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ORIGINAL RESEARCH ARTICLE

Antibody-mediated immunity induced by engineered *Escherichia coli* OMVs carrying heterologous antigens in their lumen

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Background: Outer membrane vesicles (OMVs) from Gram-negative bacteria are gaining increasing attention as vaccine platform for their built-in adjuvanticity and for their potential use as carriers of heterologous antigens. These 2 properties offer the opportunity to make highly effective, easy to produce multi-valent vaccines. OMVs can be loaded with foreign antigens by targeting protein expression either to the outer membrane or to the periplasm of the OMV-producing strain. Periplasmic expression is simple and relatively efficient but leads to the accumulation of recombinant antigens in the lumen of OMVs and the ability of OMVs carrying internalized antigens to induce antigen-specific antibody responses has been only marginally investigated and is considered to be sub-optimal.

Methods: We have systematically analyzed in qualitative and quantitative terms antibody responses induced by OMVs carrying different heterologous antigens in their lumen. *Group A Streptococcus* (GAS) Slo, SpyCEP, Spy0269 and *Group B Streptococcus* (GBS) SAM_1372 were fused to the OmpA leader sequence for secretion and expressed in *Escherichia coli*. OMVs from the recombinant strains were purified and tested for immunogenicity and protective activity.

Results: All proteins were incorporated into the OMVs lumen in their native conformation. Upon mice immunization, OMVs induced high functional antibody titers against the recombinant proteins. Furthermore, immunization with Slo-OMVs and SpyCEP-OMVs protected mice against GAS lethal challenge.

Conclusions: The efficiency of antigen delivery to the vesicular lumen via periplasmic expression, and the surprisingly high immunogenicity and protective activity of OMVs carrying internalized recombinant antigens further strengthens the potential of OMVs as vaccine platform.

Keywords: *outer membrane vesicles*; *vaccines*; *heterologous antigens*; *periplasmic expression*; Group A Streptococcus; Group B Streptococcus

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Outer membrane vesicles (OMVs) are closed spheroid particles of heterogeneous size (10–300 nm in diameter) released by all Gram-negative bacteria and generated through the "budding out" of the bacterial outer membrane. The production of OMVs was originally described as an essential step for rapid adaptation to environmental changes, but a multitude of other functions have now been attributed to OMVs, including delivery of toxins and virulence factors to host cells, inter-species and intra-species cell-to-cell cross-talk, biofilm formation, genetic transformation and defence against innate and adaptive host immune responses (1–3).

OMVs purified from several pathogens, including *Neisseria, Salmonella, Pseudomonas, Vibrio cholerae, Burkholderia*, and *Escherichia coli*, induce potent protective immune responses against the pathogens they derive from, and highly efficacious anti-*Neisseria* OMV-based vaccines are already available for human use (4–8). Such remarkable protection is attributed to 3 key features of OMVs. First, they carry surface-associated antigens, usually including protective antigens. Second, they are

readily phagocytosed by professional antigen-presenting cells, allowing efficient presentation of OMV-derived peptides. This results in efficient elicitation of antibodies and extracellular antigen mediated CD4+ T cell response against OMV proteins. For instance, mice immunized with *Salmonella* OMVs develop robust *Salmonella*-specific B- and T-cell responses, and OMVs also stimulate IFN-γ production by a large proportion of CD4+ T cells in mice previously infected with *Salmonella*, indicating that OMVs are an abundant source of antigens recognized by *Salmonella*-specific CD4+ T cells (4). Third, OMVs carry many pathogen-associated-molecular patterns (PAMPs) which, by binding to pathogen recognition receptors (PRRs), play a key role in stimulating innate immunity and promoting adaptive immune responses (9–11). An additional key feature of OMVs is the possibility to manipulate their protein content by genetic engineering. Kesty and Kuehn (12) demonstrated that *Yersinia enterocolitica* outer membrane protein Ail assembled on OMVs' surfaces when expressed in *E. coli*, and that the GFP fluorescence protein fused to the "twin-arginine transport" (Tat) signal sequence was incorporated in the OMV lumen. Furthermore, several heterologous antigens have been successfully exported to the surface of OMVs when fused to the β-barrel forming auto transporter AIDA and to the haemolysin ClyA, 2 proteins that naturally compartmentalize in *E. coli* OMVs (13–15).

For a full blown development of OMVs as vaccine platform, a number of issues are yet to be addressed. Among them there are 2 relevant questions. First, although strategies to load OMVs with recombinant antigens have been described, their general applicability remains to be demonstrated. Second, recombinant antigens can theoretically be engineered (a) to reach the lumen of OMVs, either as soluble proteins or associated to the inner leaflet of the membrane and (b) to be associated to the membrane facing the external milieu. However, how protein compartmentalization in OMVs affects antibody responses deserves further investigation.

In this work, we addressed the question whether compartmentalization of recombinant antigens in the lumen of *E. coli*-derived OMVs represents a broadly applicable approach to induce protective antibody responses. To this aim, we selected a group of heterologous antigens and we show that all of them can be incorporated in the lumen of *E. coli*-derived OMVs in a functional conformation. Furthermore, we show that mouse immunization with engineered OMVs induces functional antibodies and protective immune responses. Altogether our data strengthen the potential of OMVs as vaccine platform in that they demonstrate that complex cloning strategies to deliver recombinant antigens to the OMV surface are not strictly necessary to promote effective immunological responses.

Materials and methods

Bacterial strains and culture conditions

HK-100 and BL21(DE3) *E. coli* strains were routinely grown in Luria-Bertani (LB) broth at 37°C and used for cloning and expression experiments, respectively. When required, Ampicillin or Chloramphenicol were added to a final concentration of 100 μg/ml and 30 μg/ml, respectively.

Construction of plasmids and knockout mutants

DNA manipulations were carried out using standard laboratory methods (16).

BL21(DE3) *ΔompA* mutant was produced by replacing the *ompA* coding sequence with a Chloramphenicol (Cm) resistance cassette. Briefly, the upstream and downstream regions of the *ompA* gene were amplified from BL21 (DE3) genomic DNA with the specific primer pairs ompA-1/ompA-2 and ompA-3/ompA-4 (Table I). In parallel, the Cm cassette was amplified from pKD3 plasmid (17) using the primers CMR-for/CMR-rev. Finally, the 3 amplified fragments were fused together by mixing 100 ng of each in a PCR reaction containing primers ompA-1 and ompA-4. The obtained linear fragment, in which the antibiotic resistance gene was flanked by the upstream and downstream regions of *ompA* gene, was used to transform BL21(DE3) *E. coli* (made electro-competent by 3 washing steps in cold water). *ΔompA* mutant colonies were selected by plating transformed bacteria on LB plates containing 30μg/ml Cm. Recombination-prone BL21 (DE3) cells were obtained by the highly proficient homologous recombination system (*red* operon) (18). Briefly, electro-competent bacterial cells were transformed with 5 μg of plasmid pAJD434 by electroporation (5.9 ms at 2.5 kV). Bacteria were then grown for 1 hour at 37°C in 1 ml SOC broth and then plated on LB plates containing Trimethoprim (100 μg/ml). Expression of the *red* genes carried by pAJD434 was induced by adding 0.2% L-arabinose to the medium. Deletion of the *ompA* gene was confirmed by genomic DNA amplification using the primer pairs ompA-1/CMR-rev and CMR-for/ompA-4 (Table I).

Plasmids pET21_bla, pET21_slo, pET21_slo-dm, pET21_spycep, pET21_spy0269 and pET21_sam1372 were generated as follows. *slo, spycep* and *spy_0269* genes were amplified by PCR from M1 3348 *Group A Streptococcus* (GAS) strain (19) using primers GAS25-F/GAS25-R, spyCEP-F3/spyCEP-R3 and Spy0269-F/Spy0269-R (Table I), respectively. The gene encoding inactive Slo was cloned from plasmid pET24-slo-dm (20) using GAS25-F/GAS25-R primers. The beta-lactamase gene was amplified using pET-21b plasmid DNA as template and primers bla-omp-F and bla-omp-R. Finally, amplification of *Streptococcus agalactiae sam_1372* gene from the CJB111 genome was conducted using primers

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Table I. Oligonucleotide primers used in this study

Name	Sequence
slo-F	ACCGTAGCGCAGGCCAACAAACAAAACACTGCTAGTACAG
slo-R	GTGATGGTGATGTTACTACTTATAAGTAATCGAACCATATG
SpyCEP-F3	ACCGTAGCGCAGGCCGCAGCAGATGAGCTAAGCACAATGAGCGAACC
SpyCEP-R3	GTGATGGTGATGTTATTAGGCTTTTGCTGTTGCTGAGGTCGTTGACTTGGTTGG
Bla-omp-F	ACCGTAGCGCAGGCCCGGTAAGATCCTTGAGATTTTTCG
Bla-omp-R	GTGATGGTGATGTTATTACCAATGCTTAATCAGTGAGGC
omprev	GGCCTGCGCTACGGTAGCGAAA
nohisflag	TAACATCACCATCACCATCACGATTACAAAGA
ompA-1	GATCGGTTGGTTGGCAGAT
ompA-2	CACCAGGATTTATTTATTCTGCGTTTTTGCGCCTCGTTATCAT
ompA-3	TACTGCGATGAGTGGCAGGCGCAGGCTTAAGTTCTCGTC
ompA-4	AAAATCTTGAAAGCGGTTGG
CMR-for	CGCAGAATAAATAAATCCTGGTG
CMR-rev	CCTGCCACTCATCGCAGTA
Spy0269-F	ACCGTAGCGCAGGCCGATGATAGAGCCTCAGGAGAAACG
Spy0269-R	GTGATGGTGATGTTATCACTTAGATTCCTTACGGAACC
SAM_1372-F	ACCGTAGCGCAGGCCGACGACGCAACAACTGATAC
SAM_1372-R	GTGATGGTGATGTTAGGTTACTTTTTTGTTTTGAACTTGTTGGG
Pet-rev	CATATGTATATCTCCTTCTTAAAGTTAAAC
Slo-F-no	GGAGATATACATATGAACAAACAAAACACTGCTAGTACAG
Spycep-F-no	GGAGATATACATATGGCAGCAGATGAGCTAAGCACAATGAGCGAACC
Spy_0269-F-no	GGAGATATACATATGGATGATAGAGCCTCAGGAGAAACG
SAM_1372-F-no	GGAGATATACATATGGACGACGCAACAACTGATAC

SAM_1372-F and SAM_1372-R. Primers were designed to amplify the genes without their natural leader sequence for secretion and, in the case of SpyCEP and SAM_1372, the C-terminal cell wall–anchoring domains (aa 1614–1647 and aa 620–674, respectively) were also omitted. The polymerase incomplete primer extension (PIPE) cloning method (21) was used to insert all PCR products into plasmid pETOmpA, a pET21 derivative carrying the sequence encoding the leader peptide for secretion of *E. coli* OmpA, which was amplified using primers omprev/nohisflag (Table I). In so doing, all recombinant proteins were expressed fused to the OmpA leader sequence. Cytoplasmic expression of the proteins was accomplished by cloning the amplified genes into a pETOmpA derivative plasmid in which the *ompA* leader sequence was removed using the primers pet-rev/nohisflag (Table I) for PIPE amplification. The plasmids encoding the genes without signal peptide were named pET21_spycep-noLS, pET21_spy0269-noLS, pET21_slo-noLS and pET21_sam1372-noLS.

Expression of the heterologous proteins in BL21(DE3)ΔompA E. coli strain and OMVs preparation

pET plasmid derivatives containing the genes of interest were transformed into BL21(DE3) Δ*ompA* strain. Recombinant clones were grown in 200 ml LB medium (starting $OD_{600} = 0.05$) and, when the cultures had reached an OD_{600} value of 0.5, recombinant protein expression was induced by addition of 1 mM IPTG. After 2 hours, OMVs were collected from culture supernatants by filtration through a 0.22 μm pore size filter (Millipore) and by high-speed centrifugation (200,000 × g for 2 hours). Pellets containing OMVs were finally suspended in PBS. Total bacterial lysates were prepared by suspending bacterial cells from 1 ml cultures (centrifuged at 13,000 × g for 5 minutes) in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer, heated at 100°C for 5 minutes and loaded onto a 4–12% polyacrylamide gel (Invitrogen). Polyacrylamide gels were run in MES buffer (Invitrogen) and stained with Coomassie Blue.

Western blot analysis

Total lysates were prepared from bacteria grown in LB broth. Liquid cultures were pelleted in a bench-top centrifuge and suspended in SDS-PAGE loading buffer in an appropriate volume to normalize cell density to a final OD_{600} of 10. About 10 μl of each sample was then separated on a 4–12% SDS-PAGE (Invitrogen). For quantification of heterologous protein expression, 30 μg of OMVs were loaded onto 4–12% SDS-polyacrylamide gels along with increasing concentrations of the corresponding purified recombinant protein used as standard.

The gels were then transferred onto nitrocellulose filters by standard methods (16). The filters were blocked overnight at 4°C by agitation in blocking solution (10% skimmed milk and 0.05% Tween in PBS), followed by incubation for 90 minutes at 37°C with a 1:1,000 dilution of the required immune sera in 3% skimmed milk and 0.05% Tween in PBS. Anti-Bla and anti-Mbp antibodies were obtained from Abcam, while antibodies against Slo, SpyCEP, Spy_0269 and SAM_1372 were produced by immunizing mice with 3 doses of recombinant proteins (10 μg/dose) formulated in Alum. After 3 washing steps in PBS-Tween, the filters were incubated in a 1:2,000 dilution of peroxidase-conjugated anti-mouse immunoglobulin (Dako) in 3% skimmed milk and 0.05% Tween in PBS for 1 hour, and after 3 washing steps, the resulting signal was detected using the Super Signal West Pico chemo-luminescent substrate (Pierce).

Laser scattering analysis

The size distribution profile of OMVs was determined by dynamic light scattering based on laser diffraction method employing Malvern Zetasizer (Version 6.0, Malvern, UK). The OMV diameter was determined after dilution in PBS (1/100) and by measuring the back scattering intensity (175°) at 25°C. Three measurements (15 experimental runs per measurement) were averaged to determine the vesicles size.

Lipopolysaccharide quantification

Lipopolysaccharide (LPS) content was determined using the limulus amoebocyte lysate (LAL) assay (Thermo Scientific). Briefly, OMVs were diluted in pyrogen-free water and 50 μl were combined with 50 μl LAL reagent and incubated at 37°C for 10 minutes prior to addition of a chromogenic substrate. Absorbance was then measured at 405 nm. LPS concentration was estimated from a standard curve prepared with *E. coli* endotoxin standard.

Proteinase K protection assay

To confirm that heterologous proteins were expressed in the lumen of OMVs, proteinase K (Fermentas) was added to 15 μg of intact or solubilized (in 1% SDS) OMVs at a final concentration of 100 μg/ml, and the mixture was then incubated at 37°C for 10 minutes. After proteinase K deactivation with 10 mM phenylmethylsulfonyl fluoride (PMSF; Sigma Aldrich), samples were loaded onto a 4–12% polyacrylamide gel and Western blot analysis was performed as described above.

Functional assays

To measure SpyCEP hydrolytic activity on IL-8, OMVs expressing SpyCEP were permeabilized with 1% Triton X-100 at room temperature for 20 minutes and subsequently incubated at different concentrations with human IL-8 (50 μg/ml; Peprotech) at 37°C for 2 hours. Hydrolysis of IL-8 was followed by SDS-PAGE (18%).

Slo haemolytic activity was tested by incubating OMVs expressing the wild type form of the toxin with sheep blood erythrocytes as follows. About 50 μl of serial dilutions (in PBS + 0.5% BSA) of OMVs were dispensed into 96-well plates with U-shaped bottom. Erythrocytes were collected by centrifugation (3,000 × g for 5 minutes) from 1 ml of sheep blood, washed 3 times in PBS and finally suspended in 5 ml of PBS. About 50 μl of this suspension were added to each well and incubated at 37°C for 30 minutes. As positive control, erythrocytes were added to wells containing 50 μl of water (under these ipotonic conditions erythrocytes completely lyse) while, as negative control, blood cells were incubated with permeabilized OMVs prepared from BL21(DE3) *ΔompA* strain carrying pETOmpA vector plasmid (from now on defined as "empty OMVs" to indicate that they do not carry any heterologous antigen). Plates were then centrifuged for 5 minutes at 1,000 × g, the supernatants were transferred to 96-well flat-bottomed plates and absorbance was measured at 540 nm.

OMVs expressing β-lactamase (Bla) were incubated with the chromogenic substrate nitrocefin and the Bla activity was measured as follows. OMVs were permeabilized in 1% Triton X-100 at room temperature for 20 minutes and different aliquots were incubated with nitrocefin (0.5 mg/ml; Oxoid, Thermo Scientific, Cambridge, United Kingdom) for 30 minutes at 37°C in the dark. Hydrolysis was determined at OD_{485} and Bla concentration was calculated using a standard curve obtained with purified Bla (VWR).

Mice immunization

Five-week old CD1 female mice were immunized intraperitoneally (i.p.) on days 0, 21 and 35 with 25 μg of OMVs and, unless otherwise specified, with 20 μg or 0.5 μg of recombinant proteins formulated in 2 mg/ml Alum hydroxide as adjuvant. Mouse sera were collected before the first immunization (pre-immune sera) and 2 weeks after each immunization. In challenge studies, 3 weeks after the third immunization mice were given $2–3 \times 10^6$ colony forming unit (CFU) of M1 3348 GAS strain, via i.p. Mice survival was monitored daily for a 7-day period. All procedures were approved by the National Health Institution and Novartis Vaccines Animal Care and Ethical Committee and for humanitarian 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 25/1/99).

ELISA

Ninety six-well Maxisorp plates (Nunc, Thermo Fisher Scientific) were coated with 3 μg/ml of purified recombinant antigens in PBS. Plates were incubated for 2 hours at room temperature, then washed 3 times with TPBS (0.05% Tween 20 in PBS, pH 7.4) and blocked with

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250 µl/well of 2% BSA (Sigma–Aldrich) for 1 hour at room temperature. Each incubation step was followed by triple TPBS wash. Serum samples were initially diluted 1:100,000 in 2% BSA in TPBS, transferred to coated-blocked plates (200 µl) and serially 2-fold diluted followed by 2 hours incubation at 37°C. Then 100 µl/well of 1:2,000 diluted alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma-Aldrich) or IgM, or IgG1, or IgG2A (Southern Biotechnologies) were added and left for 2 hours at 30°C. Bound alkaline phosphatase was visualized by adding 100 µl/well of 3 mg/lL para-nitrophenyl-phosphate disodium hexahydrate (Sigma–Aldrich) in 1 M diethanolamine buffer (pH 9.8). After 10 minutes at room temperature, plates were analyzed at 405 nm in a microplate spectrophotometer and antibody titers (expressed as EU/ml) were calculated using a reference calibration curve.

Opsonophagocytosis assay

The opsonophagocytosis assay (OPA) was performed using *Group B Streptococcus* (GBS) strain CJB111 as target bacteria and HL-60 cell line (ATCC; CCL-240) differentiated into granulocyte-like cells by adding 100 mM N, N Dimethylformamide (Sigma) to the growth medium for 4 days. Mid-exponential bacteria were incubated at 37°C for 1 hour in the presence of phagocytic cells, 10% baby rabbit complement (Cederlane) and heat-inactivated mouse antisera. Negative controls consisted of reactions either with pre-immune sera, or without HL-60, or with heat-inactivated complement. For each serum sample, 6 serial dilutions (1:2) were tested. The reaction plates were incubated for 1 hour at 37°C and 300 rpm (Eppendorf Thermomixer) and the number of CFUs at the time zero (T0) and after 1 hour (T60) were compared. Bacterial killing was calculated as follows: killing (%) = [(mean CFU at T0 - mean CFU at T60)/mean CFU at T0]*100. OPKA titers were expressed as the reciprocal serum dilution leading to 50% killing of bacteria.

Results

Heterologous antigens expressed in the E. coli periplasm are incorporated into OMVs

To test whether the delivery of antigens into the OMV lumen by periplasmic expression is broadly applicable, we selected 3 proteins from GAS (the cell wall–anchored protein SpyCEP (22), the secreted toxin Streptolysin O (23) and the putative surface exclusion protein Spy0269 (19)), 1 protein from GBS (the cell-wall anchored pilus subunit SAM_1372 (24)) and 1 from *E. coli* (the periplasmic TEM1 β-lactamase, a protein shown to be incorporated into the OMVs of *Pseudomonas aeruginosa* and *Moraxella catarrhalis* (25,26)) (Table II).

Antigen selection was based on 3 criteria: their belonging to different cellular compartments, their having a measurable functional activity and their capacity to induce antibody-mediated protective immune responses in animal models. The first criterion addresses whether specific categories of proteins are more or less prone to reach the periplasm and be delivered to the OMVs. The second criterion was selected to exploit functional activity as a surrogate for antigen folding. Finally, the third criterion allows evaluating the quantity and quality of antibody responses elicited by the recombinant antigens once incorporated inside the OMVs.

All genes were cloned into the pETOmpA plasmid, a derivative of pET21b carrying the coding sequence of the *E. coli* OmpA signal peptide downstream from the T7 inducible promoter, thus allowing N-terminal fusion of the *E. coli* periplasmic secretion sequence to the protein of interest. Because of the presence of the OmpA leader sequence, all proteins (except TEM1 beta-lactamase) were cloned without their natural secretory signal. Furthermore, the C-terminal cell-wall anchoring domains of SpyCEP and SAM_1372 (amino acids 1,614–1,647 and amino acids 620–674, respectively) were also eliminated. In summary, 5 plasmids were generated, pET21_bla, pET-21_slo, pET21_spycep, pET21_spy0269 and pET21_sam1372, which were used to transform the OMV overproducer strain *E. coli* BL21(DE3) *ΔompA*. The derived strains were grown to mid-log phase and antigen expression was induced for 2 hours by addition of 1 mM IPTG. Figure 1a shows SDS-PAGE analysis of total lysates of cultures obtained with or without IPTG induction. As shown in the figure, all antigens appeared to be expressed after induction.

We next asked the question whether the proteins reached the OMV compartment. To this aim, after 2 hours' induction with IPTG, OMVs were purified from bacterial culture supernatants as described in Materials

Table II. Antigens used for expression in OMVs

Antigen	Pathogen	Functional assay	Protective immune responses
SpyCEP	*S. pyogenes*	IL-8 cleavage	Neutralizing Ab and mouse challenge
Streptolysin O	*S. pyogenes*	Haemolysis	Neutralizing Ab and mouse challenge
Spy0269	*S. pyogenes*	Not available	Mouse challenge
SAM_1372	*S. agalactiae*	Not available	Opsonophagocytic activity
R-TEM b-lac	*E. coli*	Hydrolysis of Nitrocefin	Not available

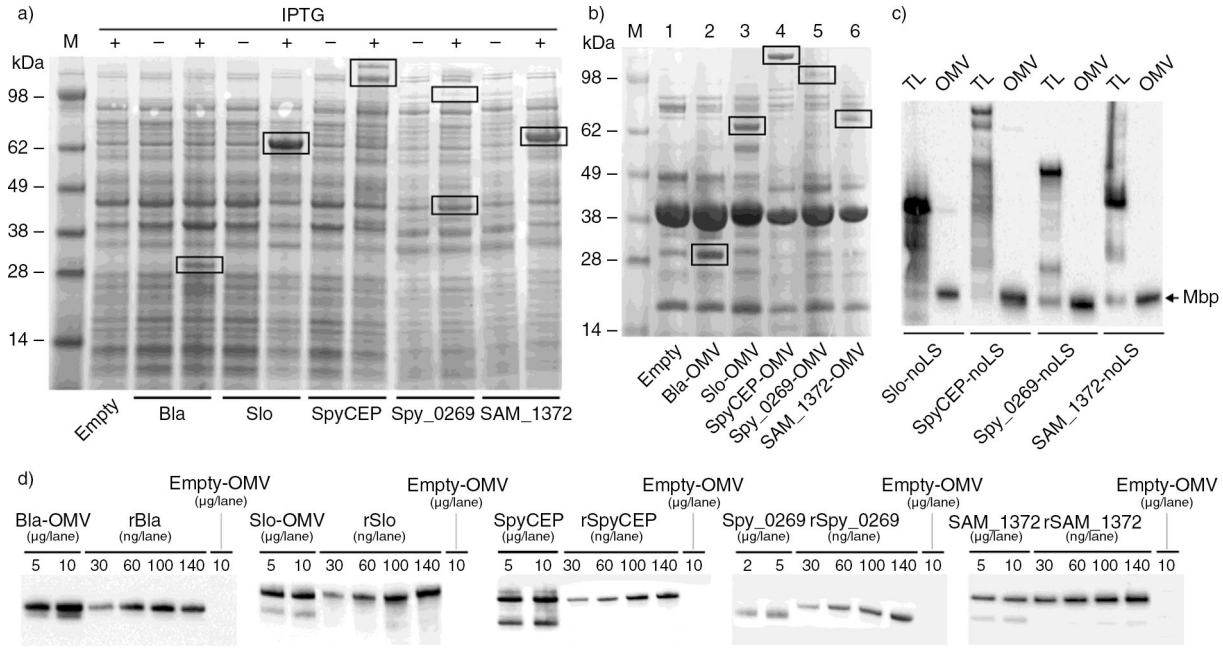

Fig. 1. Expression of heterologous antigens in OMVs – (a) SDS-PAGE analysis of *E. coli* BL21Δ*ompA* total cell extracts expressing selected antigens. Bacteria were grown to mid-log phase and recombinant protein expression induced (+) or not induced (−) with IPTG; 1 ml aliquots were collected by centrifugation, suspended in 100 µl of SDS-PAGE loading buffer and 25 µl aliquots were loaded in each lane. Bands corresponding to recombinant antigens are boxed. (b) SDS-PAGE analysis of OMV from *E. coli* BL21Δ*ompA* expressing selected antigens. OMVs were purified by ultracentrifugation of 200 ml bacterial culture supernatants and pellets were suspended in 200 µl of PBS. Aliquots corresponding to 30 µg of total OMV proteins were added with SDS-PAGE loading buffer and loaded to each lane. Bands corresponding to recombinant antigens are boxed. (c) Western Blot analysis of total cell extracts and OMVs from *E. coli* BL21Δ*ompA* expressing the selected antigens in their cytosol. Bacteria were grown to mid-log phase and protein expression was induced with IPTG. OMVs were purified from the culture supernatants by ultracentrifugation; antigen expression was assessed in total cell lysates (TL) and in the purified OMV fraction using antigen-specific polyclonal antibodies. Polyclonal antibodies against the periplasmic Maltose binding protein (Mbp) were used as a control for OMV quantitation and integrity. (d) Estimation of the amount of heterologous proteins incorporated into OMVs. Different quantities of purified recombinant proteins and OMVs expressing the protein of interest were analyzed by Western Blot. From the comparison of band intensities, the following percentages (µg of antigen expressed in OMVs/µg of total proteins $\times 100$) were estimated: Bla: 5%; Slo: 5%; SpyCEP: 10%; Spy0269: 0.5–1%; SAM_1372: 0.5%.

and Methods and analyzed by SDS-PAGE. As shown in Fig. 1b, all proteins were found in the OMVs at levels ranging from 0.5% to approximately 10% of total OMV proteins, as judged by Western Blot protein quantitation analysis in which different amounts of purified recombinant antigens were used as standard controls (Fig. 1d). The proteins that showed the lowest level of expression in OMVs were SAM_1372 and Spy0269, both representing approximately 0.5% of total OMV proteins. Bla, Slo and SpyCEP represented between 5 and 10% of total OMV proteins. Antigen fusion to the OmpA leader sequence was indispensable for protein delivery to the OMVs. In fact, when the proteins were not fused to the OmpA leader sequence (see Material and Methods), all antigens accumulated in the cytoplasm and were not found in OMVs, as judged by Western Blot analysis (Fig. 1c).

In order to assess the size and the homogeneity of the purified vesicles, they were analyzed by light scattering (see Materials and Methods). As shown in Fig. 2, vesicle size ranged from 20 to 300 nm, and the majority of vesicles had a diameter of 80–140 nm. Vesicle dimension was not affected by the presence of different recombinant antigens, and size distribution of vesicles stored at -20°C did not vary over a period of 1 month (data not shown).

Although recombinant antigens should reside in the internal space of the vesicles, the unexpected evidence that HtrA, a *Chlamydia trachomatis* protein we recently expressed in OMVs (27), was at least partially exposed on the OMV surface, prompted us to further investigate the location of the 5 antigens in OMVs. To this aim, purified OMVs were treated with proteinase K either in the presence or in the absence of 1% SDS. As shown in Fig. 3, recombinant antigens were digested by the protease only in the presence of detergent, indicating that they became protease sensitive only upon disruption of the OMV membrane.

Taken together these results demonstrate that antigen fusion to a sec-dependent leader sequence for secretion is sufficient to guarantee antigen targeting to the internal

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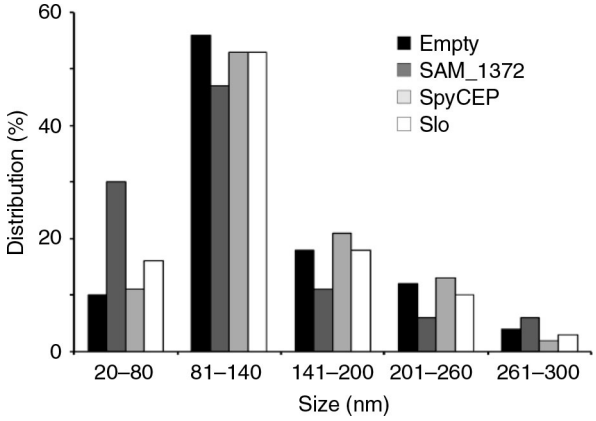

Fig. 2. Analysis of OMV dimension by light scattering – The size of OMVs released from *E. coli* BL21Δ*ompA* strain expressing SAM_1372, SpyCEP, Slo, or no antigens was determined by dynamic light scattering, measuring the back scattering intensity (175°) at 25°C. The graph represents the average size distribution from 3 independent measurements.

space of OMVs, suggesting that no major selection processes take place to determine which periplasmic protein should or should not be taken up by OMVs during vesiculation.

Heterologous antigens expressed in the lumen of OMVs preserve their native conformation

We next investigated whether recombinant antigens expressed in the lumen of OMVs had a 3D structure similar to their native conformation. This is an important prerequisite to guarantee that, when OMVs are used in immunization, the antigens can elicit functional antibody responses. As surrogate of antigen folding, we measured the functional activity of Slo, SpyCEP and R-TEM β-lactamase. In particular, Slo haemolytic activity was tested by incubating increasing concentrations of Slo-OMVs with sheep blood erythrocytes. As shown in Fig. 4a, 100% haemolysis was observed when 50 μg of OMVs (total protein content) were added to the reaction mixture. Considering that this amount of OMVs corresponds to approximately 2 μg of Slo, the kinetics of haemolysis was in line with that previously reported using purified recombinant Slo (20). The enzymatic activity of OMVs-containing SpyCEP was measured by incubating human IL-8 (50 μg/ml) with Triton-permeabilized OMVs and following the hydrolysis of IL-8 by SDS-PAGE. As shown in Fig. 4b (lane 3), IL-8 was completely cleaved after 30 minutes' incubation with 4.5 μg of OMVs (corresponding to approximately 450 ng of SpyCEP). As in the case of Slo, the specific activity of OMV-internalized SpyCEP appeared to be similar to recombinant SpyCEP purified from the *E. coli* cytoplasm (28). Finally, to determine the β-lactamase activity in OMVs, the hydrolysis of the chromogenic substrate nitrocefin was investigated after incubation with vesicles (Fig. 4c). Again, estimating that β-lactamase corresponded to approximately 5% of total OMV proteins, the specific activity of the enzyme in OMVs was similar to the one of purified R-TEM β-lactamase. Interestingly, assuming that the specific activity of R-TEM in OMVs is comparable to the specific activity of the purified enzyme, we estimated that 23 ng of β-lactamase were present in 1 μg of OMV, corresponding to 2.5% of total proteins. This number is in line with our estimation of enzyme concentration based on Western Blot analysis.

Taken together these results indicate that heterologous antigens expressed in the lumen of *E. coli* OMVs preserved their native conformation and biological activity.

Heterologous antigens expressed in the lumen of OMVs are immunogenic

Having demonstrated that the conformation of antigens delivered to the lumen of OMVs is similar to their native structure, at least as judged by functional activity, we next investigated whether, despite the fact that they are localized inside the vesicles, those antigens are capable of inducing specific antibody responses. To this aim, mice were i.p. immunized 3 times at 2-week intervals with 25 μg of OMVs and antigen-specific antibody titers were measured collecting blood samples 2 weeks after the last dose. For these experiments, the immunogenicity of Slo_{dm}-OMV, SpyCEP-OMV and SAM_1372-OMVs was investigated. Slo_{dm} is a mutated form of Slo, carrying 2 point mutations which inactivate the enzymatic activity of the antigen without affecting its immunogenic properties (20). As shown in Fig. 5, the 3 recombinant OMVs induced antigen-specific antibody responses which, in the case of Slo_{dm}-OMVs and SpyCEP-OMVs, were in the

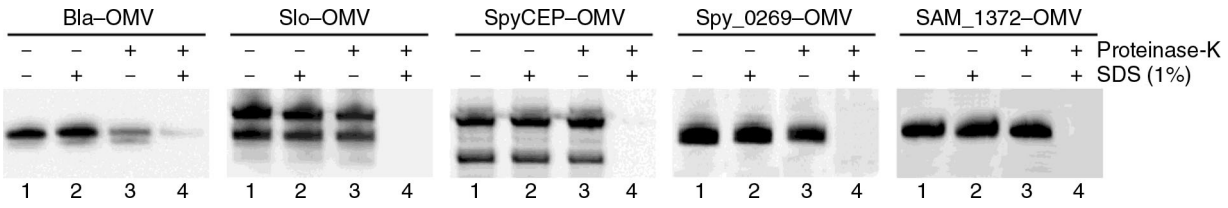

Fig. 3. Analysis of antigen localization in OMVs. – Purified OMVs expressing heterologous proteins were incubated with and without proteinase K in the presence or absence of 1% SDS. Samples were then subjected to SDS-PAGE and Western Blot analysis using antigen-specific polyclonal antibodies. The data indicate that all recombinant antigens are localized in the lumen of OMVs.

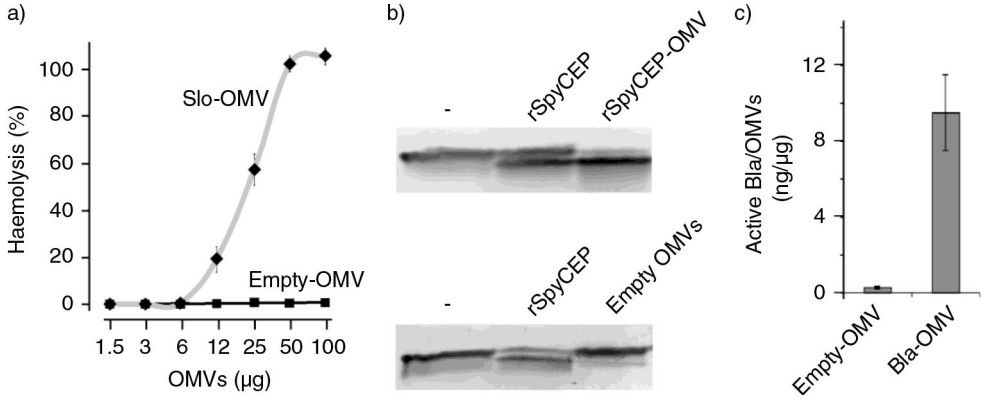

Fig. 4. Analysis of the functional activities of protein antigens expressed in the lumen of OMVs – (a) Haemolytic activity of Slo-OMVs. Aliquots of purified OMVs were incubated with sheep erythrocytes and absorbance at OD_{540} was measured from the supernatant of each sample. Haemolytic activity is expressed as percentage of OD values over the OD values obtained incubating erythrocytes with water (100% haemolysis). (b) Proteolytic activity of SpyCEP-OMVs. 10 µg of SpyCEP-OMVs and "empty" OMVs were permeabilized with Triton X-100 and subsequently incubated with IL-8. IL-8 hydrolysis was followed by Western Blot analysis using anti-IL-8 specific antibodies. (c) β-lactamase activity of Bla-OMVs – Bla-OMVs and "empty" OMVs were permeabilized with Triton X-100, and β-lactamase activity was monitored by following the hydrolysis of nitrocefin at OD_{485}. β-lactamase concentration in OMVs (ng of active enzyme/µg OMVs) was estimated using a standard curve obtained with purified β-lactamase as determined by spectrophotometry.

same range as the titers elicited by 20 µg doses of the corresponding recombinant antigens formulated in Alum. When the 20 µg doses of Slo_{dm} and SpyCEP were mixed together with 25 µg of "empty OMVs," an appreciable increase (approximately 5-fold) in antibody titers was observed highlighting the strong adjuvanticity of the vesicles. As far as SAM_1372-OMVs are concerned, they induced SAM_1372 antibody titers that were approximately 10-fold lower than those elicited by

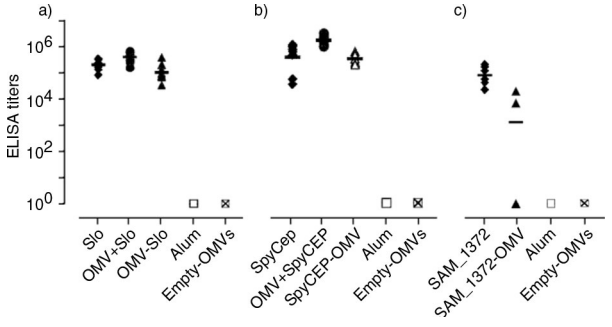

Fig. 5. IgG titers elicited in mice immunized with Slo-OMVs (a), SpyCEP-OMVs (b) and SAM_1372-OMVs (c). Groups of 6 CD1 female mice were immunized i.p. 3 times at 2-week intervals with the following immunogens formulated in Alum: 25 µg OMVs expressing the recombinant antigens (Slo-OMVs, SpyCEP-OMVs, SAM_1372-OMVs), 20 µg of purified recombinant antigen (Slo, SpyCEP, SAM_1372), 25 µg empty OMVs combined with 20 µg of purified recombinant antigen (OMVs + Slo, OMVs + SpyCEP), adjuvant alone (Alum). Sera were collected 2 weeks after the third immunization and IgG titers were analyzed by ELISA, using plates coated with the corresponding recombinant antigen (1 µg/well). Each symbol represents the ELISA value of an individual mouse. Horizontal bars represent the geometric mean values within each group.

vaccination with Alum-formulated recombinant SAM_1372 (10 µg/dose). This might reflect the relatively poor expression of this antigen in OMVs, estimated to be in the range of 0.5% of total OMV proteins (Fig. 1d). No specific antibody titers were detected when mice were immunized with empty OMVs or Alum alone as controls.

One interesting property of OMVs is their built-in adjuvanticity and their ability to skew immune responses towards a Th1/Th17 profile (4,9–11,29). Since the measurement of IgG1/IgG2A ratio is routinely used in mice as a surrogate to establish the type of immune response induced (high and low IgG1/IgG2A ratios indicating a TH2-type and a TH1-type of response, respectively (30,31)), we analyzed the isotype of antigen-specific antibodies upon immunization of mice with recombinant OMVs carrying Slo and SpyCEP. The isotype profiles were compared to the ones obtained immunizing animals with Alum-formulated recombinant proteins. As shown in Fig. 6, while purified proteins induced antibodies predominantly belonging to IgG1 isotype, IgG2A was the predominant isotype of antigen-specific antibodies obtained after immunization with recombinant OMVs. As expected for immunization regimes involving 3 doses, no antigen-specific IgM antibodies were detectable in sera from mice immunized with either purified antigens or recombinant OMVs.

Overall, these data seem to confirm the capacity of OMVs to direct the immune response towards a TH1 profile. However, to establish the precise nature of immunity induced, a direct measurement of cytokines and the characterization of T cell subpopulations will be required. In particular, it will be interesting to analyze

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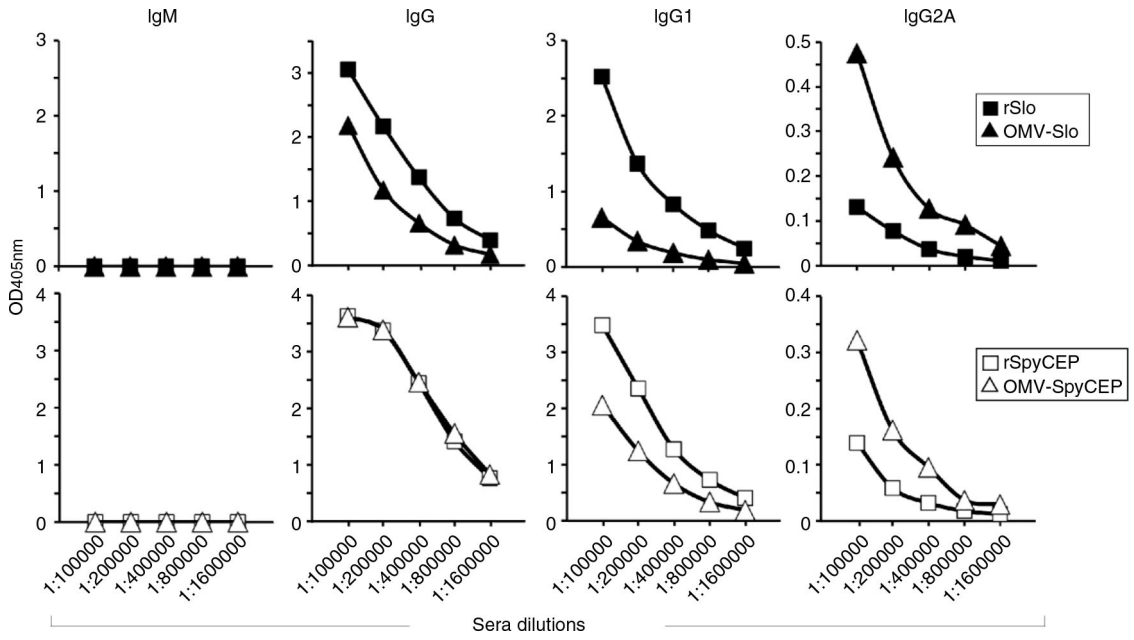

Fig. 6. Isotype analysis of antibodies elicited in mice immunized with Slo-OMVs and SpyCEP-OMVs – Sera from mice immunized i.p. 3 times with 25 μg OMVs expressing Slo or SpyCEP antigen (triangles) and with 20 μg of purified recombinant Slo or SpyCEP (squares) were serially diluted and Ig isotypes were analyzed by ELISA using plates coated with the corresponding recombinant antigens. Alkaline phosphatase-conjugated goat anti-mouse IgG, IgM, IgG1 and IgG2A were used as secondary antibodies. Graphs represent the OD values obtained for each serum dilution.

the presence of $CD4_{TH17}$ cell subset, as recently elegantly determined by Kim and co-workers (29).

Immunization with OMVs carrying recombinant antigens in their lumen induces antigen-specific functional antibodies

Having demonstrated that immunization with recombinant vesicles elicited antigen-specific antibodies, we next investigated whether these antibodies displayed functional activity. Since recombinant Slo, SpyCEP and SAM_1372 induce antibodies which neutralize Slo haemolytic activity, inhibit IL-8 cleavage and promote complement-dependent opsonophagocytosis, respectively (19,24), we analyzed whether sera from mice immunized with recombinant OMVs had similar functions. To this aim, recombinant SpyCEP was incubated with different dilutions of sera from mice immunized with either 20 μg of recombinant SpyCEP, or with 20 μg of recombinant SpyCEP+25 μg of "empty" OMVs or with 25 μg of SpyCEP-OMVs, and purified IL-8 was subsequently added to each mixture. As shown in Fig. 7a panel (i), sera from mice receiving 3 doses of each vaccine similarly inhibited IL-8 cleavage in a dose-dependent manner. Interestingly, when sera from mice immunized with 2 doses of vaccines were tested, mice given SpyCEP-OMVs had higher titers of functional antibodies with respect to mice that received either recombinant SpyCEP or recombinant SpyCEP+"empty" OMVs (Fig. 7a panel (ii)). Similarly, purified recombinant Slo was pre-incubated with different dilutions of sera from mice immunized with 3 doses of OMV-Slo and subsequently goat erythrocytes were added to the mixtures. As shown in Fig. 7b, haemolysis was effectively inhibited at serum dilutions up to 1:250 and inhibition levels were at least equal to those obtained with sera from mice immunized with 0.5 μg of recombinant Slo, a dose close to that estimated for 25 μg of OMVs (approx. 1 μg of Slo). Finally, OPK titers of sera from mice immunized with 3 doses of SAM_1372-OMVs were determined and compared to those from animals immunized with 10 μg/dose of recombinant SAM_1372. While the antibody titers induced by SAM_1372-OMVs were relatively low with respect to those induced by the purified recombinant antigen (Fig. 5), engineered vesicles elicited 5-fold higher bactericidal antibody titers compared to the recombinant antigen (Fig. 7c).

In conclusion, not only OMVs carrying heterologous antigens in their lumen induced antigen-specific antibodies but also such antibodies had potent functional activities which could exceed, in terms of either time of elicitation or potency of the immune response, those obtained with the corresponding recombinant antigens

Immunization with Slo-OMVs and SpyCEP-OMVs protects mice against S. pyogenes infection

We have previously shown that immunization with SpyCEP and Slo protected mice from GAS lethal challenge (19). Therefore, we tested whether, in addition

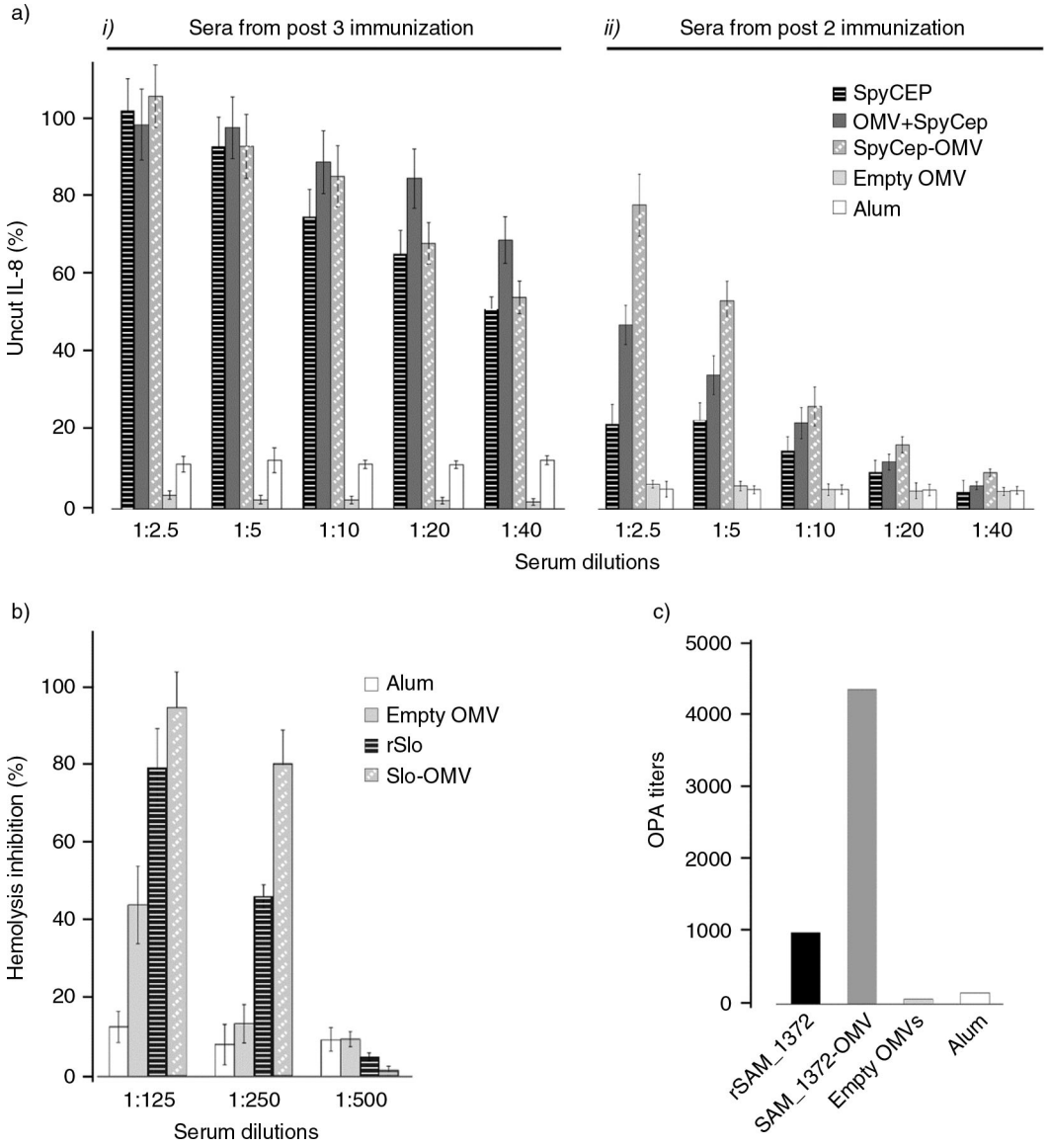

Fig. 7. Analysis of functional antibodies induced by immunization with Slo-OMVs, SpyCEP-OMVs and SAM_1372-OMVs – (a) Inhibition of IL-8 hydrolysis by sera from mice immunized with SpyCEP-OMVs. IL-8 hydrolysis was followed as described in Materials and Methods in the presence of serial dilutions of sera from mice immunized with 3 (i) or 2 (ii) doses of the different immunogens. Graphs show the percent of residual IL-8 measured after hydrolysis. Sera from each group were pooled and bars represent the geometric mean values from 3 independent experiments. (b) Inhibition of haemolytic activity of sera from mice immunized with Slo-OMVs. Haemolysis of sheep erythrocytes was measured in the presence of increasing dilutions of sera from mice immunized with the different immunogens. Haemolytic activity is expressed as percentage of OD values over the OD values obtained incubating erythrocytes with water (100% haemolysis). Sera from each group were pooled and bars represent the geometric mean values of 3 independent experiments. (c) Opsonophagocytosis activity of sera from mice immunized with SAM_1372-OMVs. OPK was carried out as described in Materials and Methods using the pools of sera from mice immunized as described in Fig. 5. Titers are expressed as serum dilutions resulting in 50% bacterial killing.

to eliciting functional antibodies, vaccination of mice with vesicles carrying Slo and SpyCEP could induce protective immunity. To this aim, mice were i.p. immunized with 3 doses of either Slo-OMVs or SpyCEP-OMVs (25 μg/dose) and subsequently i.p challenged with a lethal dose of M1-3348 GAS strain. In this sepsis model, death mainly occurs during the first 2 days, and survival is reported after day 7. As shown in Fig. 8, both OMVs induced robust protection against bacterial challenge and protection levels were at least equal to those observed by immunization with 20 μg/dose of recombinant proteins.

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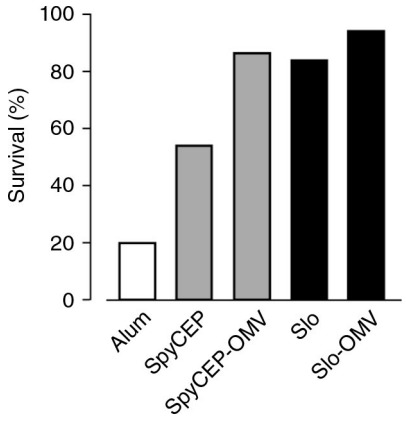

Fig. 8. In vivo protective activity of Slo-OMVs and SpyCEP-OMVs – Groups of 16 mice were immunized with 3 doses of Alum-formulated OMVs carrying heterologous antigens (25 μg total proteins), Alum-formulated purified recombinant proteins (20 μg) or Alum alone. After 2 weeks, mice were challenged intraperitoneally with a LD_{80} dose of M1 3348 GAS (2×10^6 CFUs) and survival was followed over a period of 7 days.

Discussion

Kesty and Kuehn (12) have been the first to demonstrate that the protein content of bacterial OMVs can be manipulated by targeting heterologous proteins to the outer membrane and/or to the periplasmic space of Gram-negative bacteria. In light of the potent adjuvant property of OMVs, which stimulate cell-mediated and humoral immune responses when given both systemically and mucosally, the pivotal observation by Kesty and Kuehn pointed towards OMVs as a potentially ideal platform to develop highly effective, easy to produce multi-valent vaccines.

However, for OMVs to become broadly applicable, a number of questions remain to be addressed. Among them, it is important to demonstrate the robustness of the genetic engineering strategies for antigen delivery to the OMVs and to identify the most appropriate strategy for inducing the desired immune responses (cell-mediated immunity and/or antibody responses).

Theoretically, the best way to elicit antigen-specific antibody responses using engineered OMVs is to localize recombinant antigens on the vesicle surface, thus providing the best antigen accessibility for B cell receptor binding. However, targeting proteins to bacterial outer membrane can be challenging and usually requires the construction of chimera between antigen and endogenous outer membrane proteins (13–15). The success of such fusion strategies usually inversely correlates with the size of the passenger protein. An easier approach to deliver recombinant antigens to OMVs would be to express them in the bacterial periplasm. Since periplasmic proteins get trapped in the OMV lumen during vesicle formation (2), the addition of a sec-dependent leader sequence to the N-termini of antigens should be sufficient to guarantee their incorporation into OMVs, provided that no selective periplasmic protein inclusion/exclusion occurs during vesiculation. However, luminal localization of antigens could be a sub-optimal way to induce an antigen-specific antibody response, unless membrane integrity is partially lost at the site of OMVs injection with concomitant exposure of their cargo to B cells.

In this work, we focused our attention on antigen delivery to the OMVs with the aim of establishing in a systematic manner (a) how efficiently heterologous protective antigens can be incorporated into the lumen of the vesicles and (b) to what extent antigens localized in the OMV lumen can elicit functional, antigen-specific antibody responses.

As far as the first question is concerned, our data demonstrate that all antigens selected in this study could be delivered to the *E. coli* vesicles by hooking them to the *E. coli* OmpA leader sequence for secretion. In addition, 3 heterologous antigens were previously reported to reach the OMVs via periplasmic expression (12,32,33), and we have recently achieved OMV delivery of 5 additional protective antigens from gram-positive pathogens (unpublished). In light of the foregoing results, we conclude that most bacterial antigens eliciting antibody-mediated protection can be successfully incorporated into *E. coli* OMVs via the sec-dependent pathway of periplasmic expression. This conclusion also implies that, differently from that proposed for at least some outer membrane proteins (34), vesiculation does not use a selective process for periplasmic protein incorporation in OMVs.

The high rate of success in targeting recombinant antigens to the lumen of OMVs (in essence, all heterologous antigens we have tested so far were successfully incorporated into bacterial vesicles) could appear surprising. Indeed, it has been demonstrated that binding of the leader peptide to the ATP motor protein SecA is necessary but not sufficient for protein secretion through the sec-dependent pathway and that concomitant SecA binding by conserved motifs in mature proteins is also required (35,36). This is why many cytoplasmic proteins that do not carry SecA-binding motifs cannot reach the periplasm even when fused to leader peptides. To explain the efficiency of our OMV protein delivery, it has to be pointed out that we focused our attention on heterologous bacterial antigens inducing protective antibody responses. Many of these antigens, such as those selected for this study, belong to the category of proteins that are naturally secreted or surface-exposed by the sec-dependent pathway. Therefore, these proteins are the ideal candidates to be expressed in the *E. coli* periplasm as long as an *E. coli*-compatible leader sequence is fused to their N-termini. It is in fact known that leader

sequences can be species-specific, and in particular leader sequences of Gram-negative bacteria differ from those of gram-positive species (37). In this study, we have used the OmpA leader sequence for antigen secretion, but other leader sequences are expected to work as well and indeed to achieve optimal secretion levels we are currently testing each antigen with a battery of different *E. coli* leader sequences.

Antigens inducing antibody-mediated protective activity include members of the integral membrane family and proteins that are secreted via alternative secretion pathways. Although not tested in this work, such proteins are likely to be less prone to OMV delivery through fusion to leader sequences, and other strategies might be required to achieve their expression in OMVs.

The second question we addressed in this study is whether immunization with OMVs carrying antigens in their lumen can induce antigen-specific antibodies. Theoretically speaking, localizing antigens inside the vesicles should prevent antigen-specific B cell binding and the consequent proper stimulation of antibodies. To the best of our knowledge, only 2 groups have so far analyzed immune responses against luminal recombinant antigens (32,33). Both groups have reported the elicitation of antigen-specific antibodies, but titers were relatively low and the authors reached the conclusion that, for practical vaccine application, antigens should be localized on the OMV surface. In the case of pneumococcal PspA expression in Salmonella OMVs, the authors presented evidence that the protein was released into the growth culture supernatants in addition to being localized in the periplasm/OMV lumen. Therefore, the observed PspA-specific antibodies could have been at least partially generated by the secreted protein "contaminating" the OMV preparation.

Our data unequivocally demonstrate that recombinant antigens delivered to the lumen of OMVs do induce effective antibody responses. Indeed, antibody titers were often close to those obtained with 20 μg of recombinant proteins, corresponding in some cases to 1 log higher antigen amounts than those carried in the 25 μg of OMVs doses used for mouse immunization. Most important, antibodies were characterized by a functional activity which could be greater than the one observed with sera from animals immunized with the corresponding recombinant proteins. This is particularly evident for SAM_1372-OMVs: despite the fact that the protein induced low ELISA titers (most likely because of its poor expression in OMVs) antibodies had an excellent functional activity in terms of bacterial opsonophagocytic killing.

Elicitation of high-quality antibodies translated into good protection *in vivo*, as judged by the ability of animals immunized with SpyCEP-OMVs and Slo-OMVs to survive GAS lethal challenges. At least 2 important factors contribute to such potent functional responses. First, all antigens used in this work preserved their native conformation when expressed in the lumen of OMVs, as deducible from the fact that the antigens had fully enzymatic/toxic activity. This guaranteed the elicitation of conformational antibodies capable of binding and neutralizing the antigen toxic activities. Second, and particularly important, recombinant OMVs have the intrinsic ability to co-deliver antigen and adjuvant to the same antigen-presenting cells, thus allowing the optimal activation of antigen-specific B- and T-cells (38). The relevance of antigen/adjuvant co-delivery can be appreciated when the inhibition of SpyCEP proteolytic activity by antibodies induced by 2 injections of different SpyCEP-containing vaccine formulations was compared (Fig. 7). Sera from SpyCEP-OMVs immunized animals showed a higher neutralization activity with respect to both Alum-formulated recombinant SpyCEP and SpyCEP mixed with "empty" OMVs. While the antigen-adjuvant co-delivery concept can explain how APCs taking up SpyCEP-OMVs optimally stimulate the generation of SpyCEP-specific plasma cells and memory B cells, the presentation of a luminal protein to naïve B cells necessarily requires that the integrity of OMVs is partially compromised in order to expose the protein to B cell receptors. Based on the foregoing, we propose that OMVs are taken up at the site of injection by APCs, which present the antigen-specific peptides to T cells. At the same time, a fraction of OMVs are degraded before being phagocytosed by APCs and this allows antigen presentation to naïve B cells. Antigen-specific B cells are finally stimulated to become effector and memory B cells by interaction with antigen-specific Helper T cells generated by those APCs that have taken up intact vesicles. According to this model, the ratio between intact and degraded OMVs at the site of injection is expected to be important for the generation of a sufficiently large population of antigen-specific T- and B-cells and, ultimately, a good antibody response. The extent of vesicle degradation should be determined by a number of factors, including site of immunization, formulation and nature of OMVs. In this respect, it has to be pointed out that the strains used for vesicle production and engineering usually carry specific mutations that confer an OMV-overproducing phenotype. Many of these mutations have a direct effect on the integrity of the outer membrane or on the interaction between outer and inner membranes (39). In this study we used a BL21 *E. coli* strain carrying the *ompA* gene deletion, which results in the removal of one of the major membrane proteins in *E. coli*. It is possible that the OMVs derived from this mutant are sufficiently robust/fragile to induce the good antigen-specific antibody responses we observed in our work. Other mutations could generate vesicles with different chemical–physical

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properties and this could ultimately affect immune responses both qualitatively and quantitatively.

The reactogenicity of OMVs and the implication of such reactogenicity in OMV-based vaccine development deserve one last comment. It is well known that OMVs carry large amount of LPS. We measured the LPS content in our OMV preparations and determined an LPS:protein ratio of approximately 0.5 (50 ng of LPS/100 ng of OMV proteins). This number is consistent with that recently reported by Park and co-workers (40). Such LPS concentrations are toxic in mice and 20 μg of *E. coli* OMVs injected intraperitoneally have been shown to be sufficient to rapidly kill animals (40). This is the reason why, to decrease OMV reactogenicity, throughout this work OMV immunization was carried out in the presence of Alum, which absorbs LPS and prevents its systemic dissemination. However, for human use, OMVs must be detoxified. This can be achieved in at least 2 ways. One possibility is to remove LPS using detergent extraction. For instance, the meningococcal OMV-based vaccines available on the market are produced by deoxycholate treatment of whole bacteria. A second approach is to use OMVs deriving from mutant strains that carry detoxified variants of LPS. Mutations that produce detoxified OMVs have been described for *Neisseria meningitidis* (41) and *E. coli* (42). Interestingly, a synthetic biology methodology has recently been reported that allows modulating the innate immune response of LPS by engineering the LPS biosynthetic pathway of *E. coli* (43). We are pursuing similar approaches to design OMVs with TLR4 agonist activities tailored on the basis of specific vaccine needs.

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Conflict of interest and funding

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