Bacterial outer membrane vesicles engineered with lipidated antigens as a platform for *Staphylococcus aureus* vaccine

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Bacterial outer membrane vesicles (OMVs) represent an interesting vaccine platform for their built-in adjuvanticity and simplicity of production process. Moreover, OMVs can be decorated with foreign antigens using different synthetic biology approaches. However, the optimal OMV engineering strategy, which should guarantee the OMV compartmentalization of most heterologous antigens in quantities high enough to elicit protective immune responses, remains to be validated. In this work we exploited the lipoprotein transport pathway to engineer OMVs with foreign proteins. Using 5 *Staphylococcus aureus* protective antigens expressed in *Escherichia coli* as fusions to a lipoprotein leader sequence, we demonstrated that all 5 antigens accumulated in the vesicular compartment at a concentration ranging from 5 to 20% of total OMV proteins, suggesting that antigen lipidation could be a universal approach for OMV manipulation. Engineered OMVs elicited high, saturating antigen-specific antibody titers when administered to mice in quantities as low as 0.2 μg/dose. Moreover, the expression of lipidated antigens in *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* was shown to affect the lipopolysaccharide structure, with the result that the TLR4 agonist activity of OMVs was markedly reduced. These results, together with the potent protective activity of engineered OMVs observed in mice challenged with *S. aureus* Newman strain, makes the 5-combo-OMVs a promising vaccine candidate to be tested in clinics.

vaccines | outer membrane vesicles (OMVs) | lipoproteins | adjuvants | *Staphylococcus aureus*

At the beginning of the new millennium, infectious diseases still pose increasing threats to human health. Vaccines against a considerable number of pathogens are not available yet (1) and the extensive and often improper use of antibiotics has led to the selection of antibiotic-resistant strains which in a growing number of cases have acquired resistance against virtually all available antibiotics (2). One of the most explicative example is *Staphylococcus aureus*. *S. aureus* is a commensal in humans and animals but is responsible for severe diseases when it becomes invasive. This usually occurs in patients with immunological or barrier defects, but highly pathogenic strains have recently emerged that have the ability to cause diseases in otherwise healthy individuals (3). A growing number of clinical isolates are now resistant to most antibiotics (4) and despite several decades of intense research by numerous world-class laboratories, a vaccine is still far from being available. Invasive strains express a myriad of virulent factors and more than 35 secreted immune evasion molecules, making *S. aureus* the champion of pathogens in circumventing the defense mechanisms of the mammalian immune system (5). Moreover, once phagocytosed by professional immune cells, *S. aureus* has the ability to escape the killing mechanisms, and phagocytes can become the vehicles by which the pathogen disseminates inside the host (6). Because of the above, traditional strategies to develop antibacterial vaccines, largely based on the elicitation of neutralizing and/or bactericidal antibodies, might not be sufficient for such a sophisticated pathogen, and a paradigm shift in the way the vaccine is conceptualized might be required.

In recent years bacterial outer membrane vesicles (OMVs) have emerged as a novel and flexible vaccine platform and OMV-based vaccines are already available or are being developed for human use (7, 8). OMVs are particularly attractive for their built-in adjuvanticity (9), the ease with which they can be purified (10), and the possibility of being decorated with a protein/polypeptide of interest (POI) by proper manipulation of the OMV-producing strains (11–13). With respect to this latter point, different strategies

Significance

Thanks to their potent built-in adjuvanticity, bacterial outer membrane vesicles (OMVs) represent an attractive vaccine platform. However, their full-blown exploitation relies on the availability of efficient engineering strategies. This work provides strong experimental evidence that OMVs can be successfully decorated with heterologous antigens by channeling them to the lipoprotein transport machinery. Not only did lipidated antigens accumulate in the vesicular compartment at high levels but they also interfered with the acylation process of lipid A, thus substantially reducing the lipopolysaccharide-mediated reactogenicity, a major hurdle for vaccine applications. The approach was validated with 5 *Staphylococcus aureus* antigens, and mice immunized with engineered OMVs were completely protected against *S. aureus* challenge, paving the way for development of vaccines against this important pathogen.

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Conflict of interest statement: G. Grandi, L. Fantappiè, and C.I. are coinventors of a patent on OMVs; A. Grandi and G. Grandi are involved in a biotech company interested in exploiting the OMV platform.

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have been proposed, including the delivery of the POI into the periplasmic space through its fusion to a leader sequence for secretion (14), and the use of carrier proteins to chaperone the POI in the OMV compartments (15). The ideal strategy should be flexible and should lead to the accumulation of sufficient quantities of heterologous antigens to elicit proper antigen-specific immune responses.

In this work we have tested whether the exploitation of the lipoprotein transport machinery could represent a valid alternative for OMV decoration with heterologous antigens. In Gram-negative bacteria, lipoproteins are synthesized as precursors with a N-terminal leader sequence (LS) carrying a cysteine-containing "lipobox." Once transported through the inner membrane, the cysteine is diacylated and lipoprotein precursors are cleaved upstream from the diacylated cysteine. The free NH_2 group of the cysteine is further acylated and the triacylated lipoprotein is finally transported to the outer membrane by the Lol transport machinery (16). Based on the above, the fusion of any POI to a lipobox-carrying LS can theoretically promote the lipidation of the POI and its subsequent translocation to the outer membrane. From an immunological standpoint, this should be beneficial because lipoproteins are ligands for the Toll Like Receptor 2 (TLR2) and therefore lipidated POI should further enhance the TLR2-dependent adjuvanticity properties of the engineered OMVs.

Here we show that *S. aureus* Hla_{H35L} (17), SpA_{KKAA} (18), FhuD2 (19, 20), Csa1A (21), and LukE (22), 5 extensively studied vaccine candidates shown to be highly conserved among *S. aureus* isolates and to induce protective immunity in different animal models, compartmentalize with high efficiency in the OMVs as lipoproteins. Interestingly, protein lipidation attenuates OMVs reactogenicity by modifying the lipid A structure. Furthermore, engineered OMVs elicit high antigen-specific antibody titers and the combination of the 5 engineered OMVs completely protects mice from *S. aureus* challenge. Overall, our work provides a general strategy for OMV engineering and suggests that, because of their unique adjuvanticity properties, OMVs decorated with lipidated protective antigens could represent a valid alternative for the development of an efficacious *S. aureus* vaccine.

Results

Lipidated *S. aureus* Antigens Are Incorporated into *Escherichia coli* OMVs. To test whether heterologous proteins can be incorporated into OMVs as lipoproteins, we selected 5 *S. aureus* antigens extensively described in the literature and known to induce protective activities in different animal models. In case of success, the selection of these antigens would have given us the opportunity to test whether engineered OMVs could also induce protective immune responses. Our hypothesis was that the potent adjuvanticity property of OMVs, further potentiated by an enhanced TLR2 agonistic activity, could synergize with the functional immune responses elicited by the antigens, thus allowing a robust protection against *S. aureus* infection in experimental mouse models. The selected antigens were: the mutated forms of Hla_{H35L} and SpA_{KKAA} (17, 18), LukE, 1 of the 2 components of the LukD/E leukocidin (22), Csa1A (21), and iron binding protein FhuD2 (20). In particular, SpA binds to Fcγ of immunoglobulins (Igs), thus inhibiting phagocytosis, and to the Fab portion of VH3-type B cell receptors ultimately leading to B cell apoptosis. Hla binds to lymphocytes, macrophages, alveolar epithelial cells, pulmonary endothelium, and erythrocytes and promotes their killing by forming heptameric pores of 2 nm in diameter. Finally, the leukocidin LukE induces the lysis of several immune cells by binding to the chemokine receptor type 5 (CCR5) expressed on the surface of CD4 T cells, macrophages, and dendritic cells (22). In addition to being protective, the antigens are highly conserved among a large panel of clinical isolates (*SI Appendix*, Table S1).

We first asked the question of whether the 5 selected antigens could be incorporated in the lumen of OMVs by delivering them to the periplasmic space of the OMV-producing strain (14). To this aim, we chemically synthesized the corresponding DNA sequences and we fused them to the sequence encoding the LS of the *E. coli* OmpA protein. The 5 genes were cloned in pET21b plasmid and the antigen carrying plasmids were used to transform the hypervesiculating BL21(DE3)Δ*ompA* strain. OMVs were purified from the culture supernatant of each strain and the protein content was analyzed by SDS/PAGE (Fig. 1*A*). A band corresponding to each antigen was visible in engineered OMVs and the level of expression was estimated from 2% to 10% of the total OMV protein content (Fig. 1*B*).

We next expressed the 5 *S. aureus* antigens as lipoproteins by fusing their coding sequences immediately downstream from, and in frame with, the "lipobox" cysteine of the *lpp* LS (23). The chimeras were inserted into pET21 and the 5 recombinant plasmids were used to transform the *E. coli* BL21(DE3)Δ*ompA* strain. OMVs were purified and antigen expression in OMVs was analyzed by SDS/PAGE. All 5 antigens were successfully compartmentalized in the OMVs (Fig. 1*A*). Interestingly, lipidated recombinant proteins tended to be expressed at higher level with respect to their nonlipidated counterparts. This was particularly true for FhuD2, Csa1A, and SpAKKAA, whose level of expression was from 10% to 20% of total OMV proteins (Fig. 1*B*).

To indirectly demonstrate the presence of the acyl groups at the N terminus of the recombinant antigens, vesicles were solubilized at 4 °C with a 1% water solution of Triton X-114 and subsequently the samples were warmed to 37 °C to partition Triton X-114 into 2 phases: a detergent-rich "hydrophobic" phase and a detergent-poor "hydrophilic" phase. Membrane proteins, including lipoproteins, typically partition selectively into the hydrophobic phase (24). As shown in Fig. 2, all 5 antigens engineered as lipoproteins compartmentalized in the hydrophobic phase while the periplasmic maltose binding protein (MBP) was retained in the aqueous phase. As expected, similarly to MBP, the same proteins expressed in the lumen of OMVs partitioned in the aqueous phase.

OMVs Engineered with Lipidated *S. aureus* Antigens Induce Antigen-Specific Antibodies. Having demonstrated that all 5 *S. aureus* antigens efficiently accumulated in the OMVs in the lipidated form, we next asked the question of whether the OMVs carrying acylated antigens could induce antigen-specific immune responses. To this aim, mice were immunized 3 times, in the presence or absence of alum, with different doses of FhuD2-OMVs, and anti-FhuD2 antibody titers were measured 10 d after the last immunization. As shown in Fig. 3*A*, in the absence of alum, 5 μg of recombinant OMVs was sufficient to reach the plateau of antibody titers. The same plateau was reached if as little as 1 μg of OMVs was formulated with alum. The same dose–response experiments were repeated using LukE-OMVs. Again, 5 μg of recombinant OMVs was sufficient to reach the plateau of anti-LukE IgG titers and the addition of alum increased the immunogenicity of the OMVs to the point that 0.2 μg of recombinant OMVs was enough to reach the maximum of the titers (Fig. 3*B*). We finally tested the other 3 recombinant OMVs (Csa1A-OMVs, SpA_{KKAA}-OMVs, and Hla_{H35L}-OMVs) to see whether the 5-μg OMV dose was also sufficient to reach the plateau of antibody titers. This was indeed the case as judged by the fact that 5 μg and 20 μg of OMVs elicited similar antibody titers. Interestingly, the same titers were obtained by immunizing animals with 10 μg of the corresponding purified antigens formulated with alum.

In *E. coli*, outer membrane lipoproteins are usually anchored to the inner leaflet and protruding into the periplasmic space (25). Therefore, those lipoproteins that are entrapped in OMVs during vesiculation should be oriented toward the OMV lumen. This topological organization could affect the profile of the antigen-specific IgGs. In other words, the lipidated proteins inside the vesicles might not expose the epitopes responsible for the protective activities elicited by the nonlipidated purified recombinant proteins. In an attempt to rule out this possibility, we carried out a preliminary experiment in which the purified recombinant antigens

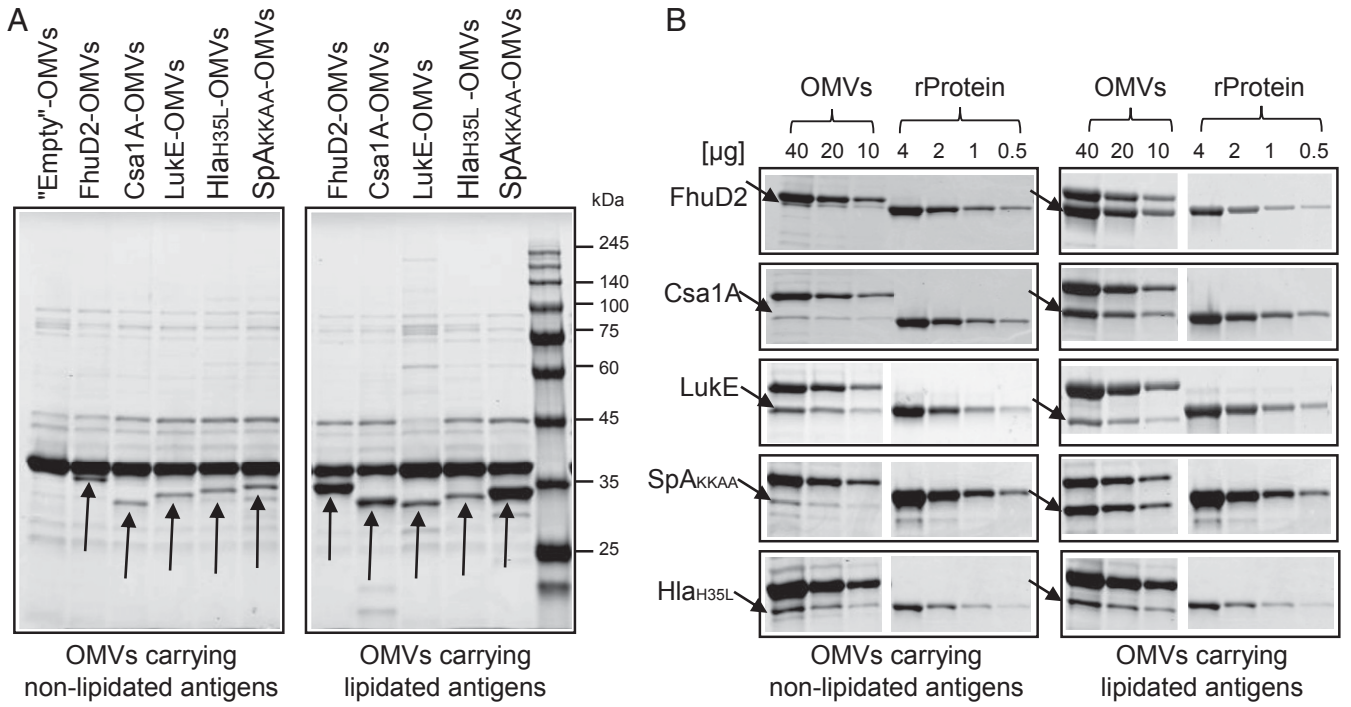

Fig. 1. Expression of Staphylococcal antigens in OMVs from *E. coli* BL21(DE3)Δ*ompA*. (*A*) *E. coli* BL21(DE3)Δ*ompA* strains expressing heterologous antigens in the periplasm or in the outer membrane as lipoproteins were grown in lysogeny (Luria-Bertani) broth (LB) at 37 °C and OMVs were purified from culture supernatants as described in *SI Appendix, Materials and Methods*. Aliquots (10 μg of total OMV proteins) were analyzed by SDS/PAGE. (*B*) The amount of heterologous proteins incorporated into the OMVs, was estimated by loading different quantities of purified recombinant proteins and engineered OMVs on SDS-polyacrylamide gels and by comparing band intensities of heterologous proteins in OMVs and of the corresponding purified proteins.

were partially digested with proteinase K, the fragments were separated by SDS/PAGE, and Western blot analysis was carried out using sera from mice immunized with either the engineered OMVs or the recombinant proteins. As illustrated in Fig. 3*D*, which reported the analysis for LukE, FhuD2, and Csa1A, no substantial differences in the recognition patterns of the 2 sera were observed.

Immunogenicity of Engineered OMVs from the *E. coli* BL21(DE3)Δ *ompA*Δ*msbB*Δ*pagP* Strain. Lipopolysaccharide (LPS) is present in OMVs at a concentration of ∼0.5 mg per milligram of total OMV proteins (14, 26). By binding to TLR4, LPS substantially contributes to the excellent adjuvanticity properties of OMVs. However, LPS abundance is also responsible for OMV reactogenicity, which has to be reduced for human use. A number of mutations in the LPS biosynthetic pathway have been described that reduce the TLR4 agonistic activity of LPS by removing acyl chains to the hexaacylated lipid A moiety. They include the inactivation of the acyltransferase genes *msbB* and *pagP*, which results in the synthesis of a LPS carrying a pentaacylated lipid A (27, 28).

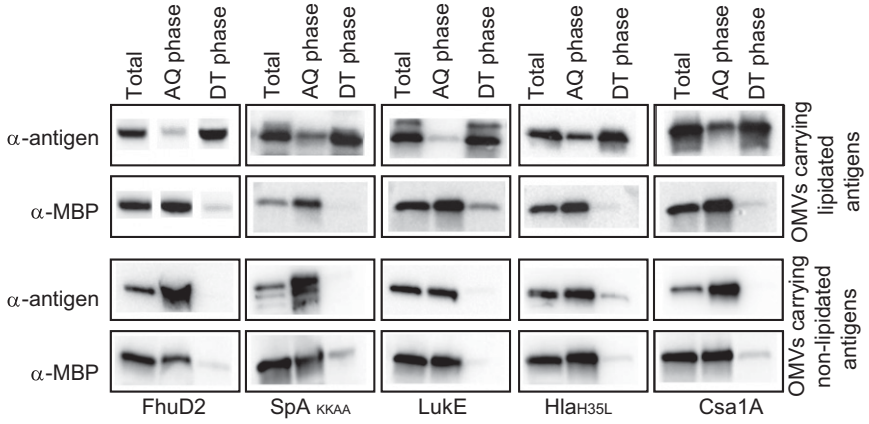

Fig. 2. Analysis of protein lipidation by Triton X-114 extraction of OMV proteins. OMVs expressing heterologous proteins in the membrane or in the lumen were dissolved by adding 1% Triton X-114 at 4 °C and subsequently aqueous and detergent phases were partitioned by centrifugation. Unfractionated total proteins from OMVs (total), hydrophilic proteins in the aqueous phase (AQ phase), and hydrophobic proteins in the detergent phase (DT phase) were precipitated with chloroform/methanol and separated by SDS/PAGE. Finally, proteins were transferred onto nitrocellulose filters and the presence of the heterologous antigens in either the aqueous or detergent phases was detected by Western blot using antigen-specific antibodies. As a control, the partitioning of the periplasmic protein MBP was also analyzed using anti-MBP antibodies.

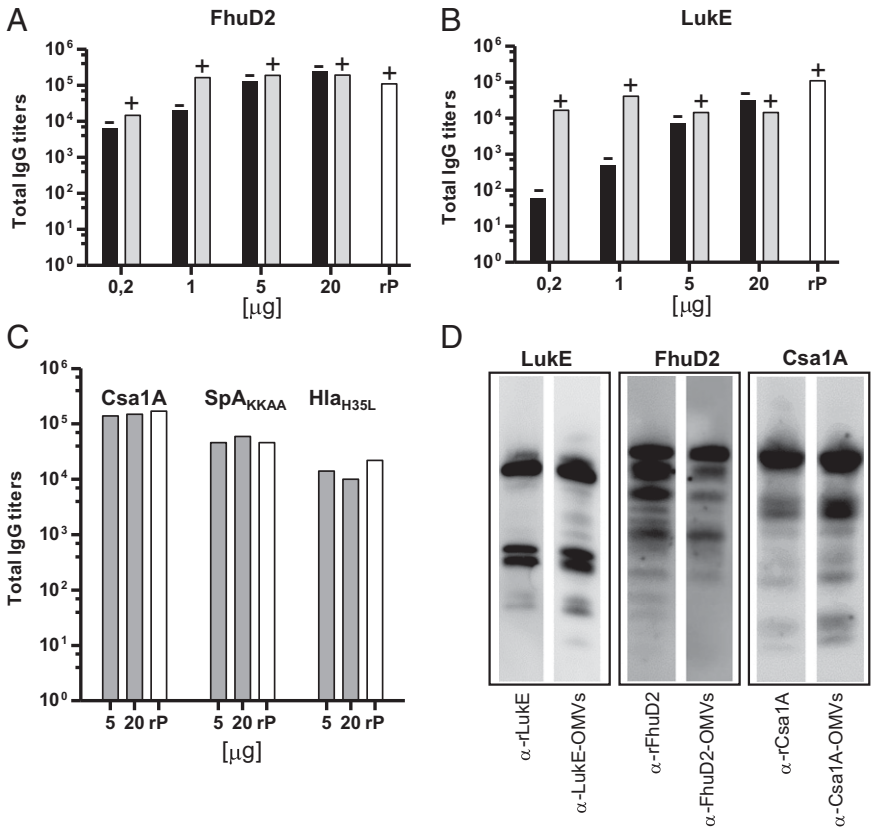

Fig. 3. IgG titers in mice immunized with OMVs expressing lipidated heterologous antigens. (*A* and *B*) Groups of 5 CD1 female mice were immunized i.p. 3 times at 2-wk interval with 0.2, 1, 5, or 20 μg of OMVs expressing lipidated FhuD2 (*A*) or LukE (*B*) antigens, formulated with (+) or without (–) alum as adjuvant. Sera were collected 10 d after the third immunization and IgG titers were analyzed by ELISA, using plates coated with the corresponding recombinant antigen (0.3 μg/well). (*C*) Groups of 5 CD1 female mice were immunized i.p. 3 times at 2-wk interval with 5 or 20 μg of OMVs expressing lipidated Csa1A, Hla$_{H35L}$, and SpA$_{KKAA}$ antigens formulated with alum, or with 10 μg of the corresponding recombinant purified protein in alum. Sera were collected 10 d after the third immunization and IgG titers were analyzed by ELISA, using plates coated with the corresponding recombinant antigen (0.3 μg/well). (*D*) Partial proteolysis of recombinant LukE, FhuD2, and Csa1A antigens. A total of 1 μg of purified recombinant antigen was partially digested in PBS with proteinase K for 30 min at room temperature. After PMSF treatment, samples were loaded on SDS-polyacryamide gel and transferred onto nitrocellulose filters for Western blot analysis. As primary antibodies, sera from mice immunized with either the engineered OMVs or the recombinant protein were used.

In view of a possible exploitation of an OMV-based *S. aureus* vaccine, we generated the *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* strain. We first verified that, when expressed in the BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* strain, the 5 *S. aureus* antigens were incorporated in the OMVs with an efficiency similar to that observed in BL21(DE3)Δ*ompA*. To this aim, the *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* strain was transformed with plasmids encoding the lipidated antigens and the OMVs purified from the culture supernatants of the 5 recombinant clones. When analyzed by SDS/PAGE, the antigen expression profiles of the vesicles were comparable to those of the OMVs purified from the *E. coli* BL21(DE3)Δ*ompA* strain (compare Fig. 4*A* with Fig. 1*A*).

Next, we analyzed the TLR4 agonistic activity of OMVs using HEK-Blue cells expressing either the murine TLR4 (mTLR4) or the human TLR4 (hTLR4) (*SI Appendix*, *Materials and Methods*). As shown in Fig. 4*B*, and according to what has been previously published (29), purified OMVs from the BL21(DE3) Δ*ompA*Δ*msbB*Δ*pagP* strain (OMVs$_{msbBpagP}$) had a mTLR4 stimulatory capacity approximately 5-fold lower than the OMVs from the BL21(DE3)Δ*ompA* strain. Unexpectedly, the recombinant OMVs$_{msbBpagP}$ carrying the lipidated antigens showed a further reduction in TLR4 stimulation. The reduction varied depending upon the expressed antigen, but in the case of SpA$_{KKAA}$-OMVs$_{msbBpagP}$ it was ∼100-fold lower than "empty"-OMVs$_{msbBpagP}$ and, in the case of Csa1A-OMVs$_{msbBpagP}$, LukE-OMVs$_{msbBpagP}$, and FhuD2-OMVs$_{msbBpagP}$ the reduction was between 10- and 20-fold lower. Even more strikingly, when hTLR4 responses were analyzed, the agonist activity of OMVs$_{msbBpagP}$ engineered with lipidated antigens was almost completely abolished (Fig. 4*C*).

Finally, we evaluated the ability of the engineered OMVs$_{msbBpagP}$ to elicit antigen-specific antibody responses. As shown in Fig. 4 *D*–*F*, despite the reduced TLR4 activity, the 5 vesicles induced antibody titers superimposable with those obtained with the OMVs derived from the BL21(DE3)Δ*ompA* strain.

The Expression of Lipidated Antigens in BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* Alters the Lipid A Structure. The unexpected reduction of TLR4 agonistic activity of OMVs$_{msbBpagP}$ decorated with lipidated antigens prompted us to characterize the lipid A structure from BL21(DE3)Δ*ompA*, *BL21(DE3)*Δ*ompA*(pET-FhuD2), BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP*, and BL21(DE3) Δ*ompA*Δ*msbB*Δ*pagP* (pET-FhuD2) strains. The LPS was purified from the 4 strains and the lipid A was analyzed by mass spectrometry. The spectra obtained for the 4 preparations are shown in Fig. 5 and a detailed description of the analysis is reported in *SI Appendix*, Fig. S1. In summary, lipid A from the BL21(DE3) Δ*ompA* strain appeared to be mostly constituted by monophosphoryl hexaacylated lipid A and its phosphorylethanolamine analog. Overall, the spectrum was essentially in line with what was reported for LPS purified from wild-type *E. coli* strains (30), even

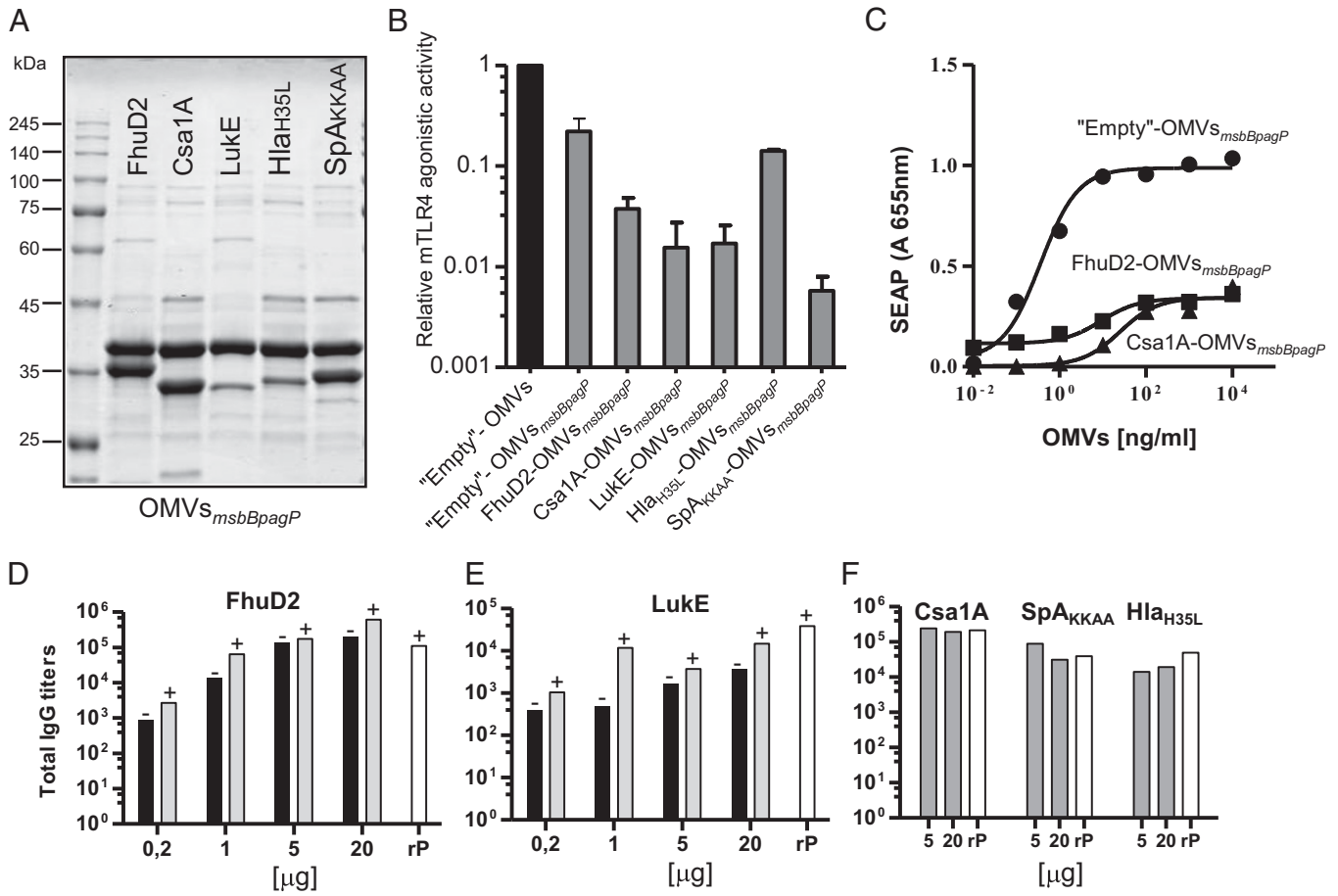

Fig. 4. Evaluation of immunogenicity and reactogenicity of antigen engineered OMVs$_{msbBpagP}$. (*A*) *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* strains expressing heterologous staphylococcal antigens as lipoproteins were grown in LB at 37 °C. At OD_{600} = 0.5, 0.1 mM IPTG was added and after 2 h, OMVs were purified from culture supernatants by ultracentrifugation. Aliquots corresponding to 10 μg of total OMV proteins were loaded to each lane. (*B*) HEK-Blue mTLR4 cell line was incubated with different amounts of OMVs (in 20 μL) purified from BL21Δ*ompA* ("empty"-OMVs) and from *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* (OMVs$_{msbB\ pagP}$) either "empty" or loaded with *S. aureus* antigens. Alkaline phosphatase activity was determined after 17 h of incubation. Relative murine-TLR4 activity is represented as ratio between EC_{50} value of each OMV over the EC_{50} value of "empty"-OMVs. Bars represent the means of 2 independent experiments. (*C*) HEK-Blue hTLR4 cell line was incubated with different amounts of OMVs purified from *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* ("empty" OMVs$_{msbB\ pagP}$) or OMVs loaded with *S. aureus* antigens (FhuD2-OMVs$_{msbB\ pagP}$ and Csa1A-OMVs$_{msbB\ pagP}$). After 17 h the levels of secreted alkaline phosphatase (SEAP) were determined by reading the OD at 655 nm. (*D–F*) Different amounts of OMVs from BL21(DE3) Δ*ompA*Δ*msbB*Δ*pagP* strains expressing lipidated *S. aureus* antigens were used to immunize groups of 5 CD1 mice. Antibody titers in pooled sera collected 10 d after the third immunization were measured by ELISA, using plates coated with the corresponding purified recombinant protein (0.3 μg/well). In the case of FhuD2 and LukE immunizations were carried out in the presence (+) or absence (–) of alum, while immunizations with OMVs decorated with lipidated Csa1A, SpA_{KKAA}, and Hla_{H35L} were carried out with alum. As control, groups of mice were also immunized with 10 μg of the corresponding recombinant proteins (rP) in alum.

though the phosphorylethanolamine analogs are usually not detected in bacteria grown in rich media. A similar profile was obtained when the lipid A from the BL21(DE3)Δ*ompA* strain expressing lipidated FhuD2 was analyzed, with the difference that the monophosphoryl hexaacylated lipid A appeared to be slightly more abundant with respect to the other species. By contrast, consistently with the inactivation of the *msbB* and *pagP* genes, the lipid A from the BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* strain had no hexaacylated lipid A species and the predominant form was the phosphorylethanolamine pentaacylated lipid A, followed by its phosphorylethanolamine analog and the tetraacylated lipid A with its phosphorylethanolamine analog. Finally, the spectrum of the BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP*(pET-FhuD2) strain showed two interesting features. First, the tetracylated monophosphoryl species was more abundant compared to the lipid A from the same strain not expressing lipidated FhuD2. Second, both penta- and tetraacylated phosphorylethanolamine analogs were completely missing. Considering that the tetraacylated monophoryl lipid A is known to have poor TLR4 agonistic activity and that phosphorylethanolamine modification slightly enhances the TLR4 agonistic activity of lipid A, these data offer an explanation of the reduced TLR4 stimulation of the OMVs$_{msbBpagP}$ decorated with the lipidated antigens (see also *Discussion*).

A Penta-Valent OMV-Based *S. aureus* Vaccine Protects Mice Against *S. aureus* Infection. Immunization with Hla_{H35L}, SpA_{KKAA}, FhuD2, Csa1A, and LukE, alone or in combination, is known to protect different experimental animals when challenged with *S. aureus* strains (17, 22, 31, 32). Therefore, considering that to be efficacious, *S. aureus* vaccines likely require the inclusion of more than one protective antigen, we tested whether the combination of the engineered OMVs decorated with the 5 *S. aureus* antigens could protect mice from *S. aureus* challenge in the sepsis model. To this aim, we first analyzed the antibody titers elicited by the 5-combo-OMVs$_{msbBpagP}$ containing 5 μg of each engineered OMV. As shown in Fig. 6, the 5-combo vaccine induced antigen-specific antibody titers that were in the same range as the titers obtained in mice immunized with the single-antigen OMVs. The only

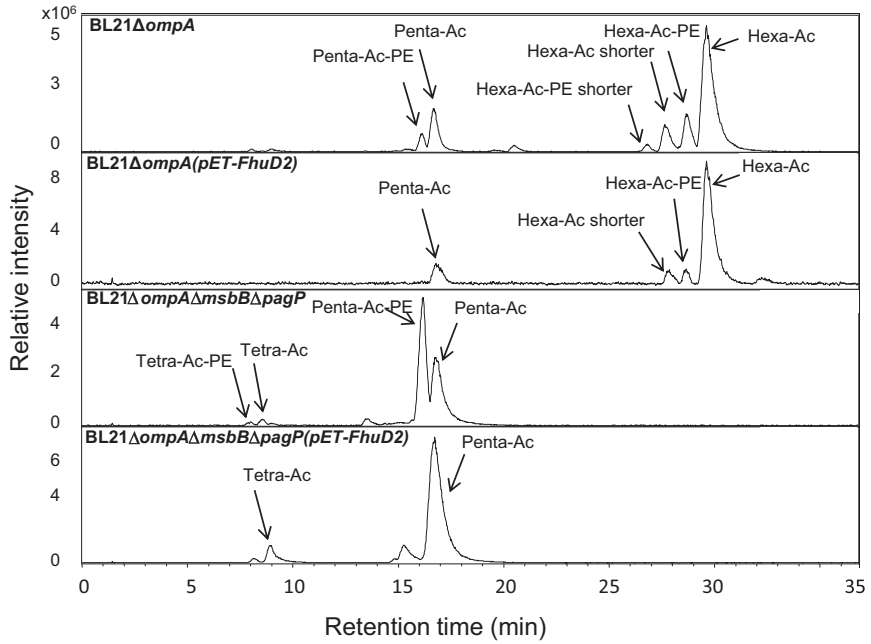

Fig. 5. Mass spectrometry analysis of lipid A from *E. coli* strains expressing lipidated FhuD2. Lipid A was purified from *E. coli* BL21(DE3)Δ*ompA*, *E. coli* BL21(DE3)Δ*ompA*(pET-FhuD2), *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP*, and *E. coli* BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP*(pET-FhuD2) strains. Purified lipid A was subjected to reverse phase liquid chromatography-electrospray ionization-mass spectrometry (RPLC-ESI-MS). Indicated peaks at different retention times were assigned as described in *SI Appendix, Materials and Methods*. The assignment of fatty acyl units was confirmed by tandem MS.

appreciable difference was observed for the anti-FhuD2 antibody titers induced by the 5-combo that were still sufficiently high (2×10^4) but approximately 1 order of magnitude lower than those induced by FhuD2-OMVs$_{msbBpagP}$ alone. Having demonstrated the good immunogenicity of the 5-combo vaccine, mice were immunized 3 times, 2 wk apart, with either 25 μg of "empty" OMVs or with the 5-combo-OMVs (5 μg of each engineered OMV). Two weeks after, mice were challenged with an i.p. injection of 2×10^8 colony-forming units (CFUs) of *S. aureus* Newman strain, a strain expressing all 5 antigens (see refs. 31, 33, and 34), and the health status of the animals was followed every day over a period of 7 d using a 1-to-4 pain scale (see *Material and Methods*). Animals that reached the pain value of 4 were killed since they reached the "near mortality point" and would have died within 24 h. On the other hand, those animals that maintained a score lower than 4, fully recovered within 7 d. Fig. 6*B* reports the cumulative data of the groups of mice immunized with empty or engineered OMVs deriving from either the BL21(DE3)Δ*ompA* strain (open symbols) or the BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* strain (closed symbols). As shown in the figure, immunization with "empty" OMVs conferred a substantial level of protection, with 50% of the animals that did not reach pain value 4 and survived. Almost 100% protection was observed in the groups of mice immunized with the 5-combo-OMVs vaccine and 70% of the animals did not experience a pain value higher than 2. No appreciable difference in protection was observed when mice immunized with OMVs derived from BL21(DE3)Δ*ompA* (open symbols) and BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* (closed symbols) were compared. The high level of protection conferred by the COMBO-OMVs vaccine in the sepsis model can be further appreciated if the data are analyzed by looking at the overall survival (Fig. 6 *B*, *Center* and *Right*). Interestingly, the Kaplan–Meier curve indicates that, differently from what was observed in previous work (31), no mice died after 24 h from the challenge. Body loss was also substantially mitigated by the COMBO-OMVs vaccine (*SI Appendix*, Fig. S2). The protective activity of the COMBO-OMVs was also tested in the kidney abscess model and in the skin infection model, two models extensively used for testing *S. aureus* vaccines (31). As shown in Fig. 6 *C* and *D*, COMBO-OMVs vaccination elicits high protection in both models. In the kidney abscess model the CFU reduction was approximately 4 orders of magnitude with respect to alum-immunized mice and in a few mice, the number of CFUs recovered from the kidneys of killed mice was below the detection limit (10^2 CFUs). Such reduction was observed in both "empty"-OMVs and COMBO-OMVs vaccinated animals, suggesting that protection was largely mediated by an innate type of response induced by the OMVs.

Discussion

The evidence that Gram-negative bacteria release OMVs dates back to the early 60s when electron microscopy was able to capture the first pictures of these curious organelles (35, 36). However, OMVs would probably have remained almost unnoticed if not for the pioneer work of 2 laboratories led by Beveridge and Kuehn, respectively, who not only shed light on many fascinating aspects of their biological role but also paved the way for their biotechnological applications, including vaccines (37, 38).

With this work, we have extended the armamentarium of strategies that can be used to decorate OMVs with heterologous antigens for vaccine applications. We show that the lipoprotein transport pathway can be exploited to deliver foreign proteins to the vesicular compartment. The unexpected feature of this strategy is its efficiency. Five proteins, selected for their relevance in *S. aureus* virulence and their potential as vaccine candidates, were successfully expressed in the OMVs at a level ranging from 5% to 20% of total OMV proteins. This level was consistently superior to what we could obtain when the same proteins were delivered to the luminal compartment of the vesicles by expressing them in the periplasm. Recent data from our laboratories show that a number of other heterologous proteins expressed as lipoproteins behave in a similar manner. Considering that the lipoprotein transport involves at least 3 additional post-translational steps with respect to periplasmic proteins and that the protein composition of OMVs does not necessarily

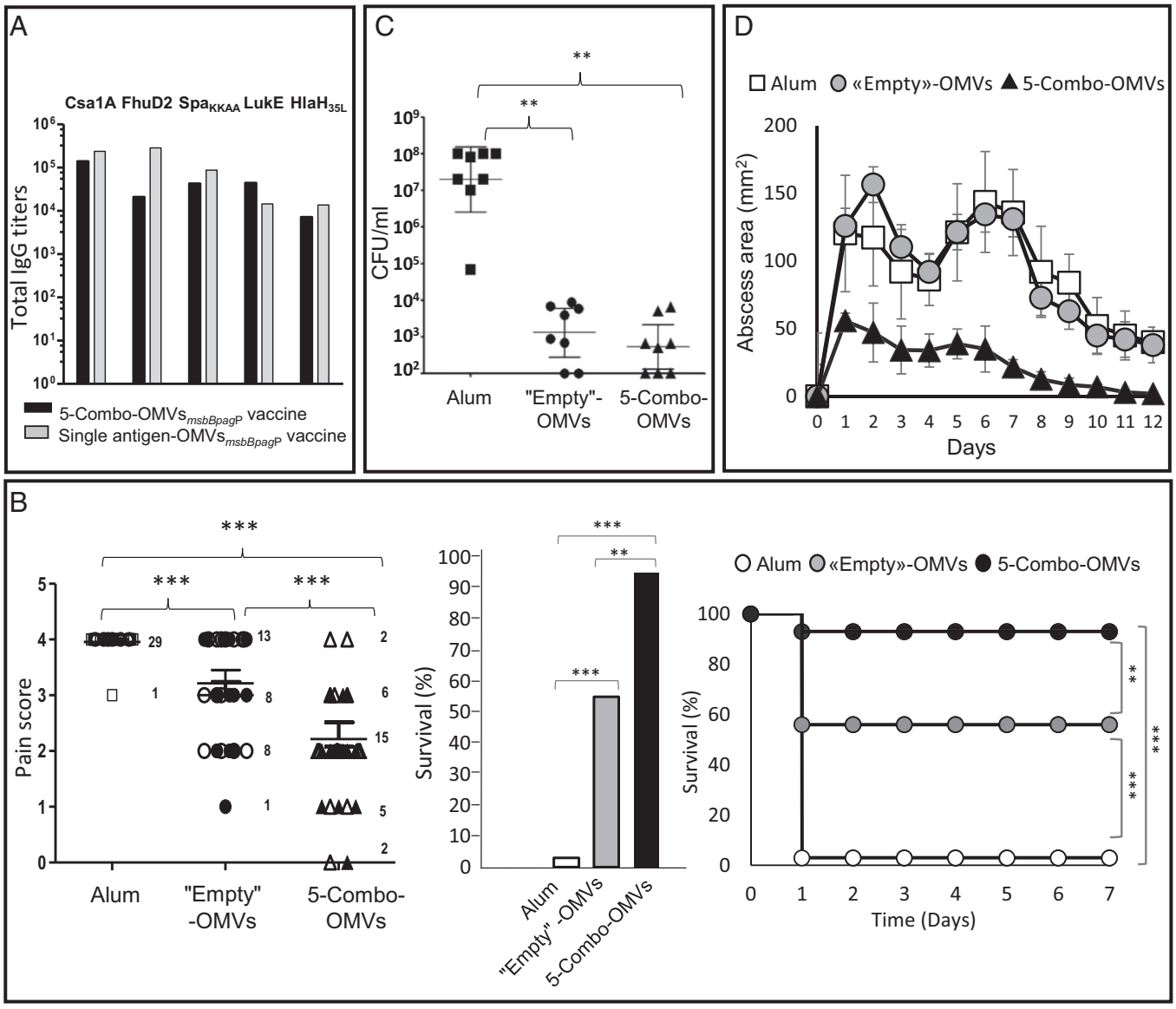

Fig. 6. Immunogenicity and in vivo protective activity of a combination of OMVs expressing *S. aureus* antigens. (*A*) Groups of 5 CD1 female mice were immunized i.p. 3 times at 2-wk interval with either 5 μg of OMVs$_{msbB\ pagP}$ expressing lipidated FhuD2, Csa1A, LukE, Hla$_{H35L}$, SpA$_{KKAA}$ antigens (gray bars) or a combination of OMVs$_{msbB\ pagP}$ expressing the 5 staphylococcal antigens (5 μg each) (black bars), formulated in alum. Sera were collected 10 d after the last immunization and IgG titers were analyzed by ELISA. (*B*) Sepsis model of infection. Four groups of mice were immunized with 2 doses of either "empty-OMV" (25 μg/dose) purified from BL21(DE3)Δ*ompA* (open circles) or from BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* (filled circles), or combinations of the 5 engineered OMVs from BL21(DE3)Δ*ompA* (open triangles) or from BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* (filled triangles). As control, mice were also immunized with alum (empty squares). After 2 wk, mice were infected intraperitoneally with a lethal dose of *S. aureus* Newman strain (4×10^8 CFUs). Protection data are reported using 3 different representation modalities. (*Left*) Pain score. Animals' health was evaluated over a period of 7 d using a 1-to-4 pain scale. A value of 4 was given to mice with: loss of weight >15%, very rough hair coat, impaired mobility. A score of 3 was given to mice with loss of weight of about 15% and rough hair coat, while scores of 2 and 1 were given to mice with a loss of weight between 6% and 14% or 0% and 5%, respectively. Means ± SEM are indicated. (*Center*) % Survival at day 7. (*Right*): % Survival as a function of time (Kaplan–Meier). See text for definition of "survival." Statistical analyses were performed using Student's *t* test with the exception of the Kaplan–Meier plot for which the log rank test was used. $^{**}P < 0.001$; $^{***}P < 0.0001$. (*C*) Renal abscess model. Eight mice per group were immunized with 3 doses of either "empty-OMV" (25 μg/dose) purified from BL21(DE3)Δ*ompA* (circles), or combinations of the 5 engineered OMVs from BL21(DE3)Δ*ompA* (triangles). As control, mice were also immunized with PBS (squares). Ten days after the last immunization, mice were infected i.v. with a sublethal dose of *S. aureus* Newman strain (1×10^7 CFUs) and 4 d afterward, mice were killed, kidneys removed and homogenized in PBS, and finally aliquots were plated on agar media for CFU determination. Bars indicate the geometric mean and the SEM for each group. (*D*) Skin model of infection. Eight mice per group were immunized with 2 doses of either "empty-OMV" (25 μg/dose) purified from BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* (gray circles), or COMBO-OMVs from BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* (black triangles). As control, mice were also immunized with alum (white squares). At 14 d after the second immunization, mice were s.c. infected with 5×10^7 CFUs of *S.aureus* Newman strain. Abscess size was monitored once per day for 12 d. Results indicate the mean ± SEM for all groups.

recapitulate what is present in the outer membrane (it has been reported that some outer membrane proteins tend to be overrepresented in the OMVs while others are missing) (39), we find the efficiency of the strategy surprising.

An important aspect to be considered in light of vaccine applications is whether OMVs with lipidated antigens embedded in their membrane are still capable of eliciting immune responses, that, in the case of OMVs engineered with other strategies, are

often better than what has been obtained with purified proteins formulated with alum (14, 40, 41). We have only marginally investigated this aspect, which surely deserves more detailed studies. By comparing the recognition pattern of the proteolytic fragments of the antigens using sera from mice immunized with the engineered OMVs and sera from mice immunized with the corresponding purified antigens, we found a substantial superimposition. Moreover, we recently found that sera from mice immunized with 10 μg of Hla_{H35L}-OMVs, which carried ∼0.5 to 1 μg of Hla_{H35L} (Fig. 2), had an anti-Hla hemolytic activity similar to the sera from mice immunized with 10 μg of purified Hla_{H35L} formulated in alum (*SI Appendix*, Fig. S3). The antihemolytic activity correlated with protection in the sepsis mouse model (*SI Appendix*, Fig. S3). This suggests that even if the lipoproteins were inserted into the OMV membrane facing the lumen (as expected for *E. coli* lipoproteins), the vesicles are partially degraded, thus exposing lipoprotein epitopes to B cell receptors. An alternative explanation is that some of the *S. aureus* antigens expressed as lipoproteins are exposed on the surface of OMVs. In support of this is the evidence that a number of lipoproteins can reach the surface of Gram-negative bacteria, either spontaneously (42) or supported by specific transport systems or "facilitators" (25), and that we recently showed that surface-exposed lipoproteins from *Neisseria* and *Aggregatibacter* are also surface exposed when expressed in *E. coli* (15). Indeed, using flow cytometry analysis we have recently confirmed that lipidated FhuD2 protrudes out of the outer membrane with high efficiency when expressed in our *E. coli* vesiculating strain (*SI Appendix*, Fig. S3).

A third interesting observation from our study is the effect that lipidated heterologous antigens can exert on the TLR4/MD2 agonistic activity of OMVs. The excellent adjuvant properties of OMVs provided by the high LPS content need be attenuated for human use. This can be achieved by inactivating the two acyltransferase enzymes encoded by the *msbB* (now renamed *lpxM*) and *pagP* genes, which catalyze the addition of secondary myristoyl and palmitoyl chains to the lipid A moiety, respectively, thus making LPS pentaacylated (29, 43, 44). When we introduced the *msbB* and *pagP* mutations in the OMV overproducing strain BL21(DE3)Δ*ompA*, the OMVs showed a 5-fold reduction in murine TLR4 activation. Interestingly, the BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* strain expressing lipidated antigens released OMVs with a further reduction in TLR4 activity, a reduction which was as high as 100-fold in the case of SpA_{KKAA}-$OMVs_{msbBpagP}$. A similar reduction was not observed in engineered OMVs from the BL21(DE3)Δ*ompA* strain. Even more striking was the effect of lipoprotein-decorated $OMVs_{msbBpagP}$ on human TLR4, whose signaling was almost abolished, as judged by the in vitro assay using hTLR4-HEK-Blue cells. Our mass spectrometry analysis of lipid A revealed that while the lipid A from BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* was predominantly a pentaacylated phosphorylethanolamine derivative (as opposed to the hexaacylated lipid A from BL21(DE3)Δ*ompA* strains), the LPS from BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP* expressing lipidated FhuD2 was constituted by pentaacylated lipid A with no phosphorylethanolamine modification and by tetraacylated lipid A. Considering that phosphorylethanolamine enhances TLR4 agonistic activity of lipid A and that pentaacylated and tetraacylated lipid A poorly stimulate TLR4, particularly human TLR4, our lipid A structural analysis is consistent with the observed TLR4 agonistic activities of the different OMVs (Fig. 4). It remains to be explained why the expression of lipidated antigens has such a profound effect on the lipid A structure. At present we can only speculate that the *ompA* mutation, by perturbing the structure of the outer membrane, triggers a stress response signal which up-regulates the expression of the lipid A phosphorylethanolamine transferase. Indeed, Gram-negative bacteria modify the LPS structure as a way to adapt to different stress conditions (45). The overexpression of lipidated proteins might compensate for the absence of OmpA, thus down-regulating the phosphorylethanolamine transferase gene. Moreover, we propose that when lipidated proteins are overexpressed in BL21(DE3)Δ*ompA*Δ*msbB*Δ*pagP*, the LpxL enzyme of the lipid A biosynthetic pathway and the acyltransferase(s) involved in lipoprotein biosynthesis compete for the same substrate and part of lipid A remains tetraacylated.

One last comment on the OMV-based vaccine here described, which we showed to be highly protective in 3 relevant mouse models. So far, 3 phase III *S. aureus* vaccine trials have been reported. They have been carried out with 3 different non-adjuvanted formulations: the 2-component CP5/CP8 glycoconjugate vaccine (46), the single-component IsdB vaccine (47), and the 4-component CP5/CP8 glycoconjugates/ClfA/MntC vaccine (48). All 3 vaccines showed promising protection in animal models but the results were largely disappointing in the clinics. While these repeated failures legitimize the concerns about the feasibility of an anti-*S. aureus* vaccine, the formulation proposed in the present study might offer 2 main advantages. First, it has been conceptualized to elicit neutralizing immune responses toward 5 antigens that play key roles in *S. aureus* virulence and immune evasion. Second, the OMV-based vaccine here proposed features high adjuvanticity properties provided by the activation of different signaling pathways, including TLR4 and TLR2 signaling. The lipoprotein-dependent TLR2 signaling is particularly relevant for protection against *S. aureus* as suggested by 3 pieces of evidence. First, *S. aureus* strains carrying the inactivation of the *lgt*, the gene responsible for the lipoprotein acylation, are surprisingly more virulent than the wild-type strain in mouse models (49). Second, $tlr2^{-/-}$ KO mice are more susceptible to *S. aureus* infections than wild-type mice (50). Third, *S. aureus* virulent strains express a number of superantigen-like toxins, 2 of which (SSL3 and SSL4) bind to TLR2, thus blocking TLR2 signaling (51). We believe that in our proposed vaccine formulation, the immune responses toward the selected antigens and the adjuvanticity of the OMV platform synergize, leading to almost 100% protection in all 3 tested models. Interestingly, in both sepsis and kidney models, where systemic invasion occurs also thanks to the "Trojan horse" role of phagocytic cells, the contribution of OMVs appears particularly relevant as indicated by the fact "empty" OMVs were also highly protective. The ability of *S. aureus* to survive inside phagocytic cells is one of the main mechanisms of pathogenesis which could not be counteracted by an adaptive immunity alone. By contrast, vaccines capable of skewing the immune responses toward a Th1/Th17 profile and, importantly, of temporarily activating a strong T cell-independent innate type of immunity, could enhance the killing capacity of phagocytic cells, thus reducing the risk of systemic dissemination of *S. aureus*. If the above is true, *S. aureus* vaccines should be administered repeatedly, particularly when the risk of infection increases, as is the case for instance of hospitalized patients.

Materials and Methods

The genes encoding *S. aureus* antigens were chemically synthesized and cloned in pET expression plasmid fused to either the *ompA* periplasmic leader sequence or the *lpp* lipoprotein leader sequence to deliver them to the lumen and to the membrane of OMVs, respectively. OMVs were purified from the culture supernatants by ultracentrifugation or tangential flow ultrafiltration. LPS was purified from bacterial cells using phenol extraction and analyzed by mass spectrometry. CD1 mice were immunized with recombinant OMVs in the presence or absence of alum and sera were analyzed by ELISA. For protection, immunized animals were challenged i.p., i.v., or in the skin with the *S. aureus* Newman strain. Experimental methods are described in detail in *SI Appendix*. All procedures were approved by the National Institution of Health and Ethical Committees of Trento University and Toscana Life Sciences Foundation, and for humane reasons animals were sacrificed at symptoms of sickness as recommended by 3Rs rules ("Refinement, Reduction, Replacement" policy towards the use of animals for scientific procedures_ 99/167/EC, Council Decision of 25 January 1999).

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1. R. Rappuoli, C. W. Mandl, S. Black, E. De Gregorio, Vaccines for the twenty-first century society. *Nat. Rev. Immunol.* **11**, 865–872 (2011).
2. R. J. Fair, Y. Tor, Antibiotics and bacterial resistance in the 21st century. *Perspect. Medicin. Chem.* **6**, 25–64 (2014).
3. S. Y. C. Tong, J. S. Davis, E. Eichenberger, T. L. Holland, V. G. Fowler, Jr, *Staphylococcus aureus* infections: Epidemiology, pathophysiology, clinical manifestations, and management. *Clin. Microbiol. Rev.* **28**, 603–661 (2015).
4. T. J. Foster, Antibiotic resistance in *Staphylococcus aureus*. Current status and future prospects. *FEMS Microbiol. Rev.* **41**, 430–449 (2017).
5. T. J. Foster, Immune evasion by staphylococci. *Nat. Rev. Microbiol.* **3**, 948–958 (2005).
6. V. Thammavongsa, H. K. Kim, D. Missiakas, O. Schneewind, Staphylococcal manipulation of host immune responses. *Nat. Rev. Microbiol.* **13**, 529–543 (2015).
7. Y. M. D. Gnopo, H. C. Watkins, T. C. Stevenson, M. P. DeLisa, D. Putnam, Designer outer membrane vesicles as immunomodulatory systems–Reprogramming bacteria for vaccine delivery. *Adv. Drug Deliv. Rev.* **114**, 132–142 (2017).
8. P. C. McCarthy, A. Sharyan, L. Sheikhi Moghaddam, Meningococcal vaccines: Current status and emerging strategies. *Vaccines (Basel)* **6**, E12 (2018).
9. T. N. Ellis, M. J. Kuehn, Virulence and immunomodulatory roles of bacterial outer membrane vesicles. *Microbiol. Mol. Biol. Rev.* **74**, 81–94 (2010).
10. J. Klimentová, J. Stulík, Methods of isolation and purification of outer membrane vesicles from gram-negative bacteria. *Microbiol. Res.* **170**, 1–9 (2015).
11. N. C. Kesty, M. J. Kuehn, Incorporation of heterologous outer membrane and periplasmic proteins into *Escherichia coli* outer membrane vesicles. *J. Biol. Chem.* **279**, 2069–2076 (2004).
12. J. L. Baker, L. Chen, J. A. Rosenthal, D. Putnam, M. P. DeLisa, Microbial biosynthesis of designer outer membrane vesicles. *Curr. Opin. Biotechnol.* **29**, 76–84 (2014).
13. M. J. H. Gerritzen, D. E. Martens, R. H. Wijffels, L. van der Pol, M. Stork, Bioengineering bacterial outer membrane vesicles as vaccine platform. *Biotechnol. Adv.* **35**, 565–574 (2017).
14. L. Fantappiè *et al*., Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen. *J. Extracell. Vesicles* **3**, 24015 (2014).
15. L. Fantappiè *et al*., Some gram-negative lipoproteins keep their surface topology when transplanted from one species to another and deliver foreign polypeptides to the bacterial surface. *Mol. Cell. Proteomics* **16**, 1348–1364 (2017).
16. S. Okuda, H. Tokuda, Lipoprotein sorting in bacteria. *Annu. Rev. Microbiol.* **65**, 239–259 (2011).
17. J. Bubeck Wardenburg, O. Schneewind, Vaccine protection against *Staphylococcus aureus* pneumonia. *J. Exp. Med.* **205**, 287–294 (2008).
18. F. Falugi, H. K. Kim, D. M. Missiakas, O. Schneewind, Role of protein A in the evasion of host adaptive immune responses by *Staphylococcus aureus*. *MBio* **4**, e00575-13 (2013).
19. R. P. N. Mishra *et al*., *Staphylococcus aureus* FhuD2 is involved in the early phase of staphylococcal dissemination and generates protective immunity in mice. *J. Infect. Dis.* **206**, 1041–1049 (2012).
20. P. Mariotti *et al*., Structural and functional characterization of the *Staphylococcus aureus* virulence factor and vaccine candidate FhuD2. *Biochem. J.* **449**, 683–693 (2013).
21. C. Schluepen *et al*., Mining the bacterial unknown proteome: Identification and characterization of a novel family of highly conserved protective antigens in *Staphylococcus aureus*. *Biochem. J.* **455**, 273–284 (2013).
22. A. N. Spaan, J. A. G. van Strijp, V. J. Torres, Leukocidins: Staphylococcal bi-component pore-forming toxins find their receptors. *Nat. Rev. Microbiol.* **15**, 435–447 (2017).
23. C. E. Cowles, Y. Li, M. F. Semmelhack, I. M. Cristea, T. J. Silhavy, The free and bound forms of Lpp occupy distinct subcellular locations in *Escherichia coli*. *Mol. Microbiol.* **79**, 1168–1181 (2011).
24. C. Bordier, Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem.* **256**, 1604–1607 (1981).
25. W. R. Zückert, Secretion of bacterial lipoproteins: Through the cytoplasmic membrane, the periplasm and beyond. *Biochim. Biophys. Acta* **1843**, 1509–1516 (2014).
26. K. S. Park *et al*., Outer membrane vesicles derived from *Escherichia coli* induce systemic inflammatory response syndrome. *PLoS One* **5**, e11334 (2010).
27. J. E. Somerville, Jr, L. Cassiano, B. Bainbridge, M. D. Cunningham, R. P. Darveau, A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. *J. Clin. Invest.* **97**, 359–365 (1996).
28. R. E. Bishop *et al*., Transfer of palmitate from phospholipids to lipid A in outer membranes of gram-negative bacteria. *EMBO J.* **19**, 5071–5080 (2000).
29. D. H. Lee *et al*., Adjuvant effect of bacterial outer membrane vesicles with penta-acylated lipopolysaccharide on antigen-specific T cell priming. *Vaccine* **29**, 8293–8301 (2011).
30. C. R. H. Raetz, C. Whitfield, Lipopolysaccharide endotoxins. *Annu. Rev. Biochem.* **71**, 635–700 (2002).
31. F. Bagnoli *et al*., Vaccine composition formulated with a novel TLR7-dependent adjuvant induces high and broad protection against *Staphylococcus aureus*. *Proc. Natl. Acad. Sci. U.S.A.* **112**, 3680–3685 (2015).
32. H. K. Kim, A. G. Cheng, H.-Y. Kim, D. M. Missiakas, O. Schneewind, Nontoxigenic protein A vaccine for methicillin-resistant *Staphylococcus aureus* infections in mice. *J. Exp. Med.* **207**, 1863–1870 (2010).
33. T. Brignoli *et al*., Absence of protein a expression is associated with higher capsule production in staphylococcal isolates. *Front. Microbiol.* **10**, 863 (2019).
34. A. Venkatasubramaniam *et al*., A critical role for HlgA in Staphylococcus aureus pathogenesis revealed by A switch in the SaeRS two-component regulatory system. *Toxins (Basel)* **10**, E377 (2018).
35. S. N. Chatterjee, J. Das, Electron microscopic observations on the excretion of cell-wall material by *Vibrio cholerae*. *J. Gen. Microbiol.* **49**, 1–11 (1967).
36. E. Work, K. W. Knox, M. Vesk, The chemistry and electron microscopy of an extracellular lipopolysaccharide from *Escherichia coli*. *Ann. N. Y. Acad. Sci.* **133**, 438–449 (1966).
37. T. J. Beveridge, Structures of gram-negative cell walls and their derived membrane vesicles. *J. Bacteriol.* **181**, 4725–4733 (1999).
38. C. Schwechheimer, M. J. Kuehn, Outer-membrane vesicles from gram-negative bacteria: Biogenesis and functions. *Nat. Rev. Microbiol.* **13**, 605–619 (2015).
39. B. K. Cahill, K. W. Seeley, D. Gutel, T. N. Ellis, *Klebsiella pneumoniae* O antigen loss alters the outer membrane protein composition and the selective packaging of proteins into secreted outer membrane vesicles. *Microbiol. Res.* **180**, 1–10 (2015).
40. E. Bartolini *et al*., Recombinant outer membrane vesicles carrying Chlamydia muridarum HtrA induce antibodies that neutralize chlamydial infection in vitro. *J. Extracell. Vesicles* **2**, 20181 (2013).
41. O. Koeberling *et al*., A broadly-protective vaccine against meningococcal disease in sub-Saharan Africa based on generalized modules for membrane antigens (GMMA). *Vaccine* **32**, 2688–2695 (2014).
42. R. J. Schulze, W. R. Zückert, *Borrelia burgdorferi* lipoproteins are secreted to the outer surface by default. *Mol. Microbiol.* **59**, 1473–1484 (2006).
43. M. K. Vorachek-Warren, S. Ramirez, R. J. Cotter, C. R. Raetz, A triple mutant of *Escherichia coli* lacking secondary acyl chains on lipid A. *J. Biol. Chem.* **277**, 14194–14205 (2002).
44. O. Rossi *et al*., Toll-like receptor activation by generalized modules for membrane antigens from lipid a mutants of Salmonella enterica serovars typhimurium and enteritidis. *Clin. Vaccine Immunol.* **23**, 304–314 (2016).
45. B. Bertani, N. Ruiz, Function and biogenesis of lipopolysaccharides. *EcoSal Plus* **8**, ESP-0001-2018 (2018).
46. A. Fattom *et al*., Efficacy profile of a bivalent *Staphylococcus aureus* glycoconjugated vaccine in adults on hemodialysis: Phase III randomized study. *Hum. Vaccin. Immunother.* **11**, 632–641 (2015).
47. V. G. Fowler *et al*., Effect of an investigational vaccine for preventing *Staphylococcus aureus* infections after cardiothoracic surgery: A randomized trial. *JAMA* **309**, 1368–1378 (2013).
48. A. S. Anderson *et al*., Development of a multicomponent *Staphylococcus aureus* vaccine designed to counter multiple bacterial virulence factors. *Hum. Vaccin. Immunother.* **8**, 1585–1594 (2012).
49. J. Bubeck Wardenburg, W. A. Williams, D. Missiakas, Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins. *Proc. Natl. Acad. Sci. U.S.A.* **103**, 13831–13836 (2006).
50. O. Takeuchi, K. Hoshino, S. Akira, Cutting edge: TLR2-deficient and MyD88-deficient mice are highly susceptible to Staphylococcus aureus infection. *J. Immunol.* **165**, 5392–5396 (2000).
51. K. J. Koymans *et al*., Structural basis for inhibition of TLR2 by staphylococcal superantigen-like protein 3 (SSL3). *Proc. Natl. Acad. Sci. U.S.A.* **112**, 11018–11023 (2015).