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Staphylococcus aureus Elaborates Leukocidin AB To Mediate Escape from within Human Neutrophils

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Methicillin-resistant *Staphylococcus aureus* (MRSA) strains of the pulsed-field type USA300 are primarily responsible for the current community-associated epidemic of MRSA infections in the United States. The success of USA300 is partly attributed to the ability of the pathogen to avoid destruction by human neutrophils (polymorphonuclear leukocytes [PMNs]), which are crucial to the host immune response to *S. aureus* infection. In this work, we investigated the contribution of bicomponent pore-forming toxins to the ability of USA300 to withstand attack from primary human PMNs. We demonstrate that *in vitro* growth conditions influence the expression, production, and availability of leukotoxins by USA300, which in turn impact the cytotoxic potential of this clone toward PMNs. Interestingly, we also found that upon exposure to PMNs, USA300 preferentially activates the promoter of the *lukAB* operon, which encodes the recently identified leukocidin AB (LukAB). LukAB elaborated by extracellular *S. aureus* forms pores in the plasma membrane of PMNs, leading to PMN lysis, highlighting a contribution of LukAB to USA300 virulence. We now show that LukAB also facilitates the escape of bacteria engulfed within PMNs, in turn enabling the replication and outgrowth of *S. aureus*. Together, these results suggest that upon encountering PMNs *S. aureus* induces the production of LukAB, which serves as an extra- and intracellular weapon to protect the bacterium from destruction by human PMNs.

Staphylococcus aureus is a Gram-positive bacterium responsible for tremendous morbidity and mortality worldwide. *S. aureus* is responsible for a large number of invasive diseases, including sepsis, pneumonia, osteomyelitis, endocarditis, and toxic shock, as well as less invasive diseases like skin and soft tissue infections (1). The impact of *S. aureus* on human health is further compounded by the emergence of multidrug-resistant strains, including methicillin-resistant *S. aureus* (MRSA). These MRSA strains, which were previously confined to hospitals, have spread into the community, resulting in a community-associated MRSA (CA-MRSA) epidemic (2). In the United States, the USA300 clone is primarily responsible for the current CA-MRSA epidemic (2–4).

The pathogenesis of USA300 is dependent on the production of a repertoire of virulence factors (5, 6). Studies comparing different *S. aureus* strains have revealed that CA-MRSA strains, including USA300, are more resistant to killing by polymorphonuclear cells (PMNs) than hospital-associated MRSA (HA-MRSA) strains (7–9). USA300 strains can produce up to five bicomponent pore-forming leukotoxins that have been implicated in the ability of USA300 to kill PMNs (10, 11). These toxins include Panton-Valentine leukocidin (LukSF-PV or PVL), gamma hemolysin (HlgAB and HlgCB), leukocidin ED (LukED), and the recently described leukocidin AB (LukAB), also known as LukGH (10, 11). These toxins are members of the β-barrel pore-forming family and are composed of two subunits commonly known as the fast (F) and slow (S) components based on their elution profiles in liquid chromatography (12). The S and F subunits are thought to form octameric pores in the plasma membrane of target cells (13), which result in osmotic imbalance and lead to the death of the target cell (10, 11).

Among the staphylococcal leukotoxins, PVL has gathered much attention in the past decade due to its linkage with CA-MRSA; however, its contribution to *S. aureus* pathogenesis has been highly contested. Studies with conflicting outcomes and conclusions (8, 14, 15) were attributed to the species specificity of PVL, wherein human and rabbit cells are highly susceptible to the toxin and murine cells are highly resistant (16). In addition to PVL, previous studies have associated HlgACB with septic arthritis and weight loss in mice (17), with the *S. aureus*-mediated inflammatory response observed in the rabbit eye *in vivo* (18), and with sepsis in mice (19, 20). In contrast to the other *S. aureus* leukotoxins, HlgACB can assemble into two different active toxins, HlgAB and HlgCB, which exert species specificity, whereby HlgAB is specific toward murine macrophages while HlgCB is specific toward human macrophages (21). Purified LukED is active toward PMNs from human, rabbits, and mice (20, 22–24). As with purified PVL (25), LukED has also been shown to induce dermonecrosis when purified toxin is injected intradermally into rabbits (22, 23). Importantly, LukED was recently found to contribute to the lethality observed upon *S. aureus* bloodstream infection of both methicillin-resistant *S. aureus* (MSSA) and MRSA strains (20, 24). The newest member of the *S. aureus* bicomponent leukotoxin family is LukAB/GH (26, 27). This toxin has been shown to be critical for the cytotoxicity of supernatants collected from both MSSA and MRSA clinical isolates toward primary hu-

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TABLE 1 *Staphylococcus aureus* strains

Strain	Background	Description	Designation	Reference
VJT 12.61	USA300 LAC	Parental strain	WT[a]	3
AH1263	USA300 LAC	Erythromycin-sensitive (Erm^s) clone	WT	30
VJT 14.26	USA300 LAC	Δ*lukAB*	Δ*lukAB*	26
VJT 22.05	USA300 LAC	*pvl*::*spec*	Δ*pvl*	This study
VJT 22.09	USA300 LAC	Δ*lukAB pvl*::*spec*	Δ*lukAB* Δ*pvl*	This study
VJT 23.51	USA300 LAC	(Erm^s) Δ*lukAB*	Δ*lukAB*	This study
VJT 32.40	USA300 LAC	(Erm^s) Δ*lukAB pvl*::*spec*	Δ*lukAB* Δ*pvl*	This study
VJT 32.58	USA300 LAC	(Erm^s) pJC111	WT	This study
VJT 32.59	USA300 LAC	(Erm^s) Δ*lukAB* pJC111	Δ*lukAB*	This study
VJT 32.60	USA300 LAC	(Erm^s) Δ*lukAB* pJC111-P_{lukAB}-*lukAB*	Δ*lukAB*::*lukAB*	This study
VJT 32.61	USA300 LAC	(Erm^s) Δ*lukAB pvl*::*spec* pJC111	Δ*lukAB* Δ*pvl*	This study
VJT 32.62	USA300 LAC	(Erm^s) Δ*lukAB pvl*::*spec* pJC111-P_{lukAB}-*lukAB*	Δ*lukAB* Δ*pvl*::*lukAB*	This study

[a] WT, wild type.

man phagocytes, is required for *S. aureus* survival in human whole blood, protects *S. aureus* from PMN-mediated growth restriction, and contributes to *S. aureus*-mediated killing of PMNs in *ex vivo* infection models (26). In addition, LukAB is responsible for *S. aureus*-mediated killing of primary human monocytes, macrophages, and dendritic cells (26). As with PVL, LukAB preferentially kills human PMNs compared to murine PMNs (28). Despite this apparent tropism toward human phagocytes, LukAB was found to be expressed *in vivo* in murine renal abscesses and to contribute to bacterial replication in renal abscesses, suggesting that this toxin contributes to the pathogenesis of USA300 during systemic infection (26). The common feature of all these toxins is their ability to target and kill human PMNs *in vitro* (10, 11).

In this study, we set out to examine the expression and production of leukotoxins as well as the contribution of these toxins to the ability of USA300 to evade killing by human PMNs. We demonstrate that USA300 differentially activates leukotoxin promoters *in vitro* in a growth medium-dependent manner impacting cytotoxicity of culture supernatants toward PMNs. In addition, we found that USA300 senses PMNs to induce the expression and production of LukAB. We show that LukAB plays an important role during USA300-PMN interaction by promoting the escape of phagocytosed bacteria, which results in improved bacterial growth during infection. Thus, these findings demonstrate that USA300 is able to adapt to the host by sensing immune cells to elaborate specific virulence factors that are critical for promoting the pathogenesis of this organism.

MATERIALS AND METHODS

Ethics statement. Blood samples were obtained from anonymous healthy donors as buffy coats (New York Blood Center). Alternatively, informed written consent was obtained from all donors as approved by the medical ethics committee of the University Medical Center Utrecht (Utrecht, The Netherlands).

Bacterial strains and culture. MRSA isolate pulsed-field gel electrophoresis type USA300 LAC (29) or a LAC strain that had been cured of the erythromycin resistance plasmid (30) was used in all experiments as the wild-type (WT) strain (Table 1). *S. aureus* strains were grown on tryptic soy broth (TSB) solidified with 1.5% agar at 37°C or in Roswell Park Memorial Institute medium (RPMI 1640; Invitrogen) supplemented with 1% Casamino Acids (RPMI+CAS) (26, 31), with shaking at 180 rpm, unless otherwise indicated.

Select LAC strains were transformed with pOS1-P_{sarA}-*sod*RBS-s*gfp*, a plasmid that constitutively and robustly produces superfolded green fluorescent protein (GFP) from the *sarA* promoter using the superoxide dismutase (*sod*) ribosomal binding site (RBS) (see Table 3) (32). These strains were cultured with RPMI+CAS supplemented with chloramphenicol at a final concentration of 10 μg/ml. Reporter strains were also cultured with the indicated media supplemented with chloramphenicol at a final concentration of 10 μg/ml.

Generation of mutant, complementation, and reporter strains. All bacteria, plasmids, and primers used in and arising from this study are listed in Tables 1, 2, and 3, respectively.

(i) Mutant strains. An isogenic mutant lacking *lukAB* and the *pvl*::*spec* mutant were constructed using the pKOR-1 plasmid as described previously (26, 33). Briefly, sequences flanking the *lukAB* locus were PCR amplified with primers 68 and 69 for the upstream fragment and primers 76 and 77 for the downstream fragment, and sequences flanking the *pvl* locus were PCR amplified with primers 428 and 429 for the upstream fragment and primers 430 and 431 for the downstream fragment. The PCR amplicons were digested with XmaI and assembled into pCR2.1 (Invitrogen). For the pCR2.1 construct containing the *pvl* fragments, the vector was digested with XmaI and ligated with the aminoglycoside-3″-adenylyltransferase (*aad*) gene also digested with XmaI to create an *aad* insertion within the *pvl* gene, as we have done previously for the generation of other mutants (32). A PCR amplicon of the joined DNA fragments was recombined into pKOR1, resulting in the pKOR-1Δ*lukAB* and pKOR-1Δ*pvl*::*spec* plasmids. Deletion of the *lukAB* and *pvl* loci was achieved by allelic replacement as described previously (33). Mutagenesis was confirmed by PCR. The Δ*lukAB* Δ*pvl* double mutant was made by transducing the Δ*lukAB* mutant with a *pvl*::*spec*-carrying phage.

The isogenic *saeQRS*::*spec* mutant used in the proof-of-concept experiment was constructed through allelic replacement of *saeQRS* using pKOR-1 as described previously (32).

(ii) Complementation strains. Integrated complementation strains were generated by cloning into plasmid pJC1111, which stably integrates into the SaPI-1 site of *S. aureus*, resulting in single-copy chromosomal complementation (20). To construct the pJC1111-P_{lukAB}-*lukAB* complementation vector, a PCR amplicon containing the *lukAB* operon with the endogenous promoter was generated using primer pair 681 and 695 and was subsequently digested and subcloned into pJC1111. The resultant plasmid was designated pJC1111-P_{lukAB}-*lukAB* and was subsequently integrated into the *S. aureus* SaPI-1 site.

(iii) Reporter strains. Transcriptional fusion reporters were generated using the pXen plasmid (Xenogen), where individual toxin promoters (P_{lukED}, P_{lukAB}, P_{hlgCB}, P_{hlgAB}, and P_{pvl}) were fused to the luciferase operon from *Photorhabdus luminescens*. The *lukED* promoter was amplified with primers 606 and 142, the *lukAB* promoter was amplified with primers 143 and 144, the *hlgCB* promoter was amplified with primers 147 and 148, the *hlgAB* promoter was amplified with primers 145 and 146, and the *pvl* promoter was amplified with primers 546 and 547.

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TABLE 2 Primers

No.	Name	Sequence	Description
681	PstI-P_{lukAB}-F	5′-CCCCTGCAGGTGTTATTTGATTTCGTTCTATG	Complementation vector
695	KpnI-*lukB*-Rahul Maurya	5′-CCCGGTACCTTATTTCTTTTCATTATCATTAAGTAC	Complementation vector
68	Δ*lukAB*-AttB1-5′	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTGATACTATGCATGACAAC	To make KO
69	Δ*lukAB*-XmaI-3′	5′-TCCCCCCGGGGATTAAATAATCAACAAAGTG	To make KO
76	Δ*lukAB*-AttB2-3′	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTGATGTCGTAATATTCAATG	To make KO
77	Δ*lukAB*- XmaI-5′	5′-TCCCCCCGGGGTAATAAAAGTTGCCTGC	To make KO
428	Δ*pvl*-AttB1-5′	5′-GGGGACAAGTTTGTACAAAAAAGCAGGCT-GCCTTGACCTACATATTTTGAAG	To make KO
429	Δ*pvl* -XmaI-3′	5′-TCCCCCCGGGTGGATAGATATACAAACTTTTGGAA	To make KO
430	Δ*pvl* -XmaI-5′	5′-TCCCCCCGGGCTAATAGTCTTTTTTTGACCATAAAA	To make KO
431	Δ*pvl* -AttB2-3′	5′-GGGGACCACTTTGTACAAGAAAGCTGGGTTACTATTGATAAAAGTATACAAGATG	To make KO
606	P_{lukE} 5′ EcoR1	5′-CCCGAATTCGATAGGTGAGATGCATACACAAC	*lux* reporter vector
141	P_{lukE} 5′ BamHI	5′-CCCCGGATCCAAGTTTCACTTTCTTTCTATATAAA	*lux* reporter vector
143	P_{lukA} 3′ BamHI	5′-CCCCGGATCCTGTTATAACCTTCTTTCGTATG	*lux* reporter vector
144	P_{lukA} 5′ EcoRI	5′-CCCCGAATTCGTGTTATTTGATTTCGTTCTATG	*lux* reporter vector
147	P_{hlgBC} 5′ EcoRI	5′-CCCCGAATTCAACAATTAATTTTTAGATGGATTC	*lux* reporter vector
148	P_{hlgBC} 3′ BamHI	5′-CCCCGGATCCAAGTTTCACTTTCTTTCTATAATTT	*lux* reporter vector
145	P_{hlgA} 3′ BamHI	5′-CCCCGGATCCAGAAATCACTTTCTTTCTATTTAAT	*lux* reporter vector
146	P_{hlgA} 5′ EcoRI	5′-CCCCGAATTCCCACTTTTTTACCTGCAACTTG	*lux* reporter vector
546	P_{pvl} 3′BamHI	5′-CCCCGGATCCTTCCTTTCTTTATAAATTTTATTAC	*lux* reporter vector
547	P_{pvl} 5′ EcoRI	5′-CCCCGAATTCTAATTGTATATGATGAATCTTAGG	*lux* reporter vector

Exoprotein production. For the production of filtered culture supernatants (exoproteins), *S. aureus* strains were grown in RPMI+CAS unless otherwise indicated. Overnight cultures were grown in 5 ml in 15-ml tubes held at a 45° angle and incubated at 37°C with shaking at 180 rpm (26). Following overnight cultures, bacteria were subcultured at a 1:100 dilution and grown as described above for 5 h. Bacteria were then pelleted by centrifugation at 4,000 rpm and 4°C for 15 min. Supernatants containing exoproteins were collected, filtered using a 0.2-μm-pore-size filter, and stored at −80°C.

SDS-PAGE of secreted proteins. Exoproteins in *S. aureus* culture supernatants were precipitated with 10% (vol/vol) trichloroacetic acid (TCA). The precipitated proteins were washed once with 100% ethanol, air dried, resuspended with 30 μl of SDS-Laemmli buffer, and boiled at 95°C for 10 min. Proteins were separated using 15% SDS-PAGE gels and stained with Coomassie brilliant blue.

Precipitated exoproteins were separated on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes. The membranes were incubated with polyclonal antibodies against LukA (26), LukS-PVL (34), and alpha-toxin (Sigma), which were detected with Alexa Fluor-680-conjugated anti-rabbit secondary antibody (Invitrogen) diluted 1:25,000. Membranes were dried and scanned using an Odyssey infrared imaging system (LI-COR Biosciences).

***In vitro* luminescence reporter assay.** To measure leukotoxin promoter activity during *in vitro* growth, reporter plasmids were constructed by fusing the leukotoxin promoters to the luciferase operon from *Photorhabdus luminescens* present in the pXen plasmid (Xenogen) (see above). The strains were grown overnight in 96-well round-bottom plates in RPMI+CAS with chloramphenicol and then subcultured 1:200 in RPMI+CAS, TSB, or Luria-Bertani (LB) broth supplemented with chloramphenicol in 96-well black clear-bottom plates. Optical density (OD) at 600 nm and luminescence readings were taken at time zero and every hour for 8 h using a PerkinElmer Envision 2103 Multilabel reader. The background fluorescence of each medium alone was subtracted from the corresponding samples.

Cytotoxicity assays. PMNs were isolated from peripheral blood mononuclear cells (PBMCs) by a Ficoll gradient (Ficoll-Paque PLUS; GE). The pellets were subsequently washed with phosphate-buffered saline (PBS), and PMNs were separated from erythrocytes with 3% dextran (Dextran 500; Pharmacosmos) in 0.9% sodium chloride (Hospira). Remaining erythrocytes were lysed with sterile water (Invitrogen) followed by ACK lysing buffer (Gibco). PMN purity was at 90 to 95% as determined by flow cytometry.

To evaluate the viability of PMNs after intoxication with *S. aureus* exoproteins, cells were plated in 96-well black clear-bottom tissue culture

TABLE 3 Plasmids

Name	Description	Resistance	Reference
pXEN1-*lux*	Luciferase reporter promoterless control	Cm	35
pXEN1-P_{lukAB}-*lux*	*lukAB* promoter driving expression of the *lux* operon	Cm	26
pXEN1-P_{lukED}-*lux*	*lukED* promoter driving expression of the *lux* operon	Cm	This study
pXEN1-P_{hlgA}-*lux*	*hlgA* promoter driving expression of the *lux* operon	Cm	This study
pXEN1-P_{hlgCB}-*lux*	*hlgCB* promoter driving expression of the *lux* operon	Cm	49
pXEN1-P_{pvl}-*lux*	*pvl* promoter driving expression of the *lux* operon	Cm	49
pJC111	Empty vector used for integration into the SaP-1 site	Cad	50, 51
pJC111-P_{lukAB}-*lukAB*	Integration vector with the *lukAB* operon	Cad	This study
pOS1sGFP-P_{sarA}-*sod* RBS	Plasmid for *sarA* P1-dependent sGFP expression using the *sod* RBS	Cm	32
pKOR1	Empty vector used for allelic replacement	Cm	33
pKOR1 Δ*lukAB*	pKOR1 vector containing *lukAB* homologous regions	Cm	26
pKOR1 Δ*pvl*::*spec*	pKOR1 vector containing *pvl* homologous regions with *aad* insertion	Cm	This study

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treated plates (Corning) at 1×10^5/well in a final volume of 100 μl of phenol red-free RPMI (Gibco) supplemented with 10% of heat-inactivated fetal bovine serum (FBS). Cells were intoxicated for 1 h at 37°C and 5% CO_2 with serial 2-fold dilutions of culture filtrate from two independent *S. aureus* cultures ranging from 20% to 0.16% (vol/vol). Healthy controls for background fluorescence were comprised of PMNs with 20% (vol/vol) *S. aureus* growth medium (RPMI+CAS). To evaluate the integrity of host cell plasma membranes following intoxication, we employed SYTOX green (Invitrogen) as previously described (26). Each well was mixed with 100 μl of PBS + SYTOX green (0.1 μM) and incubated at room temperature in the dark for 10 min. Fluorescence was measured using a PerkinElmer Envision 2103 Multilabel reader (excitation, 485 nm; emission, 535 nm).

***Ex vivo* luminescence reporter assay.** To investigate leukotoxin promoter activation during *ex vivo* infection with primary human PMNs, the luciferase reporters described above were employed. Following overnight culture in RPMI+CAS supplemented with chloramphenicol, the strains were subcultured 1:100 in RPMI+CAS with chloramphenicol for 3 h. Strains were washed twice with 1× PBS, resuspended in 1× PBS, and normalized to an OD of 1.0 at 600 nm using a spectrophotometer (Genesys 20; Thermo Scientific). PMNs were plated at 1×10^5 PMN/well in a final volume of 100 μl of phenol red-free RPMI supplemented with 10% of heat-inactivated FBS and were infected with *S. aureus* at a multiplicity of infection (MOI) of 25 in 96-well black clear-bottom plates at 37°C and 5% CO_2. OD at 600 nm and luminescence readings were taken at time zero and every 30 min for 3 h using a PerkinElmer Envision 2103 Multilabel reader.

***Ex vivo* infection assays.** To determine *S. aureus*-mediated killing of PMNs by extracellular bacteria, *S. aureus* was cultured as described above for exoprotein collection, pelleted, washed, and normalized as described above. PMNs were plated at 1×10^5 cells/well in a final volume of 100 μl of phenol red-free RPMI supplemented with 10% of heat-inactivated FBS and were infected with an MOI of 100, 50, 10, or 1 of the *S. aureus* strains in 96-well flat-bottom tissue culture treated plates at 37°C and 5% CO_2 for 1 h. Membrane disruption was evaluated using SYTOX green as described above. MOI were confirmed by serially diluting the input cultures and counting CFU on tryptic soy agar (TSA) plates.

PMN-mediated killing of *S. aureus* and subsequent growth rebound of *S. aureus* were monitored by culturing and normalizing *S. aureus* strains as described above and then opsonizing the bacteria with 20% normal human serum (NHS) (SeraCare) in phenol red-free RPMI with 10 mM HEPES buffer (RPMI+HEPES) (Invitrogen) for 30 min at 37°C with rotation. PMNs were plated at 1×10^5 cells/well in a final volume of 100 μl of RPMI+HEPES in 96-well flat-bottom tissue culture treated plates that had been coated with 20% NHS in RPMI+HEPES and subsequently washed with RPMI+HEPES. The PMNs were synchronized with an MOI of 10 of the *S. aureus* strains through centrifugation at 1,500 rpm and 4°C for 7 min and then incubated at 37°C and 5% CO_2. At 30, 60, 120, and 180 min postsynchronization the PMNs were lysed with 0.1% saponin on ice for 15 min and then serially diluted in 10-fold dilutions in TSB. Input at time zero was also saponin treated in RPMI+HEPES (equal to an MOI of 10) and serially diluted in 10-fold dilutions in TSB. Recovered bacteria were determined by counting CFU on TSA plates where the CFU at each time point were first normalized to input CFU for each strain. Input was set at 100%, and data represent the percentage of growth compared to input.

PMN membrane damage following infection with opsonized *S. aureus* was also determined by preparing PMNs and bacteria as described above, where SYTOX green was added at 30, 60, 120, or 180 min postsynchronization and fluorescence was evaluated as described above.

Tracking the location of opsonized GFP-*S. aureus* during infection of PMNs. In order to determine the location of *S. aureus* under the different *ex vivo* infection conditions described above, *S. aureus* strains constitutively expressing synthetic GFP (sGFP) were utilized to track the bacteria using flow cytometry. The strains were prepared and the PMNs were infected as described above for the two different *ex vivo* infection assays. At 0, 30, and/or 60 min postsynchronization/postinfection, the samples were fixed with fixing buffer (1× PBS + 2% paraformaldehyde + 2% FBS + 0.05% sodium azide) and analyzed using an LSR-II flow cytometer (Becton, Dickinson, BD) to measure GFP fluorescence. Lysostaphin (40 μg/ml; Ambi Products LLC) was added to the samples at 0, 30, and/or 60 min postsynchronization/postinfection in order to kill extracellular *S. aureus.* The lysostaphin was either added as a pulse for 10 min at 37°C with 5% CO_2 or added to the sample at 0 min synchronization/infection and left on the samples until the designated time point. For the microscopy experiments, polyclonal anti-LukA antibody affinity purified from rabbit sera was added along with lysostaphin at 2.5 μg/ml to block any activity of extracellular toxin, and 5 μg/ml of ethidium bromide (MP Biomedicals) was added to visualize pore formation. After lysostaphin treatment, either the cells were fixed and analyzed as described above by flow cytometry or images were captured using the Axiovert 40 CFL fluorescence microscope (Zeiss), Axiocam ICc 1 (Zeiss), and the Zen software from Zeiss. For flow cytometry analysis, fluorescence from extracellular bacteria at the indicated time points was determined by subtracting the mean GFP fluorescence of lysostaphin-treated samples from the mean GFP fluorescence of PBS-treated samples.

The amount of intracellular bacteria (those protected from lysostaphin treatment) was determined by dilution plating on TSA plates. PMNs were infected as described above; however, after lysostaphin treatment the samples were washed twice with 1× PBS and the PMNs were lysed with 0.1% saponin. The samples were serially diluted 10-fold in TSB, and recovered bacteria were determined by counting CFU on TSA plates.

Statistics. Data were analyzed using a two-way analysis of variance (ANOVA) and Bonferroni posttest (GraphPad Prism version 5.0; GraphPad Software) unless indicated otherwise. Data presented here are from one of at least three independent experiments that gave similar results unless otherwise indicated.

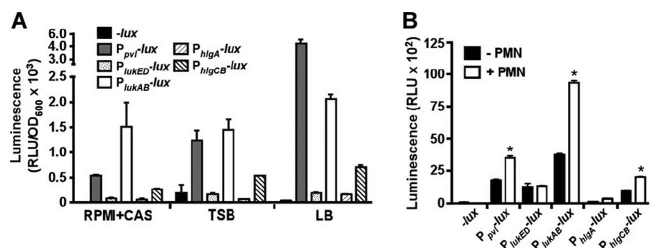

FIG 1 The *lukAB* promoter is the most activated in response to human PMNs as mimicked by certain bacterial growth media. (A) USA300 strains containing the toxin promoter *lux* reporter constructs were grown in RPMI+CAS, TSB, or LB, and luciferase activity was measured at 6 h, a time point where luciferase activity peaked. Luminescence was normalized to the culture optical density (OD). Results represent the average of results from 3 independent cultures ± standard deviation. (B) USA300 *lux* reporter strains were grown in the presence or absence of PMNs in RPMI+FBS, and luminescence was measured 2 h postincubation, a time point where luciferase activity peaked. Results represent the mean from PMNs isolated from six different donors ± standard error of the mean. *, statistical significance.

RESULTS

***S. aureus* differentially regulates leukotoxin promoters during *in vitro* growth.** To examine the differential expression of bicomponent toxin coding genes in USA300, transcriptional fusion reporters were generated in an *S. aureus* USA300 strain where each individual leukotoxin promoter was fused to the luciferase operon from *Photorhabdus luminescens* (35). Reporter strains were grown in three commonly used culture media (RPMI+CAS, TSB, and LB), and leukotoxin promoter activation was measured (Fig. 1A). We observed that the *lukAB* promoter was among the most active

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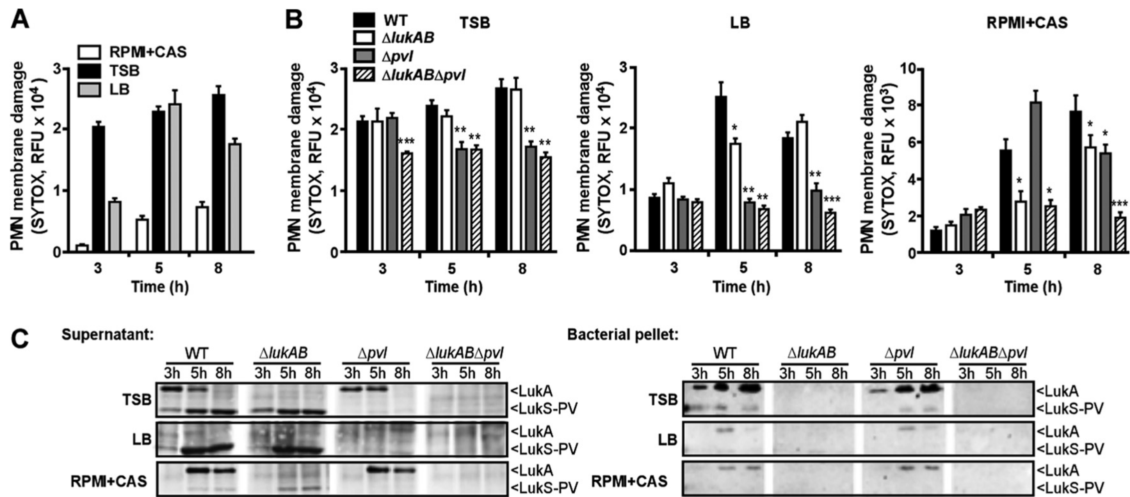

FIG 2 Bacterial growth medium influences the production and secretion of leukotoxins, affecting the cytotoxic potential of culture supernatants toward PMNs. Intoxication of PMNs for 60 min with 10% (vol/vol) culture supernatants collected from USA300 MRSA strain LAC (WT) (A and B) and isogenic strains lacking *lukAB* (Δ*lukAB*), *pvl* (Δ*pvl*), or both *lukAB* and *pvl* (Δ*lukAB*Δ*pvl*) (B) grown in indicated media and to indicated growth phases. PMN membrane damage was measured using the fluorescent dye SYTOX green. Results represent the mean from PMNs isolated from four different donors, ± standard error of the mean. (C) The abundance of LukAB and PVL in the culture supernatant and the amount of these toxins associated with the bacterial pellet under the culture conditions described above were also evaluated by immunoblotting with α-LukA and α-LukS-PV antibodies. *, statistical significance compared to the WT; **, statistical significance compared to the WT and Δ*lukAB* strains ($P < 0.05$); ***, statistical significance compared to the WT, Δ*lukAB*, and Δ*pvl* strains ($P < 0.05$).

promoters in RPMI+CAS and TSB. In RPMI+CAS, luminescence readout from the *lukAB* promoter was >200-fold above background levels (promoterless *lux* construct), at least 5-fold above those of P*lukED*, P*hlgA*, and P*hlgCB*, and approximately 3-fold above that of P*pvl*. While the P*lukAB* readout was comparable to that of P*pvl* in TSB, the two promoters exhibited the highest activity in this medium among all leukotoxin promoters tested, more than 8-fold higher than background levels. In LB medium, however, the *pvl* promoter was the most active, with >350-fold-higher luminescence over background and 2-fold-higher activity than P*lukAB* (Fig. 1A). These experiments revealed that USA300 differentially activates the leukotoxin promoters in a growth medium-dependent manner.

***S. aureus* responds to human PMNs by upregulating *lukAB*.** One common manifestation of *S. aureus* infection is the formation of abscesses (36), which provides a site where *S. aureus* is exposed to constant attack by PMNs. A potential strategy employed by *S. aureus* to avoid PMN-mediated killing could be to sense PMNs and coordinate an offensive attack through the production of leukotoxins. Consistent with this supposition, *S. aureus* has been shown to increase leukotoxin transcript levels postphagocytosis by PMNs (7). Here we investigated if extracellular *S. aureus* alters the expression of leukotoxins in response to PMNs. USA300 strains harboring the leukotoxin transcriptional reporters were grown in the presence or absence of primary human PMNs, and the activation of the luciferase fusion promoters was monitored (Fig. 1B). We observed that compared to activation in medium alone, exposure to PMNs induced the activation of the *pvl*, *lukAB*, and *hlgCB* promoters by at least 2-fold. However, the *lukAB* promoter was the most activated in response to PMN exposure, more than 2.5-fold higher than the next most active promoter, P*pvl*, and >150-fold above background (Fig. 1B). Interestingly, the patterns of leukotoxin promoter activation observed in the presence of PMNs are very similar to those seen under *in vitro* growth conditions in RPMI+CAS (Fig. 1A). These results suggest that compared to the other media tested, RPMI+CAS, a minimal growth medium, most closely mimics the conditions for *ex vivo* infections with PMNs.

A disconnect exists between promoter activation and cytotoxic potential of USA300 culture filtrates. We next evaluated the effect of growth media on the cytotoxic potential of USA300 culture supernatants toward primary human PMNs. Culture supernatants were collected from exponential (3-h), early stationary (5-h), and stationary (8-h) cultures. Incubation of PMNs with these culture filtrates revealed that the cytotoxicity of USA300 is culture medium dependent and growth phase dependent. Specifically, we observed that culture filtrates from LB and TSB were more cytotoxic than culture filtrates from USA300 grown in RPMI+CAS (Fig. 2A).

Based on the observations that cytotoxins are critical for USA300-mediated killing of PMNs (16, 26, 27, 37) and that the *lukAB* and *pvl* promoters were the most active during the *in vitro* and *ex vivo* growth conditions tested (Fig. 1), we next wanted to evaluate the contribution of these toxins to the cytotoxicity of the culture supernatants collected from the different media. We first generated isogenic strains lacking *lukAB* (Δ*lukAB*) or *pvl* (Δ*pvl*) or a double mutant lacking both *lukAB* and *pvl* (Δ*lukAB* Δ*pvl*).

Cytotoxicity assays revealed that the potent cytotoxicity observed with exoproteins collected from TSB-grown USA300 was largely independent of LukAB and PVL, although PVL does partially contribute to the toxicity observed in the supernatants collected from the early- and mid-stationary-phase cultures (Fig. 2B). Immunoblotting for the toxins with specific antibodies facilitated monitoring of toxin levels under different growth conditions (Fig. 2C). We observed high levels of LukAB in TSB culture supernatants harvested at exponential phase, but toxin levels then decreased to a point where LukAB was undetectable at stationary phase (Fig. 2C). In contrast to LukAB, the levels of PVL increased in a growth phase-dependent manner, correlating with the toxin's increased contribution to the cytotoxicity of TSB supernatants

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during stationary phase (Fig. 2B and C). Consistent with the findings of Ventura et al. (27), we found that LukAB is primarily associated with the bacterium during stationary phase when USA300 is grown in TSB. These results provide an explanation for the disappearance of LukAB from the culture supernatant and for the resulting lack of a cytotoxic role for LukAB in these supernatants (Fig. 2B and C) despite significant *lukAB* promoter activity in this medium (Fig. 1A). High levels of LukAB and PVL secreted by *S. aureus* during exponential phase in TSB could compensate for the loss of either toxin, a possible reason for the lack of a phenotype for supernatant from *lukAB* or *pvl* single mutants. However, when both toxins are absent, as with the *lukAB pvl* double mutant, a role for the toxins becomes apparent under these growth conditions, but significant cytotoxic activity remains (Fig. 2B and C). Phenol-soluble modulins (PSMs) have been shown to play a role in USA300 cytotoxicity (9), but it is unlikely that the residual cytotoxicity observed in the USA300 *lukAB pvl* double mutant is attributed to PSMs, as these cytolytic peptides would be neutralized by the serum present in this assay (38).

The cytotoxicity of LB-grown USA300 was attributable to the high levels of PVL produced in this medium, which was consistent with the *pvl* promoter being the most active in this growth medium (Fig. 2B and C and Fig. 1A). In contrast, LukAB was minimally detected in the supernatant and was not associated with the bacterium when USA300 was grown in LB (Fig. 2C). This finding was surprising, since the *lukAB* promoter is highly activated in LB (Fig. 1A), suggesting potential posttranscriptional and/or posttranslational regulation (39, 40).

The only medium that resulted in USA300 secreting copious levels of LukAB was RPMI+CAS (Fig. 2C). Cytotoxicity assays performed with culture supernatants from RPMI+CAS early-stationary-phase-grown bacteria, compared to supernatants from the other growth conditions, display the strongest role for LukAB with regard to cytotoxicity. These data are consistent with the *lukAB* promoter activation observed in Fig. 1 and with the growth conditions that led to our initial identification of the toxin (26). Interestingly, experiments with culture filtrates isolated from stationary-phase-grown bacteria in RPMI+CAS or exponential-phase-grown bacteria in TSB revealed that LukAB and PVL synergize to enhance the cytotoxic property of USA300 (Fig. 2B), consistent with the findings of Ventura et al. (27). Collectively these results demonstrate that the cytotoxic activity exhibited by exoproteins produced by USA300 is highly influenced by the growth phase and the growth medium.

USA300 utilizes LukAB to cause PMN damage before and after phagocytosis by PMNs. The data described in Fig. 1B suggest that upon encountering PMNs, USA300 undergoes an adaptive response to increase the expression of *lukAB* and *pvl*, which are potentially involved in protection from PMNs. To address this possibility, several isogenic mutants lacking *lukAB* or *pvl* or a double mutant lacking both *lukAB* and *pvl* were used to examine the role of each of these toxins with regard to USA300-PMN interaction during *ex vivo* infection. We employed two variations of *ex vivo* infection assays to study the interaction of USA300 with PMNs: an assay where PMNs and *S. aureus* are incubated together without promoting phagocytosis of the bacteria by the PMNs and an assay where bacteria are opsonized with normal human serum and then brought into close contact with PMNs through centrifugation (a process known as synchronization) to promote phagocytosis of the bacteria. First, to confirm the location of opsonized and nonopsonized *S. aureus* upon infection of human PMNs, we employed a USA300 strain that constitutively expressed GFP in *trans* from a plasmid. Extracellular bacteria were exposed to lysostaphin, a potent enzyme that kills *S. aureus* by breaking down the staphylococcal cell wall (41), or were mock treated with PBS 1 h postsynchronization/postinfection. Under lysostaphin treatment, the large majority of extracellular bacteria are eliminated, but those within PMNs are protected from lysostaphin bactericidal effect. USA300 predominantly remains extracellular when PMNs are infected without opsonization of the bacteria and without synchronization as determined by measuring GFP fluorescence associated with PMNs via flow cytometry (Fig. 3A). However, when PMNs are infected with opsonized USA300 and the samples are synchronized, the bacteria are efficiently ingested by the PMNs and thus protected from lysostaphin (Fig. 3A).

Our initial analysis involved incubating the isogenic USA300 strains with human PMNs without promoting phagocytosis, where most bacteria remained extracellular. Strains lacking *lukAB* or the double mutant lacking both *lukAB* and *pvl* exhibit similar attenuation in their ability to cause PMN membrane damage at an MOI of 50, as measured by the fluorescent dye SYTOX green (Fig. 3B). A *pvl* single mutant does not display a phenotype in this assay; however, infection at an MOI of 100 reveals a slight role for PVL-mediated killing of PMNs as evidenced by the enhanced attenuation of the *lukAB pvl* double mutant compared to the *lukAB* single mutant (Fig. 3B). These data indicate that LukAB, and to a lesser extent PVL, contribute to PMN killing by extracellular *S. aureus.*

We next examined the roles of LukAB and PVL in protecting *S. aureus* from PMN-mediated killing. For this assay, phagocytosis of *S. aureus* was promoted and bacterial burden monitored. As shown previously, PMNs kill opsonized *S. aureus* within the first 1 to 2 h followed by bacterial growth (7, 8, 42). Although there was no difference in PMN-mediated killing of the strains evaluated, we observed that the USA300 mutants that lacked *lukAB* or both *lukAB* and *pvl* exhibited impaired growth rebound compared to the WT strain (Fig. 3C). Of note, the rebound phenotype observed with the strain lacking both *lukAB* and *pvl* was attributed to LukAB since the strain lacking *pvl* was indistinguishable from the WT strain. These data demonstrate that under the conditions tested, LukAB, but not PVL, contributes to the outgrowth of phagocytosed USA300 during *ex vivo* infection with PMNs.

To confirm the predominant role for LukAB in enhancing *S. aureus* growth postphagocytosis and to determine the effect of LukAB on PMN viability postphagocytosis, we constructed a series of isogenic strains where a complementing vector carrying the *lukAB* locus, or the corresponding empty vector, was integrated into the chromosome at the *S. aureus* pathogenicity island 1 (SaPI1) site (20). Immunoblot analysis of factors secreted by these strains confirmed that integration of *lukAB* into the Δ*lukAB* strain, resulting in a Δ*lukAB*::*lukAB* strain, fully rescued the production of this toxin to a level similar to that of the wild-type strain (Fig. 3D). Using these strains, we next compared the cytotoxicity of opsonized USA300 strains to human PMNs postphagocytosis, where most bacteria are now intracellular. Assessment of PMN membrane integrity during infection with opsonized *S. aureus* using the SYTOX green dye showed that the Δ*lukAB* mutant caused significantly less damage than the LukAB-producing WT and Δ*lukAB*::*lukAB* strains (Fig. 3E). Similar results were obtained when PMN lysis was monitored with lactate dehydrogenase (LDH) (data not shown). This observation is most dramatic at 1 h

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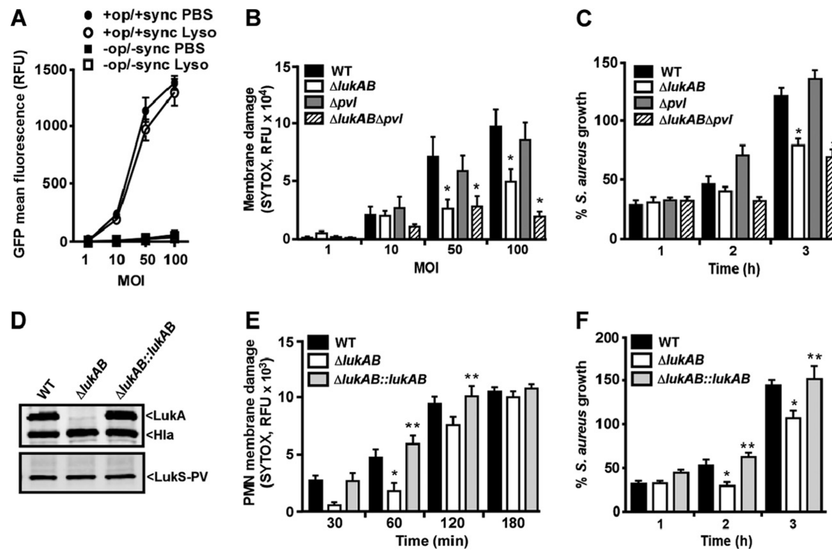

FIG 3 LukAB causes PMN damage pre- and postphagocytosis and enhances the growth of surviving USA300 cells postphagocytosis. (A) PMNs were infected with various MOI of USA300 Δ*sae* constitutively producing GFP with opsonization and synchronization (+op/+sync) or without opsonization and synchronization (−op/−sync) for 60 min. Subsequently, extracellular bacteria were eliminated with lysostaphin (Lyso) and bacteria associated with PMNs were tracked by monitoring GFP fluorescence via FACS. Results represent the mean from PMNs isolated from four different donors ± standard error of the mean. (B) Infection of PMNs with nonopsonized isogenic USA300 strains at various MOI where PMN membrane damage was monitored 60 min postinfection with SYTOX green. Results represent the mean from PMNs isolated from five different donors ± standard error of the mean. *, statistical significance compared to the WT and Δ*pvl* strains ($P < 0.05$). (C) Growth of opsonized isogenic USA300 strains upon infection of PMNs. Bacterial CFU were determined by dilution plating after lysing PMNs at 1, 2, or 3 h postsynchronization. To determine the percentage of growth following initial killing by PMNs, bacterial counts were normalized to input at time zero, which was set at 100%. Results represent the mean from PMNs isolated from five different donors, ± SEM. *, statistical significance compared to the WT and Δ*pvl* strains ($P < 0.05$). (D) Immunoblot of LukA, PVL, and Hla (alpha-toxin) protein levels produced by the indicated USA300 erythromycin-sensitive isogenic strains. (E) PMNs were infected with USA300 strains described in panel D, and membrane damage was monitored at 30, 60, 120, and 180 min postsynchronization using SYTOX green. Results represent the mean from PMNs isolated from six different donors ± standard error of the mean. (F) Growth of opsonized USA300 isogenic strains upon infection of PMNs evaluated as described in panel C. Results represent the mean from PMNs isolated from five different donors ± standard error of the mean. *, statistical significance compared to the WT; **, statistical significance compared to the Δ*lukAB* strain ($P < 0.05$).

postsynchronization, although as the infection proceeds, membrane damage occurs regardless of whether or not the strains have *lukAB*, consistent with the production of additional cytolytic factors (9, 10, 38, 43).

We also evaluated the viability of the Δ*lukAB*::*lukAB* strain and the corresponding WT and Δ*lukAB* control isogenic strains during infection with PMNs. The complemented strain was able to restore rebound of the Δ*lukAB* strain in the bacterial viability assay, confirming the contribution of LukAB to *S. aureus* outgrowth (Fig. 3F).

LukAB promotes early escape from PMNs postphagocytosis, promoting enhanced USA300 growth. Our data demonstrate that LukAB is employed by both extracellular and phagocytosed USA300 to kill PMNs during *ex vivo* infection (Fig. 3), as well as a means to promote USA300 rebound after ingestion by PMNs (Fig. 3). Interestingly, we noted subtle differences in damage to human PMNs upon infection with nonopsonized and opsonized *S. aureus*. When PMNs were infected with nonopsonized and hence nonphagocytosed bacteria at an MOI of 10 for 60 min, membrane damage by the WT was minimal and there was no difference between WT and Δ*lukAB* USA300 strains (Fig. 3B). However, when infected with opsonized, phagocytosed bacteria at an MOI of 10, LukAB-producing WT and Δ*lukAB*::*lukAB* complemented strains caused significantly more membrane damage, displaying a strong LukAB phenotype during the 60-min infection (Fig. 3E). These results suggest that LukAB is more potent in damaging PMNs after *S. aureus* internalization, where the toxin is likely to be more concentrated due to compartmentalization within the PMNs, leading us to hypothesize a possible role for LukAB in mediating the escape of *S. aureus* from PMNs. The rebound observed in the bacterial viability experiments (Fig. 3C and F) supports the idea that LukAB could promote escape of USA300 from PMNs, thus allowing the bacteria to replicate once outside the PMNs. To study LukAB in this context, the USA300 LAC WT, Δ*lukAB*, and Δ*lukAB*::*lukAB* strains described in Fig. 3D to F were transformed with a plasmid constitutively expressing GFP, enabling tracking of the location of the bacteria via flow cytometry.

Next, PMNs were infected with opsonized USA300 strains (WT, Δ*lukAB*, and Δ*lukAB*::*lukAB*) expressing GFP, followed by treatment with lysostaphin or mock treatment with PBS immediately following synchronization, as well as 30 or 60 min after synchronization. By examining the data as scatter plots of ungated samples we were able to visualize both the PMNs and the bacteria. As noted previously, immediately following synchronization, a small portion of non-PMN-associated USA300 cells remain extracellular (44), whereas most of the USA300 is PMN associated (Fig. 4A). As expected, lysostaphin treatment significantly reduced the number of extracellular bacteria, while it had only a minor effect on USA300 cells that had been ingested by the PMNs (Fig. 4A). Importantly, the fluorescence of GFP-positive PMNs and the fluorescence of non-PMN-associated extracellular bacteria are similar for all strains with or without lysostaphin treatment, indicat-

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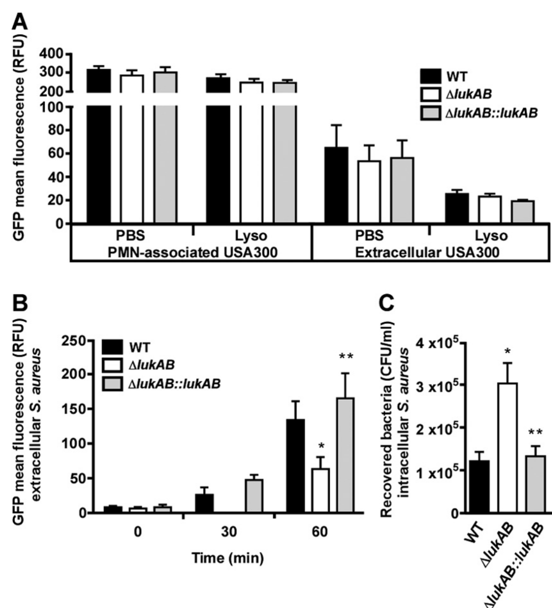

FIG 4 LukAB enhances the escape of USA300 from PMNs postphagocytosis. (A) PMNs were infected with the indicated GFP-USA300 strains at an MOI of 10, and PMNs were subsequently treated with lysostaphin immediately postsynchronization or mock treated with PBS, followed by tracking of GFP-USA300 by flow cytometry. PMN-associated GFP fluorescence or GFP fluorescence from extracellular USA300 post-PBS or lysostaphin (Lyso) treatment is shown. Results represent the mean from PMNs isolated from eight different donors ± standard error of the mean. (B) GFP fluorescence exclusively from extracellular bacteria at 0, 30, or 60 min postsynchronization as determined by subtracting the fluorescence observed with lysostaphin treatment at the indicated time points from the fluorescence observed with PBS treatment on ungated samples. Results represent the mean from PMNs isolated from eight different donors ± standard error of the mean. (C) Bacterial counts recovered from PMNs after a 60-min infection with the GFP-USA300 strains. Lysostaphin was added at 60 min postinfection and then removed through washing. PMNs were then lysed, and the released bacteria were serially diluted and plated. Counts are given in CFU/ml. Results represent the mean from PMNs isolated from six different donors ± standard error of the mean. *, statistical significance compared to the WT; **, statistical significance compared to the Δ*lukAB* strain ($P < 0.05$).

ing that the bacteria were equally phagocytosed by PMNs regardless of *lukAB* status (Fig. 4A).

The amount of extracellular bacteria at each time point during infection was determined by subtracting the GFP mean fluorescence observed with lysostaphin treatment from the GFP mean fluorescence observed with PBS treatment (Fig. 4B). Over time, the amount of extracellular bacteria increased in a *lukAB*-dependent manner, and at 60 min postsynchronization there were significantly fewer Δ*lukAB* extracellular bacteria, a phenotype that was complemented by providing *lukAB* in *trans* (Fig. 4B). This further strengthens our supposition that LukAB facilitates the escape of *S. aureus* from PMNs.

To validate the flow cytometry data, we also determined bacterial survival within PMNs following lysostaphin treatment at 1 h postsynchronization. Following treatment, lysostaphin was washed away, the PMNs were lysed, and the recovered bacteria were serially diluted and plated for CFU. We reasoned that if the Δ*lukAB* mutant is deficient in escape, a higher number of CFU of Δ*lukAB* bacteria should remain within PMNs, protected from the lysostaphin treatment. In line with that, significantly more Δ*lukAB* mutant cells than cells of the WT or complemented strain were recovered after lysostaphin treatment (Fig. 4C).

The LukAB-mediated PMN damage observed under phagocytosing conditions is solely caused by USA300 that is within PMNs. Collectively, the data presented in Fig. 4 led us to hypothesize that LukAB is mediating the escape of USA300 from within PMNs early on in infection and that the escape of the bacteria precedes PMN membrane damage or causes the damage through the escape process. However, even with opsonization and synchronization, a small percentage of bacteria are not phagocytosed at time zero (Fig. 4A and B) (44) and it is possible that this small percentage of extracellular bacteria are contributing to the observed PMN membrane damage (Fig. 3E) and subsequent escape of bacteria as the infection progresses (Fig. 3F). To address this possibility, PMNs were infected with the WT, Δ*lukAB*, or Δ*lukAB*::*lukAB* strain expressing GFP with opsonization and synchronization where lysostaphin or PBS was added immediately following synchronization and then incubated with the samples for the entirety of the 60-min infection (Fig. 5A). Under lysostaphin treatment, nearly all extracellular bacteria were eliminated at time zero, including those that were not phagocytosed initially (Fig. 4A) and those that escaped during the time of infection, thus allowing us to infer that any observed damage to PMNs under these conditions was caused by intracellular bacteria. The health of the PMNs after infection was evaluated by monitoring the forward and side scatter of the PMNs via flow cytometry. We observed that the WT and Δ*lukAB*::*lukAB* strains mediate a downward shift in both the forward and side scatter of the PMNs, indicating cell damage and death, whereas the *lukAB* mutant strain does not cause such a dramatic shift and the scatter of the majority of the cells looks similar to that of uninfected PMNs (Fig. 5A). These data show that LukAB-mediated PMN death still occurs in the constant presence of lysostaphin, suggesting that cell death is caused by intracellular, not extracellular, bacteria (Fig. 5A).

We also performed the experiment described above where we examined the damage to the PMNs using ethidium bromide, a fluorescent dye routinely used to monitor leukotoxin-mediated pore formation. In addition to constant lysostaphin, we also performed the experiment in the presence of an anti-LukA antibody that neutralizes the activity of extracellular toxin during infection (data not shown) to further ensure that the observed PMN damage was not caused by extracellular bacteria/toxin. Our observations by microscopy validated the flow cytometry data, showing that the GFP strains are equally phagocytosed at time zero postsynchronization (Fig. 5B). However, by 60 min postsynchronization the cells infected with the WT or complemented strains are highly ethidium bromide positive (Fig. 5B). In contrast, PMNs infected with the *lukAB* mutant were GFP positive at 60 min postsynchronization but ethidium bromide fluorescence remained at background levels. Ethidium bromide-positive cells were quantified by counting the number of positive cells per field, which is shown as a bar graph (Fig. 5C). Similar results were obtained when the health of the PMNs was monitored by visualizing propidium iodide incorporation via fluorescence microscopy (data not shown). Although not apparent from the overlay, we also noted that the GFP fluorescence from the WT and complement strains was dimmer and less abundant at 60 min postsynchronization compared to that of the *lukAB* mutant. This is in line with the flow

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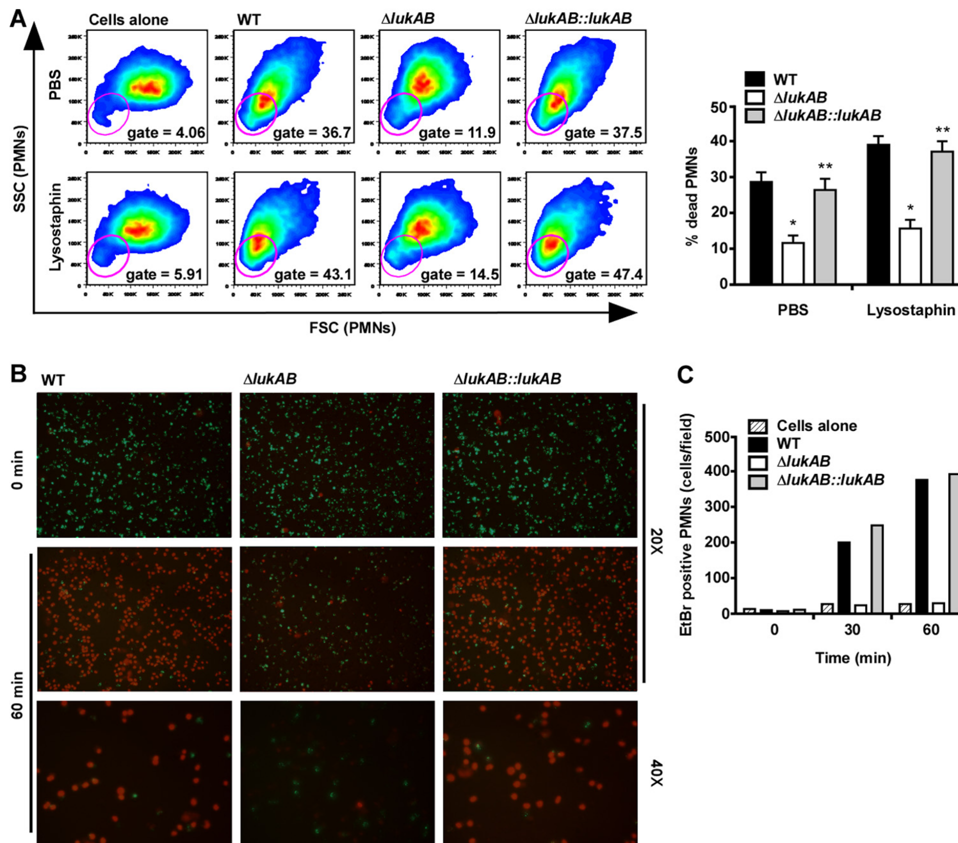

FIG 5 PMN damage caused by phagocytosed USA300 via LukAB occurs from within PMNs. (A) PMNs were infected with GFP-USA300 strains at an MOI of 10, followed by synchronization and immediate addition of lysostaphin. The 60-min infection proceeded in the presence of lysostaphin to eliminate extracellular bacteria. PMN health was monitored via flow cytometry by comparing the forward and side scatter of the cells. Scatter plots from a representative donor are shown. Dead cells are enclosed within the circular gate, and percentages of dead cells within the gate are denoted. The percentages of dead cells from PMNs isolated from eight different donors ± standard error of the mean are also represented as a bar graph. *, statistical significance compared to the WT; **, statistical significance compared to the Δ*lukAB* strain ($P < 0.05$). (B) PMNs were infected as described in panel A, in the presence of an anti-LukA affinity-purified polyclonal antibody and the fluorescent dye ethidium bromide. Images of GFP fluorescence and ethidium bromide fluorescence were captured using a fluorescence microscope at 0, 30, and 60 min postsynchronization, and representative images from 0 and 60 min are shown. For the 60-min time point, images are shown at ×20 and ×40 magnification. (C) Quantification of ethidium bromide positive PMNs per field of view obtained from images as in panel B. Results represent the average of three independent counts from infections of PMNs isolated from two donors.

cytometry data, and it supports the idea that LukAB-producing bacteria are escaping the PMNs and being killed by the lysostaphin present in the medium, thus reducing the GFP signal. Taken together, these data suggest that LukAB produced by phagocytosed *S. aureus* causes PMN membrane damage from within, promoting the escape of the bacteria from PMNs early on in infection, which results in improved bacterial growth.

DISCUSSION

S. aureus is a versatile bacterium that has evolved to thrive as a colonizer as well as a highly successful pathogen. PMNs are critical to the host response to *S. aureus* (45); they extravasate from the bloodstream and migrate to the site of infection to eliminate the bacterium. *S. aureus* has evolved an extensive armamentarium devoted to counteracting PMNs (46, 47), including the bicomponent leukotoxins LukAB, LukED, PVL, and HlgACB (10, 11). In this study, we set out to investigate the contribution of the different leukotoxins to the ability of USA300 to avoid destruction by primary human PMNs.

We show that growth media as well as growth phase strongly influence which toxins are produced *in vitro* by USA300. Importantly, our data demonstrate that regulation of leukotoxins occurs at the promoter level and localization/secretion level in a growth-medium/growth-phase-dependent manner. In particular, LukAB is unique in that it is most highly secreted by USA300 when the bacteria are grown in the minimal medium RPMI+CAS, and although highly produced in a rich medium like TSB, LukAB is predominantly associated with the bacterium under these growth conditions during stationary phase (27). The contribution of *S. aureus*-associated LukAB to avoidance of PMNs and during infection, however, remains to be determined. Overall we have found that with regard to culture supernatants, leukotoxin levels determine the extent to which a particular leukotoxin will contribute to the cytotoxicity of the strain. These findings highlight the importance of considering not only gene expression but also the level of the toxin in question when studying the contribution of leukotoxins *in vitro*.

Under the infection conditions tested here where USA300 is phagocytosed by PMNs and bacterial growth is evaluated, we find that a *pvl* mutant displays no phenotype (8); however, a *lukAB*

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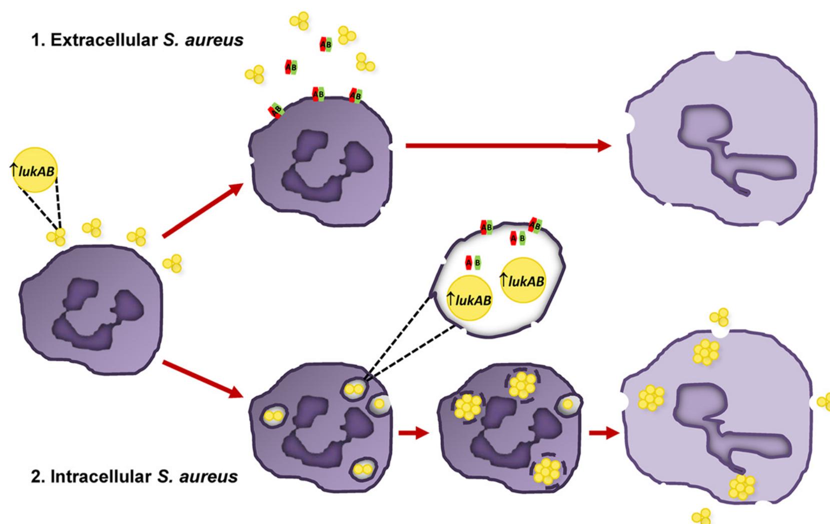

FIG 6 Model depicting the role of LukAB in *S. aureus*-PMN interactions with extracellular versus phagocytosed *S. aureus*. Upon encountering PMNs *lukAB* is upregulated. If *S. aureus* remains extracellular (pathway 1), the bacteria secrete LukAB, which forms pores in PMN membranes causing extensive membrane damage and ultimately resulting in the killing of the PMNs. However, if *S. aureus* is phagocytosed (pathway 2), we propose that LukAB is produced by *S. aureus* within the phagosome (inset) and promotes the escape of *S. aureus* from the phagosome through pore formation and breakdown of the phagosomal membrane, which ultimately results in *S. aureus* escape from the PMN and subsequent PMN death.

mutant displays an attenuated phenotype. Coupled with transcriptional fusion data pointing to *lukAB* being the most upregulated leukotoxin in response to human PMNs, these data suggest that LukAB is employed by USA300 upon first encounter with PMNs and that this response may increase the success of USA300 in establishing infections. The conditions under which PVL is predominantly utilized by *S. aureus* remain to be elucidated, but from this study it seems that the *pvl* promoter is most active under growth conditions in rich media such as TSB and LB. While it is tempting to speculate that PVL may have a larger role in established infections where nutrients are abundant, the different media used here are made up of multiple complex components, any of which could have an impact on leukotoxin gene expression.

Further investigation into the role of LukAB during *ex vivo* infection with PMNs revealed that LukAB not only is utilized by extracellular bacteria to kill PMNs but also is employed by phagocytosed bacteria in order to escape PMNs. To our knowledge, this is the first account of the involvement of a leukotoxin in the escape of *S. aureus* from PMNs. The escape of USA300 from PMNs correlates with both improved bacterial growth following initial killing by PMNs and increased USA300-mediated killing of PMNs. We found that the contribution of LukAB to the escape of USA300 from PMNs is an early phenomenon that diminishes over time, and by 3 h postinfection PMN damage occurs irrespective of LukAB. This observation is consistent with a report by Kobayashi et al. describing the escape of different USA300 strains from PMNs, where the investigators concluded that escape from PMNs by *S. aureus* was independent of toxins when evaluated 6 h postinfection (42). In light of this new role for LukAB, which may be shared with other staphylococcal factors such as delta hemolysin and beta hemolysin (48) and PSMs (38, 43), vaccine strategies that aim to promote phagocyte-mediated killing of *S. aureus* through opsonization may prove to be ineffective. Our data show that opsonization does cause PMN-mediated killing of USA300, but factors such as LukAB allow the early escape and propagation of surviving bacteria.

Collectively, the results of this study allow us to propose a model in which USA300 senses PMNs to elaborate leukotoxins to defend itself from PMN attack (Fig. 6). Moreover, the initial defense seems to be centered on the expression and production of LukAB upon exposure to PMNs. Our data demonstrate that LukAB is a versatile toxin that can be employed by extracellular bacteria to cause PMN damage during infection as well as by phagocytosed bacteria to promote the escape of USA300 from PMNs. Having a dual role, LukAB provides *S. aureus* with a PMN evasion tactic from an intra- or extracellular location. The exact mechanism of how LukAB enables USA300 escape from PMNs remains to be elucidated, but we speculate that LukAB facilitates the initial escape from the phagosome, which is likely to contribute to the virulence potential of USA300 by promoting bacterial growth.

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Ashley L. DuMont and Victor J. Torres are listed as inventors on pat-

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ent applications filed by New York University School of Medicine, which are currently under commercial license.

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