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

Carmela Irene, Laura Fantappie, Elena Caproni, Francesca Zerbini, Andrea Anesi, Michele Tomasi, Ilaria Zanella, Simone Stupia, Stefano Prete, Silvia Valensin, Enrico König, Luca Frattini, Assunta Gagliardi, Samine J. Isaac, Alberto Grandi, Graziano Guella, and Guido Grandi

*Department of Cellular, Computational and Integrative Biology (CIBIO), University of Trento, 38123 Trento, Italy; and European Research Council “OMV Vaccines” Laboratory, Toscana Life Sciences Foundation, 53100 Siena, Italy*

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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 lipiddation 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 lipided antigens in *E. coli* BL21(DE3)lompAΔmsbBΔpagP 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 lipided 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. Fantappie, 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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C.I. and L. Fantappie contributed equally to this work.

1. Present address: Antibody Discovery and Protein Engineering Unit, Medimmune/AstraZeneca, Granta Park, CB21 6GP Cambridge, United Kingdom.
2. Present address: Metabolomics Research Unit, Fondazione Edmund Mach, 38010 San Michele all’Adige, Italy.
3. To whom correspondence may be addressed. Email: guido.grandi@unitn.it.

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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 NH₂ 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 (17), SpA (18), OmpA (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 reactivity and function 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, we selected the 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 HlA135L and SpaAKKAA (17, 18), LukE, 1 of the 2 components of the LukD/E leukocidin (22), CsA1A (21), and iron binding protein Hfud2 (20). In particular, Spa binds to Fcy 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 hemoplastic 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(D3)ΔompA strain. OMVs were purified from the culture supernatant of each strain and the protein content was analyzed by SDS/PAGE (Fig. 1A). 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. 1B).

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(D3)ΔompA strain. OMVs were purified and antigen expression in OMVs was analyzed by SDS/PAGE. All 5 antigens were successfully compartmentalized in the OMVs (Fig. 1A). 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 SpaAKKAA, whose level of expression was from 10% to 20% of total OMV proteins (Fig. 1B).

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 “hydrophilic” 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. 3A, 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. 3B). We finally tested the other 3 recombinant OMVs (CsA1A-OMVs, SpaAKKAA-OMVs, and Hla135L-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, these 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
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. 3D, 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 Enterobacteriaceae**

**Immunogenicity of Engineered OMVs from the Enterobacteriaceae.** 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. 1.** Expression of Staphylococcal antigens in OMVs from *E. coli* BL21(DE3)ΔompA. (A) *E. coli* BL21(DE3)ΔompA 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.

**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.
In view of a possible exploitation of an OMV-based S. aureus vaccine, we generated the E. coli BL21(DE3)ΔompAΔmsbBΔpagP strain. We first verified that, when expressed in the BL21(DE3)ΔompAΔmsbBΔpagP strain, the 5 S. aureus antigens were incorporated in the OMVs with an efficiency similar to that observed in BL21(DE3)ΔompA. To this aim, the E. coli BL21(DE3)ΔompAΔmsbBΔpagP 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)ΔompA strain (compare Fig. 4A with Fig. 4C).

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. 4B, and according to what has been previously published (29), purified OMVs from the BL21(DE3)ΔompAΔmsbBΔpagP strain (OMVsΔmsbBΔpagP) had a mTLR4 stimulatory capacity approximately 5-fold lower than the OMVs from the BL21(DE3)ΔompA strain. Unexpectedly, the recombinant OMVsΔmsbBΔpagP carrying the lipidated antigens showed a further reduction in TLR4 stimulation. The reduction varied depending upon the expressed antigen, but in the case of SpAKAAA-OMVsΔmsbBΔpagP it was ∼100-fold lower than “empty”-OMVsΔmsbBΔpagP and, in the case of Csa1A-OMVsΔmsbBΔpagP, FhuD2-OMVsΔmsbBΔpagP the reduction was between 10- and 20-fold lower. Even more strikingly, when hTLR4 responses were analyzed, the agonist activity of OMVsΔmsbBΔpagP engineered with lipidated antigens was almost completely abolished (Fig. 4C).

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

The Expression of Lipidated Antigens in BL21(DE3)ΔompAΔmsbBΔpagP Alters the Lipid A Structure. The unexpected reduction of TLR4 agonistic activity of OMVsΔmsbBΔpagP decorated with lipidated antigens prompted us to characterize the lipid A structure from BL21(DE3)ΔompA, BL21(DE3)ΔompA(pET-FhuD2), BL21(DE3)ΔompAΔmsbBΔpagP, and BL21(DE3)ΔompAΔmsbBΔpagP(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)ΔompA strain appeared to be mostly constituted by monophosphoryl hexaacylated lipid A and its phosphoethanolamine analog. Overall, the spectrum was essentially in line with what was reported for LPS purified from wild-type E. coli strains (30), even

![Image](https://example.com/image_url)
though the phosphoryl-ethanolamine analogs are usually not detected in bacteria grown in rich media. A similar profile was obtained when the lipid A from the BL21(DE3)ΔompA 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, the tetracylated monophosphoryl species and the predominant form was the phosphoryl-ethanolamine pentaacylated lipid A, followed by its hexaacylated lipid A species and the predominant form was the monophosphoryl hexaacylated lipid A species. A similar profile was also observed for the lipid A from the BL21(DE3)ΔompAΔmsbBpagP strain grown in rich media. A similar profile was also observed for the lipid A from the BL21(DE3)ΔompAΔmsbBpagP strain grown in rich media.

Although phosphoryl-ethanolamine modification slightly enhances the TLR4 agonistic activity of lipid A, these data offer an explanation for the reduced TLR4 stimulation of the OMVs from 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 five antigens (H35L, SpA, FhuD2, LukE, and Csa1A) in mice immunized with the single-antigen OMVs.

A Penta-Valent OMV-Based S. aureus Vaccine Protects Mice Against S. aureus Infection. Immunization with HlaΔH35L, SpAΔKKAA, 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 OMVsΔmsbBpagP 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. 4. Evaluation of immunogenicity and reactivity of antigen engineered OMVsΔmsbBpagP. (A) E. coli BL21(DE3)ΔompAΔmsbBpagP strains expressing heterologous staphylococcal antigens as lipoproteins were grown in LB at 37 °C. At OD600 = 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 BL21ΔompA ("empty"-OMVs) and from E. coli BL21(DE3)ΔompAΔmsbBpagP (OMVsΔmsbBpagP) either "empty" or loaded with S. aureus antigens. Alkaline phosphatase activity was determined after 17 h of incubation. Relative mTLR4 activity is represented as ratio between EC50 value of each OMV over the EC50 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)ΔompAΔmsbBpagP ("empty" OMVsΔmsbBpagP) or OMVs loaded with S. aureus antigens (FhuD2-OMVsΔmsbBpagP and Csa1A-OMVsΔmsbBpagP). 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)ΔompAΔmsbBpagP 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, SpAΔKKAA, and HlaΔH35L were carried out with alum. As control, groups of mice were also immunized with 10 μg of the corresponding recombinant proteins (rP) in alum.
appreciable difference was observed for the anti-FhuD2 antibody titers induced by the 5-combo that were still sufficiently high ($2 \times 10^6$) but approximately 1 order of magnitude lower than those induced by FhuD2-OMVs 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 $2 \times 10^8$ 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. 6B reports the cumulative data of the groups of mice immunized with empty or engineered OMVs deriving from either the BL21(DE3)ΔompA strain (open symbols) or the BL21(DE3)ΔompAΔmsbBΔpagP 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. No appreciable difference in protection was observed when mice immunized with OMVs derived from BL21(DE3)ΔompA (open symbols) and BL21(DE3)ΔompAΔmsbBΔpagP (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. 6B, 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
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 recapitulation 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\textsubscript{EMS}, OMVs, which carried ~0.5 to 1 μg of Hla\textsubscript{EMS}, (Fig. 2), had an anti-Hla hemolytic activity similar to E. coli antigens expressed as in lipoprotein biosynthesis. (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 lipoprotein and lipopolysaccharide vaccines 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, 34, 44). When we introduced the msbB and pagP mutations in the OMV overproducing strain BL21(DE3)\textsubscript{ΔompA}, the OMVs showed a 5-fold reduction in murine TLR4 activation. Interestingly, the BL21(DE3)\textsubscript{ΔompA}\textsubscript{ΔmsbB}\textsubscript{ΔpagP} strain expressing lipoprotein antigens, with a further reduction in TLR4 agonism, a reduction which was as high as 100-fold in the case of Sp\textsubscript{KNOX} OMVs\textsubscript{subB}\textsubscript{pagP}. A similar reduction was not observed in engineered OMVs from the BL21(DE3)\textsubscript{ΔompA} strain. Even more striking was the effect of lipoprotein-decorated OMVs\textsubscript{subB}\textsubscript{pagP} 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)\textsubscript{ΔompA}\textsubscript{ΔmsbB}\textsubscript{ΔpagP} was predominantly a pentaacylated phosphorylethanolamine derivative (as opposed to the hexaacylated lipid A from BL21(DE3)\textsubscript{ΔompA} strains), the LPS from BL21(DE3)\textsubscript{ΔompA}\textsubscript{ΔmsbB}\textsubscript{ΔpagP} 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 lipoprotein 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 lipoprotein proteins might compensate for the absence of OmpA, thus down-regulating the phosphorylethanolamine transferase gene. Moreover, we propose that when lipoproteins are overexpressed in BL21(DE3)\textsubscript{ΔompA}\textsubscript{ΔmsbB}\textsubscript{ΔpagP}, 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 tetracylated.

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, the 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 it 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 lipid protein 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 human research animals were sacrificed at symptoms of sickness as recommended by 3Rs rules (“Refinement, Reduction, Replacement” policy towards the use of animals for scientific procedures, 99/176/EC, Council Decision of 25 January 1999).
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