RESEARCH ARTICLE

Staphylococcus aureus Biofilms Induce Macrophage Dysfunction Through Leukocidin AB and Alpha-Toxin

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ABSTRACT **The macrophage response to planktonic *Staphylococcus aureus* involves the induction of proinflammatory microbicidal activity. However, *S. aureus* biofilms can interfere with these responses in part by polarizing macrophages toward an anti-inflammatory profibrotic phenotype. Here we demonstrate that conditioned medium from mature *S. aureus* biofilms inhibited macrophage phagocytosis and induced cytotoxicity, suggesting the involvement of a secreted factor(s). Iterative testing found the active factor(s) to be proteinaceous and partially *agr*-dependent. Quantitative mass spectrometry identified alpha-toxin (Hla) and leukocidin AB (LukAB) as critical molecules secreted by *S. aureus* biofilms that inhibit murine macrophage phagocytosis and promote cytotoxicity. A role for Hla and LukAB was confirmed by using *hla* and *lukAB* mutants, and synergy between the two toxins was demonstrated with a *lukAB hla* double mutant and verified by complementation. Independent confirmation of the effects of Hla and LukAB on macrophage dysfunction was demonstrated by using an isogenic strain in which Hla was constitutively expressed, an Hla antibody to block toxin activity, and purified LukAB peptide. The importance of Hla and LukAB during *S. aureus* biofilm formation *in vivo* was assessed by using a murine orthopedic implant biofilm infection model in which the *lukAB hla* double mutant displayed significantly lower bacterial burdens and more macrophage infiltrates than each single mutant. Collectively, these findings reveal a critical synergistic role for Hla and LukAB in promoting macrophage dysfunction and facilitating *S. aureus* biofilm development *in vivo*.**

IMPORTANCE ***Staphylococcus aureus* has a propensity to form multicellular communities known as biofilms. While growing in a biofilm, *S. aureus* displays increased tolerance to nutrient deprivation, antibiotic insult, and even host immune challenge. Previous studies have shown that *S. aureus* biofilms thwart host immunity in part by preventing macrophage phagocytosis. It remained unclear whether this was influenced solely by the considerable size of biofilms or whether molecules were also actively secreted to circumvent macrophage-mediated phagocytosis. This is the first report to demonstrate that *S. aureus* biofilms inhibit macrophage phagocytosis and induce macrophage death through the combined action of leukocidin AB and alpha-toxin. Loss of leukocidin AB and alpha-toxin expression resulted in enhanced *S. aureus* biofilm clearance in a mouse model of orthopedic implant infection, suggesting that these toxins could be targeted therapeutically to facilitate biofilm clearance in humans.**

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Highly opportunistic pathogens possess attributes that facilitate persistent infections in part by shielding themselves from immune-mediated attack (1–3). *Staphylococcus aureus* is one such example, and in addition to its well-known arsenal of virulence determinants, biofilm formation represents another means to circumvent immune-mediated clearance in the host (4, 5). Biofilms are heterogeneous bacterial communities encased in a complex matrix composed of extracellular DNA, proteins, and polysaccharides (6–9). *S. aureus* has a propensity to form biofilms on medical devices, such as prostheses and indwelling catheters, and the organism remains a major cause of health care- and community-associated infections (10–12).

Many *S. aureus* virulence factors target innate immune pathways that are elicited during acute planktonic infection, such as phagocytosis and proinflammatory transcription factor activation (4, 13–15). Phagocytosis leads to the killing of extracellular pathogens, as well as proinflammatory cytokine and chemokine production, which collectively orchestrate local and systemic inflammatory responses and initiate adaptive immunity (16–18). Recent studies have demonstrated that biofilms formed by various bacterial species interfere with classical host antibacterial effector mechanisms (19–24). With regard to *S. aureus*, work in our laboratory and others has shown that biofilms polarize macrophages toward an anti-inflammatory phenotype by dampening proinflammatory responses and limiting macrophage invasion *in vivo* (4, 15, 25–27). This response is considered detrimental to biofilm

clearance, since polarized macrophages possess poor microbicidal activity and instead promote fibrosis (4). Similar findings of macrophage dysfunction have been reported in response to *S. epidermidis* biofilms (28–30), suggesting the existence of a conserved effort to thwart efficient biofilm recognition and clearance by the host. However, the molecules responsible for the ability of *S. aureus* biofilms to attenuate macrophage proinflammatory responses remain ill defined.

The objective of this study was to identify *S. aureus* biofilm-derived products that induce macrophage dysfunction and facilitate biofilm persistence. Quantitative mass spectrometry identified alpha-toxin (Hla) and the bicomponent leukotoxin leukocidin AB (LukAB, also known as LukGH) as potential candidates responsible for inhibition of macrophage phagocytosis and promotion of cytotoxicity, which was confirmed by using *hla* and *lukAB* mutants. A synergistic effect was demonstrated with a *lukAB hla* double mutant that also revealed decreased biofilm formation *in vivo* in a murine model of orthopedic implant biofilm infection. The reduction in macrophage phagocytosis, concomitant with enhanced cell death, likely facilitates the ability of *S. aureus* to avoid destructive host responses when organized as a biofilm.

RESULTS

***S. aureus* biofilms secrete a proteinaceous factor(s) that inhibits macrophage phagocytosis.** Our previous studies demonstrated that macrophages are unable to phagocytose *S. aureus* biofilms (4, 15); however, the mechanism responsible for this phenomenon remained to be identified. While it is known that the physical size of a biofilm is one factor that impedes phagocytosis (4), we investigated the possibility that a secreted factor(s) is also involved. In order to assess the effect of biofilm-conditioned medium on macrophage phagocytosis, we utilized fluorescent microspheres instead of live bacteria, since live *S. aureus* actively secretes factors during planktonic growth that would have been impossible to differentiate from biofilm-derived molecules. Using this approach, we were able to readily distinguish differences in phagocytosis and viability of murine macrophages exposed to fresh medium (Fig. 1A), *S. aureus* biofilm-conditioned medium (Fig. 1B), and *S. aureus* planktonic culture-conditioned medium (Fig. 1C) by confocal microscopy. Of note, similar effects of *S. aureus* biofilm-conditioned medium on macrophage phagocytosis were obtained with fluorescent microspheres and intact *S. aureus* in pilot studies (see Fig. S1 in the supplemental material), supporting the validity of this approach. Macrophage phagocytic activity was significantly reduced after treatment with conditioned medium from intact biofilms of the methicillin-resistant *S. aureus* (MRSA) clinical isolate USA300 LAC (31–34) (Fig. 1B and D), revealing a role for an extracellular factor(s). To determine whether this effect relied on an intact biofilm structure, fresh medium was added to mature biofilms that were disrupted by trituration, whereupon conditioned medium was harvested 24 h later. Treatment of macrophages with supernatants collected from disrupted biofilms had less of an impact on phagocytosis (Fig. 1D), suggesting that the putative extracellular factor(s) is enriched in intact biofilms, perhaps via a quorum-sensing system that is disturbed upon destruction of the biofilm architecture. Similarly, conditioned medium from planktonic organisms was less effective at blocking macrophage phagocytosis (Fig. 1C and D), even when cultures were grown to a high cell density (i.e., late stationary phase; data not shown), demonstrating the enrichment of this secreted factor(s) in intact biofilms. Importantly, these differences did not result from alterations in bacterial density or secreted protein levels, since titers and extracellular protein concentrations of intact biofilms, disrupted biofilms, and planktonic cultures were similar (see Fig. S2 in the supplemental material; data not shown).

Whereas little information is currently available regarding the *S. aureus* biofilm secretome, the importance of autolysis to biofilm formation has been well established (6, 35–39). To determine whether the putative biofilm extracellular factor(s) was actively secreted or a byproduct of cell lysis, mature biofilms were treated for 24 h with polyanethole sodium sulfanate (PAS) to inhibit lysis (40) or disrupted by trituration and treated with lysostaphin to artificially induce lysis. Only conditioned medium from PAS-treated biofilms maintained inhibitory activity (Fig. 1F), suggesting that *S. aureus* biofilms actively secrete a molecule(s) that impedes macrophage phagocytosis. To elucidate the chemical nature of the secreted inhibitory factor(s), conditioned medium from intact biofilms was treated with proteinase K prior to macrophage addition. Proteinase K completely ablated the inhibitory effect of *S. aureus* biofilm-conditioned medium on macrophage phagocytosis, implicating the action of a protein(s) in this phenomenon (Fig. 1H).

In addition to impaired phagocytosis, our prior report demonstrated that *S. aureus* biofilms also induced macrophage cytotoxicity (4). This could result from frustrated phagocytosis based on the inability of macrophages to physically engulf the bulky biofilm structure combined with the action of secreted toxins, such as Hla or leukocidins with known cytotoxic activity (13, 18, 41). Exposure of murine macrophages to conditioned medium from intact *S. aureus* biofilms induced significant cell death, whereas minimal cytotoxicity was observed following treatment with medium from either disrupted biofilms or planktonic *S. aureus* (Fig. 1A to C and E). Similar to the approach employed for phagocytosis, biofilms were treated with lysostaphin or PAS, where only lysostaphin prevented the cytotoxic effects of biofilm-conditioned medium (Fig. 1G), again revealing the action of an actively secreted protein based on its proteinase K-sensitive nature (Fig. 1I).

***S. aureus* biofilm-induced macrophage dysfunction is partially *agr*-dependent.** Our findings that disrupted biofilms were not as effective at blocking macrophage phagocytosis or inducing cytotoxicity suggested that quorum-sensing systems enriched during biofilm formation may regulate the putative inhibitory molecule(s). The expression of numerous virulence factors in *S. aureus*, including secreted proteases, leukocidins, and Hla, is either directly or indirectly influenced by two-component regulatory systems, such as the *agr* quorum-sensing system (42). *agr* modulates virulence factor expression and is an important regulatory switch between planktonic and biofilm lifestyles in *S. aureus* (43–47). Conditioned medium from a Δ*agr* mutant biofilm induced minimal macrophage cell death (Fig. 2B), whereas the phagocytic block was significantly attenuated but only partially influenced by *agr* (Fig. 2). Since the macrophage-inhibitory phenotypes upon exposure to *S. aureus* biofilms were partially *agr*-dependent, the Δ*agr* mutant strain was utilized for subsequent proteomic comparisons with wild-type (WT) biofilms in an attempt to identify secreted proteins enriched during biofilm growth that are capable of inducing macrophage dysfunction.

SWATH-MS as a tool to identify *S. aureus* biofilm factors that induce macrophage dysfunction. We next employed a pro-

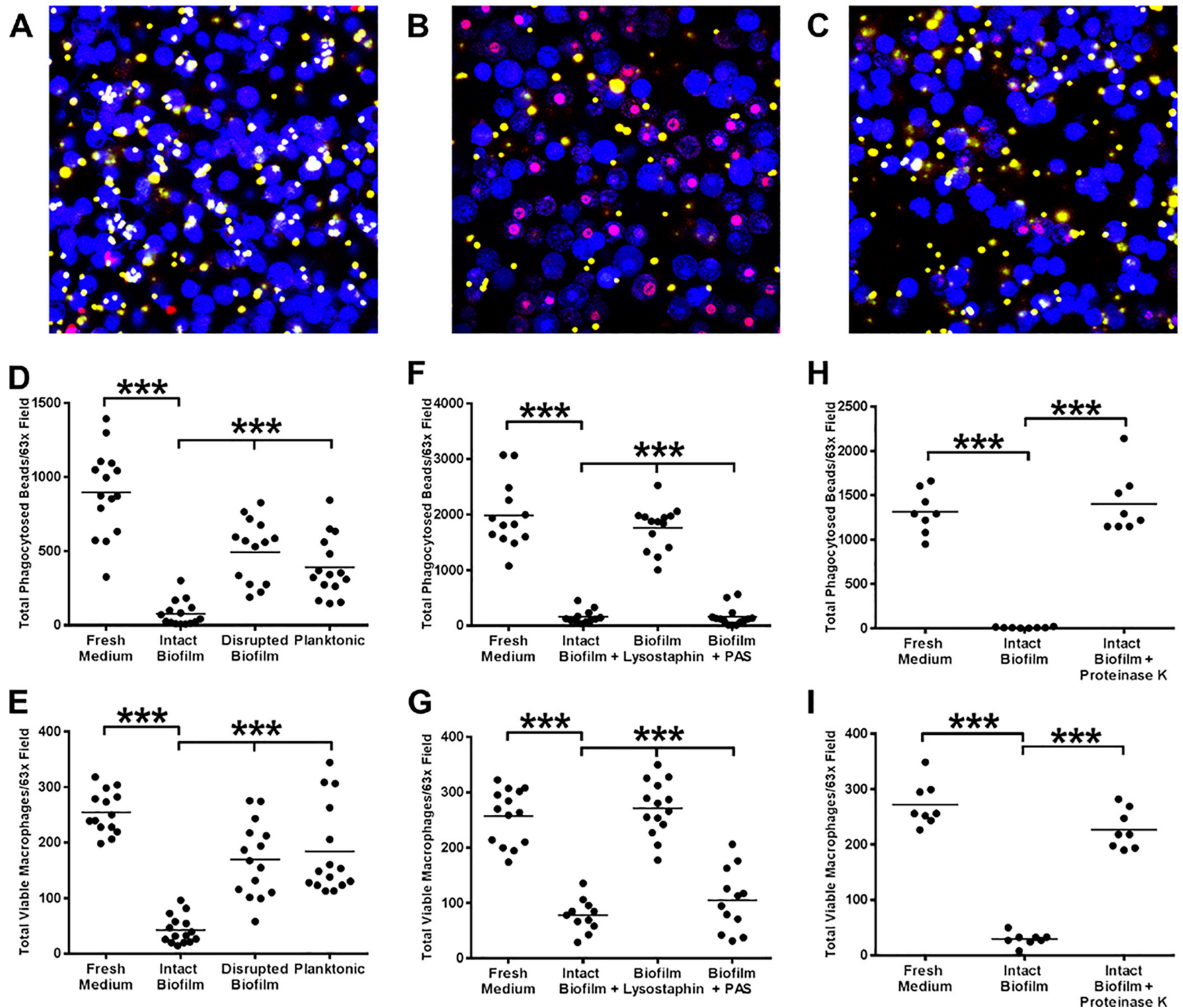

FIG 1 *S. aureus* biofilms secrete a proteinaceous factor(s) that inhibits macrophage phagocytosis. (A to C) Representative two-dimensional confocal images (×63) of bone marrow-derived macrophage phagocytosis of fluorescent microspheres (yellow-white) and cell death with PI stain (red-purple) after exposure to fresh medium (A), *S. aureus* biofilm-conditioned medium (B), or *S. aureus* planktonic culture-conditioned medium (C). (D and E) Bone marrow-derived macrophages were exposed to fresh medium or conditioned medium collected from an intact biofilm, a mature biofilm that was mechanically disrupted, or a similar number of planktonic *S. aureus* cells. After a 3-h treatment period, macrophage phagocytosis of fluorescent microspheres (D) and cell viability (E) were quantitated by confocal microscopy. (F and G) Conditioned medium from biofilms treated with either PAS (10 μg/ml) or lysostaphin (50 μg/ml) was added to macrophages to assess the relative importance of active biofilm secretion versus passive release of products via autolysis, respectively, on macrophage phagocytosis (F) and cell death (G). (H and I) Biofilm-conditioned supernatants were treated with proteinase K (10 μg/ml) prior to macrophage exposure to assess the chemical nature of the inhibitory molecule(s). Significant differences are denoted by asterisks (***, $P < 0.001$; one-way ANOVA, followed by Bonferroni's multiple-comparison test). Results are representative of at least two independent experiments.

teomic approach to identify candidate molecules that might be responsible for biofilm-mediated macrophage dysfunction. Our proteomic strategy utilized the Δ*agr* mutant strain as a comparator with WT biofilm, since the macrophage-inhibitory phenotypes were partially *agr*-dependent (Fig. 2). A second comparison between biofilm and planktonic conditions was done because the macrophage-inhibitory factors were enriched during biofilm growth (Fig. 1). To identify differentially expressed proteins in conditioned medium from WT and *agr* mutant biofilms and planktonic cultures, trichloroacetic acid (TCA)-precipitated proteins were analyzed by quantitative sequential windowed data-independent acquisition of the total high-resolution mass spectra (SWATH-MS) (48). As expected, conditioned medium from WT and Δ*agr* mutant biofilms displayed vastly different proteomic profiles, with 68 (44%) of 153 proteins significantly enriched in WT biofilms, 23% of which were either secreted proteases or known virulence factors, such as Hla (Fig. 3A and B; see Table S1 in the supplemental material). In contrast, cell wall and structural proteins were more abundant in Δ*agr* mutant biofilm-conditioned medium than in WT biofilm-conditioned medium

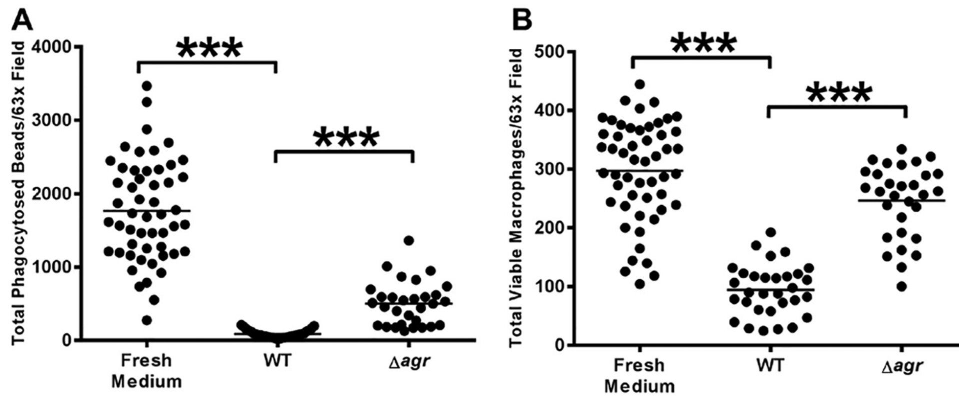

FIG 2 *S. aureus* biofilm-induced macrophage dysfunction is partially *agr*-dependent. Bone marrow-derived macrophages were exposed to fresh or conditioned medium from *S. aureus* WT or isogenic Δ*agr* mutant biofilms for 2 h, whereupon phagocytosis of fluorescent microspheres (A) and total viable macrophages (B) were quantitated by confocal microscopy. Significant differences are denoted by asterisks (***, $P < 0.001$; unpaired two-tailed Student *t* test). Results are representative of at least three independent experiments.

(Fig. 3A and B). Similarly, proteomic comparisons showed that conditioned medium from WT biofilm and WT planktonic cultures differed significantly, with 108 (36%) of 301 proteins enriched in WT biofilm, including several secreted virulence factors such as toxins and proteases (see Table S2 in the supplemental material). A functional proteomic network constructed with overlapping hits from both comparisons (WT biofilm versus Δ*agr* mutant biofilm and WT biofilm versus WT planktonic culture) identified 17 proteins, including two serine proteases and two leukocidin components, as candidates to account for the inhibitory effects of biofilm-conditioned medium on macrophage function (Fig. 3C). Additionally, Hla was significantly more enriched

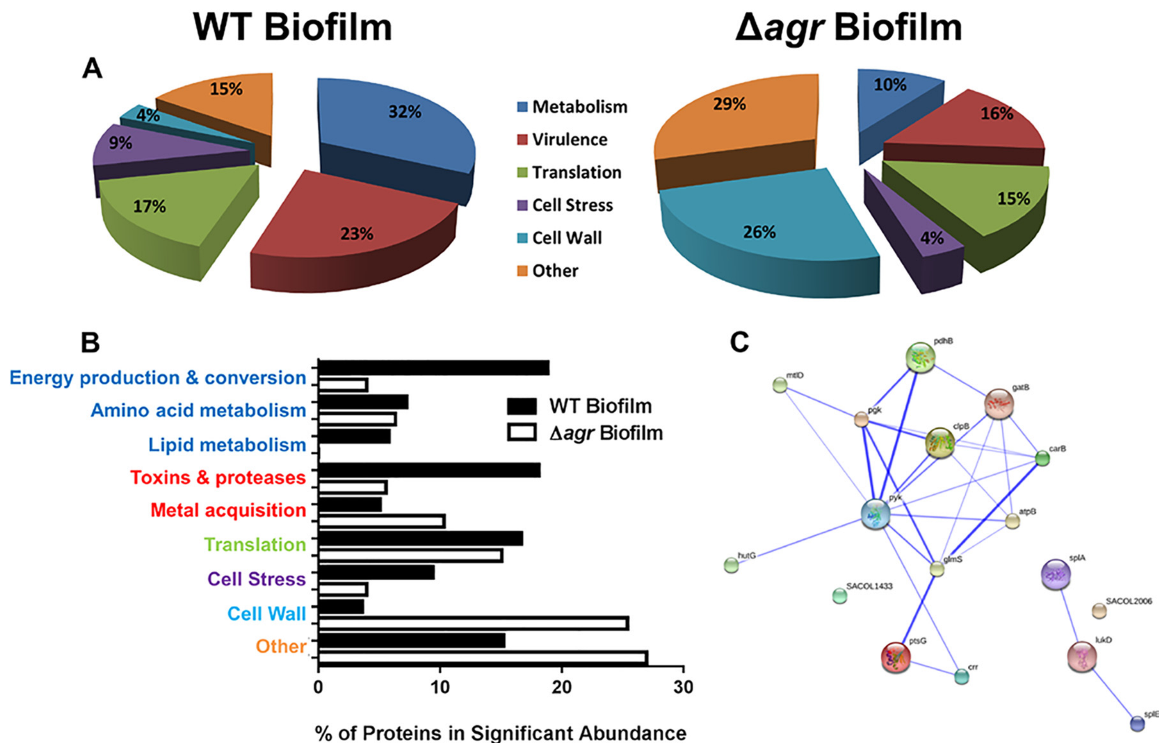

FIG 3 SWATH-MS identifies potential biofilm factors responsible for macrophage dysfunction. Conditioned supernatant from either WT *S. aureus* or a Δ*agr* mutant strain grown under planktonic or biofilm conditions was harvested and TCA precipitated for protein isolation in triplicate. Relative protein concentrations in sample sets were compared by SWATH-MS. (A) Sixty-eight (44%) of 153 identified proteins were significantly more enriched in WT than Δ*agr* mutant biofilm, with the largest percentage associated with metabolism and virulence. Eighty-five (56%) of 153 identified proteins were significantly more enriched in Δ*agr* mutant than WT biofilm, with the largest percentage of proteins falling into the functional category of cell wall proteins. (B) Direct comparison of significantly expressed proteins in WT and Δ*agr* mutant biofilms by functional category, with more-refined groups of metabolism and virulence shown. (C) Functional-association network of proteins identified by SWATH-MS as significantly more enriched in WT biofilm-conditioned supernatant than in planktonic and Δ*agr* mutant biofilm-conditioned supernatants. For all comparisons, statistical significance was assessed with a *Z* test (***, $P < 0.05$).

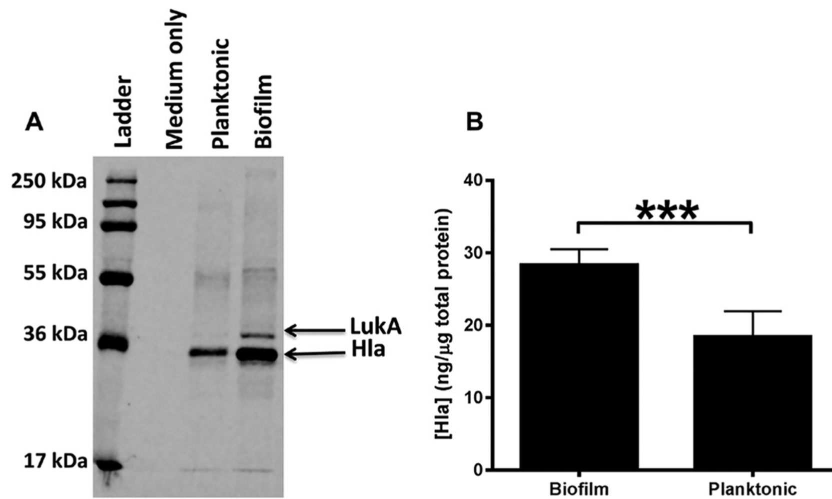

FIG 4 LukA and Hla secretion is enhanced in *S. aureus* biofilms. (A) LukA and Hla levels in *S. aureus* biofilm- and planktonic culture-conditioned media were assessed by Western blot assays. (B) Quantitation of Hla levels in conditioned medium from WT *S. aureus* biofilms and planktonic bacteria. Significant differences are denoted by asterisks (***, $P < 0.001$; unpaired two-tailed Student *t* test). Results are representative of at least two independent experiments.

in WT than in Δ*agr* mutant biofilms (see Table S1), which represented another toxin of interest for its potential role in the regulation of macrophage dysfunction. Importantly, both Hla and LukAB were significantly more enriched in biofilm-conditioned medium than in planktonic culture-conditioned medium (Fig. 4), suggesting that these two proteins may be responsible for biofilm-induced macrophage dysfunction. LukAB is a bicomponent leukotoxin also involved in *S. aureus*-mediated killing of host phagocytes (49–51). Therefore, Hla and LukAB were the focus of subsequent mechanistic studies, as they likely influence macrophage dysfunction and death in this setting.

LukAB and Hla play significant roles in biofilm-induced macrophage dysfunction. Biofilm-conditioned medium from both Δ*lukA* and Δ*lukB* mutant strains elicited minimal macrophage death, in contrast to WT biofilm-conditioned medium (Fig. 5B). Likewise, the phagocytic block induced by the Δ*lukA* and Δ*lukB* mutant strains was less pronounced than that induced by WT biofilm-conditioned medium, although phagocytosis did not return to baseline levels (Fig. 5A). Both the Δ*lukA* and Δ*lukB* mutant phenotypes could be complemented, providing direct evidence of the involvement of LukAB in the modulation of macrophage survival and phagocytosis (Fig. 5A and B). In contrast, while serine proteases were also shown to be elevated by SWATH-MS, biofilm-conditioned medium from serine protease mutants (Δ*spl* or a Δ*aur spl sspAB scpA* quad mutant), as well as another leukocidin mutant (Δ*lukD*), behaved similarly to WT biofilm-conditioned medium (data not shown).

Since LukAB did not account for the entire macrophage dysfunction phenotype and SWATH-MS identified increased Hla levels in WT biofilm-conditioned medium (see Table S1 in the supplemental material), we next examined the contribution of Hla to macrophage dysfunction. Further justification for investigating Hla stemmed from the vast literature on Hla regulation by the *agr* quorum-sensing system (41, 52–54), the finding that conditioned medium from Δ*agr* mutant biofilms was less effective at inducing macrophage dysfunction (Fig. 2), and the fact that Hla secretion was significantly increased during biofilm growth (Fig. 4). Hla inserts itself into host cell membranes and oligomerizes to form pores, leading to cell death (41, 55). Indeed, macrophage survival was significantly improved following exposure to Δ*hla* mutant biofilm-conditioned medium compared to WT biofilm-conditioned medium, which was complementable (Fig. 5D). The effects of Δ*hla* on macrophage phagocytosis were less pronounced but still reached statistical significance (Fig. 5C). Furthermore, blockade of Hla activity in WT biofilm-conditioned medium with an Hla-neutralizing antibody (Ab) phenocopied the findings with Δ*hla* mutant biofilm-conditioned medium (Fig. 5C and D). The specificity of the Hla Ab was demonstrated by its ability to inhibit the effects of purified Hla on macrophage survival and viability (see Fig. S3 in the supplemental material). Additional evidence to support the action of Hla was provided by the ability of biofilm-conditioned medium from a *S. aureus* strain that constitutively expresses *hla* (hla^{on}) to induce significant macrophage death and inhibit phagocytosis (Fig. 5C and D).

The expression of both Hla and LukAB was markedly greater in conditioned medium from WT biofilms than in conditioned medium from planktonic bacteria (Fig. 4). Therefore, to assess whether LukAB and Hla act cooperatively to effect macrophage activity, Δ*lukA* and Δ*lukB* mutant biofilm-conditioned media were treated with an Hla-neutralizing Ab (Fig. 6). Interestingly, negating the action of Hla in Δ*lukA* and Δ*lukB* mutant biofilm-conditioned media significantly improved macrophage phagocytosis over that in supernatants where Hla was active (Fig. 6A). Similar findings were obtained with a Δ*lukAB* Δ*hla* double mutant (Fig. 6A). Hla blockade in Δ*lukA* and Δ*lukB* mutant biofilm-conditioned media had no additional effect on macrophage survival, which was not unexpected since viability had nearly been restored with each of the single mutants to levels observed in fresh medium (Fig. 6B). When macrophages were treated with Δ*lukAB* mutant biofilm-conditioned medium (which still produces Hla) in combination with purified LukAB or a point mutant that lacks lytic activity ($LukAB^{E323A}$) (56), only bioactive LukAB returned both phagocytic inhibition and cytotoxicity to levels observed in WT biofilm-conditioned medium (see Fig. S4 in the supplemental

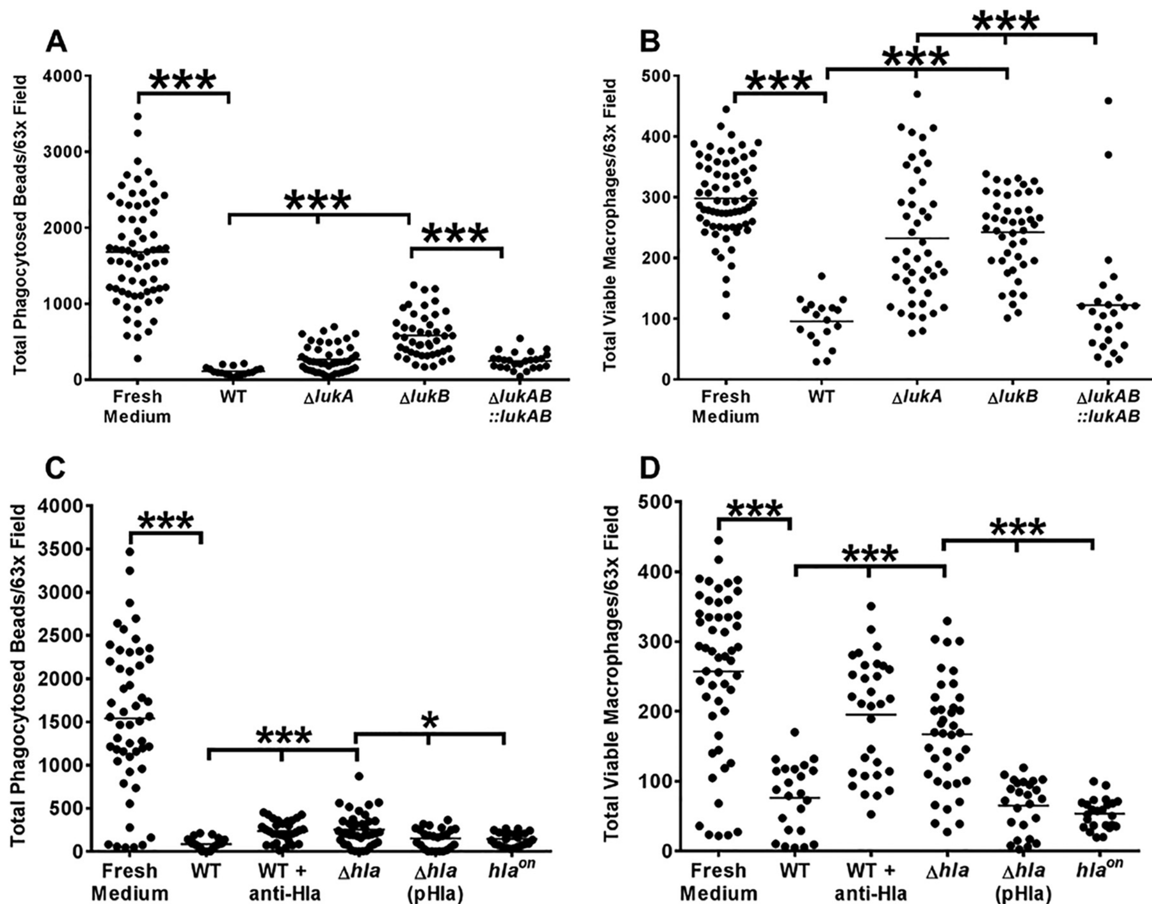

FIG 5 LukAB and Hla play significant roles in biofilm-induced macrophage dysfunction. (A and B) Bone marrow-derived macrophages were exposed to fresh or conditioned medium from WT *S. aureus*, Δ*lukA* mutant, Δ*lukB* mutant, or chromosomally complemented Δ*lukAB* mutant (Δ*lukAB*::*lukAB*) biofilms. After a 3-h treatment period, phagocytosis of fluorescent microspheres (A) and viable macrophages (B) were quantitated by confocal microscopy. (C and D) Bone marrow-derived macrophages were exposed to fresh or conditioned medium from *S. aureus* WT biofilm plus Hla Ab (α-Hla), Δ*hla* mutant, plasmid-complemented Δ*hla* mutant [Δ*hla*(pHla)], and constitutively expressed *hla* (*hla*^{on}) biofilms. After a 3-h treatment period, phagocytosis of fluorescent microspheres (C) and viable macrophages (D) were quantitated by confocal microscopy. Significant differences are denoted by asterisks (*, $P < 0.05$; ***, $P < 0.001$; unpaired two-tailed Student *t* test). Results are representative of at least three independent experiments.

material). Collectively, these results demonstrate that LukAB acts in concert with Hla to induce macrophage dysfunction.

LukAB and Hla are important for *S. aureus* biofilm formation *in vivo*. Previous work in our laboratory demonstrated that augmentation of macrophage proinflammatory activity is critical for biofilm clearance *in vivo* (15). To determine whether the identified functional role for LukAB and Hla in mediating macrophage dysfunction *in vitro* would impact biofilm formation *in vivo*, we utilized a murine model of *S. aureus* orthopedic implant biofilm infection (57–59). Similar to our *in vitro* studies revealing cooperation between LukAB and Hla, the Δ*lukAB* Δ*hla* double mutant displayed the greatest reduction in bacterial burdens in the knee joint, surrounding soft tissue, and femur at days 3 and 7 postinfection, in contrast to Δ*lukAB* or Δ*hla* mutants (Fig. 7A to C). Furthermore, macrophage infiltrates were significantly increased in mice infected with the Δ*lukAB* Δ*hla* double mutant (Fig. 7D), although they represent a minor population in this model of orthopedic implant biofilm infection, which is dominated by myeloid-derived suppressor cells (58, 59). The observed increase in macrophages may result from less cytotoxicity by the combined loss of LukAB and Hla, supporting our in vitro studies. Taken together, these results identify LukAB and Hla as important virulence factors in the modulation of bacterial persistence and macrophage infiltrates during *S. aureus* biofilm formation *in vivo*.

DISCUSSION

S. aureus subverts the host immune response by numerous mechanisms, including increased resistance to cationic antimicrobial peptides, impairment of phagocyte recruitment, interference with Ab-mediated opsonization and complement activation, and resistance to intracellular killing (13). In addition, biofilm formation further protects *S. aureus* from the host innate immune response, representing a communal virulence determinant (4, 25, 60). We have previously demonstrated that biofilm formation shields *S. aureus* from Toll-like receptor detection and interferes with macrophage activation *in vivo* (4, 15). Here we explored the genetic basis of how biofilm growth prevents macrophage phagocytosis. Our earlier study showed that macrophages are capable of phagocytosing bacteria from mechanically disrupted, but not intact, biofilms, suggesting that the size of the biofilm and/or the density of its matrix represents a physical obstacle, a phenomenon referred to as "frustrated phagocytosis" (4, 61, 62). Here we extend

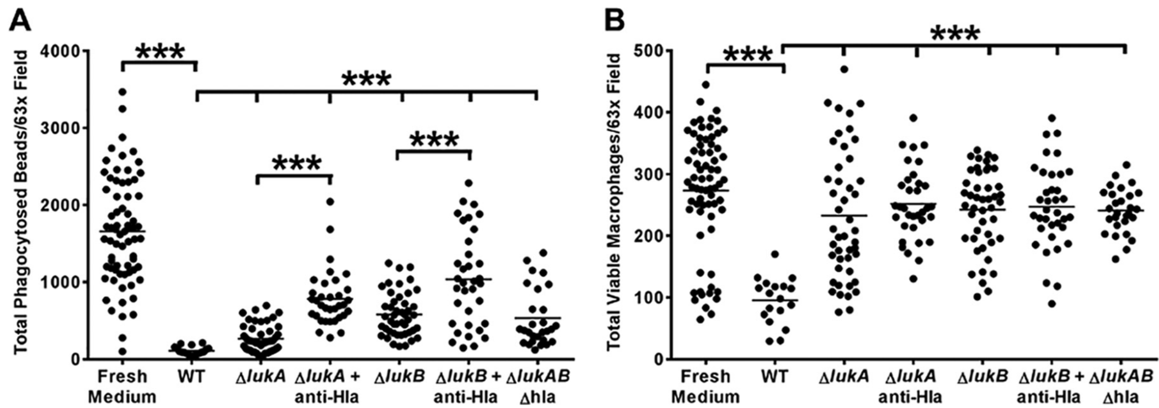

FIG 6 *S. aureus* Hla and LukAB act in concert to promote macrophage dysfunction. Bone marrow-derived macrophages were exposed to fresh or conditioned medium from *S. aureus* WT or isogenic Δ*lukA* mutant, Δ*lukB* mutant, and Δ*lukAB hla* double mutant biofilms plus Hla Ab (α-Hla). After a 3-h treatment period, phagocytosis of fluorescent microspheres (A) and viable macrophages (B) were quantitated by confocal microscopy. Significant differences are denoted by asterisks (***, $P < 0.001$; unpaired two-tailed Student *t* test). Results are representative of at least three independent experiments.

these findings to demonstrate that *S. aureus* biofilms also secrete proteinaceous factors that actively inhibit macrophage phagocytosis and induce cell death. Interestingly, this proteinaceous component was evident mainly in intact biofilms, as conditioned medium from mechanically disrupted biofilms or planktonic cultures grown to early or late stationary phase failed to prevent phagocytosis to the same extent, although the bacterial numbers and secreted protein concentrations were similar (data not shown; Fig. 1D; see Fig. S2 in the supplemental material). The preferential ability of intact *S. aureus* biofilms to inhibit macrophage phagocytosis suggested the involvement of quorum-sensing mechanisms that are enriched during biofilm growth and dissipate once the biofilm structure has been disrupted. This was confirmed by the finding that biofilm-mediated macrophage dysfunction, in

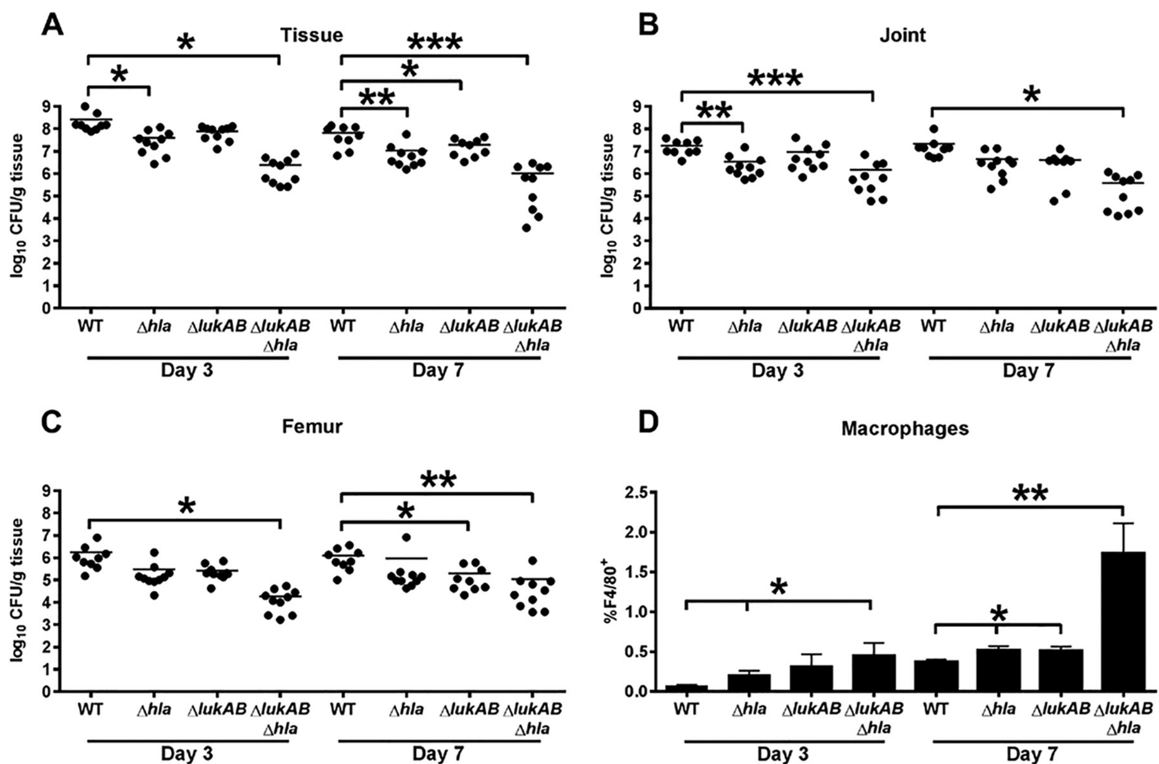

FIG 7 LukAB and Hla are important for *S. aureus* biofilm formation *in vivo*. Shown are the bacterial burdens associated with the soft tissue surrounding the knee (A), knee joint (B), and femur (C) of mice infected with WT *S. aureus* and isogenic Δ*hla*, Δ*lukAB*, and Δ*lukAB hla* mutant strains at days 3 and 7 postinfection ($n = 10$ mice per strain for each time point). Results are expressed as the number of CFU per gram of tissue to correct for differences in tissue sample size. (D) Quantitation of $F4/80^+$ macrophages infiltrating the soft tissue of mice infected with WT *S. aureus* and isogenic Δ*hla*, Δ*lukAB*, and Δ*lukAB hla* mutant strains. Significant differences are denoted by asterisks (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired two-tailed Student *t* test). Results are combined from two independent experiments.

particular, cell death, was less pronounced following exposure to conditioned medium from a Δ*agr* mutant biofilm. These observations, combined with the fact that conditioned medium from PAS-treated biofilms maintained inhibitory activity whereas lysostaphin-treated biofilms did not, strongly implied the importance of the ability of active protein secretion by the biofilm (based on proteinase K sensitivity) to inhibit macrophage phagocytosis and induce cell death.

The identification of candidate proteins responsible for inducing macrophage death and inhibiting phagocytosis was facilitated by a relatively new quantitative mass spectrometry technique, namely, SWATH-MS (48). After generating a protein library from our combined sample sets (i.e., WT biofilm, Δ*agr* mutant biofilm, WT planktonic, and Δ*agr* mutant planktonic), comparisons were performed to identify the most abundant proteins unique to WT biofilm-conditioned medium. While this list included some proteins undoubtedly released as a result of cell lysis (i.e., metabolic enzymes and ribosomal subunits), we focused on known secreted toxins and proteases that were also detected. Of the proteins on this list, LukB and its partner component LukA were shown to have a significant impact on macrophage phagocytosis and viability. LukAB is unique among leukocidins for its ability to either remain cell wall associated or be released into the extracellular milieu (49–51). A recent study has shown that human leukocytes are exquisitely sensitive to the cytolytic actions of LukAB because of its specificity for CD11b (63) mediated by the binding of a specific glutamic acid residue (323A) (56). While murine leukocytes are less sensitive to LukAB (63, 64), this toxin was still implicated in *S. aureus* pathogenesis in a murine renal abscess model (50), which was confirmed in the present study by using a murine *S. aureus* orthopedic implant biofilm model. Therefore, although human cells display a greater sensitivity to LukAB, it is clear from our report and work by others that this bicomponent leukotoxin is also active toward murine leukocytes.

In addition to LukAB, Hla also significantly contributed to biofilm-associated murine macrophage death and phagocytosis. The toxic effects of Hla are well known, and while a recent publication has demonstrated the cytoprotective effects of *S. aureus* Hla within phagosomes (65), it is important to note that this scenario is not applicable to our studies, given that our phagocytosis assay utilized microspheres and not viable bacteria. This strategy was employed to avoid confounding effects of toxins secreted by live planktonic *S. aureus* if they were used to measure macrophage phagocytosis, which could not be discriminated from biofilm-secreted molecules. Interestingly, the already potent cytolytic effects of *S. aureus* Hla were enhanced with the addition of purified bioactive LukAB. Furthermore, treatment of Δ*lukA* and Δ*lukB* mutant biofilm-conditioned media with an Hla-neutralizing Ab significantly dampened the macrophage phagocytic block. These results suggest a synergistic effect whereby the presence of LukAB enhances or accelerates Hla-mediated macrophage dysfunction, perhaps via enhanced binding, localization to the cell membrane, or regulation of intracellular signaling pathways.

Previous studies have demonstrated the importance of LukAB or Hla for *S. aureus* pathogenesis in murine models of renal abscess (50), pneumonia (66, 67), skin infection (68, 69), bacteremia (70, 71), peritonitis (69, 72, 73), and other localized infection models (41). However, it should be noted that a Δ*lukAB* single mutant displayed no attenuation of virulence in murine models of skin infection and bacteremia (64). In support of our *in vitro* findings, our study is the first to report that LukAB and Hla cooperate to regulate *S. aureus* virulence in a murine orthopedic implant biofilm infection model. While a Δ*lukAB* or Δ*hla* single mutant displayed decreased bacterial burdens in some tissues, a Δ*lukAB* Δ*hla* double mutant showed the largest reduction in bacterial numbers compared to mice infected with the isogenic WT strain. In further support of this synergistic effect, Δ*lukAB* Δ*hla* mutant-infected mice displayed the greatest increase in macrophage infiltrates. While these *in vivo* data reveal an important synergistic role for LukAB and Hla during *S. aureus* biofilm infection, it remains unclear whether these toxins directly alter macrophage survival (i.e., via cell lysis) or indirectly tailor the immune response (i.e., elicit tissue damage resulting in altered cytokine signaling to promote macrophage phagocytosis and proinflammatory activity). We also found that biofilm-conditioned medium elicited similar cytotoxic effects on proinflammatory (classically activated) and profibrotic (alternatively activated) macrophages (data not shown). We further investigated whether biofilm-conditioned medium augmented macrophage CD11b expression, which binds LukAB, but found no evidence to support this possibility. Another potential mechanism to link the synergistic effects of Hla and LukAB is the zinc-dependent metalloproteinase ADAM10, since Hla is known to recognize ADAM10 on the host cell surface (74). Once bound, Hla augments ADAM10 activity (75), which could result in increased LukAB dissociation from the bacterial cell surface and, in turn, enhanced Hla activity. However, it should be noted that this interaction may provide an explanation for our *in vivo* findings but fails to inform the apparent synergistic effect in our *in vitro* assay, since macrophages were treated with biofilm-conditioned medium cleared of bacteria. The mechanism whereby LukAB and Hla influence biofilm formation *in vivo* is an area of active investigation in our laboratory.

While the effect of *S. aureus* biofilm-conditioned medium on macrophage viability was largely LukAB/Hla dependent, it appears that part of the phagocytic block was not. SWATH-MS identified other potential candidate proteins that could act in concert with Hla/LukAB to maximally impair macrophage phagocytosis, including pyrimidine biosynthetic enzymes, phosphotransferase proteins, pyruvate kinase, and histidine metabolic enzymes. Along these lines, it is important to recognize that biofilms represent a diverse bacterial population influenced by a myriad of complex gradients (e.g., nutrient, oxygen, pH), metabolic activity, and virulence potential (76–79). For example, while our studies utilized conditioned medium collected from static biofilms, a subpopulation of planktonic or "dispersed" cells is also present at the air-liquid interface. While this was a 2- to 3-log smaller cell population than that in the biofilm (see Fig. S5 in the supplemental material), it is probably naive to disregard its impact, particularly in light of recent studies demonstrating the secretory potential of biofilm-dispersed cells (80). On the basis of this evidence, we posit that *S. aureus* biofilms prevent macrophage phagocytosis in part by inducing cell death through LukAB and Hla production (Fig. 8). However, since the phagocytic block was still evident even when macrophage viability was restored to 100% following LukAB/Hla inactivation, this suggests that additional proteins act together with Hla/LukAB to maximally inhibit macrophage phagocytic activity (Fig. 8).

Collectively, this study demonstrates that *S. aureus* biofilms have evolved mechanisms to establish persistent infections in part by actively preventing macrophage phagocytosis and eliciting cell

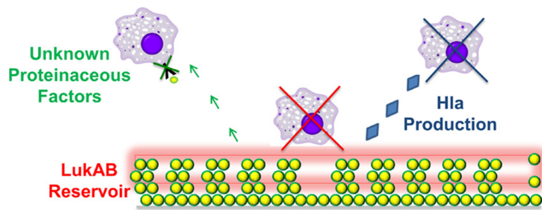

FIG 8 Proposed model of *S. aureus* biofilm-induced macrophage dysfunction. *S. aureus* biofilms produce an abundance of LukAB, which can associate with the cell surface as a toxin reservoir or be actively secreted into the extracellular milieu. Biofilms also secrete Hla, which acts synergistically with LukAB to elicit macrophage dysfunction. Other secreted proteins may also impact macrophage phagocytosis.

death that is mediated by the synergistic actions of LukAB and Hla. These findings not only identify a novel interaction for these secreted proteins but also highlight the layers of redundancy within the *S. aureus* virulence repertoire.

MATERIALS AND METHODS

Animals. Male C57BL/6 mice (8 weeks of age) were obtained from Charles River Laboratories (Frederick, MD). This study was conducted in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Institutional Animal Care and Use Committee of the University of Nebraska Medical Center.

***S. aureus* strains.** MRSA clinical isolate USA300 LAC 13C (31–34) was cured of its 27-kb LAC-p03 plasmid encoding erythromycin resistance (81) by screening for spontaneous erythromycin sensitivity as previously described (35). The isogenic LAC 13C Δ*agr* mutant strain was generated as previously reported (9). The isogenic LAC 13C Δ*hla* mutant strain was constructed by insertion mutation by site-directed mutagenesis with the pE194 erythromycin resistance cassette (*ermB*) as previously described (72), with an *hla*-complemented strain (72) and an *hla* constitutively active strain (82) included to confirm the specificity of toxin action. The USA300 JE2 Nebraska Transposon Mutant Library (NTML) (83) Δ*lukA* Δ*lukH*, Δ*lukB* Δ*lukG*, Δ*lukD*, and Δ*spl* mutants were moved to the USA300 LAC 13C background by transduction with bacteriophage φ11. In addition, allelic-replacement *lukA* and *lukB* mutants and complemented strains were used to confirm findings obtained with the transposon mutants (50). Importantly, all of the mutant strains utilized in these studies grew to comparable extents during the 6-day biofilm culture period and also secreted similar concentrations of extracellular proteins (see Fig. S2 in the supplemental material).

Preparation of biofilm-conditioned media and treatments. *S. aureus* static biofilms were formed on two-well glass chamber slides (Nunc, Rochester, NY) or 12-well plates (Becton, Dickinson, Franklin Lakes, NJ) in RPMI 1640 medium supplemented with 1% Casamino Acids (CAA; Becton, Dickinson) as previously described (4, 84, 85). Spent medium was replaced daily, whereupon conditioned medium for experiments was collected from 6-day-old USA300 LAC biofilms 24 h following the last medium change and filtered (0.2 μm). Our prior studies have established that 6-day-old *S. aureus* biofilms propagated in RPMI 1640 are mature on the basis of the presence of tower structures and thickness (4, 15). For comparisons, planktonic culture-conditioned medium was prepared by growing an overnight culture of USA300 LAC to early (12 h) or late (18 h) stationary phase. As no significant differences in phagocytosis or viability were observed when macrophages were treated with conditioned medium from either early or late stationary phase cultures (data not shown), early stationary phase supernatant was utilized throughout these studies. In some experiments, conditioned medium was collected from biofilms that were mechanically disrupted by trituration. Specifically, fresh medium was added to 6-day-old USA300 LAC biofilms and following mechanical dispersal, conditioned medium was collected 24 h later and clarified by filtration (0.2 μm). Where indicated, biofilm-conditioned medium was treated with 10 μg/ml proteinase K for 1 h at 37°C to degrade proteins. Biofilms were also either exposed to 10 μg/ml PAS or mechanically disrupted and incubated with 50 μg/ml lysostaphin (both from Sigma, St. Louis, MO) for 24 h prior to collection to assess whether proteins released by active secretion or bacterial lysis, respectively, were responsible for macrophage dysfunction. In some experiments, fresh RPMI 1640 medium was spiked with 10 μg of purified *S. aureus* Hla (Sigma) or 25 μg of purified bioactive or inactive LukAB (56) to examine effects on macrophage phagocytosis and viability. For some experiments, biofilm-conditioned medium was incubated with rabbit anti-staphylococcal Hla antiserum or control rabbit serum (both from Sigma) for 30 min prior to macrophage treatment.

Macrophage phagocytosis and cell viability assay. Mice were euthanized with an overdose of inhaled isoflurane, followed by cervical dislocation as a secondary method to ensure death prior to the harvesting of long bones to prepare bone marrow-derived macrophages as previously described (4, 86). Bone marrow-derived macrophages were labeled with 5 μM CellTracker Blue (CTB; Molecular Probes, San Diego, CA) as previously described (4, 86). CTB-labeled macrophages were added to sterile two-well glass chamber slides (5×10^6 cells/chamber) and allowed to adhere for 2 h. Next, macrophages were exposed to undiluted biofilm- or planktonic culture-conditioned medium for 2 h at 37°C, since a robust phenotype was observed under these conditions (see Fig. S1A in the supplemental material), whereupon 4.5×10^{10} green fluorescent microspheres (2.0 μm; Molecular Probes) were added for 1 h to assess phagocytic activity. Fluorescent microspheres were used instead of live *S. aureus*, since live bacteria actively secrete factors during planktonic growth that would have been impossible to differentiate from biofilm-derived molecules and pilot studies revealed that similar results were obtained with both reagents (see Fig. S1A). To test the effects of opsonization in this assay, beads were preincubated in RPMI 1640 medium with 10% fetal bovine serum for 30 min at 37°C and washed prior to macrophage addition. The extent of macrophage phagocytosis of fluorescent microspheres was similar with or without opsonization (see Fig. S1B). Macrophage viability was assessed by propidium iodide (PI) staining at the end of the 3-h incubation period. Macrophage phagocytosis and viability were assessed with a Zeiss 510 META laser scanning confocal microscope (Carl Zeiss, Oberkochen, Germany) and quantitated by determining the number of phagocytic or PI$^+$ macrophages in at least eight random fields of view (magnification, ×63) with ZEN 2009 software (Carl Zeiss). Data are expressed as either percent phagocytosis or viable macrophages, which reflects manual counts within a given experiment, or total phagocytosed beads or viable macrophages per ×63 field, where the total number of events within a given experiment was calculated by an ImageJ plugin (ImageJ 1.47v; Wayne Rasband, NIH, Bethesda, MD) based on an experimentally determined average pixel area and RGB color code specific to CTB-labeled macrophage overlap with microspheres or PI$^+$ cells. Pilot studies confirmed that identical results were obtained by this method and manual counting (data not shown).

SWATH-MS proteomics and analysis. Conditioned medium from WT and Δ*agr* mutant *S. aureus* strains grown under biofilm or planktonic conditions as described above was harvested and treated with a protease inhibitor cocktail (Roche, Basel, Switzerland), and proteins were precipitated with 20% TCA. Relative protein concentrations were compared between groups by using three independent replicates per sample by SWATH-MS as previously described (48). A Z transformation, followed by a Z test, was performed on all of the positively identified proteins (>98% confidence) with two sample sets at a time (i.e., WT biofilm versus Δ*agr* biofilm or WT biofilm versus WT planktonic) to assess significant differences in relative protein abundance as previously described (48). Identified proteins were functionally grouped by UniProt identifier utilizing David bioinformatic resource 6.7 (http://david.abcc.ncifcrf.gov/)

(87). Protein-protein interactions were predicted by using STRING 9.05 (http://string-db.org) (88, 89).

Western blot assays. Conditioned medium from WT *S. aureus* biofilm and planktonic cultures was sterile filtered and treated with a protease inhibitor cocktail (Roche). Samples were TCA precipitated overnight and suspended in 30 μl of Laemmli buffer, whereupon 5 μl of each sample was loaded onto a gel, transferred to a nitrocellulose membrane, and probed for *S. aureus* LukA and Hla.

ELISA. Conditioned medium from WT *S. aureus* biofilm and planktonic cultures was sterile filtered (0.2 μm) and analyzed for Hla concentrations by direct enzyme-linked immunosorbent assay (ELISA). Briefly, to generate a standard curve, serial dilutions of purified *S. aureus* Hla (Sigma) were diluted in carbonate-bicarbonate buffer and incubated in 96-well ELISA plates overnight at 4°C along with experimental samples. The following day, wells were washed extensively with 1× phosphate-buffered saline (PBS)–0.5% Tween and incubated with a rabbit anti-Hla Ab, followed by an anti-rabbit IgG-horseradish peroxidase Ab (both from Sigma) for detection. Plates were developed with a 3,3′,5,5′-tetramethylbenzidine substrate (Becton, Dickinson), and the reaction was halted with stop solution prior to reading at 450 nm. Hla concentrations were normalized to total protein as measured by a Pierce bicinchoninic acid protein assay.

Mouse model of *S. aureus* orthopedic implant biofilm infection. To simulate infectious complications in patients following surgical device placement, a mouse *S. aureus* orthopedic implant biofilm infection model was used as previously described (57, 58). Briefly, animals were anesthetized with ketamine-xylazine (100 and 5 mg/kg, respectively) and the surgical site was disinfected with povidone-iodine. A medial parapatellar arthrotomy with lateral displacement of the quadriceps-patella was performed to access the distal femoral trochlea. A burr hole in the femoral intercondylar notch extending into the intramedullary canal was created with a 26-gauge needle, whereupon a precut 0.8-cm orthopedic-grade Kirschner wire (0.6-mm diameter, Nitinol [nickel-titanium]; Custom Wire Technologies, Port Washington, WI) was inserted into the intramedullary canal, leaving ~1 mm protruding into the joint space. A total of 10^3 CFU of WT USA300 LAC or an isogenic Δ*hla*, Δ*lukAB*, or Δ*lukAB* Δ*hla* double mutant strain was inoculated at the implant tip. Animals received Buprenex (0.1 mg/kg subcutaneously; Reckitt Benckiser, Hull, United Kingdom) immediately after infection and 24 h later for pain relief. After this interval, all mice exhibited normal ambulation and no discernible pain behavior.

Flow cytometry. To characterize macrophage infiltrates in inflamed soft tissues surrounding the knee joint during *S. aureus* biofilm infection, tissues were excised, dissociated with the rubber end of a plunger from a 3-ml syringe, and passed through a 35-μm filter (BD Falcon, Bedford, MA). The resulting filtrate was washed with 1× PBS, and cells were collected by centrifugation (300 × *g*, 10 min), whereupon red blood cells were lysed with BD Pharm Lyse (BD Biosciences, San Diego, CA), resuspended in 1× PBS, and incubated in Fc Block (BD Biosciences) to minimize nonspecific Ab binding. Cells were stained with CD45-allophycocyanin, Ly6G-phycoerythrin (PE), Ly6C-peridinin chlorophyll protein-Cy5.5, F4/80-PE-Cy7, and CD11b-eFluor 450, and viability was determined with a LIVE/DEAD Fixable Blue Dead Cell Stain (Life Technologies, Eugene, OR). All fluorochrome-conjugated Abs were purchased from BD Biosciences or eBioscience (San Diego, CA). An aliquot of cells was stained with isotype-matched control Abs to assess the degree of nonspecific staining. The number of events analyzed per sample ranged from 10,000 to 400,000. Analysis was performed with BD FACSDiva software with doublet exclusion performed, and cells were gated on the "live" $CD45^+$ population.

Statistical analysis. The significance of differences between experimental groups was determined with either an unpaired two-tailed Student *t* test or one-way analysis of variance (ANOVA), followed by Bonferroni's multiple-comparison test, in GraphPad Prism 4 (GraphPad, La Jolla, CA). For all analyses, a *P* value of less than 0.05 was considered statistically significant.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01021-15/-/DCSupplemental.

Figure S1, TIF file, 0.1 MB.
Figure S2, TIF file, 0.2 MB.
Figure S3, TIF file, 0.2 MB.
Figure S4, TIF file, 0.1 MB.
Figure S5, TIF file, 0.2 MB.
Table S1, DOCX file, 0.02 MB.
Table S2, DOCX file, 0.02 MB.

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