm infections through improved leukocyte activation or polarization (Boelens et al., 2000; Boyce et al., 2012; Hanke et al., 2013; Li et al., 2009, 2010; Rózalska et al., 1996, Hamilton et al., 2021). In Chapter 2, we pre-recruited neutrophils to the dermis via injection of MIP-2 prior to biomaterial implantation and this greatly increased the number of neutrophils recruited to the surface and bacterial clearance, demonstrating a proof of concept immunomodulatory approach. We believe that stimulation of leukocyte recruitment or local delivery of *ex vivo* isolated leukocytes (as in Zimmerli et al., 1984) could serve as novel prophylactic strategies to improve early discovery and killing of contaminating bacteria.

It is my hope that this research provides some small insight or spark of inspiration for others in the scientific community, so that progress, and eventually therapies, may steadily advance.

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APPENDICES

APPENDIX A

FURTHER INVESTIGATION OF *S. AUREUS* VIRULENCE FACTORS AND THEIR ROLE IN
NEUTROPHIL EVASION DURING EARLY BIOFILM FORMATION

FURTHER INVESTIGATION OF *S. AUREUS* VIRULENCE FACTORS AND THEIR ROLE IN
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Brian A. Pettygrove, Jovanka M. Voyich, Philip S. Stewart

Introduction

We have previously observed that small *S. aureus* biofilm aggregates rapidly develop tolerance to killing by human neutrophils as they increase in size (Chapters 2 and 3). Once aggregates achieved approximately the same size as a neutrophil ($\sim 50 - 75 \mu\text{m}^2$), we observed little reduction in aggregate volume following phagocytosis and significantly higher rates of propidium iodide staining, indicating damage to the neutrophil membrane. In addition to large aggregates becoming mechanically more difficult for neutrophils to phagocytose (or break apart and phagocytose a smaller piece of the biofilm aggregate), we hypothesize that the concentrations of secreted virulence factors in the local environment or phagolysosome become sufficient to interfere with neutrophil function. In a strain lacking the genes for the SaeR/S two-component system, we observed deficiencies in both aggregate formation and neutrophil lysis and this strain was highly susceptible to neutrophil killing. Based on these observations, we posed two questions. 1.) Does the ability to form aggregates or lyse neutrophils play a greater role in defense against neutrophils, or do they have a synergistic effect? 2.) Which SaeR/S regulated genes are specifically required for neutrophil tolerance? We broadly sought to answer these questions by identifying isogenic mutants deficient in SaeR/S regulated genes that are either unable to aggregate or unable to lyse neutrophils.

We screened several available deletion mutants and identified a strain with a slight, albeit inconsistent, aggregation defect, $\text{LAC}\Delta\text{ecb}\Delta\text{efb}\Delta\text{sbi}\Delta\text{FnbPs}$ ($\text{LAC}\Delta 4$ -agg, for short). This strain contains deletions of fibronectin binding proteins A and B (*FnbPs*), the extracellular

complement-binding protein (*ecb*), extracellular fibrinogen-binding protein (*efb*), and the second binding protein of immunoglobulin (*sbi*). FnBPs are LPXTG-anchored surface proteins that have previously been implicated in exopolysaccharide independent biofilm formation in MRSA strains (O'Neill et al., 2008). Efb is a secreted protein known to bind to both fibrinogen and complement C3 as a method to avoid complement mediated immunity and help shield *S. aureus* from phagocytosis (Jongerijs et al., 2007; Ko et al., 2013; Koch et al., 2012). Ecb is a homolog of Efb with similar complement inhibiting interfering capabilities (Jongerijs et al., 2007). Sbi is a surface associated protein known to bind to IgG, inhibiting phagocytosis, and can additionally recruit plasminogen to the cell surface to enhance cleavage of C3/C3b (Burman et al., 2008; Guerra et al., 2017; Koch et al., 2012). A previous study demonstrated that a mutant deficient in the 5 secreted bicomponent leukotoxins (Δ LAC5) failed to lyse neutrophils and had compromised virulence in a porcine burn wound model (Bhattacharya et al., 2018). Importantly, these toxins, PVL, HlgAB, HlgCB, LukAB, and LukED, are highly regulated by the SaeR/S system in response to neutrophil derived stimuli (Liu et al., 2016; Nygaard et al., 2010; Spaan et al., 2017). The results presented here demonstrate that elimination of neutrophil lysis by deletion of the 5 bicomponent leukotoxins failed to significantly increase bacterial clearance. While the phenotype was somewhat inconsistent, preliminary results with the Δ LAC4-agg mutant suggest that a failure to aggregate increases susceptibility to neutrophil clearance.

Results

Δ LAC4-agg is slightly more susceptible to neutrophil killing

We screened several available isogenic deletion mutants using brightfield time-lapse microscopy to identify SaeR/S regulated genes that contribute to the ability to form aggregates in normal human serum (NHS). These observations suggested that the Δ LAC4-agg strain had a slight aggregation deficiency, so a constitutively expressed GFP was introduced via plasmid to

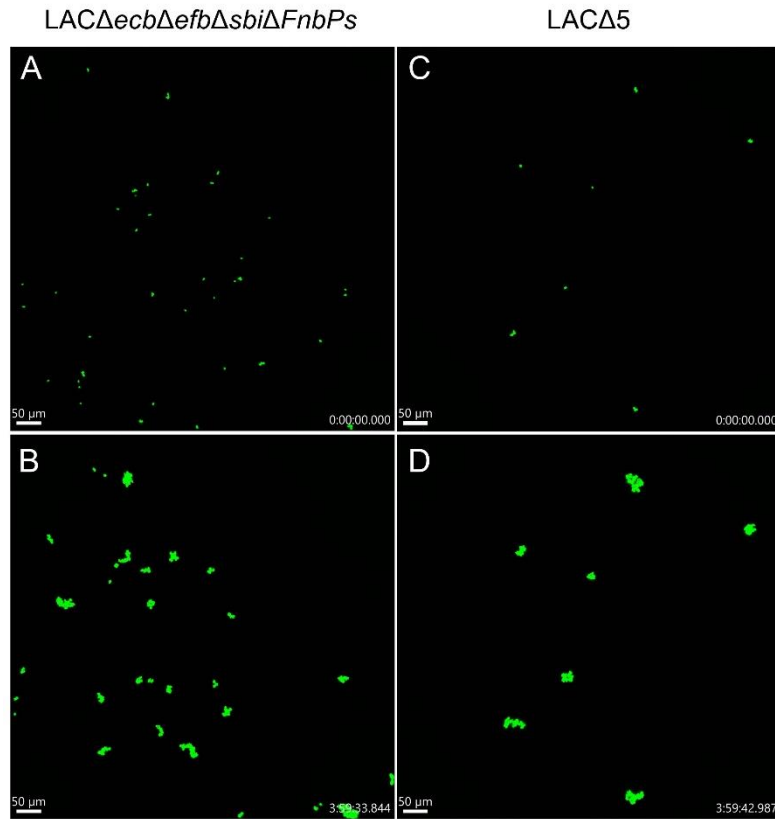


Figure 1: Differential growth of LACΔ4-agg and LACΔ5. Representative confocal microscopy images at t = 0 h (2.5 h after attachment, A, C) and t = 4 h (6.5 h after attachment, B, D). Many cells shed from LACΔ4-agg aggregates during growth leave the FOV. Scale bar = 50 μm.

allow for quantitative assessment of the strain's aggregation. The LACΔ4-agg demonstrated a propensity to shed cells from growing aggregates, resulting in smaller groups of cells drifting through the field of view (Figure 1A, 1B, Video SA1.1), albeit to a much lesser degree than the LACΔsaePQRS mutant (discussed in Chapter 3). To determine if this aggregation defect resulted in increased susceptibility to neutrophil killing, we attached cells to the surface, allowed them to grow for approximately 2.5 h in NHS, and challenged with human neutrophils. In these experiments, we compared LACΔ4-agg to LAC AH1726, a USA300 isolate that has been cured of a plasmid carrying erythromycin resistance but is commonly considered in the literature to be a WT strain. As expected, the average aggregate size at the time of discovery and phagocytosis

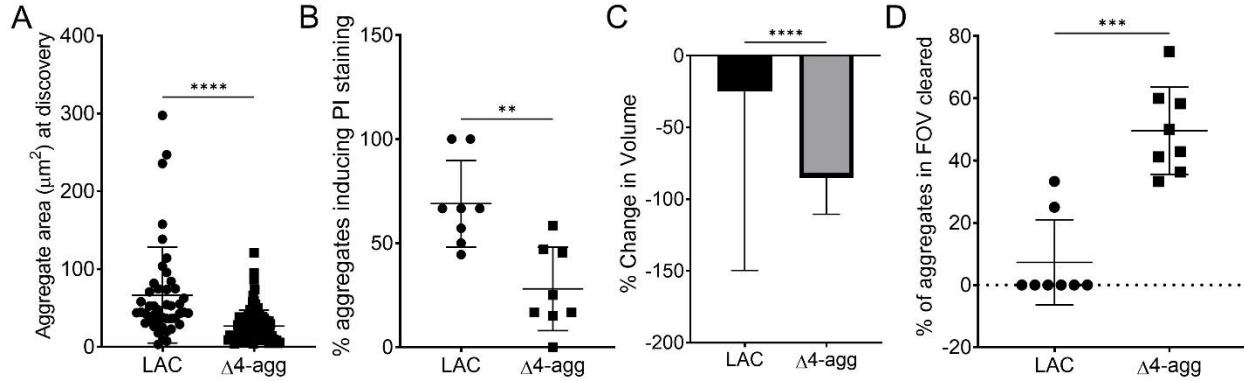


Figure 2: LAC Δ *ecb* Δ *efb* Δ *sbi* Δ *FnbPs* (LAC Δ 4-agg) forms smaller aggregates that are more susceptible to killing compared to LAC (Strain AH1726, *erm*^S). (A) Aggregate size at the time of phagocytosis by a neutrophil (N = 45 aggregates for LAC and 93 aggregates for LAC Δ 4-agg). (B) Percentage of aggregates in a FOV that induce PI staining of at least one neutrophil (N = 8 FOVs each). (C) Change in aggregate volume over the duration of an interaction compared to the aggregate size at the time of phagocytosis (N = 45 aggregates for LAC and 93 aggregates for LAC Δ 4-agg). (D) Percentage of aggregates in a FOV that were phagocytosed and had a 100% reduction in volume (N = 8 FOVs each). Error bars indicate mean $\pm$ SD. Data collected from 4 independent experiments. Mann-Whitney test ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

by a neutrophil was significantly lower for the LAC Δ 4-agg mutant compared to LAC (Figure 2A). Despite retaining the full complement of secreted toxins that may contribute to neutrophil lysis, we observed a significant decrease in the rate of neutrophil propidium iodide (PI) staining, possibly due to the smaller average aggregate size (Figure 2B). Phagocytosed LAC Δ 4-agg aggregate volumes were significantly more reduced than LAC (Figure 2C) and a higher fraction of LAC Δ 4-agg aggregates were completely eliminated from the field of view (FOV) (Figure 2D). We observed a slight increase in the number of CFUs recovered from control wells compared to WT LAC, suggesting that LAC Δ 4-agg aggregates were more effectively disrupted by vortexing and pipetting during plating (Figure 3A, 3B), an observation we have also made with a LAC Δ *saePQRS* mutant (data not shown). Overall, we observed greater killing of LAC Δ 4-agg compared to WT, suggesting a diminished ability to tolerate neutrophil clearance during early biofilm formation (Figure 3C). We additionally observed greater decreases in total GFP area within a FOV over time for the LAC Δ 4-agg mutant (Figure 3D).

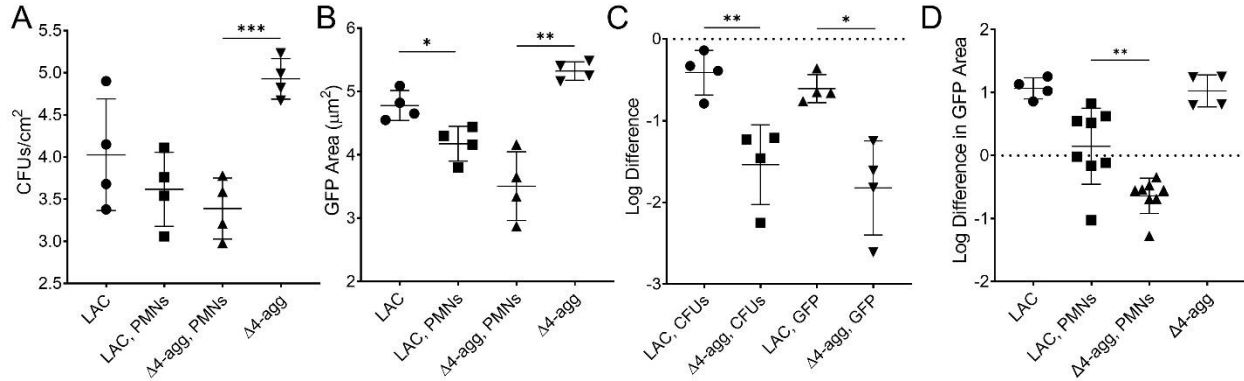


Figure 3: LAC Δ *ecb* Δ *efb* Δ *sbi* Δ *FnbPs* (LAC Δ 4-agg) is more effectively cleared from a surface compared to LAC (strain AH1726, *erm*^S). (A) Total viable bacteria recovered from the surface (N = 4 independent experiments. Unpaired t test ***p<0.001). (B) Total GFP signal remaining on the surface following neutrophil challenge, as determined by a tile scan (N = 4 independent experiments. Unpaired t test with Welch's correction *p<0.05, **p<0.01). (C) Log difference in CFUs/cm² and total GFP area following neutrophil treatment, compared to control wells (N = 4 independent experiments. Paired t test *p<0.05, **p<0.01). (D) Log difference in GFP area within a FOV over 4 h of imaging (N = 8 FOVs from 4 independent experiments. Unpaired t test with Welch's correction **p<0.01). Error bars indicate mean $\pm$ SD.

To ensure that differences that we observed between LAC AH1726 (*erm*^S) and LAC Δ 4-agg were not due to the slight difference in genetic background between LAC AH1726 and WT LAC (*erm*^R) used to generate the LAC Δ 4-agg strain, we tagged the original WT LAC strain with GFP (strain BP25, *erm*^R) and repeated the neutrophil challenge experiments. While we observed similar results between LAC AH1726 and BP25, in 3 of 4 experiments we surprisingly saw a less pronounced or no aggregation deficiency in LAC Δ 4-agg, suggesting an inconsistent phenotype for this mutant (Video SA1.2). There was still a slight decrease in the average aggregate size (Figure 4A), however this difference was less prominent than in the previous set of experiments (Figure 2A). As the strain aggregated well in most of the experiments, we tended to see only slight differences between BP25 and LAC Δ 4-agg clearance (Figures 4B – 4D). These two sets of experiments suggest that a deficiency in aggregation may lead to increased clearance, however the aggregation phenotype in this mutant is too inconsistent to conclusively demonstrate this claim.

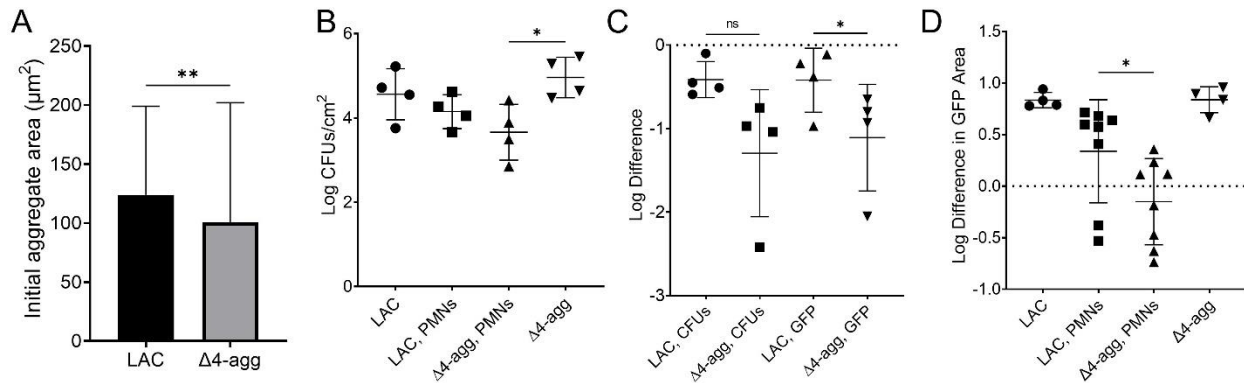


Figure 4: LACΔ*ecb*Δ*efb*Δ*sbi*Δ*FnbPs* (LACΔ4-agg) is only slightly more susceptible to killing compared to LAC (strain BP25) due to increased aggregation (A) Initial aggregate area at the start of imaging, collected from wells containing PMNs only (N = 78 aggregates for LAC and 87 aggregates for LACΔ4-agg collected from 4 independent experiments. Mann-Whitney test ** $p < 0.01$). (B) Total viable bacteria recovered from the surface (N = 4 independent experiments. Unpaired t test * $p < 0.05$). (C) Log difference in CFUs/cm² and total GFP area following neutrophil treatment, compared to control wells (N = 4 independent experiments. Paired t test * $p < 0.05$). (D) Log difference in GFP area within a FOV over 4 h of imaging (N = 8 FOVs from 4 independent experiments. Mann-Whitney test * $p < 0.05$). Error bars indicate mean $\pm$ SD.

LACΔ5 is unable to lyse neutrophils but is not more susceptible to clearance

To investigate the effect of secreted lytic toxins on *S. aureus* biofilm aggregate survival, we again grew LAC and LACΔ5 for 2.5 h in NHS to form aggregates and challenged cells with neutrophils. Importantly, LACΔ5 had no aggregation deficiency and aggregates were similar in size to LAC at the time of discovery by a neutrophil (Figure 1C, 1D, 5A). Deletion of the bi-component leukotoxins completely eliminated lysis of interacting neutrophils (Figure 5B). We observed that LACΔ5 aggregates were more not more effectively killed following phagocytosis compared to WT (Figure 5C) and saw no significant difference in the percentage of aggregates that were eliminated from a FOV (Figure 5D). Recovered CFUs and total GFP area remaining on the surface were similar between LAC and LACΔ5 (Figure 6A, 6B) and we measured no significant differences in overall clearance for either individual FOVs or across the entire surface

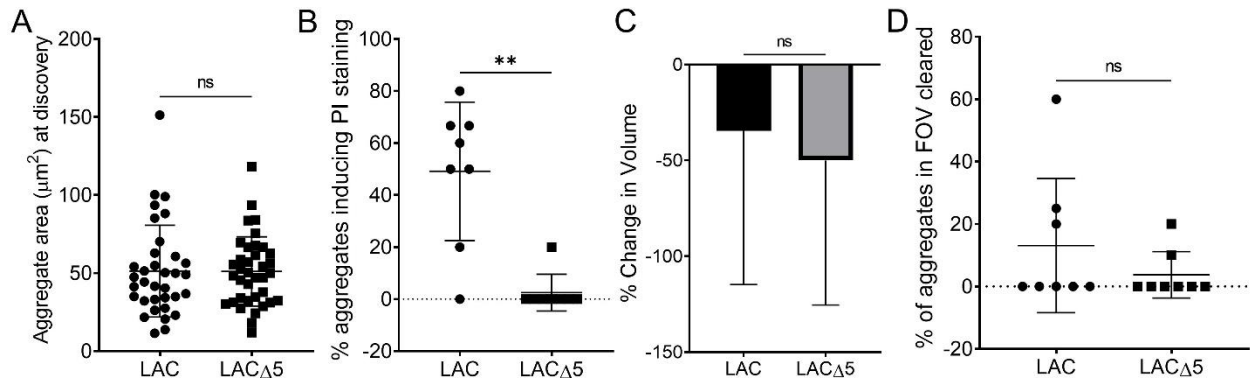


Figure 5: LACΔ5 aggregates do not lyse neutrophils but are not more susceptible to killing compared to LAC (Strain AH1726). (A) Bacterial aggregate size at the time of phagocytosis by a neutrophil (N = 34 aggregates for LAC and 37 aggregates for LACΔ4-agg). (B) Percentage of aggregates in a FOV that induce PI staining in at least one neutrophil (N = 8 FOVs each). (C) Change in aggregate volume over the duration of an interaction compared to the aggregate size at the time of phagocytosis (N = 34 aggregates for LAC and 37 aggregates for LACΔ4-agg). (D) Percentage of aggregates in a FOV that were phagocytosed and had a 100% reduction in volume (N = 8 FOVs each). Error bars indicate mean $\pm$ SD. Data collected from 4 independent experiments. Mann-Whitney test $**p < 0.01$.

(Figures 6C, 6D). These results suggest that the ability to lyse neutrophils alone is not significant driver of *S. aureus* aggregate persistence.

Discussion

S. aureus lacking the SaeR/S two-component system is deficient in the ability to lyse neutrophils and form cohesive aggregates. These smaller aggregates are highly susceptible to neutrophil clearance and suggest that SaeR/S regulated factors are essential for effective defense against neutrophil killing during early biofilm formation. We sought to identify specific SaeR/S regulated genes that contribute to neutrophil lysis and aggregate formation, respectively, to determine if either defense mechanism plays a dominant role in aggregate survival. We identified a strain lacking several SaeR/S regulated genes that displayed a mild aggregation defect in NHS, LACΔ*ecb*Δ*efb*Δ*sbi*Δ*FnbPs*. The degree to which the LACΔ4-agg mutant failed to aggregate varied from experiment to experiment, but in experiments where

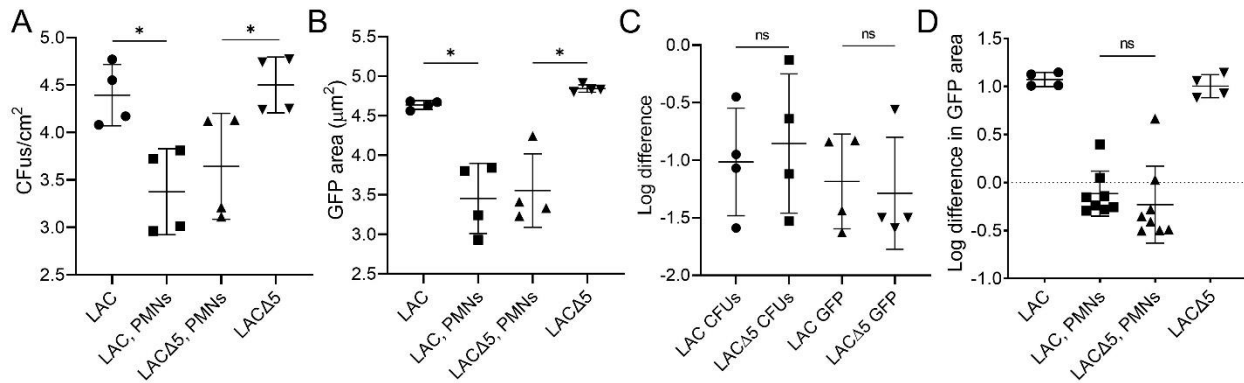


Figure 6: LACΔ5 clearance is similar to LAC (strain AH1726). (A) Total viable bacteria recovered from the surface (N = 4 independent experiments. Unpaired t test *p<0.05). (B) Total GFP signal remaining on the surface following neutrophil challenge, as determined by a tile scan (N = 4 independent experiments. Unpaired t test with Welch's correction *p<0.05). (C) Log difference in CFUs/cm² and total GFP area following neutrophil treatment, compared to control wells (N = 4 independent experiments. Mann-Whitney test). (D) Log difference in GFP area within a FOV over 4 h of imaging (N = 8 FOVs from 4 independent experiments. Mann-Whitney test). Error bars indicate mean ± SD.

cohesive aggregates failed to form, neutrophils were far more successful at killing bacteria. It is unclear why the aggregation phenotype of this strain is inconsistent, however it is possible that it is due to donor to donor variability in serum complement activity. We demonstrated in Chapter 3 that the inability of the SaeR/S mutant to form aggregates is due to complement C3 and factor B dependent activity. Three of the genes missing in the LACΔ4-agg mutant, namely *ecb*, *efb*, and *sbi*, have functions related to complement inhibition at the level of C3 or the C3b containing alternative C3/C5 convertases (Amdahl et al., 2017; Burman et al., 2008; Jongerius et al., 2007; Koch et al., 2012; Upadhyay et al., 2008). *S. aureus* produces several other SaeR/S regulated proteins that have been demonstrated to interfere with complement at the level of C3, specifically the staphylococcal complement inhibitor (SCIN) and its homologues SCIN-B and SCIN-C (Jongerius et al., 2007; Rooijackers et al., 2005, 2007) and recently the protease SplB (Dasari et al., 2021). Thus, the LACΔ4-agg mutant would be expected to have a diminished capability to interfere with the inhibitory effects of complement but may still be able to sufficiently

aggregate depending on the serum complement activity present. To our knowledge, FnBPs have not yet been demonstrated to interfere with complement function, however we have not ruled out that these proteins may be contributing to the phenotype observed in the LAC Δ 4-agg mutant. This topic will be further investigated as we seek to identify the SaeR/S regulated genes that are responsible for overcoming the ability of complement to inhibit aggregation. That line of inquiry should produce a strain defective in the ability to form aggregates while maintaining functional leukotoxin expression, thus allowing us to better interrogate the specific role of aggregate formation in survival against neutrophil challenge.

Neutrophil challenge of nascent biofilm aggregates demonstrated that a mutant deficient in the 5 bicomponent leukotoxins regulated by SaeR/S was unable to lyse neutrophils, but aggregates persisted at similar rates as WT. These data suggest that at this early stage of biofilm formation, neutrophil lysis may be less important than the ability to form a mechanically resilient aggregate. Bhattacharya et al. demonstrated that spent media from 24 h grown biofilm contained sufficient leukotoxin concentrations to induce significant neutrophil lysis (Bhattacharya et al., 2018). Additionally, LAC Δ 5 biofilms were more susceptible to neutrophil killing than WT *in vitro* and *in vivo* (Bhattacharya et al., 2018, 2020). Together these data suggest that mature biofilms produce high enough concentrations of leukocidins to induce neutrophil lysis prior to physical contact with the biofilm, protecting bacteria from phagocytosis and killing. In our system, we almost exclusively observe neutrophil lysis occurring after colocalization of bacteria and neutrophils, suggesting that the local concentration of these toxins surrounding bacterial aggregates is not yet sufficient to induce lysis before phagocytosis. LukAB specifically has been implicated in the lysis of neutrophils during phagocytosis of mature biofilm (Bhattacharya et al., 2020). The fact that the LAC Δ 5 mutant induced negligible PI staining of neutrophils indicates that the bicomponent leukocidins are the key toxins responsible for the membrane damage observed during interaction with WT bacteria.

From interrogation of the two mutants, we have gained insight that suggests that the ability to form nascent biofilm aggregates is a crucial defense against neutrophil phagocytosis and killing on a surface. While the ability to secrete high concentrations of lytic toxins has been demonstrated to be important for dense biofilms (Bhattacharya et al., 2018), our data suggests that the concentrations produced by small biofilm aggregates are not sufficient to lyse neutrophils prior to colocalization. Despite an inability to lyse neutrophils, LAC Δ 5 aggregates survived neutrophil challenge at similar levels as WT LAC. Slight defects to aggregation however, significantly increased bacterial susceptibility despite unimpaired expression of bicomponent leukotoxins. In addition to identifying the genes required for aggregation in human serum, future studies should explore strategies for disrupting biofilm aggregates to render them more susceptible to killing by immune cells.

Materials and Methods

Bacteria and neutrophil preparation

Staphylococcus aureus strain AH1726 (LAC + pCM29, courtesy of Alex Horswill), with constitutive expression of green fluorescent protein (GFP), was grown overnight in tryptic soy broth (TSB) supplemented with 10 μ g/ml chloramphenicol. Plasmid pCM29 (Pang et al., 2010) was isolated from strain AH1704 (*E. coli* BW25141) and sequentially transformed into competent *S. aureus* RN4220 and then LAC, LAC Δ *ecb* Δ *efb* Δ *sbi* Δ *FnbPs*, or LAC Δ 5 (courtesy of Victor Torres) to produce strains BP25, BP2, and BP3, respectively. Isolates were subsequently screened for GFP fluorescence and growth defects. For neutrophil challenge assays, overnight cultures were centrifuged for 5 min at 4000 rpm, rinsed, resuspended in tryptic soy broth, and serially diluted and adjusted by optical density. Cells were attached to a 4-chambered glass bottom Petri dish (Cellvis, CA, USA, Cat no. D35C4-20-1.5-N) to facilitate live-cell imaging. To attach cells, 10 μ L of diluted cell suspension was added to the surface. After 30 min of

incubation at 37° C, unattached bacteria were gently rinsed from the surface with PBS. Each chamber of the Petri dish was filled with 1 mL of 10% fresh human serum in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} to condition the surface and opsonize bacteria and incubated at 37 °C for 2.5 - 3 h to allow biofilm aggregates to form. Human neutrophils were isolated from heparinized venous blood obtained from healthy donors following a standard IRB-approved protocol. All donors provided written consent to participate in the study. Neutrophils were isolated under endotoxin-free conditions ($<25 \text{ pg ml}^{-1}$), and purity ($<1\%$ PBMC contamination) and viability ($<2\%$ propidium iodide positivity) of neutrophil preparations were assessed by flow cytometry as previously described (Voyich et al., 2005, 2009). Neutrophils were kept on ice until stained with LysoBrite™ Red (AAT Bioquest, CA, USA, Cat no. 22645) according to the manufacturer's instructions. Neutrophils were immediately added to the surface following staining and $5 \text{ }\mu\text{g/mL}$ propidium iodide was added to each well at this time. To enumerate surviving bacteria, bacteria were removed from the surface with a sterile scraper and pipetting, vortexed in dilution tubes with PBS, and plated on tryptic soy agar in triplicate. Plates without detectable bacteria were counted as 0.5 colonies prior to log transformation.

Microscopy

A Leica SP5 inverted confocal laser scanning microscope was utilized for all *in vitro* imaging. GFP-tagged bacteria and neutrophils were excited with the 488 nm and 561 nm laser lines, respectively. A LiveCell (Pathology Devices, CA, USA) environmental chamber system was utilized to maintain 5% CO_2 , 20% O_2 , 50% humidity, and 37 °C for sample incubation during imaging. 12- μm image stacks with 1- μm z-slices were recorded sequentially at 1–2 min intervals over a 4-h time course using a Leica 20x/0.7 NA dry objective lens. One field of view was imaged per control chamber and two fields of view per neutrophil treated chamber

in each experiment. Each well was then imaged using a 7×7 stitched tile scan with a 10x/0.3 NA dry objective to quantify total amount of bacteria remaining on the surface.

Image analysis

MetaMorph version 7.8.13 (Molecular Devices, San Jose, CA, USA) image analysis software was used to measure the change in bacterial biomass by quantifying the threshold area of bacterial green fluorescence over the period of 4 h. Movies were prepared with Imaris version 9.8 (Oxford Instruments, Abingdon, UK). For the analysis of individual neutrophil-bacteria interactions, the Imaris Spots feature was used to identify neutrophils and propidium iodide staining events and the Surfaces feature was used to dynamically track bacterial growth. Discovery of bacteria by neutrophils was determined by identifying the first frame in which neutrophils co-localized with a bacterial aggregate and remained co-localized in subsequent frames. Measurements of the bacterial aggregate size were recorded at the start and end of each interaction with a neutrophil, and propidium iodide staining or aggregate breakup events were cataloged. The percentage of aggregates inducing propidium iodide staining was calculated by dividing the number of aggregates that caused propidium iodide staining of at least one phagocytosing neutrophil by the total number of aggregates discovered by a neutrophil in each field of view.

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APPENDIX B

PRELIMINARY STUDIES INTO THE ROLE OF HOST PROTEINS IN NEUTROPHIL
ADHESION, MOTILITY, AND DISCOVERY OF *STAPHYLOCOCCUS AUREUS* ON AN
ABIOTIC SURFACE

PRELIMINARY STUDIES INTO THE ROLE OF HOST PROTEINS IN NEUTROPHIL
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Brian A. Pettygrove, Jovanka M. Voyich, Philip S. Stewart

Introduction

Early work in the biomaterials field sought to understand the mechanisms by which foreign bodies induce inflammation and the fibrotic foreign body response. Various reports have demonstrated potential roles for surface adsorbed fibrinogen (Fg) and possibly complement protein C3 in the recruitment and adherence of monocytes and neutrophils *in vitro* and *in vivo* (Kao, 2000; Kourtzelis et al., 2013; McNally and Anderson, 1994; Szott and Horbett, 2010; Tang and Eaton, 1993; Tang et al., 1996). We have hypothesized that the ability to adhere to and chemotax on a biomaterial surface is an essential step leading to discovery and eventual killing of surface adherent bacteria (Pettygrove et al., 2021). Thus, host proteins that adsorb onto the biomaterial surface may be critical mediators of immunity by facilitating leukocyte adherence and motility. Importantly, our lab has previously observed that neutrophils added directly to glass are not motile while neutrophils are highly motile on glass that has been conditioned with normal human serum (NHS) (Unpublished observation and Ghimire et al., 2019; Pettygrove et al., 2021). In this preliminary study, we sought to elucidate the role of serum proteins in facilitating neutrophil adhesion, motility, and discovery of bacteria on a glass surface. We observed that neutrophil motility in our system is likely dependent on neutrophil receptor MAC-1 binding of surface deposited iC3b. Surface bound Fg may also be able to facilitate motility.

Results

Heat inactivation of serum abolishes neutrophil adherence to the surface and reduces bacterial clearance.

We utilized our existing *in vitro* confocal microscopy assay of *S. aureus*-neutrophil interactions to investigate neutrophil motility and discovery of bacteria in modified serum conditions (Ghimire et al., 2019; Pettygrove et al., 2021). To broadly eliminate heat-labile serum components such as complement and any residual fibrinogen remaining in the serum, we heat inactivated the serum at 56 °C. Bacteria were attached to the surface, non-adherent cells were rinsed off, and either NHS or heat inactivated serum (HIS) was added to bathe the surface. Labeled neutrophils were added and imaged continuously for 4 h. In NHS, neutrophils attached to and migrated on the surface, leading to the discovery of *S. aureus* aggregates (Figure 1A). On a surface coated with HIS, neutrophils failed to consistently associate with the glass surface, often drifting across the field of view (FOV) and only interacting with attached bacteria when the neutrophil directly contacted an aggregate. The observed drift is likely due to slow natural convection present in the well. This resulted in most neutrophils simply entering the FOV and exiting without engaging the bacteria (Figure 1B, Video SA2.1). When neutrophil motion was tracked, the paths traveled by neutrophils in heat inactivated serum were significantly straighter than those for neutrophils in normal serum, suggesting less random walk patrolling of the surface (Figure 1C). A significantly higher fraction of neutrophils followed a nearly straight line across the FOV in heat inactivated serum compared to normal serum (Figure 1D). When neutrophils in HIS were unable to properly patrol the surface, fewer of the attached *S. aureus* cells were discovered, resulting in high densities of bacteria remaining on the surface after 4 h (Figures 1E and 1F). Phagocytosis and killing of bacteria were still occasionally observed when a neutrophil directly encountered bacteria, however this happened at a sharply reduced rate as neutrophils were far less effective at finding the bacteria (Video SA2.1). These results suggest

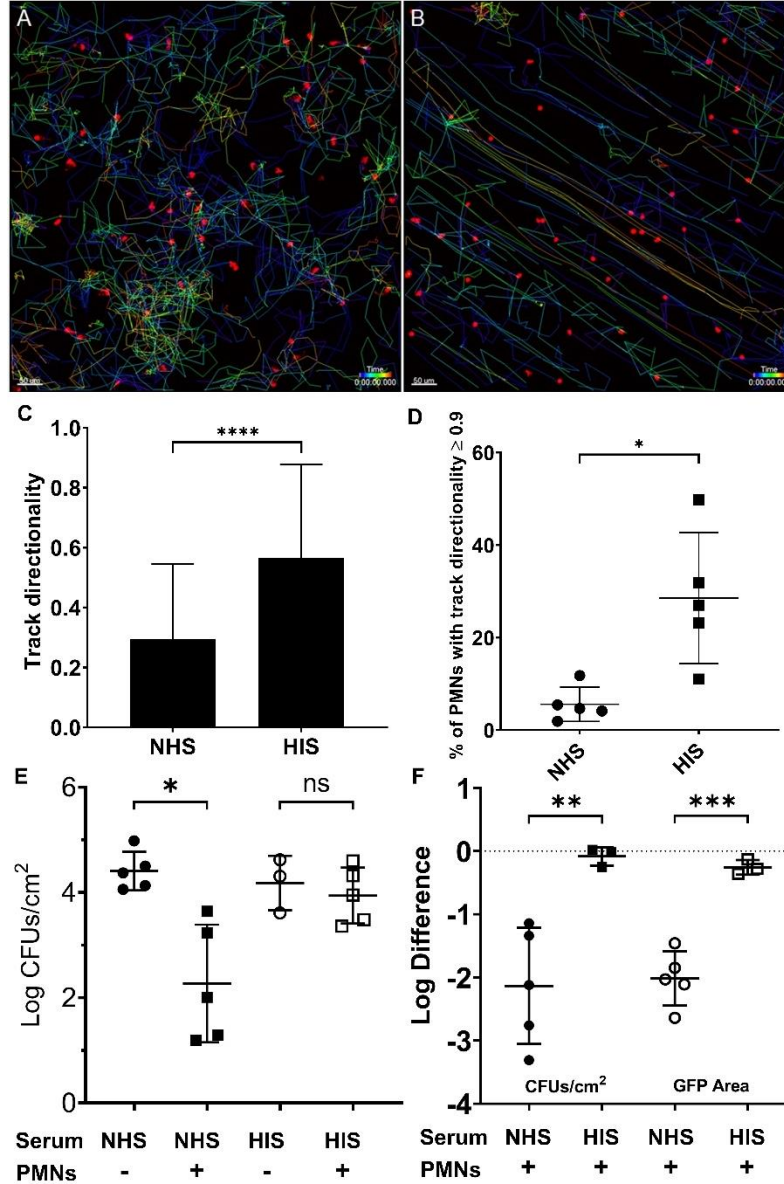


Figure 1: Heat inactivation of serum prevents proper neutrophil adhesion, motility, and clearance of attached *S. aureus*. (A) Representative image showing neutrophil (red) tracks. Colored lines indicating the positions of a given neutrophil over time in NHS and (B) in HIS. Scale bar = 50 μ m. (C) Average track straightness (track displacement divided by track length) in NHS and HIS. Error bars indicate mean $\pm$ SD (N = 3387 individual tracks for the NHS group and n = 2155 individual tracks for the HIS group from 5 independent experiments. Mann Whitney test ****p<.0001). (D) The percentage of neutrophil tracks with a calculated straightness value $\geq$ 0.9. Each point indicates the average of two FOVs. Error bars indicate mean $\pm$ SD (N = 5 independent experiments. Unpaired t test with Welch's correction *p<.05). (E) Colony forming units (CFUs) recovered from wells initially seeded with $2.49 \pm 2.01 \times 10^3$ CFUs/cm² of *S. aureus* and grown for four hours. Wells with neutrophils received $1.29 \pm 0.46 \times 10^4$ PMNs/cm² on average. Error bars indicate mean $\pm$ SD (N = 3 or 5 independent experiments. Unpaired t test with Welch's correction *p<.05, ns = Not Significant). (F) The log difference in

CFUs/cm² (left) or total GFP area remaining on the surface (right) was calculated by subtracting the amount of bacteria in the control well from the amount in the neutrophil treated well. Error bars indicate mean $\pm$ SD (N = 3 or 5 independent experiments. Unpaired t test with Welch's correction **p<.01, ***p<.001).

that host immune cells must be able to bind to and chemotax along the biomaterial surface to effectively discover and phagocytose attached bacteria as this process is an essential step in the effective clearance of the bacteria.

Identification of blood proteins that facilitate neutrophil adherence and motility

Neutrophils and other leukocytes are known to utilize the integrin $\alpha_M\beta_2$ (CD11b/CD18, MAC-1) for diapedesis, adhesion and motility (Ding et al., 1999; Moyle et al., 1994) and protection from bacterial infection (Prince et al., 2001). Both Fg and inactivated C3b (iC3b) are known ligands of MAC-1, suggesting that one or both proteins may facilitate adhesion and motility on a biomaterial surface (Diamond et al., 1993; Mazzone and Ricevuti, 1995). We conducted preliminary experiments to quantify neutrophil motility under inhibition by a selective MAC-1 inhibitor, Neutrophil Inhibitory Factor (NIF) (Moyle et al., 1994), to confirm the role of MAC-1 in motility and subsequent discovery of bacteria. To identify the protein ligands required for motility, we also investigated neutrophil motility in complement depleted conditions and in conditions with supplemental Fg. We observed vast qualitative differences in the motility between each of the tested conditions (Figure 2). Neutrophils in NHS displayed random-walk and chemotactic motility, while neutrophils in HIS failed to associate with the surface and tended to drift across the FOV, as described above (Figure 1, 2A, 2B, Video SA2.1). Neutrophils in the NIF treated wells adhered to the surface but demonstrated a dose-dependent reduction in overall motility (Figure 2C, 2D, Video SA2.2). These cells were able to move but appeared slower and to remain nearer to their initial location compared to cells in NHS. We next attempted to see if Fg added to the bulk media or coated on the glass surface prior to media

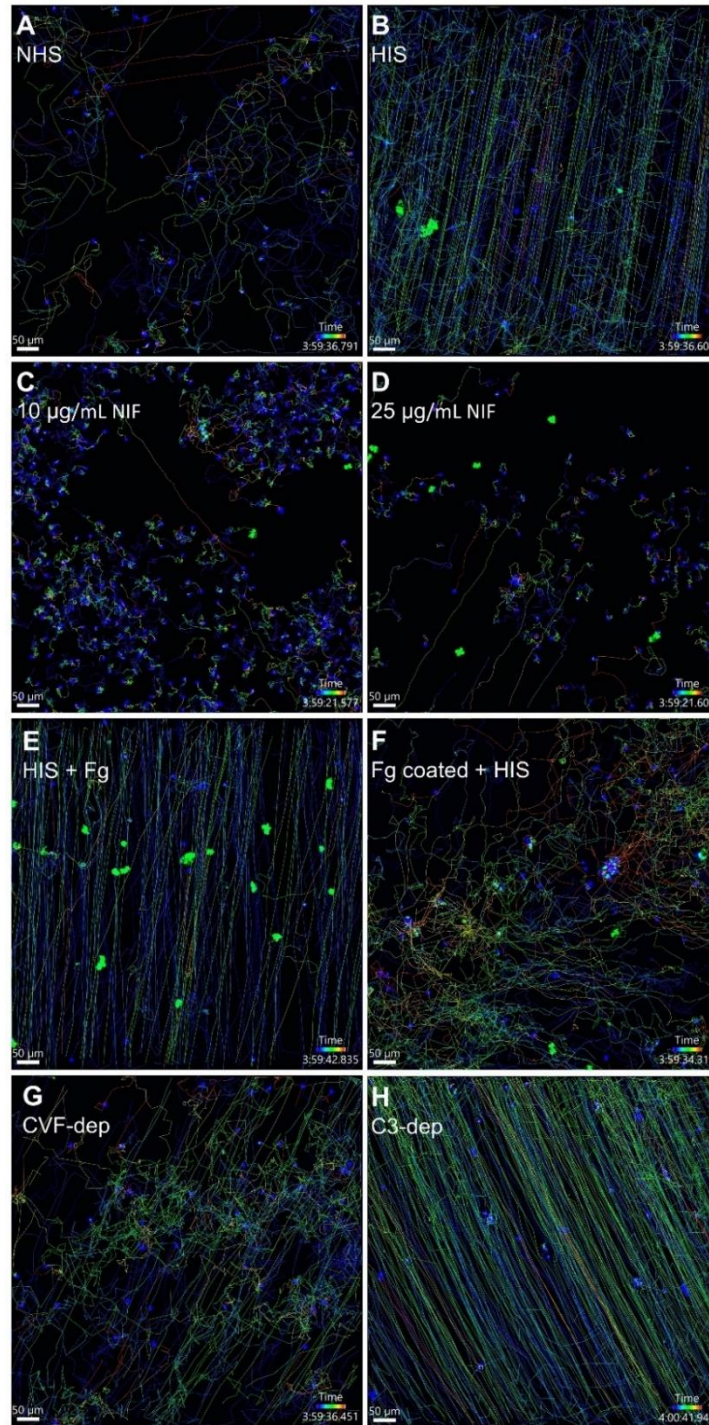


Figure 2: Neutrophil motility under tested treatment conditions. Colored lines trace neutrophil (blue) motility over 4 h. *S. aureus* shown in green. Representative images of neutrophil motility in (A) NHS, (B) HIS, (C) 10 µg/mL NIF, (D) 25 µg/mL NIF, (E) HIS + Fg added to the bulk media, (F) HIS added to a Fg coated surface, (G) CVF-depleted serum, and (H) C3-depleted serum.

addition would restore motility in HIS. Addition of Fg to the bulk media failed to restore motility and neutrophils displayed motility similar to HIS alone (Figure 2E). Conversely, pre-coating the surface with Fg appeared to restore some neutrophil motility (Figure 2F, Video S2A.3). This observation preliminarily indicates that Fg can adsorb to the glass surface to serve as a ligand for leukocyte adhesion but may not effectively adsorb in the presence of other serum proteins. Finally, we assayed neutrophil motility in serum depleted by cobra venom factor (CVF) and in C3-depleted serum. Motility appeared unaffected in CVF (Figure 2G, Video SA2.4), while neutrophils in C3-depleted serum resembled neutrophils in HIS (Figure 2H, Video SA2.5), suggesting that complement, and possibly iC3b in particular, is required for adhesion and motility.

We sought to quantify the observed differences in phenotypes by measuring neutrophil track length, displacement, and straightness (Figure 3). While the average track length was similar between neutrophils in NHS and HIS, we measured greater displacement and overall track straightness in HIS due to the “drifting” phenotype that we observed (Figure 3). We observed a significant decrease in track length and displacement upon treatment with our higher dose of NIF (Figure 3A, 3B). In keeping with their qualitative similarity to HIS, neutrophils incubated in HIS + Fg and C3 depleted serum displayed significantly higher track displacements and increased track straightness compared to NHS (Figure 3B, 3C). Conversely, while variable, we observed motility similar to NHS in serum that was depleted by CVF or on a surface that was pre-coated with Fg prior to addition of HIS (Figure 3B, 3C). Together, these results suggest that adsorbed C3 or Fg may be sufficient for neutrophil motility on glass.

Impaired motility decreases discovery and clearance of adherent bacteria

As a result of compromised motility and ineffective surveillance of the biomaterial surface by neutrophils, we observed a decrease in the efficacy of *S. aureus* clearance (Figure 4).

Importantly, successful phagocytosis is still possible when a neutrophil directly contacts a

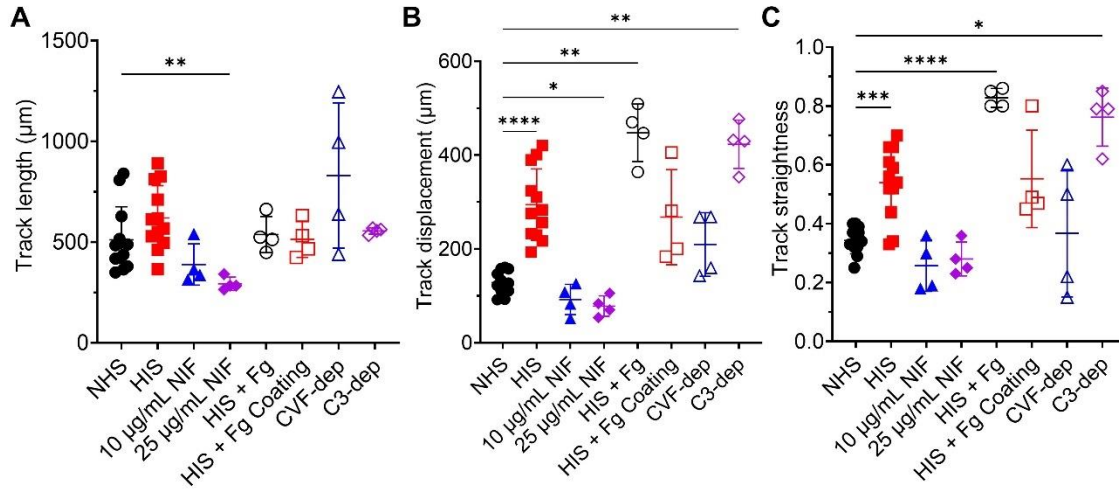


Figure 3: Neutrophil motility is dependent on MAC-1 and complement C3. (A) Average track length by individual neutrophils. (B) Average track displacement by individual neutrophils. (C) Average track straightness for individual neutrophils. Each point represents one FOV. Error bars indicate mean $\pm$ SD (N = 12 FOVs from 6 independent experiments for NHS and HIS conditions. N = 4 FOVs from 2 independent experiments for all other conditions. Welch ANOVA test with Dunnett's T3 multiple comparisons test compared to NHS condition. * p <0.05, ** p <.01, *** p <.001, **** p <0.0001).

bacterial aggregate (Mankovich et al., 2013 and Figure 4A). Under the conditions tested, specifically a high neutrophil to bacteria ratio in each well, this occurred quite frequently as neutrophils drifted through a FOV (see track densities in Figure 2B, 2E, 2H). However, in the absence of complement and the chemotactic gradient created by C5a production near the microbe surface there was delay in aggregate discovery (Figure 4B) and discovery in HIS appeared to be more dependent on chance physical contact. Importantly, as we previously observed, there is a significant decrease in killing of bacteria in HIS compared to NHS (Figure 4C). In experiments with NIF treatment, we observed a slight decrease in the number of aggregates that were discovered (Figure 4A) and a delay in aggregate discovery (Figure 4B). Aggregates that remained undiscovered grew prolifically (Figure 4C). Results for the HIS + Fg condition were quite similar to HIS alone suggesting little benefit when Fg is not effectively adsorbed to the surface (Figure 4). Interestingly, while motility was somewhat restored when Fg

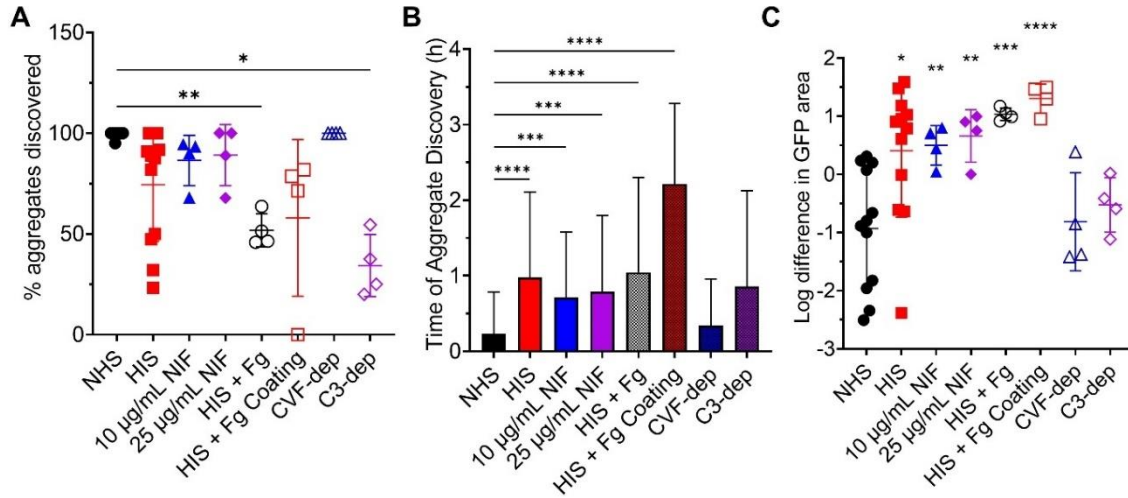


Figure 4: Interference with motility delays bacterial aggregate discovery and increases persistence. (A) Percentage of aggregates in a FOV that are discovered and phagocytosed by a neutrophil. Each point represents one FOV. Error bars indicate mean $\pm$ SD (N = 12 FOVs from 6 independent experiments for NHS and HIS conditions. N = 4 FOVs from 2 independent experiments for all other conditions. Welch ANOVA test with Dunnett's T3 multiple comparisons test compared to NHS condition. *p<0.05, **p<0.01). (B) Elapsed time after imaging start when bacterial aggregates are phagocytosed by a neutrophil. Error bars indicate mean $\pm$ SD (N = 140 and 183 events from 12 FOVs from 6 independent experiments for NHS and HIS conditions, respectively. N = 12 – 84 events from 4 FOVs from 2 independent experiments for all other conditions. ANOVA test with Dunnett's multiple comparisons test compared to NHS condition. ***p<.001, ****p<0.0001). (C) Log difference in GFP area within a FOV over 4 h of imaging. Each point represents one FOV. Error bars indicate mean $\pm$ SD (N = 12 FOVs from 6 independent experiments for NHS and HIS conditions. N = 4 FOVs from 2 independent experiments for all other conditions. Welch ANOVA test with Dunnett's T3 multiple comparisons test compared to NHS condition. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001).

was coated on the surface, we still observed slower and a decreased overall rate of aggregate discovery (Figure 4A, 4B). Overall, we observed significant bacterial persistence under this condition (Figure 4C), possibly due to the lack of complement to drive chemotaxis and effective discovery of aggregates. Interestingly, we observed that neutrophils “swarmed” towards a bacterial aggregate after it had been phagocytosed by a discovering aggregate, suggesting production of a neutrophil derived chemoattractant (Video SA2.3). Finally, in C3-depleted serum motility was not impaired and we subsequently observed effective discovery and killing of bacteria. Conversely, significant defects in discovery were observed in C3-depleted serum

(Figure 4). Importantly this experiment cannot accurately assess the impact on bacterial killing in C3-depleted serum as *S. aureus* grown in HBSS + 10% depleted serum demonstrates a significant growth defect, as described above in Chapter 3. Together, these results strongly implicate a role for MAC-1 and C3 in the successful discovery and clearance of bacteria and suggest that Fg alone is likely insufficient for effective clearance.

Discussion

The data presented in this report demonstrate a key role for surface adsorbed heat-labile serum proteins in the effective attachment to and surveillance of a biomaterial surface by human neutrophils. Neutrophils readily adhere to and chemotax on a glass surface that has been conditioned in NHS, enabling them to effectively discover and kill adherent *S. aureus*. Heat inactivation of serum greatly inhibits the ability of neutrophils to adhere to the surface and significantly impairs the timely discovery and killing of bacteria, resulting in persistence. Together, these data highlight both the importance of serum proteins adsorbed onto the biomaterial surface and neutrophil surveillance for successful host defense of a foreign body.

We found evidence to suggest that effective motility and subsequent discovery of bacteria is mediated via the MAC-1 integrin through direct inhibition with NIF or selective removal of the MAC-1 ligands Fg or iC3b. However, inhibition of MAC-1 via NIF still resulted in neutrophil adherence and slight motility that was not observed in HIS, suggesting either partial inhibition or a secondary receptor that can mediate adhesion, and possibly to some degree, motility. While CD11c is most commonly associated with dendritic cells, the CD11c/CD18 integrin has been described to facilitate neutrophil adhesion to Fg coated surfaces and can also bind to iC3b (Loike et al., 1991; Mazzone and Ricevuti, 1995). The potential role for this integrin is a matter for future investigation. The defective neutrophil motility observed in HIS supplemented with Fg suggests that other serum proteins, such as albumin, may prevent

effective Fg adsorption or block neutrophil adherence. Conversely, pre-coating the surface with Fg prior to HIS addition appears sufficient to restore neutrophil adherence and motility, although a significant defect in discovery remains, potentially due to the lack of complement components C3a and C5a to aid in neutrophil activation and chemotaxis. Interestingly, we observed qualitatively “normal” motility in CVF depleted serum. CVF binds to Factor Bb to form a highly stable C3 convertase with C5 convertase activity and specifically depletes C3 and C5 from serum (Von Zabern, 1993). This will lead to production of C3b and eventually, iC3b, which we hypothesize is serving as the ligand for MAC-1 and facilitating motility. In C3-depleted or heat inactivated serum, there is no C3b or iC3b present to bind, thus resulting in a failure to adhere to and move on the surface. These results will be further investigated and confirmed through the direct addition and adsorption of C3b or iC3b to C3-depleted serum.

The differences in discovery and clearance between CVF-depleted serum and the Fg-coated surface are slightly puzzling. While both conditions allowed for neutrophil motility, we would not expect complement mediated chemotaxis in either. It is possible that residual C3a and C5a in the CVF-depleted serum or differences in the numbers of neutrophils in the FOVs caused the observed difference and will be further scrutinized in future experiments. Importantly, much of the data presented in this report are preliminary and require refinement and additional replicates to confirm some of the initial observations. A lower number of neutrophils in each well may help to further discriminate differences in aggregate discovery between conditions and clarify the discrepancy between CVF-depleted serum and the Fg-coated surface. Additionally, blocking MAC-1 via NIF did not interfere with neutrophil adherence, a defect clearly observed in HIS, suggesting that the doses employed may not be fully blocking the receptor or there may be another receptor involved in attachment.

We additionally observed significant neutrophil swarming in our assay under complement deficient conditions, implicating a neutrophil derived chemotactic factor. We

previously have observed swarming in dermal and peritoneal implant infection models (Pettygrove et al., 2021), suggesting that it may play a significant role in the discovery and clearance of bacteria *in vivo*. The data from our peritoneal implant model also suggests that swarming may lead to preferential surveillance of specific areas of the implant surface, resulting in undiscovered bacteria on other areas of the biomaterial (Pettygrove et al., 2021, Figure 2). Previous studies have demonstrated roles for the chemoattractant LTB₄ and both LFA-1 (CD11a/CD18) and MAC-1 in *in vivo* swarming (Bogoslawski et al., 2018). Thus, we aim to further investigate the role and mechanism of neutrophil swarming both *in vitro* and *in vivo*.

While glass is not a canonical or even useful biomaterial, it does allow for effective time-lapse imaging and has produced a more robust understanding of interactions between neutrophils and surface-adherent bacteria. This data will be further supplemented by confirming these observations using more relevant biomaterials such as titanium and silicone via more traditional methods of viable plate counts and endpoint microscopy. Together, these results begin to inform the key molecular requirements for successful adherence to and surveillance of a biomaterial surface, leading to discovery and clearance of contaminating bacteria. Our findings suggest a novel strategy to reduce infection rates by engineering biomaterial coatings with selected host proteins to improve leukocyte attachment, motility, and clearance of bacteria, more effectively leveraging the host's potent immune response.

Methods

Bacteria and neutrophil preparation

Staphylococcus aureus strain AH1726 (LAC + pCM29, courtesy of Alex Horswill), with constitutive expression of green fluorescent protein (GFP), was grown overnight in tryptic soy broth (TSB) supplemented with 10 µg/ml chloramphenicol. Overnight cultures were centrifuged for 5 min at 4000 rpm, rinsed, resuspended in tryptic soy broth, and serially diluted and adjusted

by optical density. Cells were attached to a 4-chambered glass bottom Petri dish (Cellvis, CA, USA, Cat no. D35C4-20-1.5-N) to facilitate live-cell imaging. To attach cells, 10 μ L of diluted cell suspension was added to the surface. After 30 min of incubation at 37 °C, unattached bacteria were gently rinsed from the surface with PBS. Each chamber of the Petri dish was filled with 0.5 - 1 mL of 10% fresh human serum or modified serum in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} to condition the surface and opsonize bacteria. The dish was incubated at 37 °C for 30 min. Human neutrophils were isolated from heparinized venous blood obtained from healthy donors following a standard IRB-approved protocol. All donors provided written consent to participate in the study. Neutrophils were isolated under endotoxin-free conditions ($<25 \text{ pg ml}^{-1}$), and purity ($<1\%$ PBMC contamination) and viability ($<2\%$ propidium iodide positivity) of neutrophil preparations were assessed by flow cytometry as previously described (Voyich 2005, Voyich 2009). Neutrophils were kept on ice until stained with LysoBrite™ Red (AAT Bioquest, CA, USA, Cat no. 22645) according to the manufacturer's instructions.

Proteins and serum preparation

Heat inactivated serum was prepared by heating aliquots of fresh human serum to 56 °C for 20 – 30 minutes. Neutrophil inhibitory factor stock solution (R&D Systems, Minneapolis, MN, USA, Cat no. 5845-NF-050) was warmed to 37 °C and added to 10% NHS to a final concentration of 10 $\mu\text{g/mL}$ or 25 $\mu\text{g/mL}$ and mixed just prior to addition of serum to the dish. Human fibrinogen (MilliporeSigma, Burlington MA, USA, Cat no. 341576) stock solutions (42 mg/mL in sterile water) were thawed to 37 °C and added to warmed 10% HIS (4.2 mg/mL final concentration) or used to coat the dish surface directly. To coat the surface, 100 μL of fibrinogen stock solution was added to the glass surface. 50 – 100 μL of HBSS without Ca^{2+} or Mg^{2+} was gently mixed in to fully cover the surface and allowed to incubate at 37 °C for 1 hour. The

solution was then removed prior to attachment of bacteria. CVF depleted serum was produced by incubating 10 µg/mL CVF (Complement Technology, Tyler, TX, USA, Cat no. A150) with serum for 1 h at 37 °C. C3-depleted serum was obtained from Complement Technology (Cat no. A314).

Microscopy

A Leica SP5 inverted confocal laser scanning microscope was utilized for all *in vitro* imaging. GFP-tagged bacteria and neutrophils were excited with the 488 nm and 561 nm laser lines, respectively. An Okolab (Ambridge, PA, USA) Uno Stage Top Incubator with stand-alone humidity controller was utilized to maintain 5% CO₂, 20% O₂, 90% humidity, and 37 °C for sample incubation during imaging. 12-µm image stacks with 1-µm z-slices were recorded sequentially at 1–2 min intervals over a 4-h time course using a Leica 20x/0.7 NA dry objective lens. Two fields of view were imaged per chamber in each experiment.

Image analysis

MetaMorph version 7.8.13 (Molecular Devices, San Jose, CA, USA) image analysis software was used to measure the change in bacterial biomass by quantifying the threshold area of bacterial green fluorescence over the period of 4 h. Movies were prepared with Imaris version 9.8 (Oxford Instruments, Abingdon, UK). The Spots module in Imaris was utilized to track neutrophils over time, display their path, and quantify their motility. Neutrophils were automatically identified as spots based on size and manually edited and confirmed when needed. Motion was tracked using the autoregressive motion algorithm. Discovery of bacteria by neutrophils was determined by identifying the first frame in which neutrophils co-localized with a bacterial aggregate and remained co-localized in subsequent frames. Aggregates remaining in a field of view at the end of the imaging period that never co-localized with a neutrophil were considered undiscovered.

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APPENDIX C

EXPERIMENTAL DESIGNS TO STUDY THE AGGREGATION AND COLONIZATION OF
BIOFILMS BY VIDEO MICROSCOPY WITH STATISTICAL CONFIDENCE



Experimental Designs to Study the Aggregation and Colonization of Biofilms by Video Microscopy With Statistical Confidence

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The goal of this study was to quantify the variability of confocal laser scanning microscopy (CLSM) time-lapse images of early colonizing biofilms to aid in the design of future imaging experiments. To accomplish this a large imaging dataset consisting of 16 independent CLSM microscopy experiments was leveraged. These experiments were designed to study interactions between human neutrophils and single cells or aggregates of *Staphylococcus aureus* (*S. aureus*) during the initial stages of biofilm formation. Results suggest that in untreated control experiments, variability differed substantially between growth phases (i.e., lag or exponential). When studying the effect of an antimicrobial treatment (in this case, neutrophil challenge), regardless of the inoculation level or of growth phase, variability changed as a frown-shaped function of treatment efficacy (i.e., the reduction in biofilm surface coverage). These findings were used to predict the best experimental designs for future imaging studies of early biofilms by considering differing (i) numbers of independent experiments; (ii) numbers of fields of view (FOV) per experiment; and (iii) frame capture rates per hour. A spreadsheet capable of assessing any user-specified design is included that requires the expected mean log reduction and variance components from user-generated experimental results. The methodology outlined in this study can assist researchers in designing their CLSM studies of antimicrobial treatments with a high level of statistical confidence.

Keywords: microscopy, biofilm, image analysis, statistical confidence, repeatability, experimental design

INTRODUCTION

Biofilms consist of matrix embedded aggregates of microorganisms, are widespread in natural and engineered environments, and are physiologically distinct from free-living communities. Biofilm development begins with the loose association of microbes, most often on a surface, followed by a more robust cellular adhesion and aggregation (Hall-Stoodley et al., 2004). The process of biofilm development is an active area of research that has been advanced by modeling, imaging,

and “omic” based approaches (de Beer et al., 1994; Sauer et al., 2002; Rani et al., 2005; Connolly et al., 2015). One of the most important developments in the study of biofilm formation, structure, and dynamics has been the application of optical imaging, specifically confocal laser scanning microscopy (CLSM). CLSM has the ability to provide 3D information of hydrated intact biofilms non-invasively and in real-time (Stewart et al., 2009; Davison et al., 2010; Franklin et al., 2015). CLSM studies have provided valuable insight into biofilms including localization of gene expression (McLoon et al., 2011; Hung et al., 2013), analysis of extracellular material (Allesen-Holm et al., 2006; Baird et al., 2012; Gloag et al., 2013), community organization (Briley et al., 2014), flow patterns (Martínez-García et al., 2018), the diffusivity of solutes (Stewart et al., 2009), and the spatio-temporal patterns of biocide action (Bridier et al., 2011), making it a powerful tool for any biofilm researcher. However, when studying developing biofilms, the potential for increased variability within early stage biofilms in combination with phototoxicity increases the need for the development of methodological approaches that carefully consider all steps of an imaging experiment including experimental design, data collection, and the reporting of results (Lee and Kitaoka, 2018).

It is standard practice in biological research to establish the repeatability and reproducibility of a particular technique or method and this trend has extended into the biofilm field. There is increasing recognition of the importance of standardized methods in biofilm research to ensure reproducibility across labs (Malone et al., 2017). Efforts have been made to develop standard methods for several biofilm assays (Gomes et al., 2018) such as the drip-flow reactor (Goeres et al., 2009, 2020), single-tube method (Goeres et al., 2019), the CDC biofilm reactor (Goeres et al., 2005), and the 96 well MBEC assay (Parker et al., 2014). However, studies recommending standard practices for imaging-based biofilm experiments have not been similarly reported. A comprehensive review by Magana et al. (2018) found that biofilm methodology was under-represented as a percentage of all published papers in the field with only 4.9% of papers focusing on assay or method development. Conversely, for the field of genome editing, they found 23.4% of papers to be related to methodology. We have searched the biofilm literature for papers on the topic of biofilm imaging methods and observed that the majority of biofilm imaging methods papers are dedicated novel image analysis techniques and packages that assist researchers in quantifying biofilm characteristics such as volume, structure, and surface coverage (Heydorn et al., 2000; Yang et al., 2000; Daims et al., 2006; Mueller et al., 2006; Yerly et al., 2007; Milferstedt et al., 2009; Renslow et al., 2011; Almstrand et al., 2013; Sommerfeld Ross et al., 2014; Tolker-Nielsen and Sternberg, 2014; Larimer et al., 2016; Vyas et al., 2016; Baudin et al., 2017; Luo et al., 2018, 2019; Parker et al., 2018b, 2020). While these studies are essential to further the field of biofilm research by improving the quality and accuracy of data acquired through imaging, we are aware of few papers that include any recommendations for the design of biofilm imaging experiments to assure that data are representative, repeatable across experiments, and reproducible across labs (Korber et al., 1993; Lawrence and Neu, 1999; Heydorn et al., 2000; Daims and Wagner, 2011; Menzel et al., 2016). To answer questions

of interest from imaging data with a high level of statistical confidence, a quantitative assessment of variability in biofilm imaging data that specifies the requisite number of experiments and fields of view (FOV) is necessary but currently is lacking. To achieve statistical confidence, there is often an inclination to collect as many FOVs as possible. This approach has the potential to negatively impact data quality in several ways. Excessive imaging of a sample can result in photosensitivity of fluorophores and decrease data quality, although next generation imaging technologies such as light-sheet microscopy are beginning to mitigate these concerns somewhat (Power and Huisken, 2017; Qin et al., 2020). Increasing the number of FOVs being imaged can decrease the temporal resolution for time lapse microscopy, which can be a large detriment when studying rapid processes. Furthermore, advancements in imaging technology which have allowed researchers to generate massive amounts of data by imaging many FOVs over high temporal resolutions require a significant amount of time and computational resources to process and analyze collected images. Thus, identifying the correct balance between sample number and statistical confidence can make imaging-based experiments more efficient while maintaining high quality data and providing confidence in the downstream interpretation.

This paper provides guidance on designing experiments and interpreting resulting data from time-lapse visualization studies with an emphasis on early colonization biofilms. Recommendations are given for the number of experiments, FOVs and frames per hour that should be collected for imaging studies. Data were mined from 16 independent CLSM microscopy experiments that were designed to study interactions between human neutrophils (polymorphonuclear leukocytes or PMNs) and single cells or aggregates of *S. aureus* during the initial stages of biofilm formation. These data, including biological replicates (i.e., independent experiments) and varying inoculum concentrations, were then analyzed to identify the variability associated with the different levels of replication. We use the results to suggest future microscopy experimental designs. Based on the variability identified with each level of replication, results presented herein allowed us to recommend how often to take CLSM images (i.e., the temporal resolution of the sequence of images), how many FOVs to include, and how many replicate experiments to perform when studying initial attachment of biofilms using surface coverage as a quantitative response. While the presented analysis and results are specific to the data set described above, any pilot data can readily be analyzed using the approach that we present to inform experimental designs. We include our experimental design assessment tool as an Excel spreadsheet in the **Supplementary Materials**.

METHODS

Bacteria and PMN Preparation

Staphylococcus aureus strain AH2547 (HG001 + pCM29, courtesy of Alex Horswill), a known biofilm-forming strain (Pabst et al., 2016) with constitutive expression of green fluorescent protein (GFP), was grown overnight in tryptic soy broth supplemented with 10 µg/ml chloramphenicol for maintenance of the GFP carrying pCM29 plasmid. Overnight

cultures were centrifuged for 5 min at 4,000 rpm, rinsed, resuspended in phosphate buffered saline (PBS), and serially diluted. Cells were attached to a 4-chambered glass bottom petri dish (Cellvis, CA, USA) to facilitate live imaging. To attach bacteria, 10 μ L of diluted bacterial suspension were added to the surface. After 30 min of incubation at 37°C, unattached bacteria were gently rinsed from the surface with PBS. Each chamber of the petri dish was filled with 1 ml of 10% fresh human serum in Hank's Balanced Salt Solution (HBSS) with Ca^{2+} and Mg^{2+} and incubated at 37°C for 30 min. PMNs were isolated from multiple heparinized venous blood obtained from healthy donors following a standard IRB-approved protocol as described previously (Voyich et al., 2005, 2009). All donors provided written informed consent to participate in the study. PMNs were isolated under endotoxin-free conditions (<25 pg ml^{-1}) and purity (<1% PBMC contamination) and viability (<2% propidium iodide positivity) of PMN preparations were assessed by flow cytometry as previously described (Voyich et al., 2005, 2009). PMNs were kept on ice until stained with LysoBrite Red (AAT Bioquest, CA, USA) according to the manufacturers instructions. Stained PMNs were added to the appropriate wells and interactions between *S. aureus* and PMNs were imaged for 4 h. Some experiments utilized PMNs from the same donor, although PMNs from the same donor were always collected and prepared on different days.

For the antibiotic treatment assay, *S. aureus* was attached to the petri dish surface as described above, grown for 3 h in 10% human serum in HBSS, then challenged with 10 μ g/mL gentamicin and imaged for 4 h.

Microscopy

A Leica SP5 inverted confocal laser scanning microscope was utilized for all imaging. GFP-tagged bacteria and PMNs were excited with the 488 nm and 561 nm laser lines, respectively. A LiveCell (Pathology Devices, CA, USA) environmental chamber system was utilized to maintain 5% CO_2 , 20% O_2 , 50% humidity, and 37°C for sample incubation during imaging. Image stacks 12–20 μ m in size with 1- μ m z-slices were taken sequentially at 2–3 min intervals over a 4-h time course using a Leica 20x/0.7 NA dry objective lens. At least two fields of view from each chamber of the dish were generally imaged per experiment. FOVs with large numbers of bacterial objects were selected for imaging to visualize the highest number of *S. aureus*–PMN interactions.

Image Analysis

Images were analyzed as maximum projections of z-stacks at each time point. MetaMorph v 7.8.13 (Molecular Devices) image analysis software was used to measure change in bacteria biomass by quantifying the thresholded area of bacterial green fluorescence in each collected image. Log reductions (LR) in GFP area for bacteria treated with PMNs were calculated for each time point using the formula

$$LR = \log_{10}(\text{Control}) - \log_{10}(\text{Treated})$$

where the control area was an average area of the two fields of view from the control well for a given experiment and the treated area was of a single field of view of interest.

Repeatability Analysis

A linear mixed effects model (LMM) was fit to each hour of data [either $\log_{10}(\text{GFP})$ area of the controls or LRs] separately, with FOV nested in Experiment as random effects and time as a covariate. This LMM estimated the components of variance due to experiment (Var_{exp}), FOV (Var_{FOV}) and time (Var_{time}). To pool the time periods in each approximate phase of growth, an LMM was fit to data from each phase with fixed effects for $\log(\text{Inoculum})$, time period and the two-way interaction, and covariates for time as well as all 2 and 3 way interactions with the fixed effects. The serial correlation over time was modeled with an autoregressive process of order 1 [AR(1)] by fitting the LMM and then inflating the variance for the error term using the approach described in section 15.2 of Ramsey and Schafer (2013). This variance inflation factor is $c = \frac{1+r}{1-r}$ that depends on the first serial correlation coefficient $r = \frac{\sum_{t=1}^n \text{res}_t \times \text{res}_{t-1}}{\sum_{t=1}^n \text{res}_t^2 \times \text{res}_t^2}$ where res is the set of LMM residuals. Quadratic trend was fit to the variances as a function of the LR as described by Parker et al. (2018b). All LMMs were fit in R v4.0.1 package lme4 (Bates et al., 2015).

Experimental Designs

The main question of interest is how to use the repeatability results from the LMM to answer questions about microscopy experimental design. To answer this question, we focus on the precision of the mean response [either $\log_{10}(\text{GFP area})$ or LR] given by the standard error of the mean (SEM) and a one-sided 95% CI of the mean. The equation for the SEM from an experimental design with n_{exp} experiments, n_{FOV} FOVs per experiment and n_{time} per FOV is

$$SEM = \sqrt{\frac{\text{Var}_{\text{exp}}}{n_{\text{exp}}} + \frac{\text{Var}_{\text{FOV}}}{n_{\text{exp}} n_{\text{FOV}}} + \frac{\text{Var}_{\text{time}} \times c}{n_{\text{exp}} n_{\text{FOV}} n_{\text{time}}}} \quad (1)$$

Equation (1) is the standard formula for the SEM when the data are balanced and there is no serial correlation over time (i.e., when $r = 0$ and the variance inflation factor is $c = 1$). The variance inflation factor is $c > 1$ when the data over time are correlated in which case the SEM is inflated to account for that correlation. In other words, as the variance inflation factor c increases due to higher correlation over time, the SEM also increases through the third term in equation (1). We estimated the variance components Var_{exp} , Var_{FOV} , and Var_{time} in equation (1) from our data using the LMM described above. Our data can be used to estimate the SEM when the images are collected at a single time point (i.e., there is no video) by setting $n_{\text{time}} = 1$ in equation (1). If one wants to use their own data for single time point image data, then one would fit an LMM with a single random effect for experiment, retrieve variance components for Var_{exp} and Var_{FOV} , and plug in values for n_{exp} and n_{FOV} and set $\text{Var}_{\text{time}} = 0$ in equation (1).

For any growth phase given in Table 1, we can fill in the values in equation (1) to get the SEM for the mean $\log(\text{GFP area})$ for the untreated control biofilms. For example, for an experiment that is run to the early exponential phase (see row 2 in Table 1), the

TABLE 1 | Repeatability standard deviation for each phase of biofilm growth and the proportion (Prop.) of variance attributable to each of experiment, field of view, and time.

Phase	Time Period (h)	Prop. Exp	Prop. FOV	Prop. Time	Repeat. SD	First serial correlation	Inflation factor
Lag	[0, 2]	0.3207	0.6707	0.0086	0.4867	0.8313	10.9
Early	[2, 4]	0.5831	0.4083	0.0137	0.3186	0.8751	15.0
Late	[4, 8]	0.4540	0.5151	0.0309	0.2148	0.9129	22.0

The closer that the serial correlation r is to 1, the more dependent the data are over time. The variance inflation factor indicates how much the serial correlation over time increases the SEM via equation (1).

SEM is

$$SEM = \sqrt{\frac{0.583 \times 0.3186^2}{n_{exp}} + \frac{0.403 \times 0.3186^2}{n_{exp}n_{FOV}} + \frac{0.0137 \times 0.3186^2 \times 15}{n_{exp}n_{FOV}n_{time}}}$$

To fill out the previous formula:

- If an image is collected every 3 min, then 20 images are collected in each FOV over an hour-long experiment. So the number of images inputted into the formula is $n_{time} = 20$.
- If 3 FOVs are collected per experiment, then $n_{FOV} = 3$.
- If there are 2 independent experiments then $n_{exp} = 2$.

Given this setup, then the SEM in this example is

$$SEM = \sqrt{\frac{0.583 \times 0.3186^2}{2} + \frac{0.403 \times 0.3186^2}{2 \times 3} + \frac{0.0137 \times 0.3186^2 \times 15}{2 \times 3 \times 20}} = 0.191.$$

In this example the correlation over time inflates the time component of the SEM by the variance inflation factor $c = 15$ (Table 1). For these control data collected from the early exponential phase the variance component due to time is small ($\leq 1.4\%$, see Table 1), as is commonly the case, so the SEM remains about the same size whether or not the correlation due to time is accounted for [i.e., whether, in equation (1), $c > 1$ as in Table 1 or $c = 1$].

The lower limit for an upper one-sided 95% confidence interval (CI) for either the mean control response or the mean LR is calculated by

$$Mean - t \times SEM.$$

The multiplier t is the 95th percentile from a t distribution with $n_{exp} - 1$ degrees of freedom. In our results, we consider $n_{exp} = 1, 2, 3$, and 6 and provide the margin of error, $MOE = t \times SEM$, for a one-sided 95% confidence interval for each of these scenarios coupled with specifications for $n_{time} = 10$ or 40, and $n_{FOV} = 1, 2$, and 6. When $n_{exp} = 1$, we use a t distribution with 0.5 degrees of freedom to give an approximate MOE for a 1-sided 95% CI for the mean. For all SEM and MOE calculations for the mean LR, the inflation factor $c = 12$ was used. This value is the maximum possible value observed for our LR data (see Supplementary Material).

The SEM in equation (1) is closely related to the repeatability SD that quantifies the variability of the data across time points, FOV and experiments, by

$$\text{Repeatability SD} = \sqrt{\text{Var}_{exp} + \text{Var}_{FOV} + \text{Var}_{time} \times c} \quad (2)$$

Equation (2) shows that when the data over time are correlated, then the repeatability is inflated to account for that correlation. In other words, as the variance inflation factor c increases due to higher correlation over time, the repeatability SD also increases through the third term in equation (2).

RESULTS

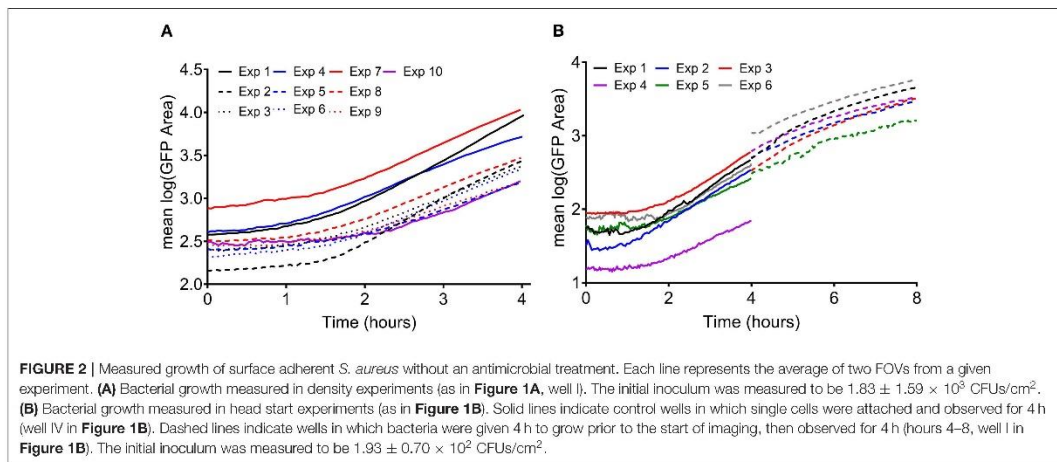
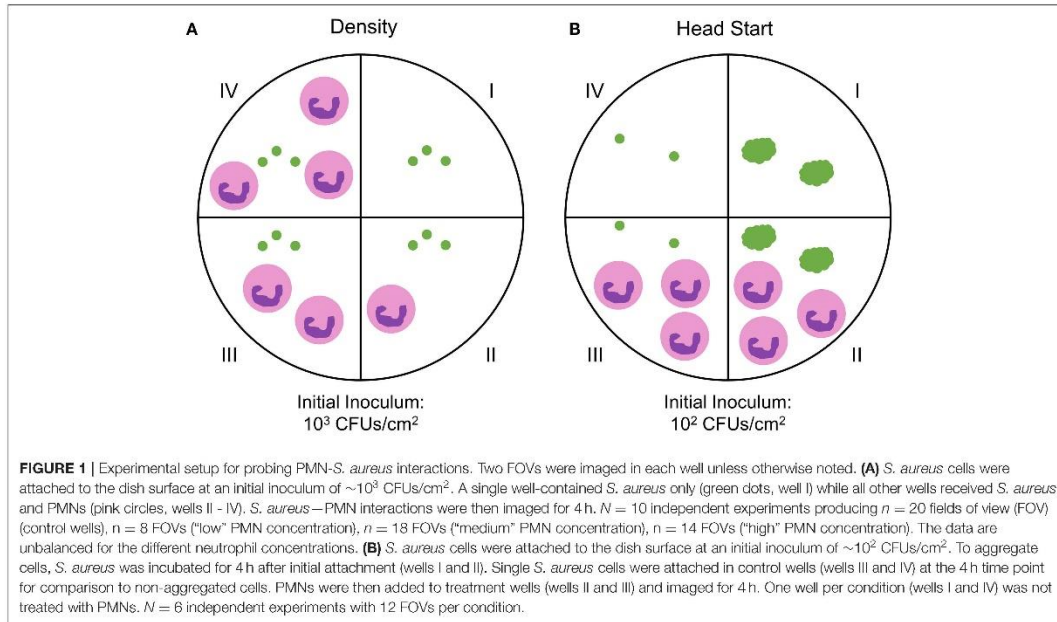
Experimental Setup and Data Acquisition

Previous and ongoing studies in our lab have utilized a time lapse imaging system to analyze interactions between human PMNs and surface adherent *S. aureus* (Ghimire et al., 2019; Pettygrove et al., 2021). Data were mined from two types of experiments that were performed to investigate the effects of PMN density and *S. aureus* aggregate size on the overall clearance of *S. aureus* from a glass surface. We will refer to these two experiment setups as “density” and “head start” experiments, respectively (Figure 1). Extensive time-lapse microscopy data of bacterial growth spanning an 8-h time frame and several corresponding treatment conditions were collected for each of the described experiments (Figures 2, 3). The overarching results and interpretation of these experiments will not be discussed at length here but are the subject of a separate manuscript (Pettygrove et al., 2021). For density experiments it was observed that a high concentration of PMNs was required to clear adherent *S. aureus* with the “medium” and “high” treatment conditions resulting in significant clearance of bacteria (Figures 3A–C). Head start experiments demonstrated that aggregated *S. aureus* becomes highly resilient to PMN clearance and bacteria will persist through PMN challenge (Figure 3D). The relative contributions of inter-experimental, inter-FOV, and inter-timepoint variances at distinct phases in the bacterial growth cycle (namely during lag phase, early exponential phase, and late exponential phase) were all calculated from the collected time lapse microscopy data.

Analysis of Untreated Biofilm

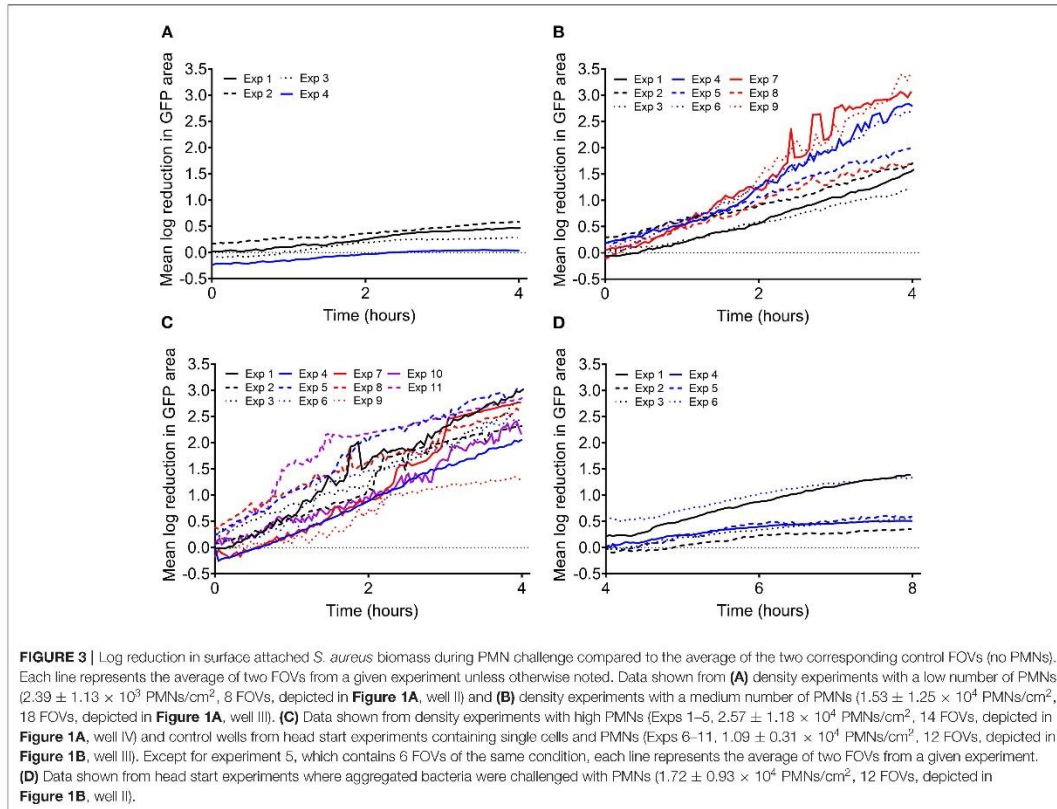
Variability of Early *S. aureus* Biofilm Colonization

Variability in observed GFP area, a surrogate for viable biomass (Schwartz et al., 2009), was analyzed over time for experiments at two inoculation concentrations for 4 h time periods (Figure 2).



Bacterial growth was consistent between experiments, containing a lag phase, early exponential phase, and in the case of the “head start” experiments (**Figure 2B**), a late exponential phase. The repeatability standard deviation (SD) was calculated (data from **Figure 2**) for each hourly period from 0 to 8 h (hours 4–8 from experiments with a head start only), broken out by the inoculation level (**Supplementary Table S1**). Although the biomass area increases dramatically over time as the biofilm

aggregates grow (**Figure 2**), the repeatability SDs and the proportions of variance were similar for the two inoculum levels within the lag or early exponential phase. The observed variance was also similar for each of the hourly periods within each phase, regardless of inoculation concentration (i.e., in **Figure 2**, the spread in the curves for the experiments is about the same over time). Thus, the data were pooled across inoculum levels and hours for each phase of growth. A single repeatability SD



and components of variance of the log(area) were calculated for each phase of growth (Table 1). Overall, variation decreased as cells progressed through exponential phase. Thus, seeding and culture conditions may affect variation more during the early periods of biofilm colonization. It is crucial to have side-by-side controls in all experiments that are highly repeatable, otherwise the log reductions can be affected (Stewart, 2015). The level of variability of the untreated controls from our experiments is acceptably small (a repeatability SD of untreated controls $< \frac{1}{2}$ log is acceptable) compared to other methods in the peer reviewed literature that have been standardized with AOAC International (Tilt and Hamilton, 1999) or ASTM International (Parker et al., 2014; Goeres et al., 2019, 2020; Allkja et al., 2021).

Design Recommendations

After calculating the variation in *S. aureus* biomass during different phases of growth (Table 1), the improvement in the standard error of the mean log₁₀(GFP Area) (SEM) was estimated under different experimental designs using the formula for SEM outlined in equation (1) in the methods. Given the SEM, we

then predicted the margin of error (MOE) for a 95% confidence interval for the mean log(GFP Area) of untreated biofilms under varied numbers of replicate experiments (1–6), FOVs (1, 2, 6) and time rates of acquisition (10, 40 images per hour) (Table 2). Larger SEM and MOE values indicate more imprecision when estimating biomass from an image. Unsurprisingly, results for early exponential phase data indicate that at least three experiments should be performed to reach a MOE below 1 log(GFP area). While one FOV may be acceptable, exceeding two FOVs per experiment yields only mild returns with regards to increasing precision, suggesting two FOVs may be optimal. Additionally, increasing the temporal resolution only negligibly increases precision, so utilizing fewer timepoints per hour for temporal studies may be advantageous to reduce fluorophore photosensitivity or phototoxicity, especially when exciting with ultraviolet light. Assessments of different experimental designs for lag and late exponential phase growth data produced similar conclusions (Table 2). Overall, the improvement in precision diminishes with increased numbers of experiments or FOVs in each experiment. However, there is a much greater decrease in MOE when more experiments are performed, even when

TABLE 2 | Expected margin of error for a one-sided 95% confidence interval for the mean $\log_{10}$ (GFP area) at different phases of growth and a variety of experimental parameters for untreated controls.

Lag	FOV	1	1	2	2	6	6
	Frames	10	40	10	40	10	40
Experiment number	1	20.0167	19.9466	16.2652	16.2221	13.1839	13.1661
	2	2.1765	2.1689	1.7686	1.7639	1.4335	1.4316
	3	0.8211	0.8182	0.6672	0.6654	0.5408	0.5401
	4	0.5723	0.5703	0.465	0.4638	0.3769	0.3764
	5	0.4639	0.4623	0.377	0.376	0.3056	0.3051
	6	0.4016	0.4002	0.3264	0.3255	0.2645	0.2642
Early exponential	FOV	1	1	2	2	6	6
	Frames	10	40	10	40	10	40
Experiment number	1	13.1355	13.0347	11.6722	11.6156	10.5849	10.5641
	2	1.4283	1.4173	1.2692	1.263	1.1509	1.1487
	3	0.5388	0.5347	0.4788	0.4765	0.4342	0.4333
	4	0.3755	0.3726	0.3337	0.3321	0.3026	0.302
	5	0.3044	0.3021	0.2705	0.2692	0.2453	0.2448
	6	0.2636	0.2615	0.2342	0.2331	0.2124	0.212
Late exponential	FOV	1	1	2	2	6	6
	Frames	10	40	10	40	10	40
Experiment number	1	8.9894	8.7664	7.6221	7.4911	6.554	6.5035
	2	0.9774	0.9532	0.8288	0.8145	0.7126	0.7071
	3	0.3687	0.3596	0.3126	0.3073	0.2688	0.2668
	4	0.257	0.2506	0.2179	0.2142	0.1874	0.1859
	5	0.2083	0.2032	0.1767	0.1736	0.1519	0.1507
	6	0.1804	0.1759	0.1529	0.1503	0.1315	0.1305

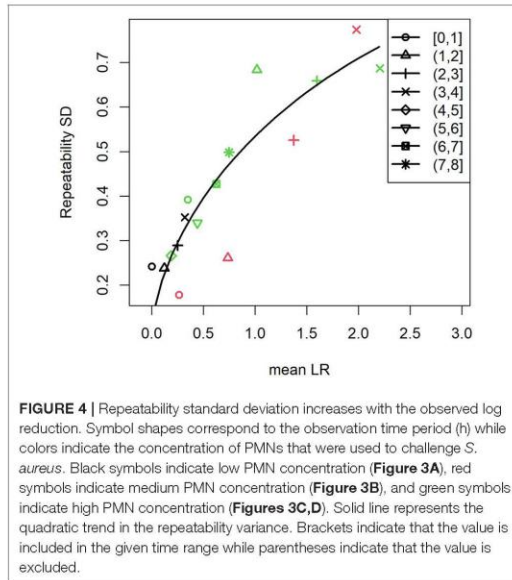
The table shows that when quantifying untreated biofilms with a mean $\log_{10}$ (GFP Area) during lag phase, at least 4 experiments with 2 FOVs each must be performed to get the error bars less than one half log. For early exponential phase, at least 3 experiments with 2 FOVs in each or 4 experiments must be performed while at least 3 experiments must be performed for late exponential phase.

fewer FOVs are imaged in each experiment compared to fewer experiments with a large number of FOVs in each. For example, when estimating the mean $\log_{10}$ (GFP area) of the untreated control biofilms during the early exponential phase at 95% confidence, consider a design utilizing two replicate experiments, two FOVs, and ten images per hour. Tripling the number of FOVs to six only yields a 9.3% reduction in the margin of error, while performing an additional experiment with two FOVs in each experiment results in a 62.3% reduction in the margin of error (Table 2). Conversely, performing six experiments with two FOVs and 10 images per hour reduces the margin of error by 81.5%, but requires a 3-fold increase in effort. Thus, our results suggest minimal benefit to utilizing a large number of FOVs with fewer experiments and instead recommend increasing the number of experiments performed when observing untreated biofilms during the early stages of colonization and development. However, the improvement in precision from performing more experiments must be weighed against the time and resource commitment required for those repetitions.

Analysis of Treated Biofilm

Variability of PMN Clearance of Nascent *S. aureus* Biofilm

By analyzing the results of experiments where surface attached *S. aureus* cells were challenged by human PMNs, we can make experimental design recommendations for studying the effect of an antimicrobial treatment on the viability of early biofilms. The two experimental setups (Figure 1) allowed us to analyze two different treatment scenarios. Experiments where the PMN density was varied (Figure 1A) can serve as an analog to changing the concentration of an antimicrobial treatment while the “head start” experiments (Figure 1B) can be modeled as application of a treatment at two different stages in biofilm development (in this case, lag and late exponential phase). It is important to note that discovery and subsequent killing of sparsely seeded *S. aureus* cells on a surface is a stochastic process that is highly dependent on the PMN concentration, unlike an antibiotic treatment that is comparatively homogeneously applied (Ghimire et al., 2019). We calculated LRs in GFP area within each treatment FOV compared to the average of the two corresponding control FOVs at each



time point. For PMN challenges against single cell *S. aureus*, as PMN density was increased, we observed higher LRs in GFP area, but increasing variability as the magnitude of the LR increased (Figures 3A–C). We similarly observed higher variability in LR in the efficacious “high PMN treatment” against single cells as compared to the relatively ineffective “high treatment” against aggregated cells (Figure 3D). Repeatability SD did not change markedly over time, but did increase with the observed LR, regardless of the experimental setup (Density vs. Head Start) (Figure 4). Intuitively, a treatment that is poorly efficacious (Figures 3A,D) will consistently result in small reductions and be more repeatable (Parker et al., 2018a). On the other hand, for a treatment that is highly efficacious and consistently kills all attached cells, we would expect a high level of repeatability as well (such a highly efficacious treatment was not tested in our experiments). Here, we observe that treatments of middling efficacy are highly variable. Thus, experiments where a treatment is expected to be highly efficacious or very ineffective will require fewer repetitions while moderately effective treatments require more. Our statistical model for the LRs explicitly accounts for differences among experiments. Because multiple donors contributed PMNs, the repeatability variance in Figure 4 may be larger than what would be expected if all PMNs were collected from the same donor. Nonetheless, the repeatability SDs for LRs that were observed in our system were <1 log and hence acceptable according to ASTM International and AOAC international (Parker et al., 2018a).

Design Recommendations

In our experiments measuring killing of attached *S. aureus* cells by human PMNs, we observed that the variance changes as a function of the LR regardless of inoculation level and time

(Figure 4 and Supplementary Table S2). As with the data from control wells, we used these results to evaluate how frequently to image, how many experiments to conduct, and how many FOVs to utilize per experiment in order to generate estimates of the mean LR with statistical confidence. The first step was to calculate the SEM for the mean LR using equation (1) from the estimated variance components for experiment, FOV and time (Supplementary Figure S1). Using these values, the expected MOE was assessed for experimental designs for treatments that have a mean LR = 0.5, 1, or 2 over a wide range of experiment numbers, FOV numbers, and temporal resolutions (Table 3). Because the variance increases as the mean LR increases (Figure 4), the SEM increases as the LR increases. Nevertheless, this does not translate into more experimental effort to precisely estimate the mean LR for moderately efficacious treatments (with mean LR = 2) compared to less efficacious treatments (with mean LRs ≤ 1) because the magnitude of the LR is less for less efficacious treatments. For an expected LR = 0.5, at least 4 experiments are required to detect a significant kill, while for LR = 1 or 2, only 3 experiments are necessary (Table 3). While the minimum number of experiments required to detect a significant kill does not change based on the number of FOVs (except when using 6 FOVs when LR = 0.5), modest improvements in the MOE are expected when at least 2 FOVs are utilized and may provide considerable benefit to the researcher. Providing an expected LR is a standard input for sample size calculations. The only difference in our approach from the standard approach is that in addition to using the expected LR as the margin of error input into the sample size calculator, we also use the expected LR to look up the variability of the LR (via Figure 4). Using the standard sample size calculator approach, the variability would be assumed to be the same for all LR values, which clearly is not the case for these efficacy data (see Figure 4 and the review in Parker et al., 2018a).

Validation of Design Recommendations

To determine how well Table 3 predicts the margin of error, we assessed a second dataset utilizing a 10 $\mu\text{g/mL}$ gentamicin treatment rather than PMN treatment. Similar to the previously described experiments, *S. aureus* was grown for 3 h, then treated with gentamicin for 4 h and imaged via confocal microscopy. We expected a LR of around 1 after 4 h of contact time and a repeatability SD of around 0.5 (Figure 4). Table 3 suggests that 3 independent experiments with 1 FOV each would be sufficient to detect a significant kill. The dataset that we analyzed contained 3 independent experiments with 3 control FOVs and 3 treated FOVs in each experiment, allowing us to assess the MOE for many possible combinations of 3 experiments with 1 control FOV and 1 treated FOV (27 possible control FOV combinations and 27 possible treatment FOV combinations yielding 729 possible LRs). We simulated 1,000 studies with this design via bootstrap sampling and calculated the mean LR, MOE, and lower 95% confidence limit for each experimental design (Supplementary Figure S2). Overall, the gentamicin treated data was less variable (mean LR = 0.80, repeatability SD = 0.12) than the neutrophil treated data (repeatability close to 0.5 according to Figure 4), resulting in MOEs much smaller than the predicted value of 0.91 (Table 3).

TABLE 3 | Expected margin of error (MOE) for a 1-sided 95% confidence interval for the mean log reduction in GFP area at a variety of experimental parameters and when LR = 0.5 (i.e., when percent reduction is 68%), when LR = 1 (i.e., when percent reduction is 90%), and when LR = 2 (i.e., when percent reduction is 99%).

LR = 0.5		FOV	1	1	2	2	6	6
		Frames	10	40	10	40	10	40
Experiment number	1		16.2871	16.2155	13.872	13.83	11.9948	11.9786
	2		1.7681	1.7604	1.506	1.5014	1.3022	1.3004
	3		0.6681	0.6651	0.569	0.5673	0.492	0.4913
	4		0.4656	0.4636	0.3966	0.3954	0.3429	0.3425
	5		0.3775	0.3758	0.3215	0.3205	0.278	0.2776
	6		0.3268	0.3254	0.2783	0.2775	0.2407	0.2403
LR = 1		FOV	1	1	2	2	6	6
		Frames	10	40	10	40	10	40
Experiment number	1		22.1521	22.1521	18.9751	18.9751	16.5211	16.5211
	2		2.4048	2.4048	2.06	2.06	1.7935	1.7935
	3		0.9086	0.9086	0.7783	0.7783	0.6777	0.6777
	4		0.6333	0.6333	0.5425	0.5425	0.4723	0.4723
	5		0.5134	0.5134	0.4398	0.4398	0.3829	0.3829
	6		0.4445	0.4445	0.3807	0.3807	0.3315	0.3315
LR = 2		FOV	1	1	2	2	6	6
		Frames	10	40	10	40	10	40
Experiment number	1		29.3802	28.4735	24.0585	23.5071	19.7287	19.5059
	2		3.1895	3.0911	2.6118	2.5519	2.1418	2.1176
	3		1.2051	1.1679	0.9868	0.9642	0.8092	0.8001
	4		0.8399	0.814	0.6878	0.672	0.564	0.5577
	5		0.6809	0.6599	0.5576	0.5448	0.4572	0.4521
	6		0.5895	0.5713	0.4827	0.4717	0.3959	0.3914

The MOE was calculated from equation (1) using the variance components from **Supplementary Figure 1** for each LR value. The table shows that when studying a treatment with a mean LR = 0.5, at least 3 experiments, each with 6 fields of view; or 4 experiments with any number of fields of view must be performed to be able to detect a statistically significant kill. When studying a treatment with a mean LR = 1 or LR = 2, at least 3 experiments must be performed to be able to detect a statistically significant kill. Because the variance due to time is 0 for LR = 1 (**Supplementary Figure 1**), there is no effect on the margin of error due to differences in the number of images acquired.

In 100% of the simulations, the design produced a statistically significant kill (**Supplementary Figure S2C**). This indicates that the recommendation of 3 experiments and 1 FOV, while conservative, was sufficient for detecting the mean LR with 95% confidence using this experimental setup (gentamicin treatment rather than PMN treatment).

DISCUSSION

Real-time CLSM non-invasively provides quantitative insight into the dynamics of living biofilms (Stewart et al., 2009; Davison et al., 2010; McLoon et al., 2011; Hung et al., 2013; Franklin et al., 2015; Ghimire et al., 2019; Pettygrove et al., 2021). Continued exploration in this area by our lab has yielded an abundance of time-lapse microscopy data that can inform the proper design of microscopy studies (Ghimire et al., 2019; Pettygrove et al., 2021). The data included are particularly suitable for assisting with the experimental design of studies investigating initial bacterial attachment, early biofilm formation, and biocidal treatments of bacteria in this stage. However, the development of criteria for

selecting the appropriate number of FOV that are required to produce representative quantitative results have been overlooked for this type of data collection. In this paper, we aimed to retrospectively utilize time-lapse data to provide quantitative guidance on the aspects of experimental design such as the numbers of experiments, fields of view, and images over time to provide an acceptable level of precision when estimating biomass on a surface.

In the presented data, variance for untreated controls representative of early biofilm formation (i.e., early colonization), depended on the growth phase of interest (lag or exponential in our data). The repeatability SD tended to decrease as cells entered exponential phase (**Table 1**). Thus, when designing an imaging experiment to study biofilm colonization, the user should primarily consider which phase of growth will be monitored and adapt the number of experiments, FOVs, and time points to include in the experimental design accordingly. The increased repeatability SD observed during the lag phase compared to early and late exponential is likely in part due to the high amounts of heterogeneity in the initial observed growth. *S. aureus* cells were

seeded onto the surface in PBS, rather than a nutrient rich media, resulting in differential recovery from lag phase for individual colonies, leading to high intra-FOV variation during lag phase as attached cells began to readjust to the media conditions. Thus, initial seeding conditions may affect variability as well and must be assessed for each system. For experiments where the measured output is a reduction in viable bacteria (GFP area in these experiments), the expected LR is required to estimate the appropriate number of experiments, FOVs, and time points. It is to be expected that the variability will be markedly different for different laboratory systems (Parker et al., 2018a; Stewart and Parker, 2019). Nonetheless, here we present a methodology for others to follow to estimate the variance in their systems and allow experimental design assessments.

As it pertains to repeatability, experimental bias is a significant obstacle for imaging studies. Sources of bias in imaging experiments can include the selection of representative regions of interest, image collection parameters, and the number of visualized FOVs (Lee and Kitaoka, 2018). Relatively arbitrary recommendations have been made for approaches to minimize bias such as visualizing a greater number of FOV, increasing the size of the visualized area, and imaging multi-well plates (Patel and McGhee, 2013; Lee and Kitaoka, 2018). There is limited suitability of some of these recommendations as it pertains to real-time biofilm imaging due to phototoxicity and the need for rapid image acquisition. As is common, the fields of view that we analyze here were not randomly chosen since the bacterial attachment was not homogenous across the surface. Statistical predictions and confidence intervals from hypothetical experimental designs, as we do here, presume that the data collected are representative of some population of all possible fields of view that could have been selected. Usually, representative data are assured by random sampling, in this case randomly selecting fields of view, wherein the population of interest is all possible fields of view. True random sampling is not typically performed in imaging studies as many fields of view are empty, especially during attachment or early biofilm formation. For the data collected here, the population of interest was all fields of view that contained some population of PMNs and biofilm aggregates, as determined by the microscopist. Fields of view were selected to maximize the number of PMN-*S. aureus* interactions that could be imaged, thus most fields of view were selected because of a high number of *S. aureus* aggregates present. While FOV selection is subjective and often dependent on the microscopist, our results are a starting point for estimating the sources contributing to the variance in observed biomass and designing microscopy experiments. There are methods, however, to reduce bias due to the subjective selection of fields of view. For example, in the ecological literature, there are adaptive sampling techniques that begin by randomly selecting locations for observation, i.e., randomly selecting a FOV (Thompson, 1990). When the ecologist (or microscopist) moves and encounters a new location (or FOV) of interest, the resulting statistics can be “adapted” to account for this deviation in sampling. Implementation of adaptive sampling that adjusts the image statistics is a matter for future research. Our lack of random sampling is a potential weakness of our work. We recommend

adaptive sampling of FOVs to minimize bias due to subjective FOV selection, but until the tools for CLSM have been developed, the next best approach to alleviate bias is to randomly select fields of view from the surface being imaged.

Our results, not surprisingly, point to the need to perform at least three experiments in most scenarios. For a given growth phase or expected LR, a substantial contribution to variance in biomass is due to differences between experiments (Table 1 and Supplementary Figure S1). Furthermore, our results indicate that a single FOV in each experiment may be sufficient, as the resulting reduction in variance from increasing FOVs is small. Most experimenters will want to collect at least two FOVs, but our results suggest that there is only minimal benefit to using more than two in most cases. Furthermore, as neutrophil challenge is a relatively heterogeneous antimicrobial treatment as compared to antibiotic therapy, it is plausible that the inter-FOV variance in our system is higher than might be observed in an antibiotic treated system. Lastly, our results demonstrate that there is not substantial increase to precision by increasing the temporal resolution of the acquired images in the video. In our variance predictions, little difference was seen between conditions utilizing 10 images per hour compared to 40 images per hour, suggesting an experimenter should utilize the temporal resolution that is best for their sample as the effect on their data quality will be minimal, barring phototoxicity. For visualizing rapid processes, high temporal resolution has an obvious benefit, however lower temporal resolution can both decrease phototoxicity and downstream image analysis burden. We observed little phototoxicity or fluorophore bleaching in our system despite a frequent image capture rate, however each experimenter should decide how rapidly to image based on the organism, fluorophores, and conditions necessary for their work. It is to be expected that a study of a different organism, even when using the same method, will produce results that have different levels and sources of variation than *S. aureus*. For example, a motile organism may enter and leave a FOV, while other organisms may have less predictable attachment mechanics. Similarly, different growth or treatment conditions would introduce different levels and sources of variability. These factors could lead to different inter-experimental, inter-FOV, or inter-timepoint variance compared to the values measured in our study. Therefore, we strongly recommend that researchers generate their own pilot data using their own methods and organisms and use the resulting variance component estimates to complete equation (1) via the included Excel tool to generate study designs. However, in the absence of available pilot data, which unfortunately is many times the case, the study designs (in Tables 2, 3) calculated from our data can serve as a starting point, with the understanding that there are likely differences between the system we present here and systems used by other researchers. Regardless of the data used, the analysis framework presented in this manuscript can help other researchers design statistically sound experiments based on a quantitative understanding of the contributors to variance in their system. Put another way, while the laboratory methodology may vary, the same statistical approach can be utilized to assess study designs.

The **Supplementary Materials** contains an Excel tool that allows the user to predict the number of experiments, FOVs, and images per hour (i.e., an experimental design) to estimate experimental parameters calculated from images of early biofilms at 95% confidence. The two parameters we focus on are the log transformed surface coverage [i.e., $\log_{10}(\text{GFP area})$] of untreated control biofilms or the LR measure of treatment efficacy. The tool requires estimates of the variance components due to experiments, FOVs and time for different experimental setups. For untreated control biofilms, the variance estimation will depend on the growth phase of interest. For treated biofilms, the variance estimation will depend on the expected LR.

As the field of quantitative imaging progresses there is a growing need for increased reporting on imaging acquisition parameters, processing, figure generation, and statistical analysis. The results reported here provide a statistical approach for deciding on appropriate replication to perform in imaging-based experiments during early biofilm colonization. Results from the presented approach also provide an efficient way for communicating methodology and statistical rigor from imaging experiments to the scientific community. While the variance observed will depend on one's specific experimental setup, these results do demonstrate the value of prioritizing experimental repetitions over high numbers of FOVs. The results (Tables 2, 3) also demonstrate the diminishing returns gained from increasing experimental repetitions and FOVs, which can help researchers decide how best to balance workload and the expected quality of data. The data mined for these analyses is derived from experiments examining sparsely seeded surfaces, thus future experiments should examine densely seeded surfaces and other measurable outputs such as volume rather than just area or surface coverage. In addition to early colonization studies there is a large component of biofilm research that is conducted on mature biofilms, hence future work should investigate the variability observed in imaging mature biofilms. Nonetheless, the statistical approach presented herein could be applied to biofilms at different life stages or different organisms and assays. We intend for this method to serve as a framework to be utilized with any pilot data examining biofilm growth to better inform the experimental design. Together, the results described in this paper can act as a roadmap to assist researchers in the design of microscopy-based studies of biofilm colonization to

ensure that acquired data is representative of the sample and statistically robust.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Montana State University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

BP designed and conducted experiments, performed data analysis, and drafted the manuscript. HS conceived the study, performed data analysis, drafted the manuscript, and supervised the study. KP performed blood draws and PMN isolations and revised the manuscript. JV and PS designed experiments, provided laboratory resources, revised the manuscript, supervised the study, and acquired funding. AP conceived the study, performed the statistical analysis, drafted the manuscript, and supervised the study. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmicb.2021.785182/full#supplementary-material>

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APPENDIX D

CO-AUTHORED PUBLICATIONS



Direct Microscopic Observation of Human Neutrophil-*Staphylococcus aureus* Interaction *In Vitro* Suggests a Potential Mechanism for Initiation of Biofilm Infection on an Implanted Medical Device

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ABSTRACT The ability of human neutrophils to clear newly attached *Staphylococcus aureus* bacteria from a serum-coated glass surface was examined *in vitro* using time-lapse confocal scanning laser microscopy. Quantitative image analysis was used to measure the temporal change in bacterial biomass, neutrophil motility, and fraction of the surface area policed by neutrophils. In control experiments in which the surface was inoculated with bacteria but no neutrophils were added, prolific bacterial growth was observed. Neutrophils were able to control bacterial growth but only consistently when the neutrophil/bacterium number ratio exceeded approximately 1. When preattached bacteria were given a head start and allowed to grow for 3 h prior to neutrophil addition, neutrophils were unable to maintain control of the nascent biofilm. In these head-start experiments, aggregates of bacterial biofilm with areas of 50 μm^2 or larger formed, and the growth of such aggregates continued even when multiple neutrophils attacked a cluster. These results suggest a model for the initiation of a biofilm infection in which a delay in neutrophil recruitment to an abiotic surface allows surface-attached bacteria time to grow and form aggregates that become protected from neutrophil clearance. Results from a computational model of the neutrophil-biofilm surface contest supported this conceptual model and highlighted the stochastic nature of the interaction. Additionally, we observed that both neutrophil motility and clearance of bacteria were impaired when oxygen tension was reduced to 0% or 2% O_2 .

KEYWORDS biofilm, neutrophil, device-related infection, immune evasion, biomaterial, joint infections

Implanted medical devices are vulnerable to localized infections (1, 2). These infections result when bacteria or yeast attach to the biomaterial surface and form a biofilm. In the biofilm state, microorganisms are protected from killing by disinfectants and antibiotics and they evade host defenses (3, 4). Consequently, device-related infections are recalcitrant to antimicrobial chemotherapy and are extremely difficult to resolve. Medical devices that are prone to foreign body infection include prosthetic joints, hernia meshes, orthopedic plates and screws, breast implants, artificial heart valves, catheters, and many others (1). As one specific example, consider knee and hip prostheses. In 2013, 27,000 revisions were performed for prosthetic joint infections, which are traumatic and expensive procedures (5). Because of the large number of

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patients who receive implanted materials in modern medicine, the collective burden to society, in terms of both economic cost and human suffering, is significant.

The innate immune system rapidly and effectively eliminates microorganisms from regions of the body where they are not normally resident; however, this process breaks down in the presence of a foreign body (6, 7). The reasons for this failure are not well understood, but this observation alone motivates exploration of approaches for restoring innate immune function in the presence of an implanted material. One mechanism of neutrophil compromise that was previously investigated is chronic activation of neutrophils that associate with a biomaterial (8–12). As the first responders to infection, neutrophils are of particular relevance and interest (13, 14).

We consider here six phenomena that may be important for successful clearance of a biomaterial adherent bacterial cell by a neutrophil. Poor clearance could result from defects at any one of these steps or from a combination of deficiencies at multiple steps. We emphasize that our focus is on the very early events after implantation, prior to the formation of mature biofilm structures that are known to be tolerant to antimicrobials and innate immunity (1–4). Here are six hypotheses for how a relatively small number of contaminating bacteria on an implanted medical device could evade clearance by neutrophils. (i) Recruitment of neutrophils to the implant surface is delayed, allowing attached bacteria a window of time in which to grow and form aggregates that are protected. (ii) Neutrophils are recruited to the biomaterial surface but do not move on or surveil the surface. (iii) Neutrophils are recruited to the implant surface and patrol it but fail to recognize attached bacteria even when encountered. (iv) Neutrophils are recruited to the surface and exhibit surveillance and discovery of attached bacteria but are unable to phagocytose adherent bacterial cells, even lone cells. (v) Neutrophils are recruited to the surface and exhibit motility, discovery, and phagocytosis of bacteria but fail to kill phagocytosed cells. (vi) Hypoxia near the biomaterial interface limits neutrophil motility and bactericidal efficacy.

Discriminating between these hypotheses likely requires direct microscopic observation of neutrophil-bacterium interactions at the single-cell level. To do this *in vivo* would require technically challenging, high-resolution intravital imaging. Such animal models for imaging individual neutrophil function on an abiotic surface have not yet been developed. We therefore turned to an *in vitro* system to perform direct microscopic video observation of human neutrophils interacting with surface-attached bacteria. This model gives access to hypotheses 2 to 6 described above but cannot measure the recruitment time of neutrophils to the implant surface (hypothesis 1). However, the *in vitro* model can be used to investigate the consequences of delayed recruitment by simply adding neutrophils at various time points after the bacterial attachment step.

In the research presented in this article, we have chosen to use *Staphylococcus aureus* as a model organism, as it is commonly implicated in prosthetic joint infections and has been the subject of extensive work on interaction with the innate immune system (14–17). Moreover, neutrophils are a key cell in eradicating *S. aureus*, as observed by the increase in susceptibility to *S. aureus* infections in individuals with neutrophil defects (18, 19). Much of the prior work investigating interaction of *S. aureus* biofilms with cells of the mammalian innate immune system has focused on mature biofilms (20–32). These studies collectively paint a picture of localized immune dysregulation, stasis, and sustained inflammation. They often implicate bacterial virulence factors and complex reciprocal signaling interactions between a bacterium and phagocyte. As we were particularly interested in the potential to prevent biofilm infections, we chose instead to focus on understanding the very early events, those that might occur in the first several hours following implantation of a contaminated biomaterial.

The goal of the work presented in this article was to characterize phenomena important in the interaction between human neutrophils and sparsely distributed bacteria adherent to an abiotic surface, simulating the situation on a medical device immediately following implantation. We used an *in vitro* system that allowed for independent experimental control of the densities of bacteria and neutrophils and also

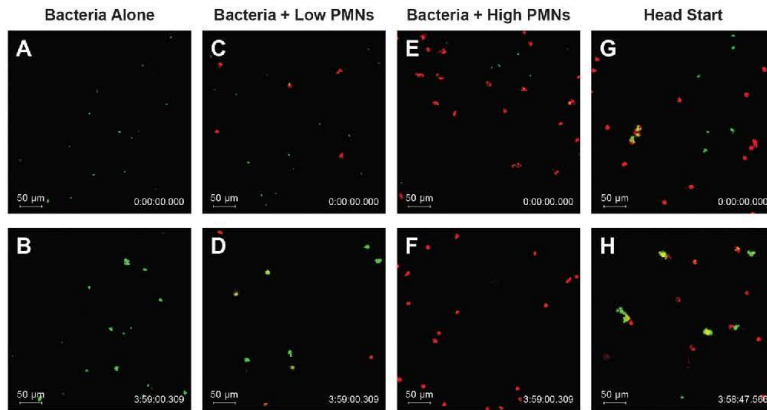


FIG 1 Neutrophil discovery and elimination of *S. aureus* attached to a serum-coated glass surface. Representative images from time 0 h (top row) and time 4 h (bottom row) from experiments with no neutrophils (A and B), low density of neutrophils (C and D), high density of neutrophils (E and F), and high density of neutrophils with preformed bacterial aggregate wherein bacteria were allowed to grow for 3 h prior to neutrophil addition (G and H) (see also Videos S1 and S4 to S6 in the supplemental material).

of the timing of neutrophil delivery. This microscopy-based system enabled direct observation of the dynamics of the two types of cells and extensive quantification of their behaviors by image analysis and suggested rate-limiting steps in the clearance of attached bacteria by these front-line leukocytes.

RESULTS

Neutrophils migrate on an abiotic surface and discover, phagocytose, and destroy single attached bacteria or very small bacterial aggregates. We imaged the interaction of newly attached green fluorescent protein (GFP)-expressing *S. aureus* with human neutrophils on a surrogate biomaterial surface *in vitro* using time-lapse confocal scanning laser microscopy. Neutrophils were stained red, enabling continuous quantitative monitoring of neutrophil motility and interactions with bacteria. *S. aureus* was attached to the glass surface as single cells or aggregates of a few cells (Fig. 1A, C, and E). Between 5×10^4 and 2×10^5 CFU were initially seeded onto the surface, resulting in attached bacterial cell densities after rinsing to remove nonadherent bacteria ranging from approximately 5×10^2 to 8×10^4 objects cm^{-2} ($1.55 \times 10^4 \pm 1.83 \times 10^4$ objects cm^{-2}). Following attachment, 20% human serum in Hanks' balanced salt solution (HBSS) was added to opsonize bacteria and condition the surface. After 30 min, HBSS was added to dilute the serum to 10%. In control experiments in which the surface was inoculated with bacteria but no neutrophils were added, the attached bacteria grew exponentially (Fig. 1A and B; see also Video S1 in the supplemental material). The measured maximum growth rate was $0.78 \pm 0.15 \text{ h}^{-1}$ ($n = 17$). Most of the bacteria remained sessile during the 4-h observation period. The green fluorescence from the plasmid-encoded GFP reliably correlated with bacterial growth, as we did not observe any outgrowth of non-GFP cells, even after 8 h in this system (see Videos S2 and S3). Additionally, after 8 h of growth, cells were scraped from the surface and plated on tryptic soy agar. All colonies remained fluorescent under UV light the following day, indicating plasmid retention.

When human neutrophils were added to the system, they moved in random walk fashion over the surface (Fig. 1C to F and Videos S4 and S5). As neutrophils encountered bacterial cells, they appeared to quickly phagocytose the bacteria. The physical association of bacteria with the neutrophil was evident in that the previously stationary bacteria began to move with the leukocyte, which usually continued to migrate. When the neutrophil surface density was relatively low, not all the bacterial cells seeded on

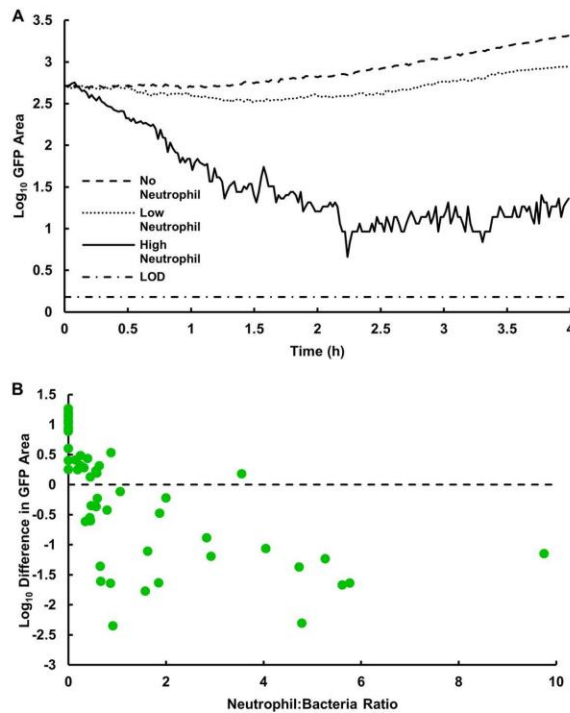


FIG 2 *S. aureus* bacterial dynamics during interaction with human neutrophils. (A) Representative experiments corresponding to bacterial growth in the absence of neutrophils, effective control of bacteria with a relatively high density of neutrophils, and an intermediate outcome at a lower density of neutrophils. Bottom line indicates the limit of detection (LOD), 1 pixel. (B) Net change in *S. aureus* bacterial area after a 4-h interaction with human neutrophils. Higher initial neutrophil numbers resulted in a net decrease in attached bacteria; $n = 55$ fields of view (17 bacteria only, 38 bacteria plus neutrophils) from 10 independent experiments.

the surface were discovered by a neutrophil in the 4-h observation window, and undiscovered bacteria continued to grow and formed small aggregates. When more neutrophils were present, they discovered most or all the bacteria on the surface. After phagocytosis of a bacterial aggregate, the GFP signal decayed, suggesting loss of viability of the bacterial cell (33).

To confirm observations in Fig. 1 and Videos S4 and S5, image analysis was used to quantify the total area of green fluorescence, as a measure of viable bacterial biomass, in each field of view over time (Fig. 2). An increase in green fluorescence implied the net growth of bacteria, whereas a decrease in green fluorescence with time implied that neutrophil clearance of *S. aureus* was more rapid than microbial growth (Fig. 2A). To capture the overall outcome from a particular field of view, we calculated the $\log_{10}$ difference in GFP area between the time that neutrophils were added and the conclusion of the observation period 4 h later. To further probe the dynamics between the *S. aureus*-neutrophil interactions, we varied both the concentration of neutrophils added to the wells and the amount of bacteria initially attached to the surface (Fig. 2B). We observed increases in bacterial biomass of up to a $\log_{10}$ difference of 1.26 (in a control experiment with no neutrophils added) and reductions up to -2.35 (an experiment with neutrophils in which the GFP area was reduced to its limit of detection). The magnitude of these decreases in bacterial biomass is comparable to the results of neutrophil challenges to planktonic bacteria at similar neutrophil/bacterium ratios (34,

35). The dependence of bacterial biomass on the neutrophil/bacterium ratio plotted in Fig. 2B was statistically significant ($P < 0.0001$). Intuitively, better control of the nascent biofilm was observed when neutrophil densities were higher and bacterial densities were lower, as there were sufficient neutrophils to discover and phagocytose each bacterial aggregate. In this set of experiments, the multiplicity of infection (number of bacterial objects at the beginning of an experiment divided by the average number of neutrophils in the same field of view over the course of the experiment) ranged from 0.10 to 7, with a mean value of 1.58 ± 1.51 .

The LysoBrite stain used to fluorescently label neutrophils did not adversely affect their ability to clear *S. aureus*. We challenged *S. aureus* with neutrophils that were either unstained or stained with LysoBrite and found that there were no significant differences in viable bacteria recovered from the surface ($P = 0.25$), total GFP area remaining on the surface ($P = 0.89$), or log differences in GFP area from a given field of view ($P = 0.61$) (see Fig. S1A to C).

Neutrophil movements over the abiotic surface are similar in the presence and absence of bacteria. Neutrophils moved over the serum-coated surface similarly in the absence and presence of bacteria. The mean track length of a neutrophil during the 4-h observation period was $1,485 \pm 514 \mu\text{m}$ in the presence of bacteria and $1,249 \pm 307 \mu\text{m}$ in the absence of bacteria ($P = 0.1368$) (see Fig. S2). A neutrophil random motility coefficient was calculated to be $2.67 \pm 0.75 \times 10^{-8} \text{ cm}^2 \text{ s}^{-1}$ by plotting the mean square displacement versus time during a 40-min window of linearity in the first hour of an experiment (see Table S1). Neutrophils gradually slowed during the 4-h experiment, and some stopped moving altogether (see Fig. S3). We hypothesized this was due to the depletion of a constituent of the medium or the accumulation of an inhibitive factor. To test for these effects, we conducted a set of experiments in which the medium was replenished halfway through an experiment (at $t = 2 \text{ h}$) and the speed in the second 2-h interval was compared (Fig. S3). Linear regression was used to compare the speeds of neutrophils in control and replacement wells both before and after medium replacement. Prior to replacement, both the slopes and intercepts of the two groups were similar ($P = 0.1587$ and $P = 0.7998$, respectively). Following replacement of the medium, the slopes of the two curves were significantly different ($P < 0.0001$). Although there was a slight increase in the speed of polymorphonuclear leukocytes (PMNs) in wells where the medium was replaced, PMN speed was not restored to levels observed during the earliest time points of the experiment ($n = 8$ fields of view, 2 independent experiments), suggesting that depletion of a nutrient in the medium is not the sole driver of the observed decrease in PMN speed. The decrease in speed over time is likely in part due to the short life span of neutrophils *ex vivo* (36).

A measure of the directionality of neutrophil movement was calculated by dividing neutrophil displacement (distance between the starting location and its location 4 h later) by the total track length of the leukocyte. This measure is bounded from a minimum of zero (the neutrophil starts and ends at the same spot) to a maximum of one (the neutrophil moves in a straight line with no change in direction). The directionality coefficient was 0.26 ± 0.10 and was not significantly different between experiments with and without bacteria ($P = 0.4453$) (see Fig. S4). To further test for a possible role of chemotaxis in the discovery of attached bacteria, the fraction of bacteria discovered by and subsequently associated with a neutrophil was plotted as a function of the fraction of the field of view that had been patrolled by neutrophils (see Fig. S5). If the discovery of bacteria was purely random, one would anticipate that this relationship would be a straight line: when neutrophils had patrolled 20% of the surface they would encounter and associate with 20% of the bacteria on the surface. In experiments in which neutrophils covered 20% of the surface, they discovered and became associated with approximately 60% of the bacteria, suggesting a chemotactic component of the discovery of surface-attached bacteria. In some cases, we observed PMNs chemotaxing toward larger *S. aureus* aggregates (see Video S6). This suggests that chemotaxis may be occurring only when aggregates are sufficiently large or when neutrophils are

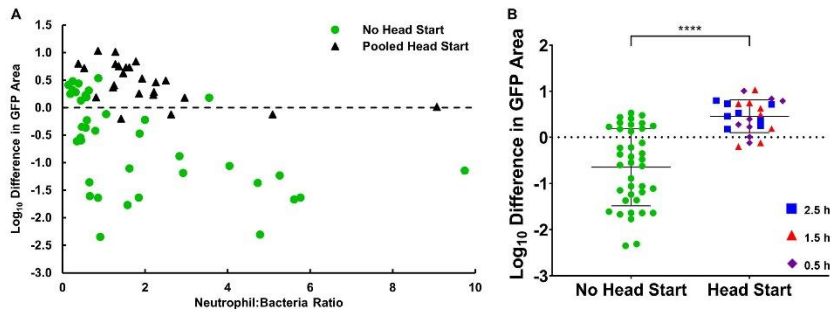


FIG 3 Larger *S. aureus* aggregates are recalcitrant to clearance by human neutrophils. (A) Net change in *S. aureus* bacterial area after a 4-h interaction with human neutrophils comparing outcomes from addition of neutrophils immediately following bacterial attachment (no head start) and after allowing bacteria to grow for 1, 2, or 3 h and form small aggregates prior to neutrophil addition (pooled head start); $n = 38$ fields of view without head starts from 9 independent experiments, $n = 24$ fields of view with head starts from 4 independent experiments ($n = 8$ fields of view per head start time). (B) Clearance of bacteria without a head start compared to pooled head starts of 1, 2, or 3 h (all time points included). Error bars represent standard deviations of the samples. ****, $P < 0.0001$ by an unpaired t test.

already quite close to the aggregate, thus not significantly changing the overall directionality of the neutrophil.

Delaying neutrophil recruitment to the surface allows bacterial aggregates to form that are recalcitrant to clearance. We noticed that bacteria that were not found by neutrophils early grew into discrete aggregates and that when these aggregates were eventually discovered by neutrophils, they appeared to be more difficult for neutrophils to destroy. To explore this effect further, “head-start” experiments were conducted, in which bacteria were allowed to grow for 1 to 3 h prior to neutrophil addition (Fig. 1G and H and Video S6). The average aggregate size within a field of view at the start of imaging was $6.54 \pm 1.11 \mu\text{m}^2$, $11.36 \pm 2.97 \mu\text{m}^2$, and $17.65 \pm 4.66 \mu\text{m}^2$ after 1-, 2-, and 3-h head starts, respectively (see Fig. S6A). However, larger than average aggregates formed, and the maximum aggregate size in a given field of view was $12.08 \pm 3.77 \mu\text{m}^2$, $31.05 \pm 12.81 \mu\text{m}^2$, and $71.88 \pm 23.59 \mu\text{m}^2$ after 1-, 2-, and 3-h head starts, respectively (Fig. S6B).

When neutrophils encountered bacterial aggregates that were similar in size or larger than the neutrophil itself, they often failed to achieve phagocytosis (Video S6). This outcome was evident in that the bacterial aggregate was not dislodged from the surface and did not move with neutrophil postencounter, as usually happened with single bacterial cells. In some cases, it was visibly apparent that the leukocyte failed to completely engulf the bacterial aggregate and the aggregate continued to grow. Occasionally, multiple neutrophils attacked the same aggregate, and in some instances, neutrophil engagement with the bacteria was strong enough to break the bacterial aggregate into smaller pieces. These observations suggest a range of outcomes, but in general, the control of bacteria by neutrophils was less effective when bacterial aggregates were preformed (Fig. 3A). The net change in bacterial area after neutrophil incubation for experiments in which neutrophil addition was delayed was a $\log_{10}$ difference of 0.46 ± 0.35 (a net increase in bacteria), whereas it was -0.65 ± 0.82 (a net decrease in bacteria) for experiments with no bacterial head start ($P < 0.0001$) (Fig. 3B). There was no significant difference between the neutrophil-to-bacterium ratio when comparing the head start and no head start data sets (data not shown, $P = 0.6487$). Thus, it appears that neutrophils must reach adherent *S. aureus* on a surface quickly, as even relatively small neutrophil-sized aggregates are recalcitrant to clearance by neutrophils.

Hypoxia reduces neutrophil efficacy against attached *S. aureus*. Inert biomaterial surfaces are not perfused by blood; therefore, oxygen availability may be reduced at the surface of an implant. Additionally, hypoxia is common in established biofilm

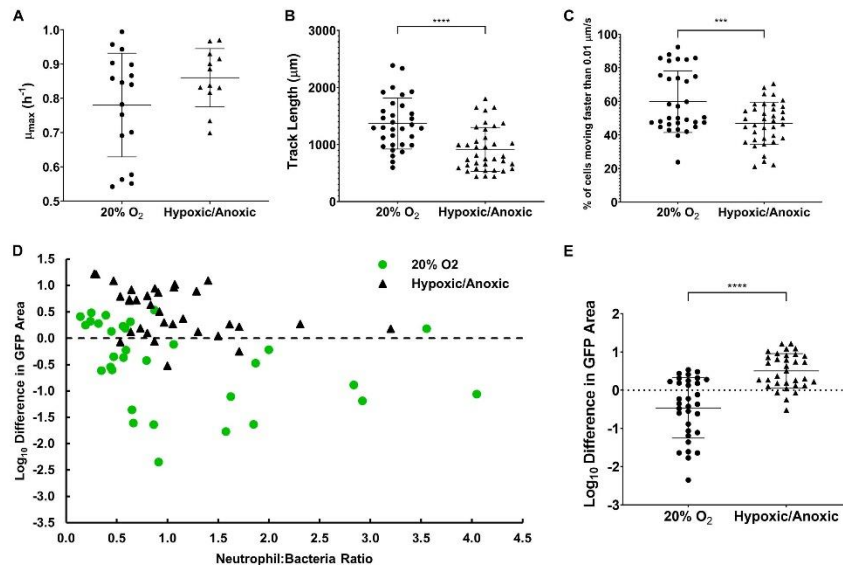


FIG 4 Oxygen is required for effective motility and clearance of adherent *S. aureus*. (A) Maximum growth rate of *S. aureus* was not different between 20% oxygen and pooled 0% or 2% oxygen conditions; $n = 17$ fields of view from 8 independent experiments and $n = 12$ fields of view from 6 independent experiments for normal oxygen and low oxygen, respectively. (B and C) Neutrophils traverse less distance and a higher fraction of neutrophils are stationary in the last ~5 min of an experiment in an environment with reduced oxygen tension; $n = 32$ from 5 independent experiments and $n = 37$ from 5 independent experiments for normal and low oxygen, respectively. (D and E) Net change in *S. aureus* bacterial area after 4 h under normal conditions ($n = 32$ from 8 independent experiments) or low-oxygen conditions ($n = 33$ from 3 independent experiments at 2% O_2 and 3 at 0% O_2). Error bars represent standard deviations of the samples. ***, $P < 0.001$; ****, $P < 0.0001$ by unpaired t tests.

infections and plausibly arises in the vicinity of a foreign body through the concerted consumption of oxygen by both leukocytes and bacteria in a diffusion-limited environment (37–41). We therefore measured the efficacy of human neutrophils in controlling attached *S. aureus* under conditions of reduced (2% O_2) or no oxygen. Reduced oxygen tension did not affect the bacterial growth rate ($P = 0.11$) (Fig. 4A). The specific growth rate of *S. aureus* under anaerobic conditions has been reported to be 44% to 45% of the value under aerobic conditions (42, 43). Our reduced-oxygen conditions were not as a whole fully anoxic. The average neutrophil track length was decreased under reduced oxygen tension ($P < 0.0001$) (Fig. 4B). While there was some variability, on average, it appeared that a higher proportion of PMNs were stationary at the end of the experiment in the low oxygen (0% and 2%) environment than in the oxic (20% oxygen) environment, suggesting that the neutrophils are unable to effectively patrol the surface without sufficient oxygen (Fig. 4C) ($P = 0.001$). Low oxygen tension diminished the antibiofilm efficacy of neutrophils (Fig. 4D and E). The difference between the oxic and grouped low oxygen conditions was statistically significant ($P < 0.0001$). These data suggest that low oxygen conditions impair neutrophil motility, reducing the chance of discovering adherent *S. aureus* on a surface. Additionally, low oxygen tension may interfere with the oxidative burst produced by neutrophils, preventing them from effectively clearing *S. aureus* that is discovered and phagocytosed (44).

Computational simulation of the neutrophil-bacterium surface interaction demonstrates variable outcomes governed by stochastic discovery of bacteria by neutrophils. We constructed a mathematical model of the interaction of bacteria and neutrophils on a two-dimensional surface. The model integrates bacterial growth, bacterial elaboration and Fickian diffusion of a chemoattractant molecule, neutrophil

movement by a combination of random motility and chemotaxis, and killing of bacteria upon discovery by a neutrophil. An illustrative example of a single simulation is provided in Fig. 5A, with the full video of this simulation available in the supplemental material (see Video S7). In such simulations, bacteria are stationary, while neutrophils move and may eventually discover the bacterial colonies. In the example in Fig. 5A, half of the bacterial colonies initially seeded in the simulation domain (12 total) were discovered and killed by a neutrophil, and the other half went undiscovered to the end of the 4-h simulation period. Every simulation is different, because the initial placements of both bacteria and neutrophils are random and also because the movement of each neutrophil is stochastic. This is illustrated with additional simulations of the case presented in Fig. 5A, in which 12 bacterial colonies and 8 neutrophils were initially placed in the simulation field (see Videos S8 and S9). We ran each case 156 times to illustrate the range of outcomes even when parameter values are fixed. Figure 5B shows 6 of the 156 outcomes for the case with 12 bacterial colonies initially and 8 neutrophils. In these 156 runs, the maximum number of bacterial colonies that survived was nine and the minimum was zero, with a mean value of 3.6. Because the undiscovered colonies continue to grow during the 4-h interval, they collectively contain an average of 40 viable bacteria at the end of the simulation, an increase from the 12 initially present. The average number of bacteria that survive the interaction with surface-associated neutrophils is predicted to be a strong linear function of the number of neutrophils present (Fig. 5C), recapitulating at least qualitatively the result shown in Fig. 2B.

DISCUSSION

How do a few microorganisms contaminating an implanted biomaterial evade host defenses long enough to establish a mature and protected biofilm? To gain insight into specific phenomena that may compromise neutrophil clearance of attached bacteria, we imaged and quantified the interaction of human neutrophils with a nascent *S. aureus* biofilm on a surrogate biomaterial surface *in vitro*. A critical conclusion from these observations is that neutrophils are able to discover, phagocytose, and destroy surface-adherent bacteria when the microorganism is present as single cells or very small aggregates and when a sufficient number of neutrophils are present. This is important because it suggests that neutrophil-mediated innate immunity has the capacity to eliminate a nascent *S. aureus* biofilm. The approximate areal density needed for protective surveillance of the surface in our experiments was on the order of 10^4 to 10^5 neutrophils per cm^2 . This surface density of neutrophils must be present at the outset of the contest in order for the leukocytes to be able to discover and destroy bacteria.

When neutrophil discovery of bacteria was delayed, either because the surface density of neutrophils was too low or because neutrophils were added after giving the bacteria a head start, undiscovered bacteria grew and formed aggregates. These relatively young bacterial biofilm aggregates were much less susceptible to clearance by neutrophils than were lone bacterial cells. This result suggests that for neutrophils to prevent biofilm formation, the time for their recruitment to the abiotic surface must be shorter than the time for the bacteria to form neutrophil-sized aggregates. Thus, there are two critical time scales that are important to quantify *in vivo*: the characteristic time for neutrophil recruitment to a biomaterial surface and the time necessary for bacteria to form aggregates that are approximately $10\ \mu\text{m}$ in diameter.

The time scale for aggregate formation by *S. aureus*, at least in the *in vitro* system used here, was 3 to 4 h. This corresponds to approximately 4 doubling times at the *in vitro* growth rate measured in this work of $0.78\ \text{h}^{-1}$. *In vivo*, this time scale may be different, likely longer, depending on the *in vivo* doubling time of the bacteria. There are very few measurements of staphylococcal growth rate *in vivo*, especially in a niche where an implanted device could dwell. Data from Fig. 1B in the article by Dastgheyb et al. (45) show a growth rate of *S. aureus* in human synovial fluid of $0.46 \pm 0.08\ \text{h}^{-1}$; 4 doublings at this specific growth rate would take 6 h. In triplicate measurements of *S. aureus* growth in our early biofilm system, we found the specific growth rate to be $0.63 \pm 0.42\ \text{h}^{-1}$ in pure bovine serum and $0.56 \pm 0.09\ \text{h}^{-1}$ in pure bovine synovial fluid.

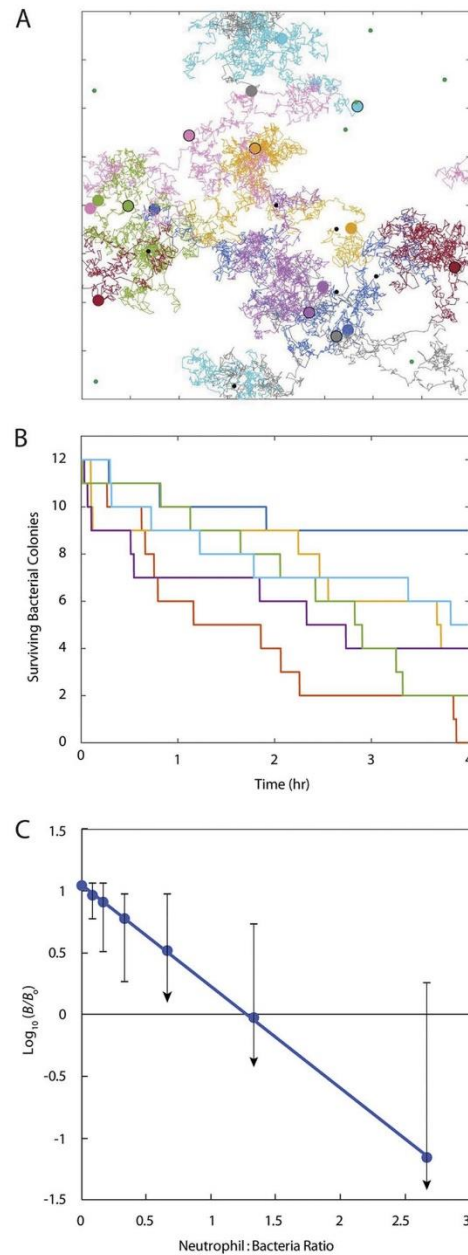


FIG 5 Mathematical model illustrates stochastic outcomes of neutrophil-*S. aureus* interactions. (A) Sample computational result showing neutrophil (larger variously colored circles) initial (with black outline) and final (no outline) locations and tracks (colors correspond to the individual neutrophil) during the course of a 4-h simulation. Bacterial colonies (small circles) are shown in green where they have escaped detection by neutrophils and remain viable or in black where they have been discovered and killed by a neutrophil. Tick marks are 100 μm apart. See full video of the simulation in the supplemental material (Video S7). (B) Decay of the number of surviving bacterial colonies for 6 of 156 repeated simulations. (Continued on next page)

The time scale for neutrophil recruitment to an implanted abiotic surface is also uncertain. Animal studies of neutrophil dynamics at sites of infection indicate that the time scale for neutrophil recruitment may range from an hour to a day (46–48).

To delve further into the possibility of delayed neutrophil recruitment to an implant surface, we extracted quantitative estimates of neutrophil recruitment times from published reports in murine models (see Fig. S7, Table S2, and related methods in the supplemental material). When a chemical stimulus was used (e.g., macrophage inflammatory protein 2 [MIP-2], zymosan, lipopolysaccharide [LPS], and peptidoglycan), the mean neutrophil recruitment time and standard deviation were 3.6 ± 2.6 h. When staphylococci were inoculated, the recruitment time was 12 ± 10.1 h. In two investigations in which an uninoculated implant (titanium or stainless steel) was examined, the neutrophil recruitment time was 21 h. While these values range widely, they appear to be consistent with the possibility of delayed recruitment. Clearly, additional research is needed to better define the characteristic times for both bacterial growth and neutrophil recruitment to a biomaterial surface *in vivo*.

This work raises the possibility that, in the earliest stage of biofilm formation, neutrophil efficacy is limited by the discovery of bacteria rather than by bacterial virulence. The stochastic nature of this interaction and the survival of occasional undiscovered bacteria are reinforced by computational modeling. Unlike the scenario of bacteria and neutrophils interacting in a homogenous well-mixed suspension (15), the interaction on a sparsely contaminated implant surface is inherently heterogeneous and probabilistically determined. If neutrophil discovery limits bacterial clearance from the surface of a biomaterial *in vivo*, then it might be possible to prevent device-related infections by boosting the recruitment and activity of neutrophils in a window of time shortly following implantation. Our *in vitro* results suggest that for such a strategy to succeed, both a sufficient number of neutrophils need to be delivered to the surface and their recruitment must be relatively rapid.

Of the six hypotheses presented above for how bacteria contaminating an abiotic surface evade neutrophil clearance, the two that are most consistent with our *in vitro* results are delayed neutrophil recruitment (hypothesis 1) and hypoxia (hypothesis 6). Hypotheses 2 to 5 are contradicted by our observation of efficient and consistent neutrophil surveillance, discovery, phagocytosis, and destruction of attached single bacteria or very small aggregates.

We hypothesized that delayed recruitment of neutrophils to a biomaterial surface provides a window of time for contaminating microorganisms to grow and establish a protected biofilm (hypothesis 1). A biomaterial is compromised in at least two important ways that could delay leukocyte recruitment. Biomaterial surfaces are not vascularized: they have no built-in system to rapidly deliver immune cells. In addition, there may be local disruption of vascularized tissue around the device as a result of the surgical implantation procedure. To ensure complete surveillance and discovery of all contaminating microorganisms, neutrophils must migrate from vascularized tissue to all the surfaces of the biomaterial. A second deficiency of a biomaterial with respect to neutrophil recruitment is that the material itself is mute in the sense that it cannot communicate with the host. Unlike vital tissue that can release chemokines in response to the presence of bacteria and thereby initiate neutrophil recruitment, abiotic implants lack any signaling capacity. The time required for neutrophils to arrive at a biomaterial

FIG 5 Legend (Continued)

simulations. All simulations used the same initial number of bacterial colonies (12) and same number of neutrophils (8), but the initial distributions of these two cell types were randomly determined for each individual repetition. Shown are simulations resulting in the maximum (dark blue) and minimum (red) number of residual bacterial colonies and four others to illustrate the range of outcomes. (C) Change in total viable bacterial numbers as a function of the neutrophil/bacterium ratio (mean from 156 repetitions of each case). Simulations all used the same initial number of bacterial colonies (12) with neutrophil numbers of 0, 1, 2, 4, 8, 16, and 32 (blue circles). Error bars denote the ranges from maximum to minimum outcomes with a downward arrow signifying complete elimination of viable bacteria as the minimum. The dotted line is a least-squares linear regression.

surface likely depends on the anatomical site of the implant, the proximity of specific locations on the implant to perfused tissue, and the number of contaminating bacteria.

We also investigated the possibility that local hypoxia in the vicinity of the implant could diminish neutrophil efficacy (hypothesis 6). The same issues mentioned above—lack of vasculature on the biomaterial surface and trauma to neighboring tissue during implantation—could reduce provision of oxygen around a biomaterial. Whereas hypoxia is widely recognized to be likely in an established biofilm infection, data on oxygen availability in the vicinity of a newly implanted biomaterial are lacking.

Improved understanding of how innate immune cell function at biomaterial surfaces is compromised and what mechanisms limit leukocyte efficacy on the surface of a foreign body may someday lead to alternative strategies for preventing device-related infections (24, 49–59). The work presented here provides insight into the pathogenesis of foreign body infections and a previously unexplored mechanism of immune evasion. We have identified that a relatively brief time window, dependent on the *in vivo* bacterial growth rate and neutrophil recruitment dynamics, exists, wherein adherent bacteria are susceptible to effective clearance by neutrophils. This short time period could possibly explain how small aggregates of contaminating bacteria on an implant surface are able to develop into biofilm infections that are recalcitrant to immune clearance and antibiotic therapy.

Areas of opportunity for future work include (i) development and application of animal models using intravital imaging to observe neutrophil-bacterium interactions with single-cell resolution, (ii) measurement of the distribution of neutrophil recruitment times to a bacterially contaminated implant surface *in vivo*, (iii) measurement of lag times and specific growth rates of surface-attached microorganisms *in vivo*, and (iv) elucidation of mechanisms of immune evasion by small (neutrophil-sized) bacterial aggregates.

MATERIALS AND METHODS

Bacterial and neutrophil preparation. *S. aureus* strain AH2547 (HG001+pCM29, courtesy of Alex Horswill), a known biofilm-forming strain with constitutive expression of green fluorescent protein (GFP), was grown overnight in tryptic soy broth supplemented with 10 μ g/ml chloramphenicol (60). Overnight cultures were centrifuged for 5 min at 4,000 rpm, rinsed, resuspended in phosphate-buffered saline (PBS), and serially diluted. Cells were attached to a 4-chambered glass bottom petri dish (Cellvis, Mountain View, CA, USA) to facilitate live-cell imaging. To attach cells, between 25 and 100 μ l of the 10^{-3} dilution was added to the surface, and PBS was added to a final volume of 500 μ l. After 30 min of incubation at 37°C, unattached bacteria were gently rinsed from the surface with PBS. Each chamber of the petri dish was filled with 500 μ l of 20% fresh human serum in Hanks' balanced salt solution (HBSS) to simultaneously coat the surface with serum and opsonize bacteria, and the dish was incubated at 37°C for 30 min. Prior to imaging, the serum concentration in each chamber was then diluted to ~10% with additional HBSS. Human neutrophils (polymorphonuclear leukocytes, or PMNs) were isolated from heparinized venous blood obtained from healthy donors according to a standard institutional review board (IRB)-approved protocol, as described previously (61). PMNs were kept on ice until they were stained with LysoBrite Red (AAT Bioquest, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Microscopy. A Leica SP5 inverted confocal scanning microscope was utilized for all imaging. GFP-tagged bacteria and PMNs were excited with the 488-nm and 561-nm laser lines, respectively. A LiveCell environmental chamber system (Pathology Devices, San Diego, CA, USA) was utilized to maintain 5% CO₂, 20% O₂, 50% humidity, and 37°C for sample incubation during imaging. Image stacks with 1- μ m z-slices were taken sequentially at 1- to 2-min intervals over a 4-h time course using a 20 $\times$ dry lens objective. At least two fields of view from each chamber of the dish were imaged per experiment.

Image analysis. MetaMorph (Molecular Devices) image analysis software was used to measure the change in bacterial biomass by quantifying the thresholded area of bacterial green fluorescence over a period of 4 h. Maximum growth rates were calculated by linear regression of all data within exponential phase. Movies were prepared with Imaris version 8.0 (Bitplane). To quantify neutrophil motion, the "ImarisTrack" module that tracks three-dimensional (3D) objects over time, displays their path, and analyzes their motion was utilized. Neutrophils were automatically identified as "spots" on the basis of size and manually edited and confirmed when needed. Motion was tracked using the Brownian motion algorithm. To quantify the fraction of a field of view "patrolled" by a neutrophil over 4 h, neutrophil tracks determined by the ImarisTrack module were set to be cylinders 10.87 μ m in diameter. The image was then imported into MetaMorph, and the percentage of the field of view covered by a neutrophil track was determined by thresholding. To determine the average track length for PMNs in a given field of view, the average speed of each neutrophil in the field of view was determined using the ImarisTrack module. The average speed for all neutrophils in a field of view was calculated for each frame, and the average track length was subsequently determined by integrating with respect to time using the trapezoidal method. This method decreased the bias of neutrophils that may have been in the field of view for only a small number of frames as opposed to simply using the track length generated by Imaris. The fraction of neutrophils moving in a given field of view

was calculated by binning neutrophils that were moving greater than $0.01 \mu\text{m/s}$. An average from the final three frames of an experiment (~ 5 to 7 min) was used to compare the numbers of neutrophils moving at the end of an experiment for the high- versus low-oxygen environment assays.

Bacterium-neutrophil surface dynamics. To assay how PMNs interact with bacteria on a surface, we varied both the density of attached *S. aureus* aggregates on the surface and the number of neutrophils added to the surface ($n = 10$ independent experiments, with up to 8 fields of view per experiment). Experimental conditions included wells with *S. aureus* only, PMNs only, and *S. aureus* and PMNs at various multiplicities of infection (MOIs).

Head-start experiments. *S. aureus* cells were attached to the surfaces of separate chambers of the dish as described above at staggered time points of 3.5, 2.5, or 1.5 h prior to the addition of PMNs. After 0.5 h, unattached *S. aureus* cells were gently rinsed from the surface, and 10% tryptic soy broth (TSB) was added to the dish. After 2.5, 1.5, or 0.5 h of incubation, the medium was gently removed with a pipette and replaced with 20% human serum in HBSS for conditioning and opsonization. Half of an hour later, the serum concentration was diluted to 10%, PMNs were added, and the dishes were imaged for 4 h. Results from the head-start experiments ($n = 4$) were pooled and compared to data obtained during previous experiments wherein bacteria were not given a head start.

Oxygen limitation experiments. Low-oxygen conditions were attained by adding N_2 to the environmental chamber to maintain O_2 levels of either 0% or 2% ($n = 3$ experiments each). Results for both the 0% and 2% conditions were pooled and compared to data obtained during previous standard experiments (20% O_2).

Directionality and mean square displacement analysis. Neutrophil directionality was calculated by dividing neutrophil track displacement by total track length using tracking data generated using the ImarisTrack module. Mean square displacement was plotted as a function of time, and an approximately 40-min-long period of linearity in the curve was used to calculate a random motility coefficient based on the slope of the curve.

Computational modeling of the neutrophil-bacterium surface interaction. Neutrophil-bacterium interactions were mathematically modeled in a three-dimensional spatial domain lying above a flat plate. The domain was periodic in the horizontal directions x and y and open above the surface of a plate ($z = 0$) in the vertical direction ($z \geq 0$). Spatial periodicity was set at $800 \mu\text{m}$ in both the x and y directions. The simulation time was 240 min in all computations. Model elements consisted of a set of nonmotile, growing bacterial colonies that emit chemoattractant (at a rate proportional to colony size) which freely diffuses in the region where $z \geq 0$ as well as a set of neutrophils which move on the plate ($z = 0$) with a combination of random and directed (via the gradient of the chemoattractant concentration) movement as in a Keller-Segel type model (62). When a neutrophil encountered a bacterial colony, the colony was eliminated and ceased to emit chemoattractant.

In each simulation, 12 bacterial colonies, each with an initial population of 1, were randomly seeded with uniform probability on the $800\text{-}\mu\text{m}^2$ plate. Coordinates $(x_j, y_j, 0)$, $j = 1$ to 12 designated colony locations for any given simulation. Note that due to horizontal periodicity, there was effectively an infinite tiling of the plane at a z of 0 with “image” colonies at locations $(x_j, y_j, 0) + (800k, 800l, 0)$, $j = 1$ to 12 , where k and l are arbitrary integers. Prior to an encounter with a neutrophil, any given colony grew at specific rate r such that colony j had population $c_j(t) = e^{rt}$.

Each colony, including the images, emitted chemoattractant at rate $2c_j(t)$, until it had an encounter with a neutrophil at, for example, $t = T_j$ at which time it ceased to emit chemoattractant and $c_j(t)$ was 0 for $t \geq T_j$. The chemoattractant concentration, denoted by $u(x, t) = u(x, y, z, t)$, satisfies the following diffusion (with sources) equation:

$$u_t = D_1 \nabla^2 u + 2\beta \sum_{j=1}^{12} \sum_{k,l \in \mathbb{Z}} c_j(t) \delta(x - [x_j + (800k, 800l, 0)]),$$

where δ is the Dirac delta function, with initial and boundary conditions

$$u(x, 0) = 2\beta \sum_{j=1}^{12} \sum_{k,l \in \mathbb{Z}} c_j(t) \delta(x - [x_j + (800k, 800l, 0)]), \quad \frac{\partial u}{\partial t}(x, y, 0, t) = 0.$$

In fact, this system can be solved analytically for $u(x, t)$ (see supplemental material).

In addition, a number N (with $N = 1, 2, 4, 8, 16$, or 32) neutrophils are also (initially) randomly seeded with uniform probability on the plate at locations $X^{(j)}(t = 0) = X_0^{(j)}$, $j = 1$ to N . Neutrophil locations $X^{(j)}(t)$ are modeled by stochastic differential equations

$$dX^{(j)}(t) = \nabla \bar{u} \left(\frac{V_{\max}}{K_m + |\nabla \bar{u}|} \right) dt + \sqrt{2D_2} dW^{(j)}(t), \quad X_0^{(j)}(0) = X_0^{(j)},$$

where $W^{(j)}(t)$ is a standard 2-dimensional Wiener process (63), and $\bar{u}(x, y) = u(x, y, 0)$ is the restriction of the analytical solution to the chemoattractant equations to the z of 0 plane. Solutions were approximated numerically using the Euler-Maruyama scheme (63). We assumed that there is a maximum speed $V_{\max}$ at which a neutrophil can move in response to the chemoattractant, imposed using a Monod-type function in the drift coefficient with half-saturation K_m . Note, again, that as a consequence of periodicity, there are effectively a doubly periodically infinite set of neutrophils. For computational purposes, we only needed to track one set: each time a neutrophil crossed the boundary of the model $800\text{-}\mu\text{m}^2$ plate, it effectively re-entered across the corresponding periodic boundary. Simulation parameters and sources were as follows: $D_1 = 0.036 \text{ mm}^2 \text{ min}^{-1}$ (comparison to raffinose, molecular weight [MW] 504 g/mole [64]); $D_2 = 0.00016 \text{ mm}^2 \text{ min}^{-1}$ (see Table S1 in the supplemental material); $K_m = 1,000$ concentration mm^{-1} (assumed); $V_{\max} = 0.012 \text{ mm min}^{-1}$ (see Fig. S2); $\beta = 0.01$ concentration min^{-1} per bacteria (assumed);

$r = 0.01 \text{ min}^{-1}$ (see "Neutrophils migrate on an abiotic surface and discover, phagocytose, and destroy single attached bacteria or very small bacterial aggregates" above).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/IAI.00745-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

SUPPLEMENTAL FILE 2, AVI file, 1.5 MB.

SUPPLEMENTAL FILE 3, AVI file, 1.5 MB.

SUPPLEMENTAL FILE 4, AVI file, 1.3 MB.

SUPPLEMENTAL FILE 5, AVI file, 1.8 MB.

SUPPLEMENTAL FILE 6, AVI file, 3.2 MB.

SUPPLEMENTAL FILE 7, AVI file, 3.8 MB.

SUPPLEMENTAL FILE 8, AVI file, 6.4 MB.

SUPPLEMENTAL FILE 9, AVI file, 6.4 MB.

SUPPLEMENTAL FILE 10, AVI file, 6.3 MB.

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APPENDIX E

LIST OF SUPPLEMENTARY VIDEOS

LIST OF SUPPLEMENTARY VIDEOS

Video S3.1: LAC Δ sae growth in normal human serum (NHS). LAC Δ sae was attached to the dish surface and grown for 4 hours in 10% NHS diluted in Hank's Balanced Salt Solution.

Video S3.2: LAC Δ sae growth in heat inactivated human serum (HIS). LAC Δ sae was attached to the dish surface and grown for 4 hours in 10% HIS diluted in Hank's Balanced Salt Solution.

Video S3.3: LAC Δ sae growth in C3-depleted human serum. LAC Δ sae was attached to the dish surface and grown for 8 hours in 10% C3-depleted serum diluted in RPMI. Video shows growth from hours 4 – 8.

Video S3.4: LAC Δ sae growth in C5-depleted human serum. LAC Δ sae was attached to the dish surface and grown for 8 hours in 10% C5-depleted serum diluted in RPMI. Video shows growth from hours 4 – 8.

Video S3.5: LAC Δ sae growth in C4-depleted human serum. LAC Δ sae was attached to the dish surface and grown for 8 hours in 10% C4-depleted serum diluted in RPMI. Video shows growth from hours 4 – 8.

Video S3.6: LAC Δ sae growth in fB-depleted human serum. LAC Δ sae was attached to the dish surface and grown for 8 hours in 10% fB-depleted serum diluted in RPMI. Video shows growth from hours 4 – 8.

Video SA1.1: LAC Δ 4-agg demonstrating a partial aggregation defect in NHS. Cells were grown for approximately 2.5 hours prior to the start of imaging. Growth was imaged for 4 hours.

Video SA1.2: LAC Δ 4-agg demonstrating no aggregation defect in NHS. Cells were grown for approximately 3 hours prior to the start of imaging. Growth was imaged for 4 hours.

Video SA2.1: PMNs fail to adhere to the dish surface in HIS.

Video SA2.2: PMNs have reduced motility in 25 μ g/mL NIF.

Video SA2.3: PMNs demonstrate surface adherence and motility on a fibrinogen coated surface. PMN swarming is observed following phagocytosis.

Video SA2.4: PMNs demonstrate surface adherence and motility in CVF-treated serum.

Video SA2.5: PMNs fail to adhere to the dish surface in C3-depleted serum.

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