Release of *Staphylococcus aureus* extracellular vesicles and their application as a vaccine platform

Xiaogang Wang¹, Christopher D. Thompson¹, Christopher Weidenmaier ²,³ & Jean C. Lee¹

Secretion of extracellular vesicles (EVs), a process common to eukaryotes, archae, and bacteria, represents a secretory pathway that allows cell-free intercellular communication. Microbial EVs package diverse proteins and influence the host-pathogen interaction, but the mechanisms underlying EV production in Gram-positive bacteria are poorly understood. Here we show that EVs purified from community-associated methicillin-resistant *Staphylococcus aureus* package cytosolic, surface, and secreted proteins, including cytolysins. Staphylococcal alpha-type phenol-soluble modulins promote EV biogenesis by disrupting the cytoplasmic membrane; whereas, peptidoglycan cross-linking and autolysin activity modulate EV production by altering the permeability of the cell wall. We demonstrate that EVs purified from a *S. aureus* mutant that is genetically engineered to express detoxified cytolysins are immunogenic in mice, elicit cytolysin-neutralizing antibodies, and protect the animals in a lethal sepsis model. Our study reveals mechanisms underlying *S. aureus* EV production and highlights the usefulness of EVs as a *S. aureus* vaccine platform.
Staphylococcus aureus is a pathogenic bacterium that causes a wide spectrum of human infections, ranging from mild skin lesions to invasive, life-threatening infections. The pathogenesis of S. aureus infections is attributed to a wide array of virulence determinants, including surface proteins and glycopolymers, as well as multiple secreted proteins, such as superantigens, leukotoxins, hemolysins, and proteases. Although several specific export pathways have been described in S. aureus, the secretome often includes proteins that lack export signals and have typical cytoplasmic functions. The mechanisms by which cytoplasmic proteins are excreted by S. aureus have attracted recent interest, and there is increasing evidence that these proteins may be secreted within extracellular membrane vesicles (EVs).

EVs are nano-sized, spherical, bilayered membrane vesicles with a cargo that includes diverse proteins, polysaccharides, nucleic acids, and lipids. EV formation by Gram-negative bacteria was first observed by electron microscopy in the 1960s, and these bacteria secrete what are now referred to as outer membrane vesicles (OMVs). The generation of OMVs occurs by phospholipid accumulation in the outer leaflet of the outer membrane, followed by the formation of outer membrane protrusions that pinch off to form vesicles. OMVs likely play important roles in bacterial pathogenesis due to packaging of multiple virulence factors, and the ability of OMVs to serve as immune modulators by inducing innate and adaptive immune responses.

Recent work has described the production and release of EVs from Gram-positive bacteria and fungi. Because of the thick peptidoglycan (PGN) structure typical of Gram-positive microbes, EV biogenesis is a complex and poorly understood process. Toyofuku et al. recently reported that membrane vesicle formation in Bacillus subtilis was a result of prophage-encoded endolysins that generated holes in the PGN, facilitating EV release. EVs from Gram-positive organisms play important roles in host-pathogen interactions, as supported by reports that EVs contain biologically active toxins, exhibit cytotoxicity, and elicit proinflammatory mediators. Moreover, toxin-positive S. aureus EVs elicit skin barrier disruption in mice with characteristic atopic dermatitis-like skin inflammation. The toxicity of staphylococcal EVs has hampered a relevant study of their immunogenicity and potential use as a vaccine platform.

Despite repeated efforts to develop experimental vaccines and immunotherapeutics against S. aureus, neither have proven effective in preventing staphylococcal infections in humans. Mice immunized with native S. aureus EVs produced a robust T-cell response and were protected against lung infection, but EV toxicity was not addressed in this study. The development of EVs as a S. aureus vaccine platforms will require characterization of the mechanisms of EV biogenesis to enable consistent production with adequate quality assurance.

In this study, we generate EVs from a predominant community-acquired, methicillin-resistant S. aureus (CA-MRSA) clone in the United States. Our study reveals distinct mechanisms that facilitate EV production at multiple stages. Phenol-soluble modulins (PSMs) act at the membrane level to facilitate vesicle budding at the cytoplasmic membrane; whereas, cell wall porosity is modulated by PGN cross-linking and production of autolysins. Our results demonstrate the cytotoxicity of native S. aureus EVs for multiple cell types. By genetically engineering a non-toxic S. aureus mutant to over-produce detoxified cytolysins, we show that engineered EVs (eng-EVs) are immunogenic, non-toxic, and

**Fig. 1** Extracellular vesicles from S aureus JE2. The bacteria were cultivated in TSB. a Ultrathin sections of S. aureus JE2 examined by TEM revealed EVs (indicated by a red arrow) released from the cell wall. b Crude EVs (red arrows) pelleted by ultracentrifugation from the JE2 culture supernatant were imaged by TEM. c EVs were purified by density gradient ultracentrifugation (Optiprep), and fractions were visualized by silver-stained SDS-PAGE. d Fractions 3–8 were pooled; OptiPrep was removed by dialfiltration, and the samples were imaged by TEM. EVs were not visualized in fractions 9–11. Scale bar, 100 nm.
protect mice against *S. aureus* lethal sepsis. Our investigation describes a vaccine platform and provides the basis for further studies on the impact of EVs on the pathogenicity of *S. aureus* and other Gram-positive pathogens.

**Results**

**Isolation of *S. aureus* EVs.** Ultrathin sections of JE219 cells examined by transmission electron microscopy (TEM) revealed vesicle-like structures released from the *S. aureus* cell surface (Fig. 1a). We isolated the EVs by concentrating the culture supernatants to remove molecules <100 kDa before ultracentrifugation to pellet the EVs, shown in Fig. 1b. To remove non-membranous proteins, protein aggregates, and denatured EVs, Optiprep-based density gradient centrifugation was performed on the crude EV preparations. Consecutive Optiprep fractions (10 µl) were subjected to SDS-PAGE. Little silver-stained material was recovered from fractions 1 and 2 (Fig. 1c). Samples with similar protein banding patterns (fractions 3–8 and 9–11) were pooled, dialyzed, and examined by TEM. EVs were observed in fractions 3–8 (Fig. 1d), but not from fractions 9–11, indicating that EVs were distributed in fractions containing 20–35% Optiprep.

**Protein composition of *S. aureus* EVs.** A proteomic analysis of purified JE2 EVs by liquid chromatography–tandem mass spectrometry (LC–MS/MS) identified 165 proteins (Supplementary Data 1), including alpha toxin (Hla), leukocidin subunits (LukS-PV, LukF-PV, LukE, LukD, HlgB, and HlgC), adhesins (ClfA, ClfB, SdrD, SdrE, EfB, and Ebh), MntC, proteases, and immune evasion factors (Sbi, phenol-soluble modulins, catalase, CHIPS, and SodA).
Other proteins of interest included penicillin-binding proteins, autolysins (Atl, Sle1, and other putative autolysins with predicted N-acetylmuramoyl-L-alanine amidase activity), proteins involved in iron acquisition, and multiple other lipoproteins. Bioinformatic analyses revealed that 46% of EV proteins were cytoplasmic (n = 76), 16% were extracellular proteins (n = 27), 16% had an unknown localization (n = 27), 12% were membrane proteins (n = 19), and 10% were cell wall associated proteins (n = 16).

Phenol-soluble modulins promote EV release. *S. aureus* secretes PSMs, which are a family of amphipathic, alpha-helical, surfactant-like peptides that are proinflammatory and show cytolytic activity against neutrophils, erythrocytes, epithelial cells, and endothelial cells. Alpha-type PSMs are required for mobilizing lipoproteins from the staphylococcal cytoplasmic membrane, a process essential for activating TLR2, as well as the export of cytoplasmic proteins, consistent with the membrane-damaging activity of PSMs. Because the cargo of *S. aureus* EVs is enriched for both lipoproteins and cytoplasmic proteins, we evaluated whether PSM peptides were critical for EV generation.

We measured EV production by the WT USA300 LAC strain (the parent strain of JE2), as well as LAC Δ*psma*, Δ*psmβ*, and Δ*psmaΔpsmβ* mutants. Dot immunoblot analysis revealed that deletion of *psma* genes reduced EV production (Fig. 2a). Likewise, protein assays and nanoparticle tracking analysis (NTA) indicated that the *psma* mutant showed significantly reduced *S. aureus* EV yield (Fig. 2b) and particle number (Fig. 2c), respectively. The Δ*psma* and Δ*psmaΔpsmβ* double mutant produced comparable levels of EVs (Fig. 2b), indicating that PSMa peptides play the dominant role in this phenotype. Complementation with pTXα expressing PSMA1-4 genes, but not the pTXα vector alone, restored EV production to the Δ*psma* mutant (Fig. 2b, c). Mutation of the *psma* genes significantly reduced *S. aureus* EV size (Fig. 2c, d); whereas, the Δ*psmβ* mutant produced EVs of intermediate size compared to that of wild-type (WT) LAC. We transduced pTXα,PSMA1-4 into strain JE2, and its EV yield (protein content) increased from 184 ± 12 to 650 ± 17 ng ml⁻¹ (n = 3). Nonetheless, electron micrographs of JE2 (pTXα,PSMA1-4) showed intact bacterial cells producing abundant EVs (Fig. 2f). Significant differences in bacterial numbers recovered from JE2 cultures with or without pTXα,PSMA1-4 were not observed (Supplementary Fig. 1a), indicating minimal impact of EV formation on bacterial viability.

PGN cross-linking modulates EVs production. Unlike OMVs produced by Gram-negative microbes, *S. aureus* cytoplasmic membrane-derived EVs must traverse a PGN cell wall structure before cellular release. To determine whether the degree of PGN cross-linking affected *S. aureus* EV biogenesis, we cultured *S. aureus* JE2 in medium with a sublethal concentration (0.2 µg ml⁻¹) of penicillin G (PenG) that has been shown to decrease PGN cross-linking. Treatment with a sublethal concentration (0.1 µg ml⁻¹) of erythromycin (Em) served as an antibiotic control that has no effect on PGN cross-linking. Compared to EVs recovered from untreated cultures or cultures incubated with Em, the EV yield from PenG-treated cultures was distinctly higher (Fig. 3a). When the EV protein content was quantified from a fixed volume of culture left untreated or treated with sublethal antibiotic concentrations, we observed a 10-fold increase in EV yield from PenG-treated cultures (Fig. 3b). EV production had little effect on bacterial viability since differences were not observed in bacterial numbers recovered from JE2 cultured with or without PenG (Supplementary Fig. 1b).

*S. aureus* penicillin-binding protein 4 (PBP4) is a carboxypeptidase that is essential for secondary cross-linking of PGN, and a *Δpbp4* mutant shows a significant reduction in PGN cross-linking. As predicted, both dot blot (Fig. 3c) and EV protein yield assays (Fig. 3d) showed increased EV production by JE2Δpbp4, and the protein yield was threefold higher than the wild-type JE2 strain. We also measured EV production in MRSA isolates MW2, COL, and their Δ*pbp4* mutants; the relative increase in EV yield in the mutant strains (Fig. 3c, d) was consistent with that of JE2Δpbp4.

WTA is a PGN-anchored glycopolymer that is major component of the *S. aureus* cell wall and plays a critical role in cell wall homeostasis. The *tagO* gene encodes an N-acetyl glucosamine-phosphate transferase enzyme that catalyzes the first step in WTA biosynthesis and, and deletion of *tagO* gene abrogates *S. aureus* WTA production. Compared to the WT strains JE2, COL, and Newman, *tagO* mutants showed an enhanced signal in the dot immunoblot assay for EV production (Fig. 3e). Likewise, quantitative analysis of EV protein yield showed that all three *tagO* mutants produced significantly more EVs than the parental isolates (Fig. 3f). Thus, WTAs negatively modulate *S. aureus* EV production, consistent with reports showing that *tagO* mutants are characterized by diminished PGN cross-linking. The WTA backbone is decorated with ester-linked D-ala residues, which confer a zwitterionic charge to the polymer. As shown in panels e and f of Fig. 3, production and yield of EVs by the Δ*delA* mutant were similar to that of the parental strain Newman.

To determine whether EV size was affected by reduced PGN cross-linking, the size distribution of purified EVs was measured by dynamic light scattering (DSL). Treatment of JE2 cultures with PenG or mutation of *pbp4* or *tagO* resulted in a significant increase in the size distribution of EVs (Fig. 3g), as well as an increased EV average size (Fig. 3h) compared to untreated WT EVs. Because enhanced EV production and yield associated with reduced PGN cross-linking might be a result of larger EVs that would carry an increased cargo load, we quantified EVs by nanoparticle tracking analysis. As shown in Fig. 3i, treatment of JE2 cultures with PenG or mutation of *pbp4* or *tagO* resulted in suspensions containing significantly greater numbers of EV
particles per ml compared to untreated WT EVs. Electron micrographs of bacterial cells treated with PenG (Fig. 3j) or carrying a pbp4 mutation (Fig. 3k) showed EVs being released or budding, respectively, from the cell membrane. Taken together, our data indicate that S. aureus EV production is inversely proportional to the degree of PGN cross-linking.

Autolysin Sle1 promotes the release of EVs. Atl and Sle1 belong to a family of PGN hydrolases that plays a critical role in separation of daughter cells30,31, and Atl modulates the excretion of a subset of staphylococcal cytoplasmic proteins3. To determine whether PGN-hydrolases facilitate the release of EVs by altering the thick Gram-positive cell wall, we compared EV production from isogenic atl and sle1 mutants with that of strains JE2 and Newman. Although both mutants showed reduced EV production (Fig. 4a), the reduction in yield was only significant in the sle1 mutants (Fig. 4b). Likewise, NTA revealed that only the sle1 mutant yielded a significantly lower EV concentration compared to WT JE2 (Fig. 4c). Complementation with pSle1 expressing the sle1 gene, but not the pOS1-hprK vector alone, fully restored EV production to the JE2 Δsle1 mutant (Fig. 4b, c). Both atl and sle1 mutants exhibited significantly reduced EV size compared to WT JE2 EVs (Fig. 4d, e).

Bacteriophages also produce PGN hydrolases called endolysins, which degrade the bacterial cell wall from within, resulting in cell lysis and release of progeny phages. Recently, Toyofuku et al.14 reported that prophage-encoded endolysins mediate the formation and release of EVs from Bacillus subtilis by generating a hole in the cell wall, leading to cell death. To investigate whether prophages or prophage-encoded endolysins are involved in S. aureus EV production, we analyzed S. aureus strains NCTC 8325 carrying φ11, φ12, and φ13 and 8325-4, which is cured of all three prophages32. Plating culture filtrates of NCTC 8325 on lawns of recipient strain RN4220 resulted in the formation of plaques, whereas culture filtrates of 8325-4 yielded no plaques (Supplementary Fig. 2a). EV yields and NTA revealed that NCTC 8325 and prophage-free strain 8325-4 produced comparable level of EVs (Supplementary Fig. 2b and 2c), indicating that prophage mobilization is not essential for the generation of S. aureus EVs.

Effects of the S. aureus capsule on EV release. To determine whether the presence of capsular polysaccharide (CP) production impacted S. aureus EV biogenesis, we evaluated a number of isogenic CP+ and CP− strains. As shown in Fig. 5a, the CP phenotype had no obvious impact on the EV dot blot signal derived from WT or CP− mutants of strains Newman (CP5+) or 6850 (CP8+). Similarly, USA300 strain 923 (complemented to restore CP5 production) produced CP513, but there was no effect on the EV signal levels achieved by dot blotting (Fig. 5a). Likewise, CP+ and isogenic CP− strains of Newman, 6850, and 923 produced comparable protein yields of EVs (Fig. 5b), indicating that CP did not modulate S. aureus EV production.

To investigate whether CP antigens were associated with S. aureus EVs, we performed CP immunoblots on EVs prepared from strains Newman (CP5+), MN8 (CP8+), and USA300 FPR3757 (CP−). CP antibodies react with surface-associated CP on intact EVs, whereas intravesicular CP would only be detected in sonicated EV preparations. Figure 5c shows that CP5 was only detected in sonicated, but not intact Newman EVs; whereas, CP8 was detected in both intact and sonicated MN8 EVs. EVs from CP− FPR3757 were non-reactive. Thus, both CP5 and CP8 were associated with EVs produced by CP+ S. aureus, although only CP8 was surface exposed.

Detoxified EVs as a multicomponent vaccine platform. Multiple antigens were packaged within JE2 EVs, including lipoproteins, cytolytic toxins, surface proteins, and enzymes (Supplementary Data 1). If the toxicity of the EVs were
eliminated, JE2 EVs could serve as a multicomponent S. aureus vaccine candidate. To repress the expression of cytolytic toxins, we mutated the S. aureus global regulator agr in strain JE2. We subsequently deleted spa (the gene encoding protein A) since an agr mutant overexpresses Spa, which binds to the Fcγ domain of immunoglobulin and dampens antibody development by cross-linking the Fab domain of V\textsubscript{H}1\textsubscript{3}-type B-cell receptors\textsuperscript{14}. The JE2 agr mutation significantly inhibited mRNA expression of hla (encoding alpha toxin) and the genes encoding all nine leukocidin subunits (Supplementary Fig. 3a). The JE2agrΔspa double mutant served as our S. aureus EV vaccine producing host strain. EVs from JE2, but not the JE2agrΔspa mutant, contained native Hla and LukE (Supplementary Fig. 3b) as assessed by western blotting. When we analyzed the protein content of JE2agrΔspa EVs by LC–MS/MS, many of the extracellular proteins present in JE2 WT EVs were not detected in JE2agrΔspa EVs. However, some antigens such as MntC and PhuD2 that protect mice against experimental S. aureus infections\textsuperscript{35,36} were present in EVs from the mutant strain. Neither protein A nor the toxins Hla, Panton-Valentine leukocidin (Luk-PY), LukED, HlgCB, SelX, or PSms were detectable in EVs purified from the JE2agrΔspa mutant (Supplementary Data 2). Although LukAB was still present in EVs from JE2agrΔspa, there was ≥86% reduction in the number of peptides detected in the mutant strain (Supplementary Data 1 and 2). Moreover, as indicated below, EVs recovered from the mutant strain showed no residual toxicity toward human leukocytes.

We immunized mice with 5µg EVs from JE2agr or JE2agrΔspa mutants; control mice were given phosphate buffered saline (PBS). EVs from both mutants elicited a serum antibody response against sonicated WT EVs, although the antibody level elicited by Δagr EVs was higher than that elicited by ΔagrΔspa EVs (Supplementary Fig. 4a). To examine the antigen profiles from EVs that elicited antibody responses after immunization, a bacterial lysate from USA300 strain FPR3757 was subjected to SDS-PAGE and immunoblotted with sera pooled from mice immunized with either Δagr EVs or ΔagrΔspa EVs. Sera from ΔagrΔspa EVs-immunized mice reacted with more bacterial antigens than sera from Δagr EVs-immunized mice (Supplementary Fig. 4b), suggesting that ΔagrΔspa EVs elicited a greater diversity of antibodies than Δagr EVs. The immunized mice were then challenged with strain FPR3757, a heterologous USA300 isolate. Immunization of mice with EVs from JE2agrΔspa, but not EVs from JE2agr, provided significant protection against lethal sepsis (Supplementary Fig. 4c). Immunization with higher doses of JE2agrΔspa EVs mixed with alum did not significantly enhance immunogenicity (Supplementary Fig. 4d).

**Engineered EVs protect mice against lethal sepsis.** To enhance the protective efficacy of detoxified EVs from JE2agrΔspa, we engineered JE2 to package non-toxic Hla\textsubscript{H35L} and the LukE monomer within eng-EVs. LukED, detected in 82% of blood isolates and 61% of nasal isolates\textsuperscript{38}, targets human and murine neutrophils, macrophages, T cells, dendritic cells, NK cells, and erythrocytes\textsuperscript{39}.

We expressed non-toxic Hla\textsubscript{H35L} and LukE in strain JE2agrΔspa under control of the spa promoter, which is enhanced in an Δagr genetic background\textsuperscript{40}. Thus, mRNA levels of Hla\textsubscript{H35L} and LukE expressed in JE2agrΔspa were dramatically increased compared to expression in JE2agrΔspa or JE2agrΔspa with the empty vector (Supplementary Fig. 3c). Both Hla\textsubscript{H35L} and LukE were detected by western blot in eng-EVs isolated from recombinant strain JE2agrΔspa (pHla\textsubscript{H35L}-LukE) (Supplementary Fig. 3b).

The relative toxicity of EVs prepared from WT strain JE2 and JE2agrΔspa vs. eng-EVs from JE2agrΔspa (pHla\textsubscript{H35L}-LukE) was assessed by incubating EVs in vitro with three different cell types. A549 cells are susceptible to Hla-mediated cytolyis, and WT strain JE2 EVs were toxic for A549 cells at concentrations as low as 1 µg ml\textsuperscript{−1}. In contrast, JE2agrΔspa mutant EVs and the
eng-EVs from JE2ΔagrΔspa (pHlaH35L-LukE) exhibited negligible toxicity (Supplementary Fig. 5a). HL60 cells are susceptible to the cytolysis induced by Δ and the leukocidins HlgAB and LukED39. JE2 EVs exhibited significant hemolytic activity, whereas no hemolysis resulted from Δ mutant EVs or eng-EVs (Supplementary Fig. 5c). These data demonstrate that the eng-EVs were non-toxic in vitro for mammalian cells.

We immunized mice on days 0, 14, and 28 with 5 µg eng-EVs from JE2ΔagrΔspa (pHlaH35L-LukE) or with 5 µg EVs from the JE2ΔagrΔspa mutant; control mice received 5 µg bovine serum albumin (BSA). Whereas sera from mice immunized with both eng-EVs and ΔagrΔspa EVs, but not BSA, reacted by ELISA with ΔagrΔspa mutant EVs or eng-EVs (Supplementary Fig. 5c). These data demonstrate that the recombinant proteins packaged within S. aureus EV are immunogenic.

To examine whether the antibodies elicited by the eng-EV vaccine were functional, toxin neutralizing assays were performed. Sera from mice immunized with eng-EVs effectively neutralized Hla at dilutions ranging from 1:20 to 1:80 (Fig. 6d), whereas serum neutralizing antibodies were low or undetectable in the BSA or ΔagrΔspa EV groups. Similarly, sera from mice immunized with eng-EVs, but not BSA or ΔagrΔspa EVs, were able to effectively neutralize LukED at dilutions ranging from 1:10 to 1:20 (Fig. 6e). Sera from mice immunized with eng-EVs also neutralized leukocidin HlgAB (Fig. 6f), but not PVL-SF or HlgCB leukotoxins.

The immunized mice were challenged with USA300 strain LAC or USA500 strain NRS685, a PVL-negative MRSA bacteremia isolate. We chose the latter strain because the PVL-S and PVL-F subunits can interact with LukE and LukD to form inactive hybrid complexes, which have been shown to influence LukED-mediated S. aureus virulence in mice41. As shown in Fig. 6g, h, immunization with eng-EVs provided significant protection against both S. aureus isolates in the lethal murine sepsis model. JE2ΔagrΔspa EVs were not protective against the USA500 strain (Fig. 6h).

**Discussion**

The production of membrane vesicles represents a secretory pathway common to mammalian cells, fungi, and bacteria that allows cell-free intercellular communication12–44. Microbial EVs encapsulate cargo that include lipids, proteins, glycans, and nucleic acids, which have been shown to play roles in microbial physiology, pathogenesis, and the transmission of biological signals into host cells to modulate biological processes and host innate immune responses42,43,45,46. In Gram-negative bacteria, EVs are generated by pinching off the outer membrane, but the mechanism(s) by which EVs escape the thick cell walls of Gram-positive bacteria, mycobacteria, and fungi are unknown. Once shed, S. aureus EVs can undergo cholesterol-dependent fusion with host cell membranes to deliver their toxic cargo47. S. aureus EVs are produced in vivo during experimental pneumonia in mice47. In this report, we demonstrate unique properties associated with EV production by JE2, a S. aureus USA300 strain representative of the prevalent CA-MRSA clone in the US. Similar to EVs characterized from other S. aureus isolates47–49, JE2 EVs encapsulate an array of bacterial antigens, including lipoproteins, exotoxins, and cytoplasmic proteins.

In this report, we evaluated putative factors that modulate the membrane and PGN related steps of EV release. PSMs are a group of small alpha helical peptides that have surfactant-like properties and potent cytolytic activity for leukocytes, epithelial cells, and endothelial cells20. PSMα peptides are 20–22 amino acids in length; whereas, PSMβ peptides are 43–45 amino acids in length. In our studies, PSMα peptides, but not PSMβ peptides, supported the generation of EVs from S. aureus. EVs from the PSMα mutant were less abundant and smaller in size compared with WT EVs. Chatterjee et al.30 reported that an S. aureus mutant that lacks the PSM transporter protein accumulates PSMs intracellularly, causing cytoplasmic membrane perturbations.

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**Fig. 6** Immunogenicity and protective efficacy in mice of engineered-EVs. Antibody levels in sera (diluted 1:100) from mice immunized with eng-EVs were analyzed on ELISA plates coated with a JE2 sonicated EVs, b Hla, or c LukE. Data were expressed as mean ± s.e.m. The neutralizing activity of sera from mice immunized with BSA or different EV preparations was determined by either incubating serial dilutions of sera with d Hla, e LukED, or f, or HlgAB for 1 h at 37 °C before adding target cells. Control cells were incubated with toxins but no sera. Data are expressed as percent neutralization ± s.e.m. Mice (n = 5) immunized with different JE2 EV preparations were challenged IV with 8 × 10⁷ CFU S. aureus LAC (g) or 2 × 10⁸ NRS685 (h). Survival (comparing EV-immunized vs. BSA-immunized mice) was analyzed with the log-rank test.
Surfactants or surfactant-like proteins with amphipathic helical structures have been shown to insert into lipid monolayers and generate local deformation. PSMs, due to their surfactant-like activity, as well as amphipathic helical structure, may enhance membrane curvature under cytoplasmic turgor pressure, resulting in membrane disruption and the formation of EVs. Although EVs from Gram-negative bacteria arise from the outer membrane rather than the plasma membrane, the biogenesis of OMV production is also thought to be due to perturbations in the outer leaflet of the membrane due to specific phospholipid accumulation therein. Recently, Ebner et al. reported that *S. aureus* PS Ma peptides-induced the cellular release of cytoplasmic proteins, lipids, nucleic acids, and ATP into culture supernatants, and that this effect was mediated by the membrane-damaging activity of the PS Ma peptides. Because PS Ma peptides promote EV production, and EVs encapsulate cytoplasmic proteins, lipids, and nucleic acids within a bilayered membrane, we postulate that these released cellular components are associated with and are likely contained within EVs.

The *S. aureus* cell envelope is comprised of a thick, highly cross-linked PGN layer, proteins, and glycopolyomers like lipo-teichoic acid, WTA, and CP. Highly cross-linked PGN serves as a barrier for EV biogenesis since treatment of *S. aureus* with a sublethal concentration of PenG or genetic inactivation of pbp4 or tagO resulted in a significant increase in EV production and size. This inverse correlation between PGN cross-linking and EV yield was also observed with *S. aureus* strains MW2, COL, and Newman. WTA has been shown to be critical for PGN-cross-linking by regulating PBP4 localization to the septation site. A secondary mechanism by which WTA regulates EV production is via its ability to control the activity of Atl and Sle1—not only by preventing their binding to *S. aureus* cell wall PGN, but also by creating an acidic milieu that limits Atl PGN hydrolase activity. Consequently, autolytic activity is not localized to the septum area in a *tagO* mutant but is spread throughout the cell surface, likely facilitating EV release. Schlag et al. reported that a *tagO* mutant showed an altered cell surface with bobble- and hairy-like protrusions, which may represent EVs. Although we do not yet fully understand the mechanism(s) of EV generation in Gram-positive bacteria, it seems logical that a poorly cross-linked cell wall or a cell wall lacking WTA would lessen the barrier to EV release and generate larger EVs as a result of larger pores within the PGN structure.

Autolysins that cleave the PGN barrier also impact the biogenesis of *S. aureus* EVs. Atl and Sle1 localize to the septum during cell division where they exhibit peptidoglycan hydrolase activity, resulting in separation of the daughter cells. Atl localized only at the external (surface exposed) edge of the septum, while Atl modulates LukED-mediated shedding of cytoplasmic proteins in *S. aureus*.

Although both autolysin activities are localized to the *S. aureus* septum region, JE2 EVs are not confined to the septal region (Fig. 2f), and EVs have been visualized by others surrounding the bacterial surface. A recent report demonstrated differential roles for Atl and Sle1 during cell division and separation. Whereas Sle1 could be visualized over the entire septal surface, Atl localized only at the external (surface exposed) edge of the septum. How autolysins modulate EV release from the cell wall or whether this process is spatially or temporally regulated remains to be determined.

We reported that *S. aureus* CP was shed from broth-grown *S. aureus* cells, and it is feasible that EVs could serve as a vehicle to liberate CP from the cell envelope. The *S. aureus* capsule was reported to hinder EV release in this pathogen, whereas no effect was observed on EV yield in strains with or without the hyaluronic capsule of *Streptococcus pyogenes*. Whether these streptococcal CPs are present as EV cargo in these pathogens was not addressed. Although EV yield varied among different isolates, we recovered similar quantities of EVs from isogenic *S. aureus* strains that varied only in CP production. The glucuronoxylomannan capsule of *Crytococcus neoformans* has been identified as a component of EVs from this fungal pathogen and polysaccharide A from *Bacteroides fragilis* was shown to be packaged into OMVs that were capable of inducing immunomodulatory signaling in dendritic cells. Ongoing studies in our laboratory will address whether *S. aureus* EV-host cell interactions impact the pathogenesis of staphylococcal disease.

We considered that *S. aureus* EVs could serve as a vaccine platform if their cytotoxicity was abrogated, and this was accomplished by purifying EVs from an *ΔagrΔspa* mutant of strain JE2. To enhance the protective efficacy of the *ΔagrΔspa* EV vaccine, non-toxic *Hla*Δ448 and LukE were expressed in JE2ΔagrΔspa under the control of the *agr*-derepressed *spa* promoter. Immunization with purified non-toxic *Hla*Δ448 has been shown to prevent lethal pneumonia and lethal peritonitis and reduce the incidence of necrotic skin abscesses. *S. aureus* leukocidins comprise a family of pore-forming cytolysins produced by *S. aureus* that target monocytes, lymphocytes, neutrophils, and macrophages—the very cells responsible for resolution of bacterial infection. These “eng-EVs” elicited antibodies in the sera of immunized mice that reacted with Hla and LukE by ELISA and neutralized the cytolytic activity of Hla, LukED, and HlgAB in vitro.

Immunization with eng-EVs provided significant protection against lethal sepsis provoked by USA300 strain LAC, a virulent PVL+ isolate. Because of a report that the presence of PVL modulates LukED-mediated *S. aureus* virulence in mice, we challenged another group of immunized mice with USA500 strain MR685, a PVL-negative MRSA bacteremia isolate. Immunization with eng-EVs, but not *ΔagrΔspa* EVs, protected 50% of the mice against MR685 lethal sepsis. Protective efficacy against additional *S. aureus* strains and in additional infection models remains to be evaluated. Overexpression of additional antigens that have been shown to protect mice against experimental *S. aureus* infections, such as MntC and FhuD, in second-generation eng-EVs may yield a more broadly protective vaccine. LC–MS/MS analysis of EVs from both WT JE2 and the *ΔagrΔspa* mutant strain contained multiple lipoproteins. As a predominant TLR2 ligand, lipoproteins have been increasingly used as adjuvant components because they are potent activators of host innate immunity and can mediate humoral and cell mediated immune responses. The self-adjuvanting composition of eng-EVs may provide it with a unique advantage over purified component vaccines.
In summary, we have generated, purified, and characterized EVs isolated from S. aureus USA300, the predominant CA-MRSA clone in the United States. Our study revealed that S. aureus PSMs are central for EVs generation by targeting the cytoplasmic membrane. Likewise, the Sle1 autolysin was shown to be critical for the release of EVs from the S. aureus cell wall. Whereas mutations in Atl or CP production did not affect EV yield, PBP4 and WTA promoted PGN cross-linking and consequently diminished EV production. Our study elucidates certain mechanisms whereby S. aureus produces and sheds EVs (Fig. 7) and will ultimately further our understanding of bacterial physiology and pathogenesis. We designed and created eng-EVs as a vaccine platform against S. aureus infection. Detoxified EVs that over-produced HlaH35L and LukE were immunogenic, elicited toxin neutralizing antibodies, and protected mice in a lethal sepsis model, indicating that these naturally produced recombinant EVs were immunogenic in mice and showed protective efficacy in a sepsis model of S. aureus infection.

Methods

Bacterial strains and plasmids. S. aureus isolates (listed in Supplementary Table 1) were cultivated overnight with aeration in tryptic soy broth (TSB; Difco) at 37 °C. Escherichia coli strain XL-1 (Agilent), used in DNA cloning experiments, was grown at 37 °C in Luria Broth (LB; Difco). S. aureus—E. coli shuttle vector pCU1 was used for cloning and expression of appropriate genes in S. aureus, and pOSI—hprK was used for pSle1 complementation studies. Antibiotics were added in the following concentrations: PenG 0.2 μg ml−1, ampicillin (Amp; 100 μg ml−1), Em 5 μg ml−1, chloramphenicol (Cm; 10 μg ml−1), kanamycin (Kan; 50 μg ml−1), and tetracycline (Tet; 5 μg ml−1).

DNA manipulation. Fey et al. derived S. aureus JE2 from the USA300 strain LAC by curing it of plasmids, rendering it sensitive to Em. The agr mutation (Δagr−tetM) was transduced from S. aureus RN6911 to WT JE2 using bacteriophage φ80a with selection for Tet resistance. To construct the ΔagrΔspa double mutant, the spa mutation was transduced from JE2 (spa::ermB) to JE2Δagr by φ80a transduction. The pspa mutation was transduced from JE2 (ϕ80Em::ermB) to WT MW2 and COL by φ80a transduction with selection for Em resistance. All mutants were confirmed by PCR using the primers listed in Supplementary Table 2. ELISA results confirmed the phenotype of the Δspa mutant, and the agr mutant lost its hemolytic phenotype. To construct the WTA mutants, the tagO mutation was transduced from SA113ΔtagO (ϕR8tagO) to WT JE2 and COL with φ80a with selection for Em resistance. Mutants were confirmed by PCR and acquisition of resistance to lysis by φ80a. To complement the JE2Δspa mutant, a DNA fragment of 1005 bp containing the sle1 gene was amplified from JE2 genomic DNA using the primer pair listed in Supplementary Table 2. The Sle1 expression plasmid was constructed by cloning the sle1 gene under the control of the hprK promoter into the E.coli—S. aureus shuttle vector pOS1. pSle1 was transformed into RN4220 by electroporation and then transduced to JE2Δspa by φ80a transduction.

To construct a shuttle vector for expression of HlaH35L and LukE, the spa promoter, hlaH35L, and lukE genes were amplified from S. aureus strains JE2, DU1990 (pHlaH35L), and FPR3757, respectively, using the primers listed in Supplementary Table 2. To drive the expression of hlaH35L, its sequence was fused to the 3′ terminus of the spa promoter containing the ribosome binding site by overlapping PCR. The Pspa−hlaH35L fusion sequence was cloned into the shuttle plasmid pCU1 with restriction enzymes HindIII and Sall. The amplified lukE sequence containing a ribosome binding site was inserted into pCU1 with restriction enzymes Sall and EcoRI. The resulting plasmid pCU1-Pspa−hlaH35L-lukE was verified by enzyme digestion and DNA sequencing. To construct the ΔspaΔagr expressing non-toxic HlaH35L and LukE, pCU1-Pspa−hlaH35L-lukE was transformed into RN4220 by electroporation and then transduced with φ80a to JE2ΔspaΔagr, selecting for Em resistance.

Isolation and purification of EVs. S. aureus was cultivated in TSB with shaking to an OD600nm of 1.2. The culture supernatant was filtered and concentrated 25-fold with a 100-kDa tangential flow filtration system (Pall Corp.). The retentate was filtered again before centrifugation at 150,000×g for 3 h at 4 °C to pellet the vesicles and leave soluble proteins in the supernatant. The EV pellet was suspended in 40% Optiprep density gradient medium (Sigma) and overlaid with gradient layers of Optiprep ranging from 35 to 10%. After centrifugation at 139,000×g for 16 h at 4 °C 1 ml fractions were removed sequentially from the top of the gradient. Each fraction was subjected to SDS-PAGE and stained with a thermo Fisher silver staining kit. Fractions with a similar protein profile on SDS-PAGE were pooled.

Fig. 7 Proposed mechanisms underlying EV production by Staphylococcus aureus. EVs are generated from the cytoplasmic membrane, and this process is promoted by S. aureus PSM peptides, which have surfactant-like activity, causing membrane disruption. Membrane-derived EVs must also traverse the peptidoglycan barrier, and the extent of cell wall cross-linking modulates the efficacy of EV production. Autolysins, such as Sle1, facilitate EV release by hydrolyzing peptidoglycan, particularly at sites of active cell division. We mutated S. aureus to render its EVs non-toxic, and then genetically engineered the mutants to package detoxified antigens in EVs. These recombinant EVs were immunogenic in mice and showed protective efficacy in a sepsis model of S. aureus infection.
and the OptiPrep medium was removed by dialfiltration with PBS using an Amicon Ultra-50 Centrifugal Filter Unit. The dialfiltrated retentate was filtered (0.45 µm) and stored at −80 °C. EV concentrations were determined by using a Protein Assay Dye Reagent (Bio-Rad). EV samples were evaluated with a Nanobrook ZetaPALS potential analyzer (Brookhaven Instruments Corp.), and the data for size distribution and particle diameter were generated with ZetaPALS particle sizing software. Nanoparticle tracking analysis was performed by purifying EVs from 100 µl bacterial cultures, as described above. The number of EV particles recovered from individual cultures (and suspended in 1 mL PBS) was determined using a Cell_profiler kit (Promega). A 2% rabbit erythrocyte suspension was mixed with EVs or 1 µg mL−1 Hla in a 96-plate well for 1 h at 37 °C. The erythrocytes were pelleted by centrifugation, and hemolysis was recorded by measuring the OD 545 nm of the sample supernatants. For leukocidin cytotoxicity, samples were serially diluted and mixed with toxin concentrations yielding ~75% cell lysis (12.5 µg mL−1 LukED, 2.5 µg mL−1 PV, 1 µg mL−1 HlgAB, or 2 µg mL−1 HlgCB (1:1 S and F subunits)). Samples were pre-incubated with leukocidins for 30 min at RT before the addition of neutrophils (1 × 106 cells). After 2 h at 37 °C in 5% CO2, the cells were collected by centrifugation and suspended in fresh medium. Cell cytotoxicity was evaluated using a CellTiter kit (Promega). Percent neutralization was calculated using the formula: [% cytotoxicity of (serum + toxins) − % cytotoxicity of (serum + toxins)]..

Data availability. Data supporting the findings of this manuscript are available from the corresponding author upon reasonable request. Mass spectrometry proteomics data were deposited in the ProteomeXchange Consortium (http://proteomecentral.proteomeexchange.org) via the PRIDE partner repository with the data set identifier PXD007953.

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Author contributions
X.W. initiated the project, and X.W., C.W., and J.C.L designed experiments. X.W. performed the experiments with the assistance of C.D.T. All authors analyzed the data, and X.W., C.W., and J.C.L. wrote the manuscript. All authors reviewed the manuscript.

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