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Salmonella Paratyphi A Outer Membrane Vesicles Displaying Vi Polysaccharide as a Multivalent Vaccine against Enteric Fever

G. Gasperini,[a] R. Alfini,[a] V. Arato,[a] F. Mancini,[a] M. G. Aruta,[a] P. Kanvatirth,[b] D. Pickard,[b] F. Necchi,[a] A. Saul,[a] O. Rossi,[a] F. Micoli,[a] P. Mastroeni[b]

[a]GSK Vaccines institute for Global Health, Siena, Italy
[b]University of Cambridge, Cambridge, United Kingdom

F. Micoli and P. Mastroeni contributed equally to the work. P. Mastroeni is the lead applicant on the Grant that funded this work.

ABSTRACT Typhoid and paratyphoid fevers have a high incidence worldwide and coexist in many geographical areas, especially in low-middle-income countries (LMIC) in South and Southeast Asia. There is extensive consensus on the urgent need for better and affordable vaccines against systemic *Salmonella* infections. Generalized modules for membrane antigens (GMMA), outer membrane exosomes shed by *Salmonella* bacteria genetically manipulated to increase blebbing, resemble the bacterial surface where protective antigens are displayed in their native environment. Here, we engineered *S.* Paratyphi A using the pDC5-*viaB* plasmid to generate GMMA displaying the heterologous *S.* Typhi Vi antigen together with the homologous O:2 O antigen. The presence of both Vi and O:2 was confirmed by flow cytometry on bacterial cells, and their amount was quantified on the resulting vesicles through a panel of analytical methods. When tested in mice, such GMMA induced a strong antibody response against both Vi and O:2, and these antibodies were functional in a serum bactericidal assay. Our approach yielded a bivalent vaccine candidate able to induce immune responses against different *Salmonella* serovars, which could benefit LMIC residents and travelers.

KEYWORDS OMV, GMMA, vaccine, Vi, *Salmonella*, enteric fever, typhoid fever, *S.* Paratyphi, *S.* Typhi

Salmonella enterica serovars Typhi (*S.* Typhi) and Paratyphi (*S.* Paratyphi) subtypes A, B, and C cause enteric fevers, a major global health concern. *S.* Typhi (typhoid fever) causes an estimated 14.9 million cases annually and 116,800 associated deaths with postantimicrobial relapses in up to 10% of patients and chronic carriage in up to 6% of treated individuals (1); *S.* Paratyphi causes an estimated 3 million paratyphoid fever cases and approximately 19,000 deaths annually (1). These diseases coexist in many geographical areas, especially in low-middle-income countries (LMIC). *S.* Typhi incidence is high in South and Southeast Asia as well as Africa; an increasing incidence of *S.* Paratyphi A has been reported over the past 2 decades in different parts of Asia, including Nepal (2), Cambodia (3), and China (4).

Current treatments for *S. enterica* infections are hampered by the emergence of multidrug-resistant strains (5–8).

Vaccines are a powerful tool against systemic *Salmonella* infections. Several vaccines have been licensed for the prevention of typhoid fever; however, no vaccine is available against paratyphoid fever (7, 9). The licensed *S.* Typhi Ty21a live typhoid vaccine is safe but gives moderate protection after multiple dosing (10). Typhoid conjugate vaccines (TCV), in which *S.* Typhi Vi capsular polysaccharide is covalently linked to carrier proteins, offer several potential advantages over earlier generations of vaccines, especially enhanced immunogenicity and the ability to induce immune responses in

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Address correspondence to F. Micoli, francesca.x.micoli@gsk.com, or P. Mastroeni, pm274@cam.ac.uk.

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infants (11). Similar strategies are currently being investigated for the development of a paratyphoid vaccine, including live attenuated (LAV) and nonliving vaccines (12). Some recent LAV candidates (e.g., the *aroC-ssaV* M01ZH09 and WT05 mutants) showed insufficient immunogenicity (13). LAV can cause lethal infections in immunocompromised hosts (14–17); therefore, conjugate vaccines would represent a much safer alternative. Recently, an O-antigen (OAg) glycoconjugate based on the immunodominant O:2 factor was proposed as a vaccine against *S*. Paratyphi A infections (18, 19).

A bivalent formulation would probably be the wiser choice to induce antibody responses that can potentially protect against both *S*. Typhi and *S*. Paratyphi A. Moreover, such a vaccine combination would increase the commercial attractiveness of the *S*. Paratyphi A component, especially considering the disproportionate incidence of the two diseases.

Recently, general modules for membrane antigens (GMMA) were proposed as an alternative delivery system for the OAg (20). GMMA are outer membrane vesicles (OMV) naturally shed by Gram-negative bacterium specifically engineered to increase blebbing and obtained through a simple and robust manufacturing process, possibly leading to affordable vaccines (21–23). GMMA contain mainly outer membrane proteins and lipopolysaccharides (LPS), together with luminal periplasmic proteins. GMMA are highly immunogenic and induce T-cell-dependent, boostable, isotype-switched, highly functional IgG profiles (24). This is crucial, given the importance of the quality of the antibody response in protection against salmonelloses (25, 26). Compared to traditional glycoconjugate vaccines, GMMA have the added value of combining multiple antigens in a single vaccine component, including polysaccharides and proteins possibly contributing to clinical protection. Indeed, GMMA from *S*. Typhimurium and *S*. Enteritidis are protective in animal models (24), and a *Shigella sonnei* GMMA-based vaccine was recently shown to be well tolerated and immunogenic in healthy adults and populations in areas where it is endemic (27–29).

In this study, we explored the possibility of inducing functional immune responses against *S*. Paratyphi A O:2 OAg and *S*. Typhi Vi polysaccharide antigen using GMMA from *S*. Paratyphi A as a delivery system.

RESULTS

Generation and characterization of OMV expressing Vi- and OAg-specific antigen. With the aim of engineering a *S*. Paratyphi A GMMA-producing strain that also would display the *S*. Typhi Vi antigen, *S*. Paratyphi A strain NVGH2041 (ParA O:2 Vi$^-$), lacking the *tolR* gene for increased outer membrane blebbing, was transformed with pDC5-*viaB*; this is a plasmid that contains the entire *viaB* locus from *S*. Typhi and, therefore, all genes needed for Vi production and anchoring to the membrane (30). Simultaneous surface exposure of both Vi and O:2 on the bacterial surface of the resulting strain, indicated as ParA O:2 Vi$^+$, was confirmed by flow cytometry using specific anti-O:2 and anti-Vi sera (Fig. 1). ParA O:2 Vi$^+$ and O:2 Vi$^-$ were both recognized by the anti-O:2 serum, indicating that the presence of Vi does not hinder the binding of antibodies to OAg. GMMA were produced from ParA O:2 Vi$^+$ and O:2 Vi$^-$ strains and were fully characterized through a panel of analytical methods. Both sets of GMMA had a similar size (average size of 72 and 83 nm in diameter; Table 1), as determined by dynamic light scattering (DLS), and a similar OAg/protein (wt/wt) ratio, as determined by high-performance anion exchange chromatography-pulsed amperometric detection (HPAEC-PAD). The amount of Vi (in micrograms) in ParA O:2 Vi$^+$ GMMA was ~10 times lower than the amount of OAg. To determine whether such a low Vi amount was due to heterologous expression of the *viaB* locus in *S*. Paratyphi A, *S*. Typhi BRD948 (Typhi O:9 Vi$^+$) and its isogenic Δ*tviB* mutant (Typhi O:9 Vi$^-$) were included as benchmarks in our analysis. Both *S*. Typhi strains display OAg containing the immunodominant O:9 factor, but only the native BRD948 is Vi$^+$ due to the presence of the *viaB* locus in the chromosome. Similar to what is seen for ParA O:2 Vi$^+$, surface exposure of both Vi and O:9 was detected on the bacterial surface of *S*. Typhi O:9 Vi$^+$ (Fig. 1). Naturally released OMV were produced from *S*. Typhi strains and compared to GMMA obtained

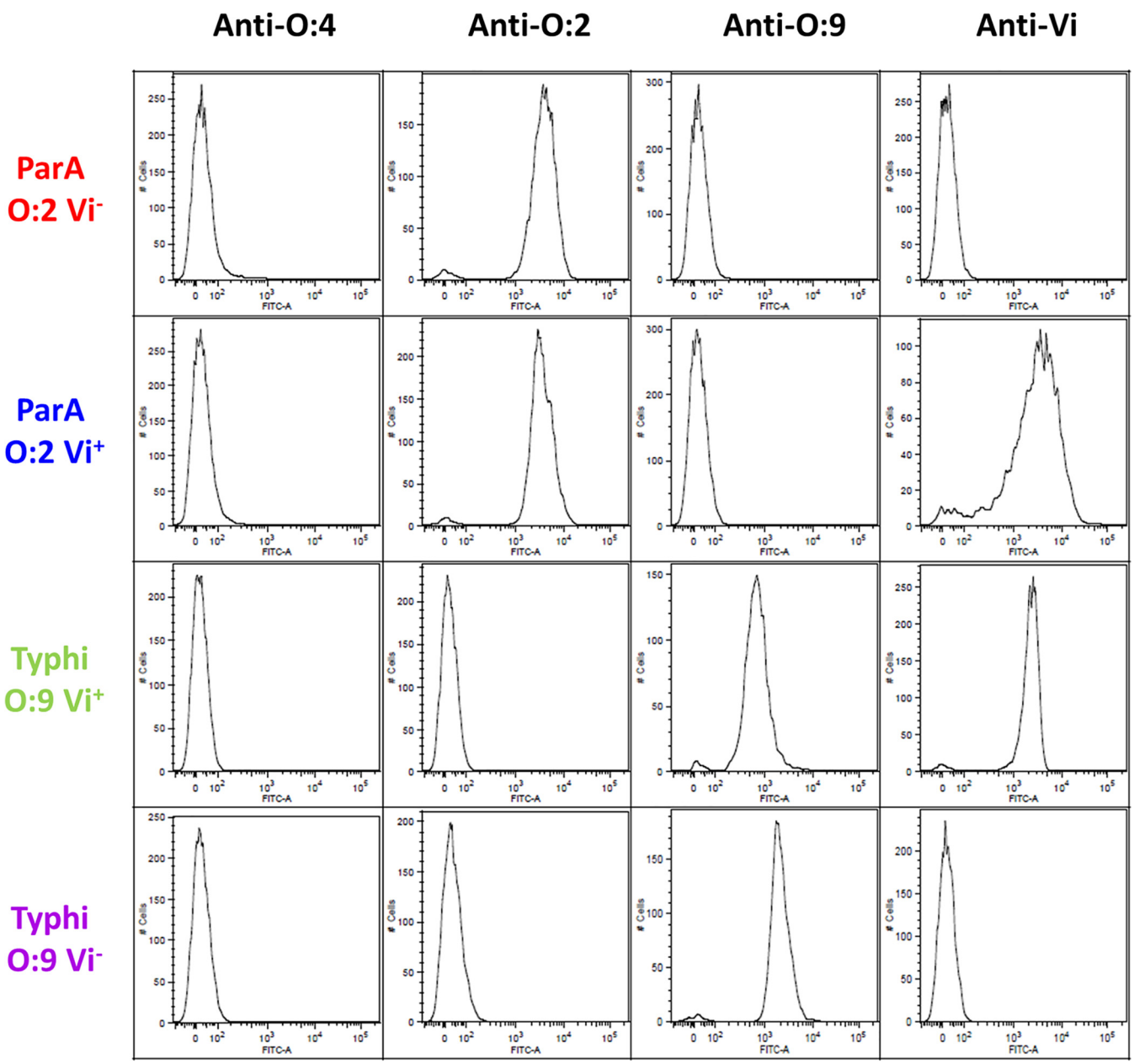

FIG 1 Display of polysaccharide antigens on the bacterial surface. Flow cytometry analysis of surface polysaccharides of ParA O:2 Vi$^-$, ParA O:2 Vi$^+$, *S.* Typhi O:9 Vi$^+$, and *S.* Typhi O:9 Vi$^-$. Flow cytometry was performed using rabbit anti-O:2, -O:9, and -Vi polyclonal serum, followed by Alexa Fluor 488-conjugated secondary antibodies. Bacteria stained with a rabbit anti-O:4 polyclonal serum were included as a negative control.

from *S.* Paratyphi A strains. OMV had more heterogeneous size than GMMA, with an average size of 81 and diameter of 133 nm and a higher polydispersion index (Table 1). The OAg/protein (wt/wt) ratio in *S.* Typhi O:9 Vi$^-$ OMV was similar to that measured in *S.* Paratyphi A GMMA, while Typhi O:9 Vi$^+$ OMV showed a higher OAg/protein (wt/wt) ratio than the other preparations (Table 1). Importantly, the Vi/protein (wt/wt) ratio in Typhi O:9 Vi$^+$ OMV was comparable to that of ParA O:2 Vi$^+$ GMMA (Table 1).

Immunogenicity of GMMA/OMV in a preclinical murine model. To test the possibility of inducing immune responses against both Vi and OAg with OMV/GMMA vaccine candidates, 4 groups of six C57BL/6 mice were immunized subcutaneously with vesicles prepared from ParA O:2 Vi$^+$, ParA O:2 Vi$^-$, Typhi O:9 Vi$^+$, and Typhi O:9 Vi$^-$. All animals received a booster vaccination on day 28, and sera were collected from individual animals on day 42. Each mouse received a dose equivalent to 0.5 μg of Vi

TABLE 1 GMMA and OMV analytical characterization

Antigen	Vi/OAg[a] (wt/wt) ratio, %	Vi/protein[a] (wt/wt) ratio, %	OAg/protein[a] (wt/wt) ratio, %	*Z* avg diam[b] (nm)	Polydispersion index[b]
ParA Vi$^+$ GMMA	9.6	4.2	43	83	0.14
ParA Vi$^-$ GMMA	NA	NA	47	72	0.13
S. Typhi Vi$^+$ OMV	10.1	5.0	50	133	0.41
S. Typhi Vi$^-$ OMV	NA	NA	164	81	0.35

[a]OAg content and Vi content were measured by HPAEC-PAD analysis and protein content by micro-BCA analysis, and reported ratios were calculated. NA, not applicable.
[b]GMMA diameter and polydispersion index were calculated by DLS.

antigen; this dose also resulted in the administration of similar amounts of OAg (Table 2). The immunogenicity of GMMA/OMV was assessed by measuring total IgG against Vi (Fig. 2A), O:2 (Fig. 2B), and O:9 (Fig. 2C). ParA O:2 Vi$^+$ and ParA O:2 Vi$^-$ GMMA induced similar levels of anti-O:2 IgG, confirming that the display of Vi at the surface of the vesicles did not hinder the ability to induce anti-OAg IgG responses (Fig. 2B). Immunization with ParA O:2 Vi$^+$ resulted in the induction of anti-Vi antibodies (Fig. 2A), showing that the Vi antigen was delivered in immunogenic form when using Vi$^+$ vesicles. Interestingly, the anti-Vi response induced by the vesicles from *S.* Paratyphi A engineered to display Vi using episomal expression of the *viaB* locus was comparable to that of OMV from the naturally Vi$^+$ serovar Typhi (Fig. 2A). This indicated that immune responses to Vi can be induced using vesicles produced from strains engineered for the heterologous display of Vi. Moreover, *S.* Typhi O:9 Vi$^+$ and *S.* Typhi O:9 Vi$^-$ also induced similar levels of anti-O:9 IgG (Fig. 2C), once again confirming the lack of immune interference between Vi and OAg. Next, we tested the functional activity of resulting sera in a serum bactericidal assay (SBA) using bacterial strains displaying O:2, O:9, or Vi. Sera from mice immunized with ParA O:2 Vi$^+$ and ParA O:2 Vi$^-$ GMMA showed similar bactericidal activity against the O:2-displaying *S.* Paratyphi A test strain (Fig. 2E). Thus, the display of Vi on the surface of *S.* Paratyphi A GMMA does not affect the ability to induce functional antibody responses capable of mediating bactericidal activity. We confirmed the ability of ParA O:2 Vi$^+$ GMMA antisera to exert SBA against a *C. freundii sensu lato* (s.l.) strain, displaying the Vi antigen but not any other *Salmonella*-specific OAg determinants (Fig. 2D). As previously observed in enzyme-linked immunosorbent assay (ELISA), the functional activity of anti-Vi antibodies induced by ParA O:2 Vi$^+$ GMMA was comparable to that induced by Typhi O:9 Vi$^+$ OMV (Fig. 2D). This shows that vesicles from ParA O:2 Vi$^+$ and Typhi O:9 Vi$^+$, but not from their Vi$^-$ counterparts, can induce functional antibodies able to activate complement deposition and exert Vi-specific SBA. Finally, sera from mice immunized with Typhi O:9 Vi$^+$ and Typhi O:9 Vi$^-$ OMV also showed similar bactericidal activity against the O:9-displaying *S.* Enteritidis test strain (Fig. 2F).

DISCUSSION

The possibility to deliver multiple antigens and to confer protection against multiple *Salmonella* serovars is becoming increasingly important in light of the awareness of the geographical coexistence of multiple *Salmonella* diseases, such as typhoid and paratyphoid fever.

TABLE 2 Mouse immunogenicity study: antigens and doses

Group	Dose (μg)			
	Protein	Vi	O:2	O:9
ParA Vi$^+$ GMMA	12.0	0.5	5.2	0
ParA Vi$^-$ GMMA	11.0	0	5.2	0
S. Typhi Vi$^+$ OMV	9.9	0.5	0	4.9
S. Typhi Vi$^-$ OMV	3.0	0	0	4.9
Saline	0	0	0	0

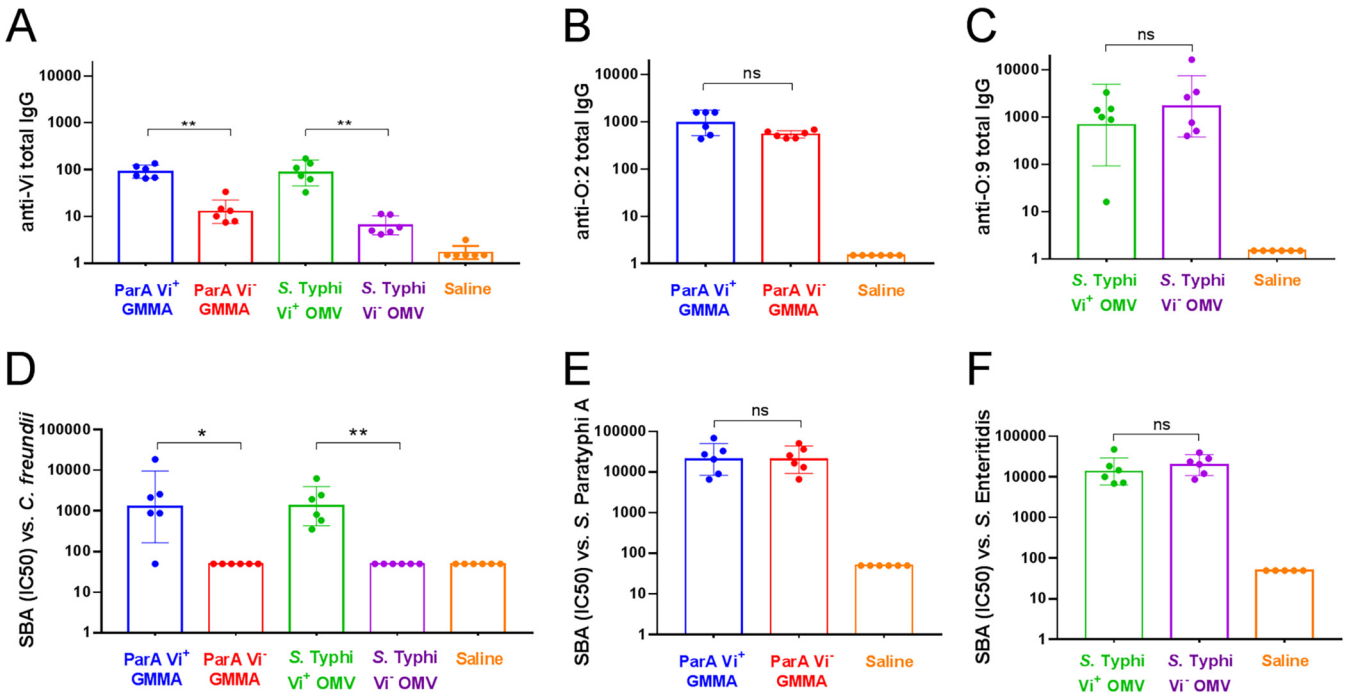

FIG 2 Immunogenic (ELISA) and functional (SBA) assessment of vaccines. Total anti-O:2, anti-O9, and anti-Vi ELISA IgG (top) and SBA titers (IC_{50}) against *S.* Paratyphi A (O:2-positive), *S. Enteritidis* (O:9-positive), and *C. freundii* s.l. (Vi-positive) strains (bottom) are shown. Unpaired, nonparametric *t* test (Mann-Whitney) was used to determine the statistically significant differences between groups (ns, not significant; *, $P < 0.033$; **, $P < 0.002$).

In the present study, we explored the possibility of producing a vesicle-based bivalent vaccine candidate against enteric fever based on GMMA delivering the Vi polysaccharide from *S.* Typhi and the somatic O-antigen from *S.* Paratyphi A. Different GMMA preparations were obtained at small scale and characterized, ensuring the reproducibility of the main analytical characteristics. However, additional work will be needed for the process scale-up, including the evaluation of lot-to-lot consistency. We show that an *S.* Paratyphi A GMMA engineered to display the Vi antigen from *S.* Typhi can induce both anti-Vi and anti-O:2 antibodies. This indicates that Vi does not render the underlying O-antigen inaccessible for recognition by the immune system. Furthermore, the immune responses induced by the O and Vi antigens are functional against both O:2$^+$ and Vi$^+$ target strains in a serum bactericidal assay, further supporting their potential for broad protective activity.

A typhoid-paratyphoid vaccine would be a great asset for LMIC and travelers, given that no paratyphoid vaccines are currently licensed. Glycoconjugates are a well-established bacterial vaccine approach and have been proposed as strategies against both *S.* Typhi and *S.* Paratyphi A (12, 20). More recently, GMMA have been proposed as an alternative delivery system for OAg (20) and are particularly attractive when multicomponent preparations are required and when impoverished communities are the vaccine target population. Compared to traditional glycoconjugates, GMMA show similar or better immunogenicity and a simpler manufacturing process (31), representing a promising alternative for the development of affordable multicomponent vaccines against *Salmonella* serovars (24).

S. Typhi OMV, naturally displaying the Vi antigen, were included in this study as internal controls and compared to *S.* Paratyphi A GMMA engineered to display the heterologous Vi polysaccharide. No differences were observed either in the amount of Vi found on the resulting vesicles or in the immunogenicity and functional activity of anti-Vi antibodies elicited upon immunization. Moreover, *S.* Typhi OMV could induce both anti-Vi and anti-O:9 antibodies, similar to what was observed with ParA O:2 Vi$^+$ GMMA inducing both anti-Vi and anti-O:2 antibodies. These OMV therefore induced

responses that would target both Vi$^+$ *S.* Typhi (anti-Vi and anti-O:9 antibodies) and Vi$^-$ *S.* Typhi isolates, which occur in the field and are reported to be able to cause disease (32). Our previous work found that the Vi antigen is rapidly downregulated once the bacteria reach an intracellular location in the infected tissues, with the majority of the bacterial population becoming Vi$^-$ and no longer displaying a target for the immune response (33). Finally, these OMV would also target *S.* Enteritidis, which shares the O:9 antigen with *S.* Typhi.

In summary, our work shows that it is possible to deliver both O and Vi antigens using vesicle-based vaccine platforms, inducing strong and functional antibody responses against different polysaccharides. Moreover, the presence of protein antigens on *Salmonella* OMV/GMMA may represent an added value for GMMA vaccines compared to other polysaccharide-based formulations. In conclusion, bacterial outer membrane vesicles represent a flexible, affordable, and highly immunogenic platform for the development of multivalent *Salmonella* vaccines.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella* Paratyphi A NVGH308 (displaying the O:2 OAg [34]) is the isolate that has been engineered with a Δ*tolR* mutation to increase outer membrane blebbing (i.e., GMMA production), resulting in strain NVGH2041. Serovar Paratyphi A does not naturally produce the Vi antigen; heterologous display of Vi in *S.* Paratyphi A Δ*tolR* strain NVGH2041 was obtained through episomal expression of the *viaB* locus using the pDC5-*viaB* plasmid, a gift from Andreas Baumler, University of California-Davis (30). These strains are referred to as ParA O:2 Vi$^-$ and ParA O:2 Vi$^+$, respectively. Attenuated *S.* Typhi BRD948 (Ty2 Δ*aroC* Δ*aroD* Δ*htrA* mutant naturally displaying the O:9 OAg and Vi antigen [35]) and *Salmonella* Typhi BRD948 Δ*tviB* (displaying O:9 but not Vi [36]) strains were used as *S.* Typhi OMV-producing strains (Typhi O:9 Vi$^+$ and Typhi O:9 Vi$^-$, respectively). All strains were grown at 30°C in liquid Luria-Bertani (LB) medium in rotary shakers for 16 h. For OMV/GMMA production, overnight cultures were diluted in HTMC medium (15 g/liter glycerol, 30 g/liter yeast extract, 0.5 g/liter $MgSO_4$, 5 g/liter KH_2PO_4, 20 g/liter K_2HPO_4) to an optical density at 600 nm (OD_{600}) of 0.3 and grown at 30°C for 8 h with a liquid-to-air volume ratio of 1:5. A supplement of a mixture of aromatic amino acids (Aro mix; 0.04 g/liter phenylalanine, 0.04 g/liter tryptophan, 0.01 g/liter para-aminobenzoic acid, and 0.01 g dihydrobenzoic acid) and 0.04 g/liter tyrosine was used for the *S.* Typhi strains (35). The ParA O:2 Vi$^+$ strain was grown in the presence of 100 μg/ml ampicillin to retain plasmid pDC5-*viaB* expression.

Flow cytometry analysis. To monitor the display of the O and Vi polysaccharide antigens on the surface of OMV/GMMA-producing strains, bacteria were grown for 16 h in liquid culture and analyzed by flow cytometry. Bacteria were pelleted at 4,000 $\times$ *g* for 5 min, washed with phosphate-buffered saline (PBS), and fixed using Cytofix fixation buffer (BD Biosciences) for 30 min. Fixed bacteria were then blocked with PBS containing 3% (wt/vol) bovine serum albumin (BSA) for 15 min and incubated for 1 h with rabbit polyclonal sera against O:2, O:9, or Vi (Denka Saiken), diluted 1:500 in PBS plus 1% (wt/vol) BSA. Rabbit polyclonal serum against O:4 (Denka Seiken) was used as a negative control. Samples were incubated with Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes) diluted 1:500 in PBS plus 1% BSA for 30 min. Flow cytometry analysis was performed using a FACSCanto II flow cytometer (BD Biosciences).

OMV/GMMA production. OMV and GMMA were purified from the culture supernatant of each bacterial strain and characterized as previously described (23, 37). Bacteria were pelleted by centrifugation at 5,000 $\times$ *g* for 45 min. Cell-free supernatants were collected, filtered through 0.22-μm Stericup filters (Millipore), and ultracentrifuged at 175,000 $\times$ *g* for 2 h at 4°C using an SW32Ti rotor (Beckman Coulter). Pellets containing OMV/GMMA were resuspended in PBS, ultracentrifuged at 175,000 $\times$ *g* for 2 h, resuspended in PBS, filtered, and stored at 4°C until use.

Analytical characterization of GMMA/OMV. GMMA/OMV were characterized in terms of antigen composition and size. A micro-bicinchoninic acid (BCA) kit (Thermo Scientific) was used for GMMA/OMV total protein quantification using BSA as a reference standard and by following the manufacturer's instructions. The sugar monomers constituting the Vi and O polysaccharide repeating units were quantified through HPAEC-PAD, as previously described (38, 39). Particle size distribution of GMMA/OMV was evaluated by DLS, as previously reported (23, 40).

Animal experiments. Female C57BL/6 mice were purchased from Envigo UK and used when over 6 weeks of age (mean weight 20 $\pm$ 3 g). The mice were housed in specific-pathogen-free containment facilities and were allowed water and food *ad libitum*. Six mice per group were vaccinated subcutaneously at days 0 and 28 with either GMMA or OMV diluted in saline and normalized to contain approximately 5 μg of OAg per dose and 0.5 μg of Vi per dose (in case of Vi-positive OMV/GMMA), as reported in Table 1. A separate control group of mice received saline. Individual sera were collected at day -1 (pooled sera) and at day 42 (individual sera). All animal experiments were performed in accordance with good animal practice as defined by the relevant international (Directive of the European Parliament and of the Council on the Protection of Animals Used for Scientific Purposes, Brussels 543/5) and local (University of Cambridge) animal welfare guidelines. This research was regulated under the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012 following ethical review by the University of Cambridge Animal Welfare and Ethical Review Body (AWERB).

Assessment of anti-Vi and anti-OAg specific total IgG by ELISA. Anti-OAg and anti-Vi antigen-specific IgG levels were measured 2 weeks after the second immunization (day 42) by ELISA as previously reported (41). Briefly, 96-well round-bottom MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 100 μl/well antigen overnight at 4°C. OAg purified from *S.* Paratyphi A (O:2) or *S. Enteritidis* (O:9) and Vi purified from *C. freundii* s.l. were used at 15 μg/ml and 2 μg/ml in carbonate or at 1 μg/ml in phosphate buffer, respectively (38, 42). Plates were blocked with PBS plus 5% fat-free milk (Sigma) for 2 h at room temperature (RT) and then washed 3 times with PBS plus 0.05% Tween 20 (PBS-T). Serum samples were diluted 1:100 and 1:4,000 in PBS-T supplemented with 0.1% BSA (diluent buffer), and both dilutions were assayed in triplicate. After incubation for 2 h at RT, plates were washed three times with PBS-T and incubated at 25°C for 1 h with anti-mouse goat IgG-alkaline phosphatase (Sigma), diluted 1:6,000, 1:8,800, and 1:2,600 (for Vi, O:2, or O:9, respectively) in diluent buffer. After washing three times with PBS-T, plates were developed by adding the alkaline phosphatase substrate (SIGMAFAST N2770; Sigma) and read at 405 nm and 490 nm using an ELx 800 reader (BioTek). ELISA units were expressed relative to a mouse antigen-specific antibody standard serum curve composed by 10 standard points and 2 blank wells (run in duplicate on each plate), with the best five-parameter fit determined by a modified Hill plot. One ELISA unit is defined as the reciprocal of the dilution of the standard serum that gives an absorbance value equal to 1 in this assay.

Assessment of serum bactericidal activity by SBA. Individual mouse sera collected at day 42 were heat inactivated (HI) at 56°C for 30 min prior to being tested in a serum bactericidal assay based on luminescent readout against *Salmonella* Paratyphi A NVGH308, *Salmonella Enteritidis* CMCC3014, and Vi-positive *Citrobacter freundii sensu lato* strain 3056 (43, 44). SBA was performed in 96-well round-bottom sterile plates (Corning). Dilutions of HI test sera were incubated for 3 h in the presence of exogenous complement (baby rabbit complement [BRC]) and bacteria as previously described (43). Briefly, an adequate volume of reaction mixture containing the target bacterial cells (around 100,000 CFU/ml), BRC (50% for *S. Enteritidis*, 20% for *S.* Paratyphi A, and 5% for *C. freundii* s.l.), and buffer (PBS) was added to SBA plates containing HI serum dilutions and incubated for 3 h at 37°C. At the end of the incubation, the plates were centrifuged for 10 min at 4,000 $\times$ *g*, the supernatant was discarded to remove ATP derived from dead bacteria, and live bacterial pellets resuspended in PBS were transferred to a white round-bottom 96-well plate (Greiner) and mixed 1:1 (vol/vol) with BacTiter-Glo reagent (Promega). The reaction mixture was incubated for 5 min at RT in an orbital shaker, and the luminescence signal was measured using a luminometer (Viktor). A 4-parameter nonlinear regression was applied to raw luminescence for all the serum dilutions tested, as previously described (45). The SBA titer is reported as IC_{50}, defined as serum dilutions giving 50% inhibition of the ATP level in the negative-control well. Titers below the minimum measurable level of luminescence were arbitrarily given an IC_{50} of 50, representing half of the first dilution of sera tested (i.e., 100). GraphPad Prism 7 software (GraphPad Software) was used for fitting and IC_{50} determination.

Statistical analysis. Unpaired, nonparametric *t* test (Mann-Whitney) was used to determine the statistically significant differences between groups, using GraphPad Prism 7 software (GraphPad Software).

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Conceived and designed the experiments: G.G., A.S., O.R., F. Micoli, and P.M. Performed the experiments: G.G., R.A., V.A., F. Mancini, M.G.A., P.K., P.M., and D.P. Analyzed the data: G.G., R.A., V.A., F. Mancini, M.G.A., P.K., F.N., A.S., O.R., F. Micoli, and P. M. Contributed to the writing of the manuscript: G.G., O.R., F. Micoli, and P.M. All authors had full access to the data and approved the final manuscript.

G.G., R.A., V.A., F. Mancini, M.G.A., F.N., O.R., and F. Micoli are employees of the GSK group of companies. A.S. was employed by the GSK group of companies at the time of the study, owns GSK shares, and is listed as an inventor on patents owned by the GSK group of companies. This does not alter the authors' adherence to all journal policies on data and material sharing.

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