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Staphylococcal Biofilms and Immune Polarization during Prosthetic Joint Infection

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Abstract

Staphylococcal species are a leading cause of community- and nosocomial-acquired infections, where the placement of foreign materials increases infection risk. Indwelling medical devices and prosthetic implants are targets for staphylococcal cell adherence and biofilm formation. Biofilm products actively suppress proinflammatory microbicidal responses, as evident by macrophage polarization toward an anti-inflammatory phenotype and the recruitment of myeloid-derived suppressor cells. With the rise in prosthetic hip and knee replacement procedures, together with the recalcitrance of biofilm infections to antibiotic therapy, it is imperative to better understand mechanisms of crosstalk between biofilm-associated bacteria and host immune cells. This review describes the current understanding of how staphylococcal biofilms evade immune-mediated clearance to establish persistent infections. The findings described herein may facilitate the identification of novel treatments for these devastating biofilm-mediated infections.

INTRODUCTION

Skin and nasal colonization with *Staphylococcus epidermidis* or *S. aureus* are known risk factors for invasive infections [1–3]. Prosthetic joint infections (PJIs) likely originate from colonization with small numbers of bacteria, which may provide a window of opportunity for survival if the pathogen does not elicit an initial vigorous immune response to mediate clearance. Staphylococcal invasion at the surgical site followed by adherence to the prosthesis frequently results in biofilm formation [4–6]; a community of bacterial cells encased within a self-produced matrix composed of proteins, polysaccharides and extracellular DNA [5,7]. The biofilm matrix supports the three-dimensional organization of bacteria while also acting as a barrier against host immune cell invasion. Biofilm development *in vitro* is a well-characterized process involving an array of proteins required for attachment, maturation, and dispersal [8,9]; however, less is understood about staphylococcal biofilm development *in vivo*. In addition to limited treatment options due to genetically-acquired antibiotic resistance, staphylococcal biofilm infections are characterized by inherent antibiotic tolerance due to their dampened metabolic state and decreased susceptibility to phagocytosis [10]. As such, biofilm-mediated PJIs often require

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surgical revision and replacement of a new prosthesis, which is associated with an increased frequency of infection recurrence and significant morbidity [11].

New approaches to prevent and treat biofilm-associated infections include anti-adhesive medical device coatings, therapies which disrupt the biofilm matrix, and antimicrobials targeting biofilm-specific bacterial processes [12]. Until recently, the immune response to staphylococcal biofilm has remained largely unexplored. This scope of this review includes recent advances in our understanding of the effects of staphylococcal biofilm on immune cell function, with particular consideration of macrophage dysfunction and preferential recruitment of myeloid-derived suppressor cells (MDSCs) [13–17]. In addition, staphylococcal biofilm products responsible for modifying immune cell function will be discussed. Findings from these studies may unveil a potential two-pronged strategy that limits bacterial biofilm development and coordinates a productive immune response to these infections. Once such strategies become available, there is promise that the rate of biofilm-mediated infections will be abated.

1. IMMUNE RESPONSE TO STAPHYLOCOCCAL BIOFILMS

With the rise in acquired antibiotic resistance by staphylococcal species leaving few treatment options, deciphering the innate immune response during device-associated biofilm infections could lead to novel therapeutic strategies. Staphylococcal biofilm infections have recently been described to induce an anti-inflammatory response, characterized by the recruitment of alternatively-activated macrophages and MDSC expansion. These two events, described in detail below, polarize the local environment toward an anti-inflammatory and pro-fibrotic milieu, thereby contributing to the chronic nature of biofilm infections. The findings described below raise the possibility of future approaches aimed at targeting immune cell activity to a mount a productive microbicidal response to clear PJIs.

Alternatively-activated macrophages

Considered among the first lines of innate immune cellular defense against bacterial infections, resident tissue macrophages can be classically-activated (pro-inflammatory) or alternatively-activated (anti-inflammatory) depending on the inflammatory milieu. We will not utilize the M1/M2 nomenclature in this review to describe macrophage activation states, since the original intent of the M1/M2 dichotomy was to describe macrophage responses to well-controlled *in vitro* conditions [18], which is clearly not the case *in vivo*. This is reflected by many reports demonstrating that macrophages can simultaneously possess gene expression profiles indicative of both M1 and M2 states [19,20]. Instead, we will refer to general functional attributes of macrophages in response to biofilm-associated bacteria, keeping in mind that responses are likely more complicated. Classically-activated macrophages are essential effectors during planktonic bacterial infection, in part, through their robust pro-inflammatory cytokine production, phagocytosis, and killing by generation of reactive oxygen and nitrogen species. The pro-inflammatory macrophage response is characterized by iNOS, TNF-α, IL-1β, and IFN-γ expression, which promote bacterial clearance, whereas anti-inflammatory mediators, such as arginase-1 (Arg-1), IL-4, and IL-10 attenuate macrophage microbicidal activity and promote a pro-fibrotic environment,

facilitating bacterial persistence. Recent studies by our laboratory and others have shown that staphylococcal biofilms evade Toll-like receptor 2 (TLR2) and TLR9 recognition and skew host leukocytes toward an anti-inflammatory, pro-fibrotic response, evidenced by increased Arg-1 and decreased iNOS expression [21–24]. This alternatively-activated response induces robust fibrosis surrounding the biofilm, effectively preventing macrophage invasion and phagocytosis of biofilm-associated bacteria, favoring biofilm persistence [15,25,26]. Although proinflammatory cytokine production is detected during *S. aureus* biofilm infections, this response is clearly not sufficient to mitigate biofilm growth or survival [13–15,27]. This suggests a primary defect in the phagocytes that normally clear bacteria, which is supported by the preferential recruitment of MDSCs into staphylococcal biofilms that possess anti-inflammatory properties by preventing macrophage proinflammatory activity and T cell activation.

To circumnavigate the anti-inflammatory milieu induced by *S. aureus* biofilms, an activated macrophage adoptive transfer strategy was employed by Hanke *et al.* in a catheter-associated model of *S. aureus* biofilm infection [25]. Since earlier work revealed the inability of resident tissue macrophages to invade the biofilm proper, this approach addressed whether the direct injection of proinflammatory activated macrophages (IFN-γ + peptidoglycan) into biofilms would transform the inflammatory milieu to facilitate biofilm clearance. Injection of activated macrophages during early biofilm formation augmented proinflammatory cytokine production, reduced macrophage Arg-1 expression, and limited *S. aureus* biofilm development. Proinflammatory macrophage polarization was also shown to increase phagocytosis and killing of *S. aureus* biofilms *in vitro.* Furthermore, treatment of mice with the C5a receptor (CD88) agonist EP67, which invokes macrophage proinflammatory activity, augmented macrophage infiltration and proinflammatory mediator expression in biofilm-infected tissues, which translated into reduced *S. aureus* biofilm growth [25]. Taken together, these findings demonstrate that *S. aureus* biofilms interfere with antibacterial effector mechanisms of resident tissue macrophages, which is an essential step for biofilm development. However, if proinflammatory macrophages gain access to sites of *S. aureus* biofilm (i.e. by direct inoculation), they are capable of exerting antibacterial activity that manifests as improved biofilm clearance. This supports an important role for *S. aureus* in thwarting early macrophage activation to establish chronic *S. aureus* biofilm infections.

Myeloid-Derived Suppressor Cells (MDSCs)

Suppression of proinflammatory responses by MDSCs have been demonstrated in a number of cancer and bacterial infection models [28–31]. MDSCs are a heterogeneous population of immature monocytes and granulocytes and are functionally characterized by their ability to suppress T cell activation in an antigen-dependent or -independent manner (reviewed in [32]). Depending on the local tissue microenvironment, infiltrating MDSCs can either maintain their suppressive properties or differentiate into mature neutrophils, macrophages, or dendritic cells. Our laboratory was the first to report MDSC recruitment ($CD11b^{+}Ly\text{-}6G^{+}Ly\text{-}6C^{high}$) in a mouse model of *S. aureus* PJI, which was confirmed by the identification of MDSC-like cell populations in human PJIs, including those caused by *S. epidermidis* [13,15]. Subsequently, other groups have reported MDSC recruitment together

with immunosuppressive activity in mouse models of *S. aureus* cutaneous infection [33] and to the kidneys following *S. aureus* sepsis [17].

In assessing the functional role of MDSCs in establishing the suppressive environment surrounding *S. aureus* biofilms, an antibody-mediated depletion strategy was used to target Ly-6G^{+} cells. Any responses would be attributed MDSC activity, since although Ly6G is also expressed on neutrophils, our prior work has shown that few neutrophils are recruited to *S. aureus* biofilms. Importantly, Ly6G treatment would leave CD11b^{+}Ly-6C^{high} Ly-6G^{-} monocytes intact, which was predicted to enhance monocyte/macrophage proinflammatory and bactericidal activity in the context of reduced MDSC inhibitory signals. This was indeed the case, where Ly6G-depleted animals displayed significantly increased proinflammatory mediator production (i.e. IL-1β, G-CSF) that translated into reduced *S. aureus* burdens in a mouse PJI model [13]. To directly demonstrate enhanced monocyte/macrophage microbicidal activity in the context of MDSC depletion, mice were treated with Gr-1 antibody, which targets both Ly-6G^{+}Ly-6C^{high} MDSCs and Ly-6G^{-}Ly-6C^{high} monocytes. This approach resulted in significantly higher *S. aureus* biofilm burdens, which was attributed to the fact that although MDSC inhibition was removed, effector phagocytes (i.e. monocytes/macrophages) were also reduced, leading to unchecked biofilm expansion [13]. These data demonstrate that MDSCs actively suppress monocyte/macrophage proinflammatory activity, which is important for biofilm persistence. Interestingly, Gr-1^{+} depletion in a *S. aureus* sepsis model had no effect on bacterial burdens [17]; however, this is not a biofilm infection and mature macrophages are not present in the bloodstream, and as such, the modes of bacterial clearance differ.

A role for both IL-12 and IL-10 signaling has been reported to shape the anti-inflammatory biofilm milieu by promoting MDSC recruitment; however, the kinetics of cytokine involvement differ [14,15]. Namely, IL-12 deficiency leads to an early reduction in MDSCs, whereas IL-10 affects later MDSC recruitment into *S. aureus* biofilms (Figure 1). As a result of reduced MDSC infiltrates, monocyte influx was increased in both IL-12 and IL-10 knockout (KO) mice and these monocytes displayed increased proinflammatory activity that translated into decreased *S. aureus* biofilm burdens. Importantly, adoptive transfer of wild-type MDSCs into IL-12 and IL-10 KO animals returned biofilm growth to levels seen in wild-type animals [14,15]. Adoptive transfer of MDSCs also exacerbated infection outcome in a mouse model of *S. aureus* sepsis, again supporting the functional role of MDSCs in promoting bacterial expansion [33].

T cells

The role of T cells in mediating staphylococcal biofilm PJIs remains unclear. Our laboratory has found minimal T cell infiltrates in a mouse *S. aureus* PJI model [14,15] and T cells were undetectable in human PJIs compared to tissues recovered from aseptic orthopedic revisions, where a prominent T cell population was observed [15]. The relative paucity of T cells in both human PJIs and our mouse PJI model could be explained by the robust MDSC infiltrate associated with these infections based on their ability to inhibit T cell proliferation/activation [13]. In contrast to our studies, other work has reported a role for Th1 and Th17 cells in response to *S. aureus* biofilms [34]. These discrepancies may be explained by the distinct

model systems employed; namely the use of a tibial implant pre-coated *in vitro* with an established biofilm compared to our approach where a sterile orthopedic implant is infected *in vivo* using a 200-fold lower bacterial dose. Indeed, we recently reported that increasing the infectious inoculum from 10^3 to 10^5 CFU in the mouse PJI model altered leukocyte recruitment and inflammatory mediator production as well as biofilm growth/clearance [27]. This highlights the need to carefully consider infectious doses when examining inflammatory attributes of biofilm infection, in particular to their relevance in terms of modeling events that might occur during native PJI in humans.

Part 1 Conclusions

In contrast to planktonic bacteria, the immune response to staphylococcal biofilms is suppressive and polarizes macrophages toward an anti-inflammatory state. This is due, in part, to MDSC recruitment that attenuates monocyte/macrophage activation. Importantly, similar leukocyte infiltration patterns are observed between the mouse PJI model and human PJI tissues, including increased MDSC-like cells and few T cells. Limiting MDSC influx and/or their immunosuppressive action may offer a new therapeutic strategy to thwart chronic staphylococcal biofilm infections.

While biofilm invasion and phagocytosis by neutrophils appear to be less affected than macrophages [21,35,36], studies have suggested that neutrophils recovered from human implant/endoprosthesis infections are less phagocytic and contribute to infection persistence [37]. This may result from inhibition of opsonophagocytic killing of *S. epidermidis* biofilms compared to planktonic cells [38]. In the S. *epidermidis* biofilm matrix, polysaccharide intracellular adhesion (PIA) has been shown to play an important role in preventing macrophage [23] and neutrophil phagocytosis [39]. Another model of S. *epidermidis* biofilm formation on peritoneal dialysis catheters reported defective macrophage function that was linked to reduced IFN-γ production 40]. With regard to *S. aureus*, it is interesting that the biofilm transcriptome remained relatively unaffected in the face of neutrophil challenge, whereas a large percentage of genes (~ 95%) were significantly downregulated upon macrophage exposure [36]. These experiments demonstrate that *S. aureus* biofilms differentially modify their gene expression patterns depending on the leukocyte subset encountered. The finding that biofilms were more responsive following macrophage addition rather than neutrophils is in agreement with the preferential recruitment of macrophages but minimal neutrophils during biofilm formation [13–16,21,25–27]. While the anti-inflammatory response appears to be driven, in part, by biofilm products, the specific effectors and their mechanism(s) of action have yet to be fully elucidated.

2. BIOFILM EFFECTORS OF IMMUNE CELL DYSFUNCTION

In general, PJIs associated with more virulent organisms, such as *S. aureus*, often present within the first 3 months after surgery, whereas complications triggered by less virulent organisms, such as *S. epidermidis*, can manifest as chronic infections months or years post-surgery [11,41]. This dichotomy may reflect a passive defense strategy in *S. epidermidis* rather than the broad arsenal of toxins and other virulence determinants that *S. aureus* possesses [42]. Interestingly, as mentioned above, co-culture of macrophages with *S. aureus*

biofilm resulted in a global suppression of *S. aureus* transcription, whereas only a few genes were up-regulated [36]. This suggests that *S. aureus* may "hide" from the immune system by globally repressing gene expression [43]; however it is clear that proteins and other molecules released from *S. aureus* biofilm also effect macrophage function [16]. Identification of candidate molecules responsible for inhibiting macrophage proinflammatory properties represents an essential step in understanding host-pathogen interactions during PJI. The second part of this review focuses on secreted factors that induce immune cell dysfunction, with a particular focus on macrophages.

Toxins

S. aureus produces a wide array of cell wall-associated and secreted virulence factors that interfere with antimicrobial effectors of the immune system [44,45]. Two well-studied toxins include α-toxin (Hla), which acts on red blood cells and leukocytes by binding to ADAM10 [46,47] and the bi-component leukocidins LukAB [48], LukED [49], and Panton-Valentine leukocidin (PVL, consisting of LukFS) [50], which bind to specific leukocyte surface receptors. *S. aureus* also produces enterotoxins, such as toxic shock syndrome toxin (TSST); however, they will not be discussed in this review. As mentioned above, *S. epidermidis* does not produce many toxins [51], but rather relies on a thick biofilm matrix and extensive polysaccharide network for immune evasion. For this reason, the remainder of this section will focus on *S. aureus* biofilm components and their effects on antimicrobial immune mechanisms.

While biofilm size and matrix density represent physical obstacles for macrophage invasion, our laboratory recently demonstrated macrophage phagocytosis was significantly attenuated following exposure to *S. aureus* biofilm-conditioned medium [16]. Restoration of phagocytosis was achieved following the treatment of biofilm supernatant with proteinase K, indicating that a secreted proteinaceous factor(s) actively inhibits macrophage phagocytosis of *S. aureus* biofilm. Mutation in the accessory gene regulator (*agr*) locus also alleviated the macrophage phagocytic block, suggesting that the putative proteins were regulated in an *agr*-dependent manner [16]. Subsequent experiments demonstrated that LukAB and α-toxin act in a synergistic manner to prevent macrophage phagocytosis and are also important for inhibiting macrophage invasion *in vivo* in a mouse PJI model, which translates into biofilm persistence [16]. However, it is clear that additional extracellular factors released from *S. aureus* biofilms play a role in thwarting macrophage activation, since the phagocytic block was not completely reversed following simultaneous inhibition of α-toxin/LukAB activity [16]. This is not unexpected, given the extensive arsenal of virulence determinants expressed by *S. aureus*, which may represent viable therapeutic targets to augment macrophage microbicidal activity.

Intracellular components

Cell death and lysis is an essential mechanism in staphylococcal biofilm development, as demonstrated by mutation in genes responsible for autolysis [52,53] or treatment with an inhibitor of autolysis, polyanethol sulfonate (PAS) [54,55]. Intracellular molecules, such as DNA, are important components of the biofilm matrix [5], and while this represents a potential pathogen-associated molecular pattern (PAMP) to trigger proinflammatory activity

via TLR9, we have previously shown that *S. aureus* biofilms evade TLR9-mediated recognition of bacterial DNA [21]. The potential of secreted molecules from *S. aureus* biofilm to induce macrophage dysfunction was recently addressed by Scherr *et al* [16]. Namely, treatment of macrophages with conditioned medium from mature *S. aureus* biofilms prevented macrophage phagocytosis [21]. *S. aureus* biofilms treated with lysostaphin were no longer able to inhibit macrophage phagocytosis, suggesting that the bioactive molecule(s) was not released following cell lysis. This was confirmed by the fact that conditioned medium from PAS-treated biofilms was still capable of blocking macrophage phagocytosis and was proteinase K sensitive, indicating that secreted proteins are responsible for the observed macrophage dysfunction [16]. However, unpublished observations from our laboratory also suggest that small molecules released via autolysis can escape the biofilm matrix and effect immune cell activity, since conditioned medium from PAS-treated biofilms can also alter macrophage gene expression profiles. Together, these findings suggest that large bacterial PAMPs capable of eliciting robust proinflammatory activity (i.e. eDNA released during autolysis) are buried within the biofilm matrix and are inaccessible to macrophages based on their inability to invade the biofilm, whereas smaller molecules diffuse from the biofilm and, as such, are capable of interacting with proximal immune cells to trigger a non-productive immune response typified by phagocytic impairments and cell death. Therefore, therapeutics targeting biofilm matrix disruption hold interesting potential for augmenting leukocyte microbicidal activity and biofilm clearance.

Part 2 Conclusions

S. aureus biofilms interfere with microbicidal immune responses, in part, by polarizing macrophages toward an anti-inflammatory, pro-fibrotic phenotype. While gene expression profiles in *S. aureus* biofilms are transiently, but globally repressed following macrophage exposure [36], secreted proteins enriched during *S. aureus* biofilm growtn, including α-toxin and LukAB, are capable of inducing macrophage dysfunction [16]. Other potential proteins or molecules responsible for inhibiting macrophage anti-biofilm activity remain to be identified. Additionally, further investigation into the role of biofilm molecules that are actively secreted or released following bacterial lysis that promote MDSC recruitment/activity will shed light on critical biofilm processes that contribute to the establishment of chronic PJIs.

FINAL REMARKS

The work discussed in this review details recent advances in the molecular crosstalk between staphylococcal biofilms and host immune cells (Figure 1). Microbial-immune cell interactions have proven to be important factors during biofilm infections, and these studies help advance the knowledge of how bacteria manipulate host immune responses. Staphylococcal biofilms have evolved effective mechanisms to establish chronic infections, in part, by actively preventing macrophage phagocytosis and proinflammatory activity. Evidence suggests that this is due to the action of MDSCs recruited to the site of staphylococcal PJI, which inhibit monocyte/macrophage proinflammatory action and biofilm clearance. The role of MDSCs on mediating T cell suppression during biofilm formation remains unclear; however, they may play a significant role in the failure to induce

protective adaptive immunity during staphylococcal PJIs. Preventing the suppressive activity of infiltrating MDSCs may prove to be a novel therapeutic strategy to thwart PJIs; however it remains unclear whether biofilm products directly contribute to MDSC accumulation by arresting their maturation or if staphylococcal biofilms cooperate with MDSCs to inhibit immune effector function. These questions represent areas of active investigation in our laboratory.

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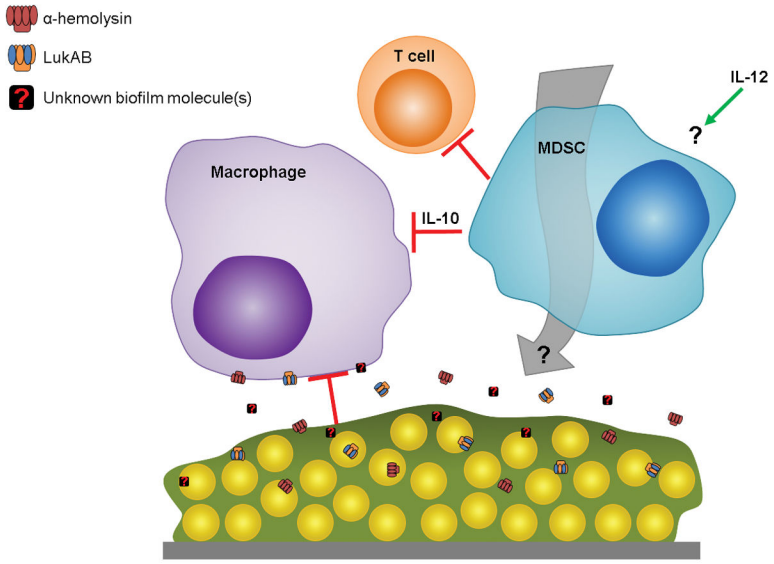

Figure 1. Model for *S. aureus* biofilm immune evasion

Toxins secreted from *S. aureus* biofilms, including α-toxin (Hla) and LukAB, inhibit macrophage microbicidal function and induce cell death. Additional unidentified molecules released from staphylococcal biofilms, either via active secretion or following bacterial cell lysis, likely contribute to maximize inhibition of host antimicrobial activity. MDSC recruitment by a yet unknown mechanism(s) that requires IL-12 suppresses T cell activity and induces a local anti-inflammatory milieu characterized by IL-10 production. Biofilm-mediated immune polarization results in biofilm persistence and chronic disease.