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Supplementary Information for

Staphylococcus aureus biofilms release leukocidins to elicit extracellular trap formation and evade neutrophil mediated killing

Mohini Bhattacharya, Evelien T. M. Berends, Rita Chan, Elizabeth Schwab, Sashwati Roy, Chandan K. Sen, Victor J. Torres and Daniel J. Wozniak

Daniel J. Wozniak

Email: Daniel.Wozniak@osumc.edu

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Supplementary Information Text

Supplementary Materials and Methods

Bacterial strains and growth conditions. Unless stated, all studies were performed in the USA300LAC background (AH1263, provided by Dr. Alex Horswill Carver College of Medicine, University of Iowa, Iowa) and strains were grown in trypticase soy broth (TSB) supplemented with dextrose (2.5g/L, Becton Dickinson) (See Table S2 for more details). The previously published AHLAC protease knockout strain was also generously provided by Dr. Horswill[23].

We are grateful to Drs. Kenneth Bayles, Marat Sadykov and Todd Wildhem for generating the GFP-labeled USA300LAC strain. We acknowledge past (Francis Alonzo, Pauline Yoong, and Tamara Reyes-Robles), and present (Ashira Lubkin and William Souse) members of the Torres lab in creating leukocidin mutants. We would also like to thank Brian Rhea and Dr. Shomita S. Mathew-Steiner for their help with processing of porcine burn wound sections. Transposon mutants used in these studies were obtained from the Network for Antimicrobial Research in *Staphylococcus aureus* at the University of Nebraska, Medical Center[51]. The presence of transposon insertions for these mutants was verified by plating onto TSA + 5 µg/mL erythromycin before use and performing PCR reactions with primers specific for the transposon[51] as well as the respective genes (S3 Table). Double mutants of *lukSF* and either *hlgA, hlgB,* or *hlgC* were constructed by transducing the USA300LAC (JE2) *hlgA::bursa, hlgB::bursa,* or *hlgC::bursa* strains from the Nebraska Transposon Mutant Library into the AH1263 LACΔ*lukSF* strain using phage 80α. Mutants were confirmed by PCR and Western blot. For detailed information on various mutants and how they were generated please refer to Table S2.

Neutrophil isolation. Neutrophils were isolated from whole blood using previously published methods[50]. Briefly, heparinized blood derived from healthy human donors by venipuncture was collected in saline and layered into a Ficoll-Paque gradient. Tubes were then centrifuged at 404 x g for 40 minutes at 23^{o}C (without brake) after which pellets were allowed to sediment in a 1:1 solution of 3% Dextran and 0.9% saline. Spent media were collected and centrifuged at 335 x g for 10 minutes. RBCs in pellets were then lysed with sterile water and 1.8% sodium chloride was used to restore isotonicity. Tubes were centrifuged at 233 x g for 3 minutes. Pellets containing 95-97% neutrophils were re-suspended in HBSS and counted in a hemocytometer chamber, using trypan blue exclusion[52].

Spent media collection. To generate spent media for neutrophil killing assays, isolated colonies were used to inoculate 5 mL TSB and grown for 16- 18 hours at 37^{o}C under shaking (200 RPM) conditions. These were then allowed to grow to an optical density (OD at 600 nm) of 0.5- 0.7, equivalent to an exponentially growing *S. aureus* culture and diluted to OD_{600} of 0.1 for inoculation as planktonic (5 mL/15mL test tube) or biofilm cultures (tissue culture treated 96-well plates, 200 µL/well)[48]. Planktonic (shaking/200 RPM) and biofilm (static) cultures were grown for 24 hours, centrifuged and filter sterilized (0.22 µm). Protein concentrations were measured using a Bradford assay.

LIVE- DEAD killing assays. Neutrophils were incubated with bacterial spent media for 30 minutes and stained with a 1:1 mixture (by volume) of Syto-9 (3.34 mM) and propidium iodide (20 mM) for 15 minutes (Thermo Fisher), after which the ratio of fluorescence generated by Syto -9 (Ex 485nm/ Em 525nm) and propidium iodide (Ex 525nm/ Em 630nm) was measured using a standard fluorometer. %Dead neutrophils were calculated using 0.1% SDS treated cells as a control. For microscopy, 4 X 10^{6} cells/mL were stained with 100 µM Cell Tracker Blue (Thermo

Fisher/ 30min) followed by a 15-minute incubation with spent media and 15 min staining with 2 µM Calcein AM (Invitrogen) and 4 µM Ethidium homodimer- 1 (Invitrogen). Neutrophils treated with 1 µg/mL phorbol 12-myristate 13-acetate (PMA) were used as a positive control. Cell suspensions were centrifuged and mounted onto glass slides using Prolong Gold Antifade mounting medium (Thermo Fisher).

Biofilm formation assays. To test for the ability of strains to form biofilms, cultures were grown for 16-18 hours at 37ºC under shaking conditions (200RPM) and diluted in fresh media (TSB+glucose (2.5g/L, Becton Dickinson)). Once cultures reached exponential phase (O.D of 0.5 @600nm) these were seeded into 96-well tissue culture plates and grown for another 16- 18 hours under static conditions (37ºC) to facilitate biofilm formation. Supernatants were then removed, and biomass attached to the bottom of the well was measured using a previously published crystal violet staining assay[53]. Briefly, biomass was incubated with 0.1% crystal violet and incubated for 20 minutes. Cells were then washed with water and incubated with 95% ethanol with shaking, for an additional 20 minutes to slough biomass off the walls of the wells. Optical density of biomass was then measured at 565nm.

Polyacrylamide Gel Electrophoresis and Liquid Chromatography-Mass Spectrometry. To visualize proteins, present in biofilm supernatants, cultures were grown as previously described. Supernatants from planktonic and biofilm cultures were then filter sterilized and desalted in HBSS using Bio- Rad 10DG desalting columns. Desalted supernatants were then concentrated by lyophilization and amount of proteins was estimated before and after desalting, as well as after lyophilization was estimated using a Bradford assay. Proteins were applied to SDS-PAGE using a 12% Tris-Glycine SDS-Polyacrylamide gels under denaturing conditions. Detection and staining of proteins in polyacrylamide gels was done with Coomassie Brilliant Blue R250. Following

staining, with Coomasie blue reagent bands were cut out from gels in matched pairs (WTP and WTBF) de-stained and trypsin digested. The peptides were then extracted for mass spectrometric analyses. Mass spectrometry analysis was carried out using a Thermo Fusion Orbitrap HPLC MS-MS system, by the Campus Chemical Instrument Center at The Ohio State University.

Western Blot Analysis. The Torres Lab at the Langone Medical Center, New York University, New York, carried out all western blot analyses. Briefly, Freeze-dried pellets were reconstituted in 1x SDS loading buffer and boiled for 10 minutes. Samples were loaded on a 12% SDS-PAGE gel and subsequently transferred to a nitrocellulose membrane. The membranes were probed with a rabbit polyclonal antibody against LukF-PV, previously produced[54]. As a secondary antibody, AlexaFluor-680-conjugated anti-rabbit (Invitrogen) was used after which membranes were imaged using an Odyssey CLx Imaging System (LI-COR Biosciences).

Construction of the USA300-*ftsAZ::gfp* reporter strain. For generation of the USA300-*ftsAZ::gfp* reporter strain the pWSE-1 plasmid was used, a derivative of the plasmid pCL25[55]. The *ftsAZ::gfp* reporter fusion was made by replacement of the cadmium-inducible promoter in front of the gene encoding the short half-life GFPaav in the plasmid pEM64[56], a derivative of the pBK123[57], with 0.6 kb *Sph*I-*Bam*HI DNA fragment containing *ftsAZ* promoter. The resulting plasmid was designated as pEM69. Then, a 1.8-kb PCR fragment of the pEM69 containing the *ftsAZ::gfp* reporter fusion was amplified using primers FTSZ-f (5'-CATGCTAATCAAAAGTGTTGAGATACACAAGC) and *Sac*I-*blaZ*-r (5'-CAAGAGCTCGCCTGTCACTTTGCTTGATATATGAG). Following digestion with the restriction endonuclease *Sac*I, the PCR product was ligated into the plasmid pCL25 hydrolyzed with restriction endonuclease *Sac*I and *Sma*I. The resulting pWSE-1 plasmid was introduced into the strain CYL316 with following integration at the L54a *attB* site located within the *geh* gene in

S. aureus chromosome as previously described[55]. The USA300-*ftsAZ::gfp* strain was constructed by bacteriophage Φ11-mediated transduction of the integrated pWSE-1 plasmid as described.

Citrullinated histone staining. For imaging of neutrophils with antibodies against citrullinated histone proteins, 4 X 10^6 cells/mL were stained using the LIVE-DEAD protocol described above, centrifuged and fixed using 2% paraformaldehyde. Cells were blocked using 1% bovine serum albumin (60min, RT/overnight, 4^{o}C). Cells were centrifuged at 149 x g and washed with phosphate buffered saline +0.1% Tween-20 (n=3). Cells were centrifuged and re-suspended in rabbit polyclonal primary antibody against histone H3 (citrulline R2) (Abcam ab174992). Cells were incubated (120min, RT/overnight, 4^{o}C) and washed as described above. Alexa Fluor 488 goat anti-rabbit protein was used as a fluorescent secondary antibody (120min, RT/Invitrogen). Cells were mounted onto glass slides using Prolong Gold Antifade mounting medium (Thermo Fisher).

Myeloperoxidase/ Elastase antibody staining. For imaging of neutrophils with antibodies against MPO and NE, 4X10^6cells/mL were stained using the LIVE-DEAD protocol described above, after which cells were centrifuged and fixed using 2% paraformaldehyde. Fixed cells were then blocked using 1% bovine serum albumin for 1 hour at room temperature (or overnight at 4^{o}C). Blocked cell suspensions were centrifuged at 233 x g and washed with phosphate buffered saline containing 0.1% Tween-20 for a total of 3 washes. Cells were then centrifuged and re-suspended in primary antibody against either myeloperoxidase or neutrophil elastase (Abcam ab9535/ ab21595). Cells were incubated for 2 hours at room temperature (or overnight at 4^{o}C) and washed as described above. Alexa Fluor 488 goat anti-rabbit protein was used as a fluorescent secondary antibody. Cells were incubated for an additional 2 hours at room temperature (or overnight at 4^{o}C)

washed and mounted onto glass slides using prolong golf antifade reagent. Slides were then imaged at 600X using confocal laser scanning microscopy.

Porcine full thickness burn-wound model. White female Yorkshire pigs (70-80 lb, ~11 weeks old) were obtained from Hartley Farms, Circleville, Ohio. (Male pigs were excluded to prevent potential uro-genital obstruction caused by dressings. The use of female pigs maximizes the area available for creating burns, as the dressing can be wrapped farther caudad). Six 2X2 inch burn wounds were created on the backs of two pigs, using previously established methods[11]. To prevent cross contamination wild type and Δ5 strains were infected on separately housed pigs (n=2 each). Briefly, 3 days after wounding pigs using an electrically heated burning device, 10^7 bacteria were spread over the surface of each wound (n=6 per animal). Wounds were then covered using a semi-occlusive dressing as previously described[11]. 8mm punch biopsies (n=3 per wound) and a longitudinal full thickness section, were collected at 7, 14 and 35 days after infection. Each wound healed separately and were considered experimental replicates. Punch biopsies were homogenized and resuspended in 1 mL of phosphate buffered saline to calculate cfu/g of tissue in triplicate. Longitudinal sections were utilized for all antibody staining procedures. Samples were coded, and data collection performed in a blinded fashion.

Antibody staining of porcine tissue sections. To stain for the presence of bacteria as well as for citrullinated histone proteins, biopsies taken across the length of the wound were embedded in paraffin and sectioned (100 µm). These sections were mounted onto glass slides and blocked using 1% bovine serum albumin for 16-18 hours (4ºC). Slides were then incubated with primary antibodies against citrullinated histones (Abcam 174992) and *S. aureus* (Abcam 37644) for 2 hours at room temperature. Slides were then washed with phosphate buffered saline containing 0.1% Tween 20. Goat anti- rabbit Alexa Fluor 488 (Invitrogen) was used as a secondary fluorescent

antibody for citrullinated histones while a goat anti-mouse IgG Alexa Fluor 647 (Abcam 150115) was used to visualize *S. aureus*. Finally, slides were stained with 1µg/mL DAPI for 30 minutes and visualized using confocal microscopy. Co-localization analyses and calculation of Pearson's coefficients was performed using Imaris software version x 64 8.4.1 using ten fields of view from 3 independent longitudinal sections. Lastly, results from immunofluorescence were corroborated with a blinded gross analysis of the differences in levels of bacterial and histone staining by six independent individuals, using triplicate, stained longitudinal sections.

Scanning Electron Microscopy

Processing and imaging of samples was performed as previously described [1, 2].Briefly, following glutaraldehyde fixation and dehydration with graded ethanol series, the samples were treated with hexamethyldisilazane (HMDS, Electron Microscopy Sciences, Hatfield, PA (EMS)) and left overnight to dry. Before scanning, samples were mounted on an aluminum stub and coated with gold-palladium (Au-Pd). Imaging of samples was done using FEITMNOVA nanoSEM scanning electron microscope (FEITM, Hillsboro, OR) equipped with a field-emission gun electron source.

Quantification and Statistical Analysis. All statistical analysis was carried out using Graph Pad Prism Ver 5.0b. One-way analysis of variance with Tukey's least significant difference was a post-hoc test where appropriate. Bartlett's test was used to determine the extent of variability between sample populations. Variance between groups was found to be similar. Results for all experiments represent triplicate conditions performed in at least 3 independent experiments. Sample sizes for neutrophil killing assays were chosen based on the standard deviation of a pilot experiment performed in triplicate to achieve approximately a specified half width of the 95% confidence interval (α=0.05).

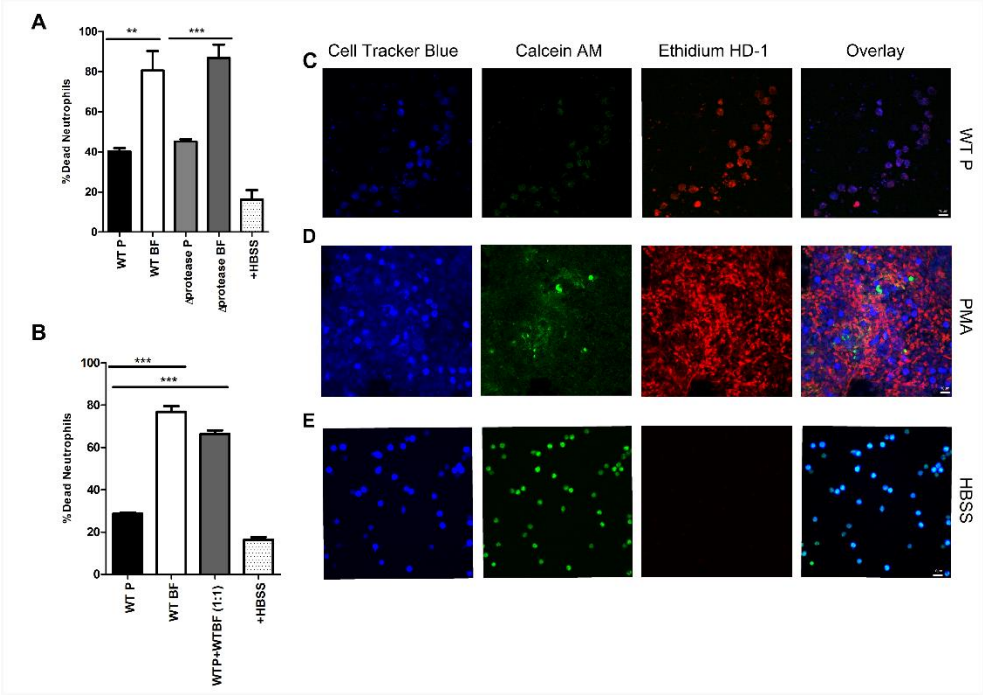

Fig. S1. Planktonic and biofilm spent media show distinct neutrophil killing activities. Killing of neutrophils treated with WTP and WTBF spent media compared to an isogenic Δprotease (*Δaur, ΔsspAB, ΔscpA, Δspl*:erm) strain(A). LIVE-DEAD analysis of neutrophils treated with WTP+WTBF spent media mixed 1:1 by volume for 60 minutes (B). Neutrophils treated with planktonic spent media (C) in comparison to neutrophils treated with 1µg/mL phorbol myristate acetate (PMA) or HBSS as positive and negative controls respectively (D, E). Percentage cell death for all neutrophil killing assays was calculated in comparison to neutrophils treated with 0.1% sodium dodecyl sulfate as a positive control and Hank's Balanced Salt Solution(HBSS) as a negative control (+HBSS). Results represent an average of six independent experiments ±s.e.m. **P<0.01, ***P<0.001 using one- way analysis of variance and a Tukey's post hoc analysis.

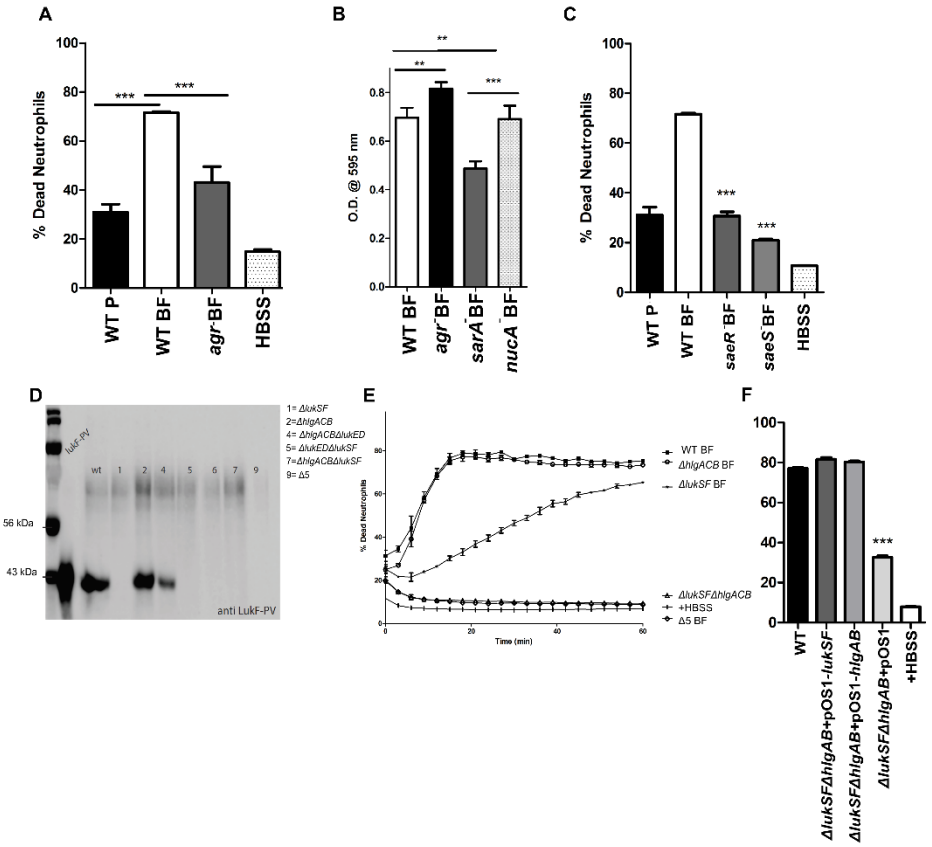

Fig. S2. Neutrophil killing requires *agr* regulated proteins. LIVE-DEAD assay showing neutrophil death after incubation with biofilm spent media from wild type and an isogenic Δ*agr* strain (A). Crystal violet assay measuring biofilm biomass of respective strains. Δ*nucA* and Δ*sarA* biofilms were used as positive and negative controls respectively (B). LIVE-DEAD assays for *saeR::bursa* and *saeS::bursa* transposon mutants (C). Western blot analysis of PVL LukF-PV subunit protein released in biofilm spent media. Molecular markers for each subunit are shown 56kDa = LukS, 43kDa= LukF (D). Kinetic assay showing percentage dead neutrophils every 3 minutes for a 60-minute incubation with biofilm spent media from indicated strains (E). Neutrophil killing assays similar to A, with spent media from wild type biofilms, the *ΔlukSFΔhlgAB* strain overexpressing LukSF (Δ*lukSF*Δ*hlgAB*+pOS1-*lukSF*) or HlgAB (Δ*lukSF*Δ*hlgAB*+pOS1-*hlgAB*). Neutrophil killing with biofilm spent media from a

ΔlukSFΔhlgAB containing an empty vector (Δ*lukSF*Δ*hlgAB*+pOS1) is shown for comparison (F). Percentage cell death for all neutrophil killing assays was calculated in comparison to neutrophils treated with 0.1% sodium dodecyl sulfate as a positive control and Hank's Balanced Salt Solution(HBSS) as a negative control (+HBSS). Results represent an average of 6 independent experiments ±s.e.m. **P<0.01, ***P<0.001 using one- way analysis of variance and a Tukey's post hoc analysis. Significance for panels C, F are shown in comparison to WT.

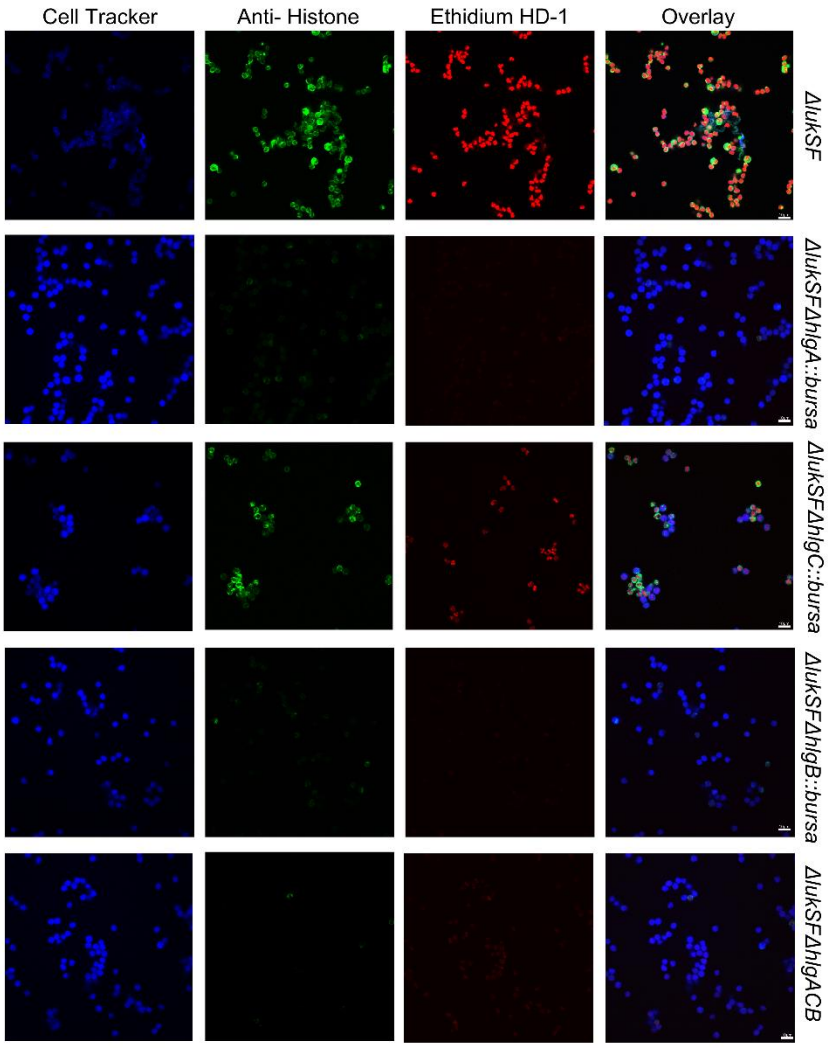

Figure S3. HlgAB and not HlgCB contributes to neutrophil extracellular trap formation. Confocal laser scanning microscopy of neutrophils treated with spent media from biofilms of indicated strains. Neutrophils were pre-stained with Cell Tracker Blue (cytosol) and labelled with an anti-histone H3 (citrulline R2) antibody. Alexa Fluor 488 (secondary antibody/ green) and ethidium homodimer-1(DNA/ red) were used to visualize cells. Images were captured at a 600X magnification and represent the majority population phenotype of six independent experiments performed in triplicate.

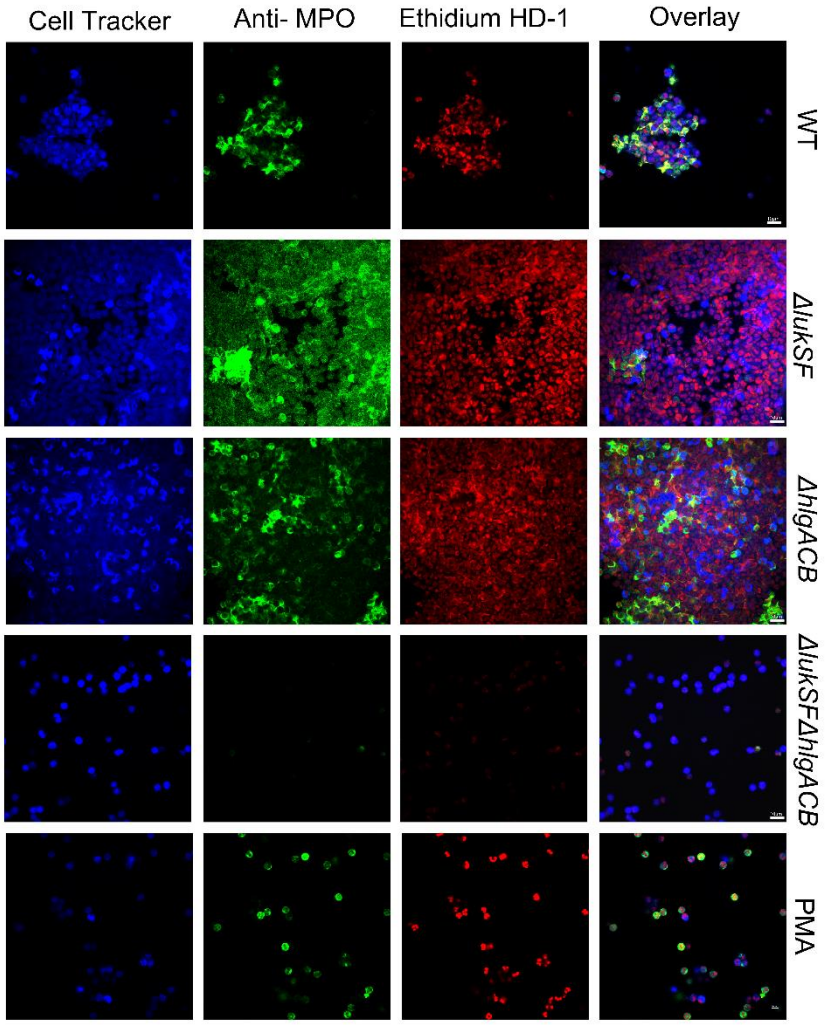

Figure S4. Neutrophils treated with biofilm spent media release myeloperoxidase. Confocal laser scanning microscopy of neutrophils treated with spent media from biofilms of indicated strains. Neutrophils pre-stained with Cell Tracker Blue were incubated with spent media for 30 minutes, and treated with anti- neutrophil myeloperoxidase antibody, stained with Alexa Fluor 488 secondary (green) and ethidium homodimer-1(DNA/dead). Images were captured at a 600X magnification and represent the majority population phenotype of 6 independent experiments. Neutrophils treated with 1µg/mL phorbol myristate acetate (PMA) are shown as a positive control.

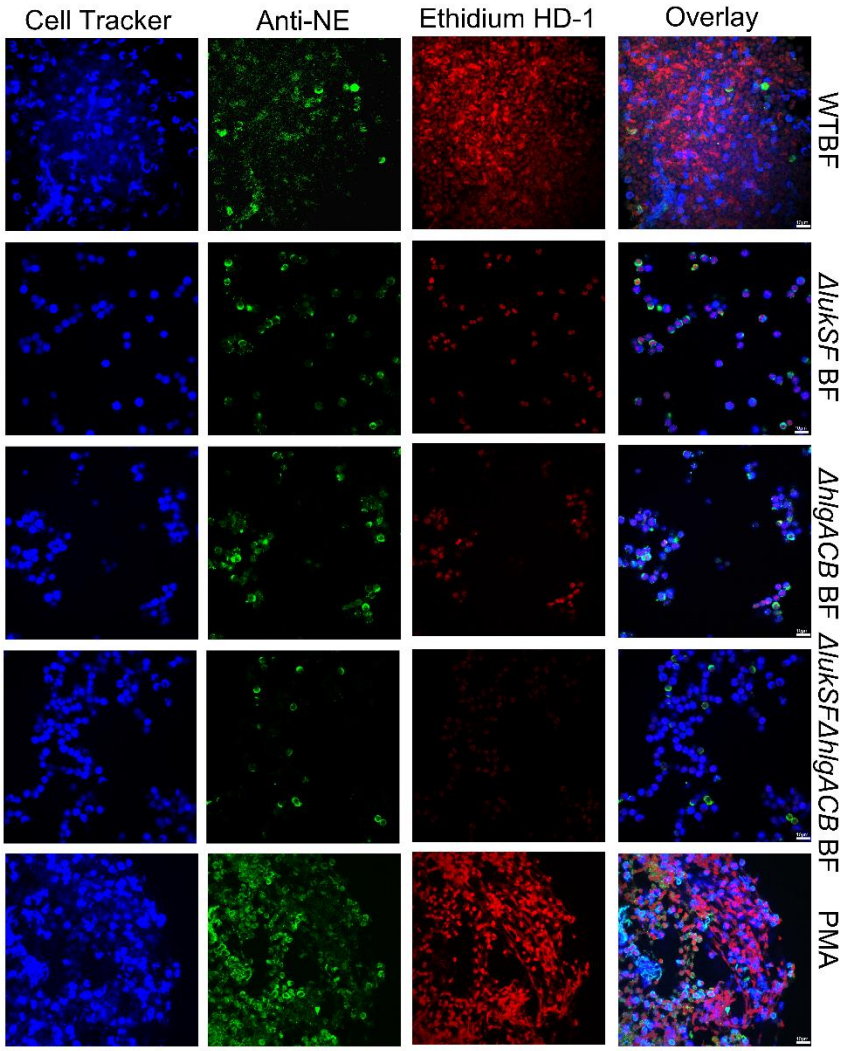

Figure S5. Neutrophils treated with biofilm spent media release neutrophil elastase. Confocal laser scanning microscopy of neutrophils treated with spent media from biofilms of indicated strains. Neutrophils pre-stained with Cell Tracker Blue were incubated with spent media for 30 minutes and treated with an antibody against neutrophil elastase. Cells were stained using an Alexa Fluor 488 (green) secondary and ethidium homodimer-1. Images were captured at a 600X total magnification and represent the majority population phenotype of 6 independent experiments. Neutrophils treated with 1µg/mL phorbol myristate acetate (PMA) are shown as a positive control.

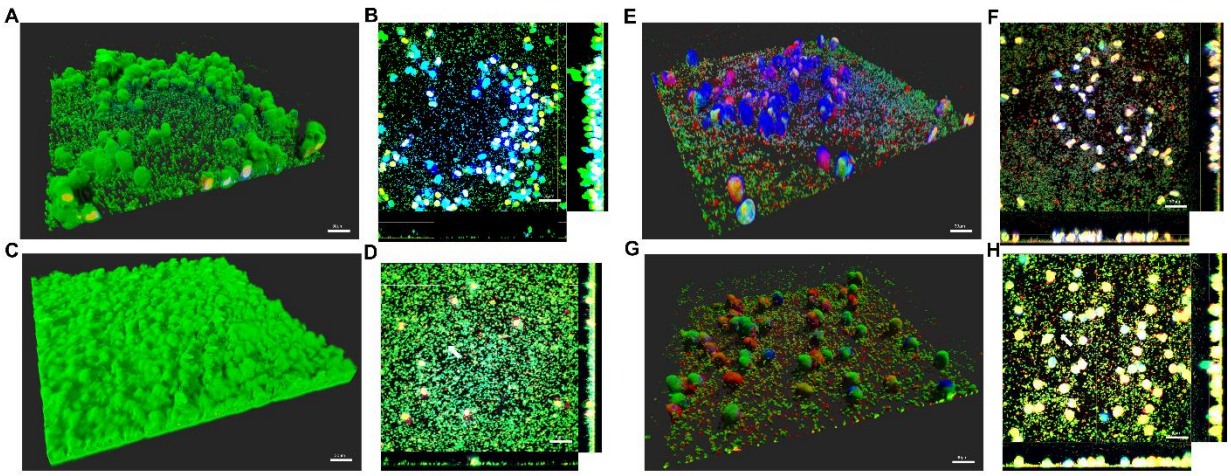

Figure S6. Leukocidins are required for biofilms to survive interaction with neutrophils. Neutrophils were stained with Cell Tracker Blue for 30 minutes and then incubated with WT 24-hour *S. aureus* biofilms for 30 mins (A, B), or 2 hours (C, D). Viable bacteria were stained with Syto- 9 (green), DNA of membrane damaged or dead cells were stained with ethidium homodimer-1 (red). Images were collected as a Z-stack, representing the thickness of the biofilm (left panels). Sections through the biofilm were imaged to assess penetration of neutrophils into the biofilm biomass (right panels). Similar experiments performed with the isogenic Δ5 strain, lacking all 5 leukocidins (E-H). Images are representative of six independent experiments performed in triplicate.

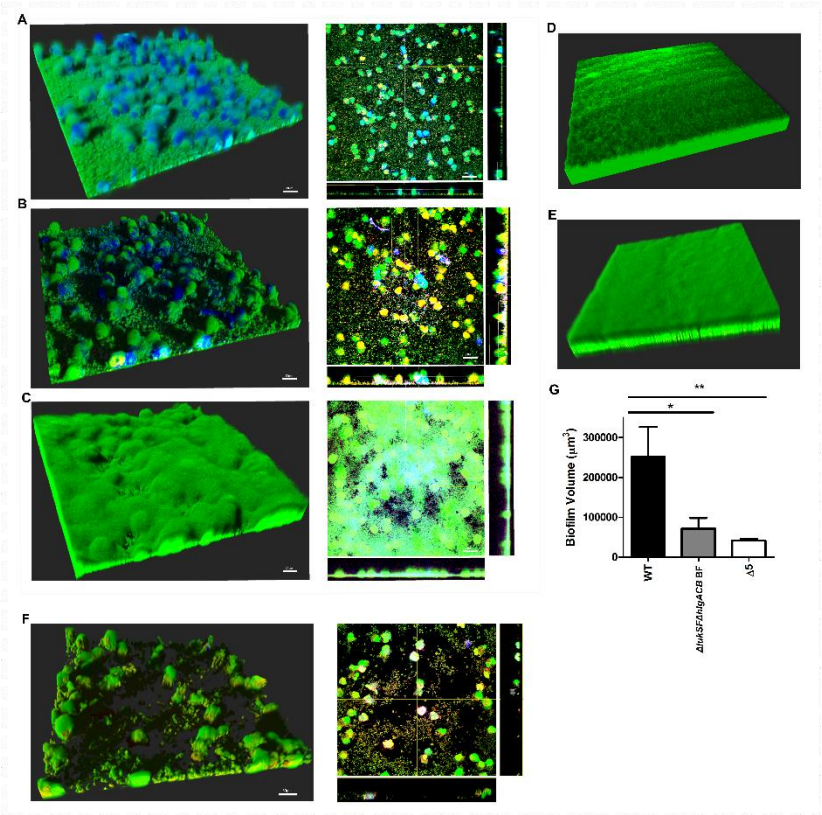

Figure S7. NET induction does not affect biofilm bacterial survival. Experiments were performed as described for Figure 4. Biofilms of a USA300LAC13C::P*ftsAZ*:*gfp* strain were grown for 24 hours and incubated for 30 mins (A), 1 hour (B) or 2 hours (C) with $4X10^6$ neutrophils/ mL. Neutrophils were pre- incubated with Cell Tracker Blue and fixed with 2% paraformaldehyde after incubation periods. Ethidium homodimer-1 was used to stain for DNA of dead/damaged neutrophils. Images were collected as a Z-stack, representing the thickness of the biofilm (left) growing in respective flow cells. Sections through the biofilm were imaged to assess penetration of neutrophils into the biofilm (right). Images are representative of three independent experiments. Negative control showing biofilms of WT USA300LAC (D) and Δ5 strains (E) incubated without neutrophils and stained with Syto 9 (green) and EthHD-1 (red). Experiments similar to those performed in A-C, with the *ΔlukSFΔhlgACB* strain (F). Volume quantification of

WT, *ΔlukSFΔhlgACB* and Δ5 biofilms, after treatment with neutrophils for 2 hours (G). Imaris software version x 64 8.4.1 was used for quantification of biofilm volume. Results represent an average of quantification from 5 independent experiments ±s.e.m. **P<0.01, ***P<0.001 using one- way analysis of variance and a Tukey's post hoc test.

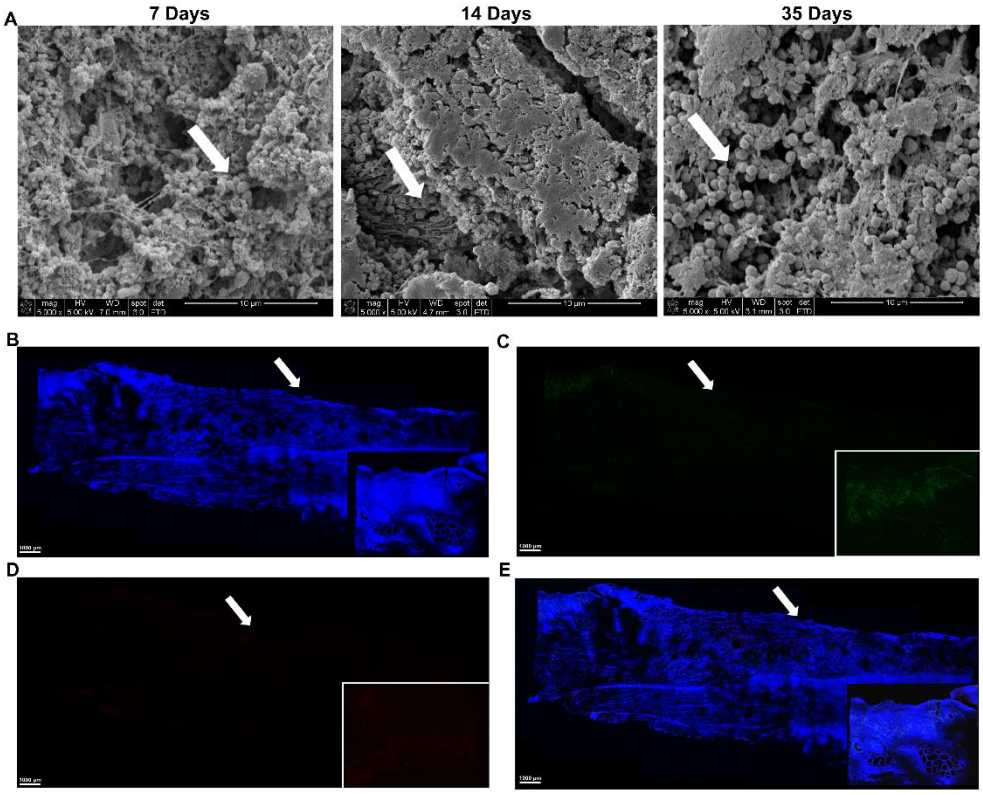

Figure S8. *S. aureus* forms biofilms in porcine full-thickness burn wounds where PVL and HlgAB elicit NET formation. Scanning electron microscopy images of bacterial communities taken from wound beds of pigs infected with wild-type *S. aureus* USA300LAC at 7, 14 and 35 days post inoculation (White arrows highlight bacterial aggregates) (A). Longitudinal sections taken across the wound bed of pigs infected with a *ΔlukSFΔhlgACB* strain, at day 7 post inoculation. Sections were stained with 4',6'-diamidino-2-phenylindole (DAPI) (B), an anti-citrullinated histone antibody (green) (C) and an anti- *S. aureus* antibody (red) (D) and visualized at 100X total magnification. Overlay of images shown in B-D (E). Images were taken at 600X total magnification, from surface (white arrows) of corresponding wounds B-E. Insets show zoomed in images of the wound bed stained with respective dyes. Results represent an average of two independent infections per strain, performed in triplicate ±s.e.m.

Table S1. Proteins identified in spent media

Biofilm protein	Accession Number	Molecular Weight (kDa)	Planktonic protein	Accession number	Molecular weight (kDa)
Thermonuclease (*nuc*)	A0A0H2XHI2_STAA3	25	Triacylglycerol lipase	A0A0H2XI72_STAA3	76
Autolysin (*atl*)	A0A0H2XIJ3_STAA3	137	Triacylglycerol lipase	A0A0H2XG41_STAA3	77
Immunoglobulin G binding protein A (*spa*)	A0A0H2XJH7_STAA3	56	Uncharacterized protein	A0A0H2XIL0_STAA3	15
Lipoteichoic acid synthase (*ltaS*)	LTAS_STAA3	74	Alpha hemolysin	A0A0H2XEW5_STAA3	36
Probable transglycosylase (*isaA*)	ISAA_STAA3	24	Enolase	ENO_STAA3	47
V8 protease (*sspA*)	A0A0H2XFC3_STAA3	36	Putative surface protein	A0A0H2XIW0_STAA3	16
Cysteine protease (*sspB*)	A0A0H2XGH9_STAA3	45	Uncharacterized protein	A0A0H2XG24_STAA3	19
Clumping factor A (*clfA*)	A0A0H2XG16_STAA3	97	Glyceraldehyde-3-phosphate dehydrogenase	A0A0H2XHX6_STAA3	36
Alkyl hydroperoxide reductase subunit C (*ahpC*)	AHPC_STAA3	21	Uncharacterized protein	A0A0H2XJY4_STAA3	20
Ear protein (ear)	A0A0H2XJB4_STAA3	20	Chaperone protein DnaK	DNAK_STAA3	66
Triosephosphate isomerase (*tpiA*)	TPIS_STAA3	27	Immunodominant staphylococ	ISAB_STAA3	19

			cal antigen B		
Superoxide dismutase [Mn/Fe] 1 (*sodA*)	SODM1_STAA3	23	Putative cell wall surface anchor family protein	A0A0H2XJV9_STAA3	49
Panton-Valentine leukocidin, LukF-PV (*lukF-PV*)	A0A0H2XFK0_STAA3	37	5'-nucleotidase, lipoprotein e(P4) family	A0A0H2XKC1_STAA3	33
Bacterial non-heme ferritin (*ftnA*)	FTN_STAA3	20	N-acetylmuramoyl-L-alanine amidase domain protein	A0A0H2XI01_STAA3	69
30S ribosomal protein S1 (*rpsA*)	A0A0H2XJ39_STAA3	77	Putative staphylococcal enterotoxin	A0A0H2XIS3_STAA3	23
50S ribosomal protein L17 (*rplQ*)	RL17_STAA3	14	CHAP domain family	A0A0H2XHA3_STAA3	17
Fructose-bisphosphate aldolase class 1 (fda)	ALF1_STAA3	33	Chemotaxis inhibitory protein	CHIPS_STAA3	49
Penicillin-binding protein 3 (*pbp3*)	A0A0H2XIC6_STAA3	43	Dihydrolipoyl dehydrogenase	A0A0H2XIQ2_STAA3	33

Table S2. Bacterial strains used in this study

Strain	Description	Source/Reference
AH1263 LAC	Erm^S USA300 parent strain	(Wormann *et al.*, 2011)
JE2	Erm^s USA300 parent strain	(Fey *et al.*, 2013)
AH LAC *agr:tet*	AH LAC transduced with *agr::tetM*	(Kiedrowski *et al.*, 2011)
AH LAC *Δ*protease	AHLAC *ΔaurΔsspABΔscpAΔspl::erm*	(Wormann *et al.*, 2011)
AH LAC *ΔlukSF* (VJT 44.10)	AH LAC transduced with the *lukSF:spec* mutation	(Yoong and Torres, 2015)
AH LAC *ΔhlgACB* (VJT 29.98)	AH LAC transduced with the *hlgACB:tet* mutation	This Study (Francis Alonzo)
AH LAC *ΔlukED* (VJT 30.01)	AH LAC transduced with the *lukED::kan* mutation	(Yoong and Torres, 2015)
AH LAC *ΔlukEDΔhlgACB* (VJT 39.10)	AH LAC *ΔhlgACB* transduced with the *lukED::kan*	This Study (Tamara Reyes-Robles)
AH LAC *ΔlukSFΔlukED* (VJT 44.11)	AH LAC Δ*lukED:kan* transduced with the *pvl:spec* mutation	(Yoong and Torres, 2015)
AH LAC *ΔlukSFΔlukEDΔhlgACB* (VJT 38.84)	AH LAC Δ*lukEDΔhlgACB* transduced with the *lukSF:spec* mutation	This study (Pauline Yoong)
AH LAC *ΔlukSFΔhlgACB* (VJT 38.83)	AH LAC Δ*hlgACB::tet* transduced with the *lukSF:spec* mutation	This study (Pauline Yoong)
AH LAC *ΔlukAB* (VJT 23.51)	AH LAC Δ*lukAB* created using the pKOR1 Δ*lukAB* plasmid	(DuMont, Yoong, Surewaard, *et al.*, 2013)
AH LAC *ΔΔΔΔ* (VJT 47.15)	AH LAC Δ*lukSF*Δ*lukED*Δ*hlgACB* also harboring a clean deletion of *luk*AB using the pKOR1-Δ*lukAB* plasmid	(Blake *et al.*, 2018)
AH LAC *ΔlukSFΔhlgA* (VJT 56.09)	AH LAC Δ*lukSF::spec* transduced with phage 80α lysate from JE2 *hlgA::bursa* to get a Δ*pvl*Δ*hlgA* double mutant.	This study (ETMB and RC)
AH LAC *ΔlukSFΔhlgB* (VJT 56.10)	AH LAC Δ Δ*lukSF::spec* transduced with phage 80α lysate from JE2 *hlgB::bursa* to get a Δ*pvl*Δ*hlgB* double mutant.	This study (ETMB and RC)

AH LAC *ΔlukSFΔhlgC* (VJT 56.11)	AH LAC Δ*lukSF::spec* transduced with phage 80α lysate from JE2 *hlgC::bursa* to get a Δ*lukSF*Δ*hlgC* double mutant.	This study (ETMB and RC)
AHLAC pOS1-p*lukAB*-his-*lukSF*	*lukSF* under the control of the *lukAB* promoter, introduced on the multicopy number plasmid pOS1, in the *ΔlukSFΔhlgACB* strain	This study (ETMB, RC), (DuMont, Yoong, Day, *et al.*, 2013)
AHLAC pOS1-p*lukAB*-his-*hlgAB*	*hlgAB* under the control of the *lukAB* promoter, introduced on the multicopy number plasmid pOS1, in the *ΔlukSFΔhlgACB* strain	This study (ETMB, RC), (DuMont, Yoong, Day, *et al.*, 2013)

Table S3. Primers used in this study

Genes	Forward Primer	Reverse Primer
lukS- PV	GGAAAGGCCACCTCATTGCT	GATGGCGCTGAGGTAGTCAA
lukF- PV	TCTCTGCCATATGGTCCCCA	AACGGCTTATCAGGTGGAGG
lukA	ACCGCTGGCAATTGTGTCAT	CAGCACCGGATGATATTGGGA
lukB	ACCAGACCAATAGCCCCAGA	ATCGTGGAGGCTTAACTGGA
lukE	TGCTGAACCTGTTGGACCAT	TGCACCTTTAGCATCTCCGA
hlgA	ACTTCCAATCAGCGCCATCA	TGGACCAGTTGGGTCTTGTG
hlgC	CTTACTTGCCCCTCTTGCCA	ATTCGCTTTGACGCCCCATA
hlgB	ATAGCGCAACACCAAATGCC	TAATTTGCGCCTGCCCAGTA

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