Lipopolysaccharide Modifications of a Cholera Vaccine Candidate Based on Outer Membrane Vesicles Reduce Endotoxicity and Reveal the Major Protective Antigen

Deborah R. Leitner, Sandra Feichter, Kristina Schild-Prüfert, Gerald N. Rechberger, Joachim Reidl and Stefan Schild
Infect. Immun. **2013, 81(7):2379. DOI: 10.1128/IAI.01382-12. Published Ahead of Print 29 April 2013.**

IAI Journals.ASM.org

Lipopolysaccharide Modifications of a Cholera Vaccine Candidate Based on Outer Membrane Vesicles Reduce Endotoxicity and Reveal the Major Protective Antigen

Deborah R. Leitner, Sandra Feichter, Kristina Schild-Prüfert, Gerald N. Rechberger, Joachim Reidl, Stefan Schild

Institute of Molecular Biosciences, University of Graz, Graz, Austria

The causative agent of the life-threatening gastrointestinal infectious disease cholera is the Gram-negative, facultative human pathogen *Vibrio cholerae*. We recently started to investigate the potential of outer membrane vesicles (OMVs) derived from *V. cholerae* as an alternative approach for a vaccine candidate against cholera and successfully demonstrated the induction of a long-lasting, high-titer, protective immune response upon immunization with OMVs using the mouse model. In this study, we present immunization data using lipopolysaccharide (LPS)-modified OMVs derived from *V. cholerae*, which allowed us to improve and identify the major protective antigen of the vaccine candidate. Our results indicate that reduction of endotoxicity can be achieved without diminishing the immunogenic potential of the vaccine candidate by genetic modification of lipid A. Although the protective potential of anti-LPS antibodies has been suggested many times, this is the first comprehensive study that uses defined LPS mutants to characterize the LPS-directed immune response of a cholera vaccine candidate in more detail. Our results pinpoint the O antigen to be the essential immunogenic structure and provide a protective mechanism based on inhibition of motility, which prevents a successful colonization. In a detailed analysis using defined antisera, we can demonstrate that only anti-O antigen antibodies, but not antibodies directed against the major flagellar subunit FlaA or the most abundant outer membrane protein, OmpU, are capable of effectively blocking the motility by binding to the sheathed flagellum and provide protection in a passive immunization assay.

The secretory diarrheal human disease cholera is caused by the Gram-negative, motile, curved rod bacterium *Vibrio cholerae* (1). Cholera is transmitted via the fecal-oral route and is characterized by its ability to cause explosive outbreaks. In Asia, the outbreaks peak seasonally before and after the monsoon rain, while in other areas additional factors, such as natural disasters, can contribute to devastating cholera epidemics as was recently observed in Haiti (2–5). Cholera is a major cause of secretory diarrhea in adults, but particularly infants and young children show a high mortality rate in developing countries, where diarrheal diseases remain the second most common cause of death (6, 7). The burden of cholera is difficult to determine because of gross underreporting, but the WHO estimates that 3 to 5 million cases occur per year (8, 9). Treatment of cholera consists essentially of an oral or intravenous rehydration therapy, sometimes in combination with antimicrobial agents (5, 10). While the rehydration therapy is highly effective, the availability of the necessary supplies, trained health care staff, and adequate sanitation are often limited during the explosive outbreaks. Hence, besides the therapeutic approach, the further investigation and development of alternative strategies for prevention, such as affordable vaccines, should be a desired goal of the ongoing research.

Currently, the only globally licensed cholera vaccine consists of killed whole-cell *V. cholerae* O1 supplemented with purified recombinant cholera toxin B subunit (11–14). Despite its benefits for travelers in high-risk areas, the vaccine is considered unsatisfactory for broad use in developing countries due to its short shelf life, high cost, and need for cold-chain distribution (15, 16). Closely related reformulations with lower production costs are now marketed. However, only one of them meets the recommended WHO standards, and they still require a cold storage temperature, which could be a big challenge for their broad use in the future (17–20). In addition, live attenuated *V. cholerae* vaccines and conjugate vaccines might provide interesting alternative approaches but are still under development and have not been commercialized so far (21–30). The intensive ongoing research activity in the field highlights the demand for a better cholera vaccine.

We recently started to investigate the potential of outer membrane vesicles (OMVs) derived from *V. cholerae* as an alternative approach for a vaccine candidate against cholera (31–33). OMVs are naturally released by various Gram-negative bacteria and predominantly contain outer membrane components with periplasmic compounds entrapped in the lumen (34, 35). Although we are only beginning to understand the physiological role and biogenesis of OMVs, they are basically nonliving facsimiles of the donor bacterium and can be seen as delivery vehicles for important surface antigens in their native conformation. We demonstrated that immunization of mice by mucosal routes (e.g., intranasal [i.n.] or intragastric) with OMVs derived from *V. cholerae* induced a specific, long-lasting, high-titer immune response (33). The suckling neonates of the primary immunized female mice were protected

Received 7 December 2012 **Returned for modification** 21 January 2013
Accepted 16 April 2013
Published ahead of print 29 April 2013
Editor: S. M. Payne
Address correspondence to Stefan Schild, stefan.schild@uni-graz.at.
Supplemental material for this article may be found at http://dx.doi.org/10.1128/IAI.01382-12.

doi:10.1128/IAI.01382-12
The authors have paid a fee to allow immediate free access to this article.

against oral challenge with *V. cholerae*, indicating the induction of a protective immune response upon immunization with OMVs. This indirect protection assay was used, since adult mice are successfully colonized by *V. cholerae* only after pretreatment with antibiotics to decrease the bacterial gut flora. Further characterization revealed that this protective immune response relies upon the transfer of the acquired immunoglobulins (Ig) from the primary immunized female mice to the offspring via breast milk (32). In addition, the OMV vaccine candidate proved to be highly stable and immunogenic without the requirement of additional adjuvants (31–33). Thus, a cold chain or accessory buffer solutions are unlikely to be required for the OMV vaccine candidate. However, protection against both clinically relevant serogroups O1 and O139 was achieved only by immunization with a mixture of O1 and O139 OMVs. Since O139 has evolved from O1, the two serogroups are closely related but differ in the composition of the lipopolysaccharide (LPS). Hence, the data suggest that antibodies directed against the LPS play a crucial role in protection, although immunization with OMVs also induces a strong immune response against several surface proteins present in the OMVs. Interestingly, we were able to correlate the observed protection in the *in vivo* model with the ability of anti-OMV antibodies to inhibit motility of different *V. cholerae* strains *in vitro*, suggesting that inhibition of motility contributes to protection (31). Noteworthy, *V. cholerae* has a single polar flagellum that is covered by an outer membrane sheath including LPS molecules (36, 37). Thus, we currently propose a model by which antibodies directed against the LPS may block motility by binding to the sheathed flagellum. This inhibition of motility and agglutination of the bacteria is likely to be the critical factor for protection *in vivo*, since flagellar motility is crucial to access the mucosa at the initial stages of colonization (38–42).

Naturally, OMVs contain a relative large amount of LPS, including a hexa-acylated lipid A moiety that potently activates Toll-like receptor 4 (TLR-4) and can result in septic shock (43). Especially parenteral administration routes exhibit a low level of tolerance against the lipid A endotoxin (44–46). The relatively simple administration as well as the robust induction of secretory Ig titers currently argues for immunization with *V. cholerae* OMVs by mucosal routes. However, an ideal vaccine candidate should not be limited to these immunization routes. Moreover, a general reduction of endotoxicity to minimize adverse effects might be necessary for a safe application of the OMV vaccine candidate in humans, as highlighted by a variety of other vaccine candidates containing LPS (47–55). For example, the *Neisseria meningitidis* OMV vaccines, used to control outbreaks in Scandinavia and New Zealand, include an additional step to remove most of the LPS by detergent extraction (56, 57). As will become evident throughout this study, the LPS itself provides the major protective antigen for the *V. cholerae* OMV vaccine candidate. Hence, a simple detergent extraction to substantially decrease the amounts of LPS is not applicable, and an alternative strategy is required.

Here, we present immunization data using LPS-modified OMVs of *V. cholerae*, which allowed us to improve and identify the major protective antigen of the vaccine candidate. Therefore, we constructed defined, in-frame deletion mutants of well-characterized genes involved in the LPS biosynthesis pathway. Subsequently, these genetically engineered *V. cholerae* strains served as OMV donors. Our results indicate that reduction of endotoxicity can be achieved without diminishing the immunogenic potential of the vaccine candidate by genetic modification of lipid A. Subsequently, we attempted to develop a serogroup-independent OMV vaccine by the use of OMVs lacking the O antigen, which is the most important discrimination marker between the serogroups O1 and O139. However, our results indicate that the generation of antibodies against the O antigen portion of the LPS is crucial for the protective immune response.

TABLE 1 Strains and plasmids used in this study

Strain or plasmid	Relevant genotype and description	Reference
E. coli		
DH5αλpir	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*argF-lac*)*U169 deoR recA1 endA1 hsdR17*(r_K^- m_K^+) *supE44 thi-1 gyrA69 relA1*, λ*pir*R6K, Ap^r	103
SM10λpir	*thi thr leu tonA lacY supE recA*::RPA-2-Te:: Mu λpirR6K, Km^r	104
V. cholerae		
WT (O1)	AC53, wild-type *V. cholerae* strain serogroup O1; biotype, El Tor; serotype, Ogawa; spontaneous Sm^r mutant of E7946; clinical isolate from Bahrain, 1978; *hapR*$^+$, Sm^r; used for previous immunization studies (31–33, 105)	106
O139	MO10, wild-type *V. cholerae* strain serogroup O139; 1992 clinical isolate from India; *hapR*$^+$, Sm^r	107
Δ*waaL*	AC53Δ*waaL*, Sm^r	This study
Δ*msbB*	AC53Δ*msbB*, Sm^r	This study
Δ*waaL* Δ*msbB*	AC53Δ*waaL* Δ*msbB*, Sm^r	This study
Plasmids		
pCVD442	*ori6K mobRP4 sacB*, Ap^r	61
pBK	pBAD18-Kan, araBADp cloning vector, Km^r	108
pBA	pBAD18, araBADp cloning vector, Ap^r	108
pΔmsbB	pCVD442::Δ*msbB*, Ap^r	This study
pΔwaaL	pKEK229::Δ*waaL*, Ap^r	76
pwaaL	*waaL* from *V. cholerae* in pBK, Km^r	109
pmsbB	*msbB* of AC53 in pBA, Ap^r	This study
pSSH2188	pBAD/Myc-HisA::*flaA* (six-His)	66
pET15b-cI-TcpA		67
pOmpU	*ompU* of AC53 in pMal-p2X (six-His), Ap^r	This study

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains and plasmids used in this study are listed in Table 1; oligonucleotides are listed in Table S1 in the supplemental material. *V. cholerae* AC53, a spontaneous streptomycin (Sm)-resistant mutant of the clinical isolate E7946 (O1, El Tor Ogawa), or *V. cholerae* MO10 (Sm-resistant clinical isolate, O139) were used as wild-type (WT) strains representing serogroup O1 [here referred to as WT (O1)] and serogroup O139, respectively. *Escherichia coli* strains DH5αλpir and SM10λpir were used for genetic manipulations. If not noted otherwise, strains were cultured in Luria-Bertani (LB) broth or on LB broth agar plates with aeration at 37°C. To induce TcpA expression, *V. cholerae* was grown in AKI broth (0.5% sodium chloride, 0.3% sodium bicarbonate, 0.4% yeast extract, 1.5% Bacto peptone) for 4 h without shaking, followed by 4 h with aeration at 37°C (58, 59). If required, antibiotics or other supplements were used in the following final concentrations: Sm, 100 μg/ml; ampicillin (Ap), 50 μg/ml in combination with other antibiotics or 100 μg/ml; kanamycin (Km), 50 μg/ml; arabinose (Ara), 0.02%; IPTG (isopropyl-β-D-thiogalactopyranoside), 1 mM; glucose (Gluc), 0.2%; sucrose (Suc), 10%.

Construction of in-frame deletion mutants and expression plasmids. The isolation of chromosomal DNA, the PCR assays, the purification of plasmids or PCR products, and the construction of suicide and

expression plasmids, as well as the subsequent generation of deletion mutants, were carried out as described previously (60). To construct the suicide plasmid pΔmsbB, upstream and downstream fragments of the gene were PCR amplified using the oligonucleotide pairs msbB_XbaI_1 and msbB_XhoI_2 or msbB_XhoI_3 and msbB_SphI_4, digested with the appropriate restriction enzyme (New England BioLabs) indicated by the name of the oligonucleotide, and finally ligated into the SphI/XbaI-digested pCVD442. Deletion mutants were generated using derivatives of the suicide vector pCVD442 in combination with the method described by Donnenberg and Kaper (61). Briefly, the respective suicide plasmid was mobilized via conjugation into *V. cholerae*, and integration of the plasmid on the chromosome was selected by isolating Sm- and Ap-resistant (Sm^r Ap^r) colonies. Subsequently, sucrose selection was applied to obtain Ap^s colonies and correct deletions were confirmed by PCR (data not shown). To construct the expression plasmid pmsbB, the respective gene was PCR amplified using the oligonucleotides msbBcomp_SacI_1 and msbBcomp_XbaI_2, digested with SacI and XbaI, and ligated into pBA, which has been digested similarly. The expression plasmid pOmpU was constructed using the oligonucleotide pairs OmpU_NcoI_1 and OmpU_HindIII_2 for amplifying the respective gene and the appropriate restriction enzyme (New England BioLabs) indicated by the name of the oligonucleotide for digestion. Finally, the PCR-amplified fragment was ligated into the NcoI/HindIII-digested pMAL-p2X. Ligation products were transformed into DH5αλpir, and Ap^r colonies were characterized by PCR for the correct constructs (data not shown).

Preparation of OMVs and LPS. OMVs from late-exponential-phase LB broth cultures of *V. cholerae* were isolated as described previously (33). OMVs from AKI cultures of *V. cholerae* were isolated accordingly. The protein and LPS concentration of the OMV preparations was estimated with the modified Lowry protein assay (Thermo Scientific Pierce) or by the Purpald assay (see "KDO determination" below). All OMV amounts given below are by protein or LPS amount. LPS of *V. cholerae* cultures was purified as described previously (31). The concentration of the *V. cholerae* LPS was determined by comparison to a standard curve of commercially available *Escherichia coli* O26:B6 LPS (Sigma). LPS was separated on a 15% gel and visualized by silver staining (62). A ChemiDoc XRS system (Bio-Rad Laboratories) was used for documentation.

KDO determination. The Purpald assay, adapted for 96-well microtiter plates, was used to quantify the LPS content in OMV samples (63). Briefly, serial dilutions of 3-deoxy-D-*manno*-octulosonic acid (KDO) (Sigma) standard and OMV samples were incubated with 50 μl of 32 mM $NaIO_4$ for 25 min. Following addition of 50 μl of Purpald reagent (Sigma), the reaction was allowed to proceed for 20 min and stopped by adding 50 μl of 64 mM $NaIO_4$. After addition of 20 μl of 2-propanol, the absorbance at 550 nm was determined.

Protein analysis by MS. The respective protein band was excised from the gel and tryptically digested according to the method of Shevchenko et al. (64). Peptide extracts were dissolved in 0.1% formic acid and separated on a nano-high-performance liquid chromatography (HPLC) system (Ultimate 3000; Dionex, Amsterdam, Netherlands). Samples of 70 μl were injected and concentrated on the loading column (LC Packings C18 PepMap, 5-μm particle size, 100-Å pore size, 300 μm inside diameter [i.d.] by 1 mm) for 5 min using 0.1% formic acid as an isocratic solvent at a flow rate of 20 μl/min. The column was then switched to the nanoflow circuit, and the sample was loaded onto the nanocolumn (LC-Packings C18 PepMap, 75-μm i.d. by 150 mm) at a flow rate of 300 nl/min and separated using the following gradient: solvent A, water, 0.3% formic acid; solvent B, acetonitrile-water, 80:20 (vol/vol), 0.3% formic acid; for 0 to 5 min, 4% B; after 40 min, 55% B; then, for 5 min, 90% B; and finally 47 min of reequilibration at 4% B. The sample was ionized in a Finnigan nano-ESI source equipped with NanoSpray tips (PicoTip Emitte; New Objective, Woburn, MA, USA) and analyzed in a Thermo-Finnigan LTQ linear ion trap mass spectrometer (Thermo, San Jose, CA, USA). The tandem mass spectrometry (MS/MS) data were analyzed with SpectrumMill Rev. 03.03.084 SR4 (Agilent, Darmstadt, Germany) software using a *V. cholerae* proteome database (ftp://ftp.jcvi.org/pub/data/Microbial_Genomes/v_cholerae_el_tor_n16961/annotation_dbs/v_cholerae_el_tor_n16961.pep).

Hydroxylated fatty acid analysis of the LPS. To determine differences in the hydroxy-fatty acid content of the WT (O1) and Δ*msbB* mutant LPS, cultures (125 ml) of the respective strains were grown to the late exponential phase. Cells were harvested by centrifugation, and the pellet was washed twice with 40 ml phosphate-buffered saline (PBS). Phospholipids were removed from the samples by two washing steps according to the method of Folch et al. (65), followed by four washing steps with chloroform-methanol (2:1, vol/vol). Removal of phospholipids was confirmed by analysis of the supernatants after the second and sixth washing steps using an RP-UPLC-QTOF-MS system (Waters, Manchester, United Kingdom) (see Fig. S1 in the supplemental material). The remaining pellet was resuspended in methanol and transferred to a Pyrex glass tube, and the supernatant was removed after centrifugation. The fatty acids were transesterified using 2 ml boron trifluoride in methanol and 0.5 ml toluene. After 2 h of incubation at 100°C, 1 ml ice-cold water was added, and methyl esters were extracted two times with 2 ml hexane. The samples were dried under a stream of nitrogen, and the hydroxyl groups were silylated by incubating with 60 μl BSTFA [*N*,*O*-bis(trimethylsilyl)trifluoroacetamide]–pyridine (1:2, vol/vol) at room temperature for 30 min. Gas chromatography (GC)-MS was performed using the Trace GC and DSQ-MS (Thermo Scientific, Vienna, Austria). One microliter of sample solution was injected (injector temperature, 250°C) and separated on a 60-m DB5-MS column (gradient from 80 to 300°C) (Agilent, Waldbronn, Germany). MS data were acquired in positive electron impact (EI) ionization mode (70 eV; source temperature, 280°C; mass range, 50 to 700 *m/z*). Data analysis was performed using the XCalibur 1.4 software (Thermo). The peaks (see Fig. S2 in the supplemental material) in the extracted ion chromatograms of *m/z* 287 (= M-15) for trimethylsilyl (TMS)-3-hydroxylauroylmethylester and *m/z* 315 (= M-15) for TMS-3-hydroxymyristoylmethylester were integrated, and the ratios of the peak areas were calculated (see Fig. S3 in the supplemental material).

Purification of proteins and immunization. The His-tagged proteins FlaA and TcpA were purified using the expression plasmids pSSH2188 and pET15b-cI-TcpA as described previously (66, 67). For the expression of OmpU, cultures of *E. coli* DH5αλpir containing pOmpU were grown in 500 ml LB containing 100 μg/ml ampicillin for 4 h and induced with 1 mM IPTG for another 4 h. Cells were pelleted and resuspended in binding buffer (20 mM sodium phosphate, 0.5 M NaCl, 10 mM imidazole [pH 7.4]) with 8 M urea. His-tagged OmpU was purified using a HisTrap HP column (GE Healthcare) according to the manufacturer's instructions. In order to generate high-titer IgG1 antisera against FlaA and OmpU, the respective purified His-tagged proteins (100 μg protein per immunization) were used to intraperitoneally (i.p.) immunize mice on days 0, 14, and 28. Finally, sera were collected on day 40.

Polymyxin B assay. Overnight cultures of *V. cholerae* were subcultured 1:10,000 into LB. Samples of 100 μl bacterial culture were placed into wells of a 96-well plate, and 11.1 μl of the 1:2 serially diluted peptide solution (at a concentration 10 times higher than the final concentration) was added. After 18 h of incubation in a humid chamber at 37°C, the optical density at 600 nm (OD_{600}) was measured using the FLUOstar Omega Microplate Reader (BMG Labtech).

TNF-α, IL-1, and IL-6 induction assays. For tumor necrosis factor alpha (TNF-α), interleukin 1 (IL-1), and IL-6 induction assays, a confluent layer of RAW macrophages was stimulated with OMVs (100 ng/ml) derived either from WT (O1) or from Δ*msbB* for 6 h before the RNA was isolated. Nonstimulated RAW macrophages grown in parallel served as a control. To isolate the RNA, RAW macrophages were washed once with PBS and suspended in 600 μl TRIzol/well (Invitrogen). Following chloroform extraction, RNA was precipitated by isopropanol. An aliquot of the RNA (1 μg) was used as the template to synthesize cDNA using the iSCRIPT Select cDNA Synthesis kit (Bio-Rad Laboratories) according to the manufacturer's instructions. Quantitative reverse transcription-PCR (qRT-PCR) experiments were performed in triplicates with SYBR

GreenER qPCR SuperMix for the ABI Prism instrument (Invitrogen), utilizing a Rotor-Gene 600 and Rotor-Gene 600 Series Software 1.7 (GenXpress) according to the manufacturer's instructions. The sequences of the oligonucleotides used in this study are listed in Table S1 in the supplemental material. All measured mRNA levels were normalized to the expression of the housekeeping gene RPLP0 (36B4). Values above or below 1 indicate induction or repression, respectively, of the respective gene in OMV-stimulated RAW macrophages compared to nonstimulated RAW macrophages.

Animals. Female BALB/c mice (Charles River Laboratories) were used in all experiments in accordance with the rules of the ethics committee at the University of Graz and the corresponding animal protocol, which has been approved by the Austrian Federal Ministry of Science and Research Ref. II/10b. Mice were housed with food and water *ad libitum* and monitored under the care of full-time staff. All animals were acclimated for 1 week before any procedures were carried out and were approximately 9 weeks old at the start of the immunization.

OMV immunization protocol and neonatal challenge with *V. cholerae*. Nine-week-old female mice were i.n. immunized at days 0, 14, and 28 with 25 μg of OMVs in PBS as described previously (32, 33). Mice were briefly anesthetized by inhalation of 2.5% isoflurane gas prior to all immunizations. A group of sham (PBS)-immunized control mice were housed in parallel with immunized mice for the duration of each experiment. Two independent immunization rounds were performed with at least three mice per group. Both immunization rounds revealed no differences in the induced protective immune response in the respective immunization groups. Mice were mated on day 41, and 5- to 6-day-old neonates were challenged with *V. cholerae* as previously described (32, 33). Neonatal mice from a given litter were split into two groups and infected with either *V. cholerae* O1 or O139 to obtain challenge data for both serogroups. Briefly, for the challenge, the 5- to 6-day-old neonates of immunized and sham-immunized mice were separated from their dams for 1 h, anesthetized by inhalation of 2.5% isoflurane gas, and orally infected with approximately 6×10^4 CFU/mouse (exact input doses ranged from 3.4×10^4 to 2.3×10^5 CFU/mouse), which is about 40-fold (O139) or 300-fold (O1) the 50% infective dose (ID_{50}) (31–33). Infected mice were given back to their respective dams. At 24 h postinfection, neonates were sacrificed, and the small intestine was removed from each neonatal mouse by dissection and mechanically homogenized in 1 ml LB with 20% glycerol. Appropriate dilutions were plated on LB plates supplemented with Sm to determine viable counts. The limit of detection was 10 CFU/small intestine.

In addition, a passive protection study using purified antisera in combination with the infant mouse model was performed to assess the protective quality of antisera directed against certain surface antigens in more detail. The anti-IgG1 titer of each serum was determined via enzyme-linked immunosorbent assay (ELISA) using the appropriate coating antigen (OMVs, FlaA, OmpU, or LPS) to allow an adjustment to equal concentrations in the assay. For the inoculum, a *V. cholerae* O1 culture was adjusted to an OD_{600} of 0.003 and subsequently mixed in a 1:2 ratio with appropriate dilutions of anti-WT (O1) OMVs, anti-Δ*waaL* Δ*msbB* OMVs, anti-O1 antigen (BD Biosciences), anti-FlaA (this study), or anti-OmpU serum (this study) to achieve a final IgG1 titer of 9 μg/ml. After 30 min of incubation at room temperature, neonatal mice born to naive dams were infected with 50 μl of the respective inoculum, resulting in an infectious dose of 10^5 CFU/mouse. At 24 h postinfection, neonates were sacrificed and the colonization was determined as described above.

Collection of samples. To monitor the immune response, blood samples were collected from immunized and sham-immunized mice on days 0, 14, 28, and 38 by lateral tail vein nick as well as by cardiac puncture at day 86. The samples were processed as described previously and stored at −70°C (32, 33).

Dot blot analysis. Different amounts of LPS were absorbed on a nitrocellulose membrane (Amersham) and incubated for 2 h at 37°C. After 1 h of blockage with 5% skim milk in TBS (10 mM Tris-Cl, pH 7.5, 150 mM NaCl), the membrane was washed once in TBS before the diluted mouse serum (1:500 in 5% skim milk) was added to the membrane and incubated overnight at 4°C. The membrane was washed three times with TBS for 5 min each and incubated with a secondary antibody (horseradish peroxidase-conjugated goat anti-mouse IgM plus IgG plus IgA [H+L]; Southern Biotech) diluted 1:7,500 in skim milk (5% in TBS) for 2 h at room temperature. Subsequently, the membrane was washed three times in TBS for 5 min each, and luminol-enhanced chemiluminescence was detected using a ChemiDoc XRS system (Bio-Rad Laboratories).

SDS-PAGE and immunoblot analysis. To separate proteins, the standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) procedure in combination with 15% gels and the Prestained Protein Marker Broad Range (New England BioLabs) as a molecular mass standard was used. Approximately 5 μg protein was loaded for each sample. Proteins were stained according to the procedure of Kang et al. (68) or transferred to a nitrocellulose membrane (Amersham) for immunoblot analysis, which was performed as described previously (69).

ELISA. The quantification of different Igs and the half-maximum total Ig titers to OMVs and the determination of the half-maximum total Ig titers to LPS were carried out essentially as described previously (32, 33, 69). Briefly, appropriate OMVs (5 μg/ml in PBS, pH 7.4) or LPS (7 μg/ml in PBS, pH 7.4) served as coating material as well as appropriate Ig isotype standards (BD Biosciences). Horseradish peroxidase-conjugated goat anti-mouse antibodies (BD Biosciences) were used as secondary antibodies. Plates were developed using the TMB peroxidase substrate reagent set (BioLegend) according to the manufacturer's manual. Optical densities were calculated at 450 nm with a FLUOstar Omega microplate reader (BMG Labtech).

Motility assay. The motility assay was essentially performed as previously described (31). Briefly, the anti-IgG1 titer of each serum derived from mice immunized with OMVs, purified His-FlaA, or His-OmpU as well as the commercially available anti-O antigen serum (BD Biosciences) was determined via ELISA using the appropriate coating antigen (OMVs, FlaA, OmpU, or LPS) to allow an adjustment to equal concentrations in the assay. Sera of sham-immunized mice were diluted by the lowest dilution factor needed for the immune serum to obtain 6 μg/ml anti-IgG1 in the same experiment. *V. cholerae* was taken from an LB plate supplemented with Sm, resuspended in LB broth at an OD_{600} of 0.16, and mixed with heat-inactivated serum (56°C, 30 min) in a 1:2 ratio to achieve a final IgG1 titer of 6 μg/ml. After 10 to 30 min of incubation at room temperature, 5 μl of the culture was placed on a slide under a coverslip and viewed with a 40× objective by dark-field microscopy (Olympus CH-2). Motile bacteria appeared as lines or swirls, whereas nonmotile bacteria appeared as bright dots. The images were taken using a 456-ms exposure (TCA-3.0C; Mueller, Germany). To analyze the viability of the bacteria after treatment with serum samples, *V. cholerae* was mixed with the respective sera as described for the motility assay (see above). After 10 to 30 min of incubation at room temperature, samples were rigorously vortexed to dissolve potential aggregates, and appropriate dilutions were plated for CFU counts.

Statistical analysis. Data were analyzed using the Mann-Whitney U test or a Kruskal-Wallis test followed by *post hoc* Dunn's multiple comparisons. Differences were considered significant at P values of <0.05. For all statistical analyses, GraphPad Prism version 4.0a was used.

RESULTS

OMVs derived from Δ*msbB* and Δ*waaL* mutants are defined by alterations in the LPS. MsbB (also referred to as LpxN) was recently characterized as a functional secondary lipid A acyltransferase of *V. cholerae*, which is required for transferring a 3-hydroxylaureate to the 3′ position of the glucosamine disaccharide (70). Consequently, an *msbB* mutant strain lacks a 3-hydroxylaureate of lipid A, resulting in LPS that is underacylated compared to that of WT (O1). In general, such underacylated LPS has been demonstrated to exhibit less endotoxicity due to reduced stimu-

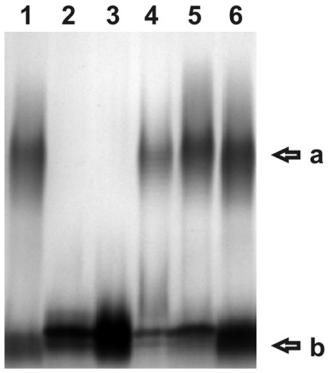

FIG 1 SDS-PAGE analysis of full-length LPS and lipid A-core OS precursor of *V. cholerae* WT (O1) and mutants used in this study. Shown are the isolated surface polysaccharides separated by SDS-PAGE (15%) and silver stained from WT (O1) pBK (lane 1), Δ*waaL* Δ*msbB* pBA (lane 2), Δ*waaL* Δ*msbB* pmsbB (lane 3), Δ*waaL* Δ*msbB* pwaaL (lane 4), Δ*msbB* pBA (lane 5), Δ*msbB* pmsbB (lane 6). Arrows indicate the positions of the full-length LPS (a) and the lipid A-core OS precursor (b).

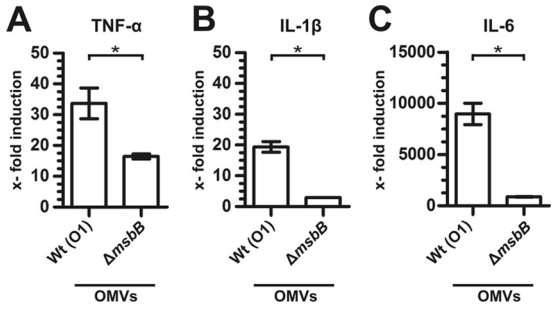

FIG 2 OMVs derived from Δ*msbB* lead to reduced endotoxicity. Shown is the induction of TNF-α (A), IL-1β (B), and IL-6 (C) in RAW macrophages after treatment with OMVs (100 ng/ml) derived from WT (O1) or Δ*msbB* compared to the nonstimulated control group and normalized to the housekeeping gene 36B4. RNA of stimulated or nonstimulated macrophages was isolated after 6 h of incubation with OMVs and analyzed via qRT-PCR. Each data set represents the mean and standard deviation from at least four independent experiments. Significant differences between the data sets are marked by asterisks ($P < 0.05$; Mann-Whitney U test).

lation of TLR-4 (70–74). We constructed a Δ*msbB* strain to test if the OMVs with underacylated LPS exhibit less endotoxicity but retain their immunogenicity.

The hydroxyl group of the 3-hydroxylaureate transferred by MsbB serves as the site of a glycine modification, which in turn confers resistance to polymyxin B (75). Consequently, *msbB* mutants have been demonstrated to exhibit an increased polymyxin B sensitivity (70, 71). In concordance with these reports (70, 71, 75), the Δ*msbB* mutant with the empty vector control displayed a higher sensitivity to the antimicrobial peptide polymyxin B than did WT (O1) with the empty vector control (see Fig. S4 in the supplemental material). This defect was partially complemented by the expression of *msbB* in *trans*. An incomplete rescue of this nature is not surprising, given the fact that expression levels from inducible plasmids rarely match the same levels detected in the wild type and slightly altered amounts of membrane associated enzymes might already affect membrane integrity. Accordingly, partial complementation has been reported previously for other LPS biosynthesis gene products (76–78).

Furthermore, we visualized the LPS of WT (O1) and Δ*msbB*, both containing the control plasmid, as well as the complementation strain by SDS-PAGE and silver staining (Fig. 1). The WT (O1), Δ*msbB*, and Δ*msbB* pmsbB strains exhibited similar migration patterns of the LPS bands as well as comparable levels of the fully synthesized LPS (Fig. 1, lanes 1, 5, and 6). Finally, we determined the ratio of 3-hydroxylaureate to 3-hydroxymyristate in phospholipid-purified LPS samples derived from WT (O1) and the Δ*msbB* mutant by mass spectrometry to reveal the fatty acid missing in the Δ*msbB* mutant. A proportional reduction of 3-hydroxylaureate for the Δ*msbB* mutant LPS compared to the WT (O1) LPS was observed (see Fig. S3 in the supplemental material). This result is consistent with a comprehensive lipid A analysis of a Δ*msbB* mutant reported previously by Hankins et al. (70), which already demonstrated that MsbB transfers a 3-hydroxylaureate to the 3′-linked primary acyl chain of *V. cholerae* lipid A. In summary, the LPS of the Δ*msbB* mutant lacks one fatty acid in lipid A, while further synthesis of the LPS and the attachment of the O antigen are not affected.

To investigate the endotoxicity, RAW macrophages were stimulated *in vitro* with purified OMVs derived either from WT (O1) or the Δ*msbB* mutant. Subsequently, RNA was isolated from the OMV-stimulated macrophages and assayed for the induction of inflammatory markers like TNF-α, IL-1β, and IL-6 via qRT-PCR (Fig. 2). Nonstimulated RAW macrophages grown in parallel served as a control. As expected, the expression of all three cytokines, which are part of the inflammatory response pathway activated by LPS, was greatly induced by WT (O1) OMVs, ranging from 30-fold for TNF-α to 20-fold for IL-1β and 9,000-fold for IL-6. In contrast, the inflammatory response after stimulation with Δ*msbB* OMVs was much weaker and reached only 10 to 50% of the level detected for WT (O1) OMVs. This indicates a significant lower endotoxicity of the Δ*msbB* OMVs due to its underacylated, detoxified LPS.

In addition to the Δ*msbB* mutation, we created a second OMV donor strain and additionally deleted *waaL*, encoding the functional O antigen ligase of *V. cholerae* (79). Hence, the LPS of the Δ*waaL* Δ*msbB* mutant consists of an underacylated lipid A and lacks the O antigen. The latter was confirmed by visualization of the LPS by SDS-PAGE and silver staining (Fig. 1, lane 2). The Δ*waaL* Δ*msbB* mutant shows only the lipid A core oligosaccharide (lipid A-core OS) without any O antigen attached. The expression of *waaL* in *trans*, but not that of *msbB*, restored the attachment of the O antigen and the presence of fully synthesized LPS (Fig. 1, lanes 3 and 4).

Furthermore, we analyzed the protein profile of the purified OMVs derived from WT (O1), Δ*msbB*, and Δ*waaL* Δ*msbB* by SDS-PAGE in combination with Kang's staining method (Fig. 3). In comparison to the protein profile of the WT (O1) OMVs, the Δ*msbB* OMVs lack one band of approximately 28 kDa and the Δ*waaL* Δ*msbB* OMVs exhibit an increase of some high-molecular-weight bands and two protein bands at approximately 40 kDa (Fig. 3, lanes 1, 2, and 3). Such changes might indicate a role of the LPS in the sorting of the OMV cargo, which is currently being investigated in our laboratory. In summary, although the mutant strains display distinct differences in their OMV protein profiles, the most abundant protein bands between 25 and 46 kDa are present in all OMVs analyzed (Fig. 3).

Immunization with OMVs derived from Δ*msbB* and Δ*waaL* Δ*msbB* induces a robust immune response. To characterize the

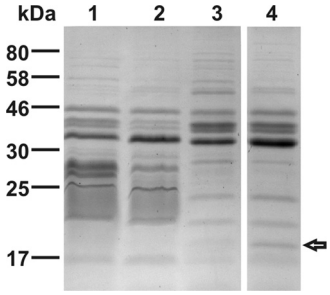

FIG 3 Protein profile comparisons of OMVs used for immunization in this study. Depicted are the protein profiles of purified OMVs derived from WT (O1) (lane 1), Δ*msbB* (lane 2), and Δ*waaL* Δ*msbB* (lane 3), as well as Δ*waaL* Δ*msbB* grown in AKI broth (lane 4). Samples were separated by SDS-PAGE (15%) and Coomassie blue stained according to the method of Kang et al. (38). The arrow highlights the band present only in Δ*waaL* Δ*msbB* grown in AKI broth, which was identified as TcpA by mass spectrometry. Lines to the left indicate the molecular masses of the protein standards in kDa.

immunogenic properties of OMVs derived from the WT (O1), Δ*msbB*, and Δ*waaL* Δ*msbB* strains, we i.n. immunized female mice with 25 μg (protein equivalents) per immunization of the respective OMVs according to our standard immunization protocol, which consists of an initial immunization followed by two boosts at days 14 and 28 (33). As will become evident below, the LPS present in the OMVs plays a crucial role for induction of a protective immunity. Thus, we also quantified the LPS content of the OMVs using a Purpald assay (63), which revealed comparable amounts of LPS in OMV samples derived from WT (O1), Δ*msbB*, and Δ*waaL* Δ*msbB* (see Table S2 in the supplemental material). Hence, the OMV samples derived from WT (O1) and mutant strains used for the immunization studies contained similar amounts of protein as well as LPS. Mice sham immunized with PBS served as a nonvaccinated control group. The temporal immune responses and the half-maximum total Ig titers in sera of immunized or nonvaccinated female mice were monitored by ELISA using WT (O1) OMVs as coating material. As an example, the progression of the temporal IgG1 response is shown in Fig. 4 for all immunization groups. At day 0, the median IgG1 titers to WT (O1) OMVs were relatively low and showed no significant differences between the groups tested. In general, the IgG1 titers of all immunization groups increased during the vaccination period,

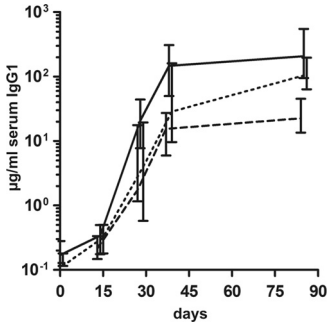

FIG 4 Temporal immune responses to OMVs. Shown are the median titers over time of IgG1 antibodies to OMVs derived from WT (O1) in sera from mice intranasally immunized with WT (O1) OMVs (solid line), Δ*msbB* OMVs (dotted line), and Δ*waaL* Δ*msbB* OMVs (dashed line) ($n \geq 6$ for each group). The error bars indicate the interquartile range of each data set for each time point.

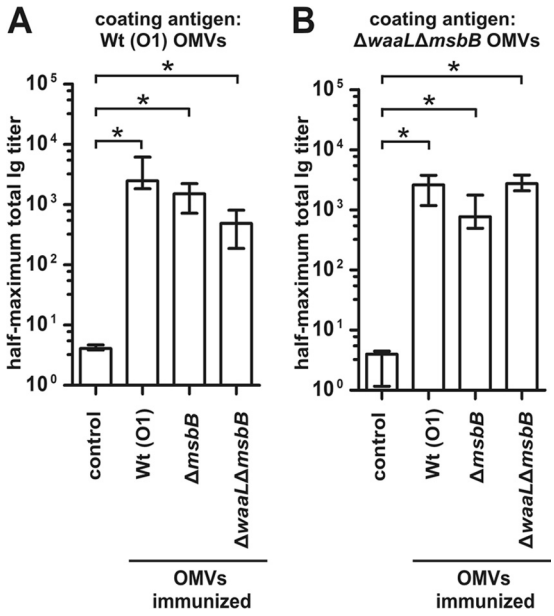

FIG 5 Half-maximum total Ig titers to OMVs. Shown are the median half-maximum total Ig titers to OMVs derived from WT (O1) (A) as well as Δ*waaL* Δ*msbB* (B) in sera collected at day 86 from mice intranasally immunized with WT (O1) OMVs, Δ*msbB* OMVs, and Δ*waaL* Δ*msbB* OMVs and from the nonvaccinated control group ($n \geq 6$ for each group). The error bars indicate the interquartile range of each data set. Significant differences between the data sets are indicated by asterisks ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons).

with the highest level detected on day 86. Noteworthy, the mice immunized with WT (O1) or Δ*msbB* OMVs showed similar median IgG1 titers at the endpoint of the experiment, while the median IgG1 titer of the mice immunized with Δ*waaL* Δ*msbB* OMVs was significantly lower ($P < 0.05$; Mann-Whitney U test). The antibody titers of the nonvaccinated control group were monitored at the beginning and the end of the experiment and remained below the limit of detection for both time points (data not shown).

Additionally, we determined the half-maximum total Ig titers against WT (O1) and Δ*waaL* Δ*msbB* OMVs in sera collected from immunized mice as well as from nonvaccinated mice at day 86 (Fig. 5A and B). The quantification of the total immune response allowed us to compare the overall immunogenicity and the effectiveness of each tested vaccine candidate. The median half-maximum total Ig titers of all immunization groups were significantly higher than those of the nonvaccinated control group ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons). Consistent with the temporal immune response, the mice immunized with Δ*waaL* Δ*msbB* OMVs showed a significant 4-fold-lower level of the half-maximum total Ig-titers compared to the WT (O1) OMV immunization group ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons). It should be noted that these Ig titers were determined by ELISA using WT (O1) OMVs as coating material. Based on the observed alterations in the protein profile and LPS in the double mutant, it could be speculated that mice immunized with Δ*waaL* Δ*msbB* OMVs developed a slightly different specificity of the immune response compared to the mice immunized with WT (O1) OMVs. For example, Δ*waaL* Δ*msbB* OMV-immunized mice might have raised

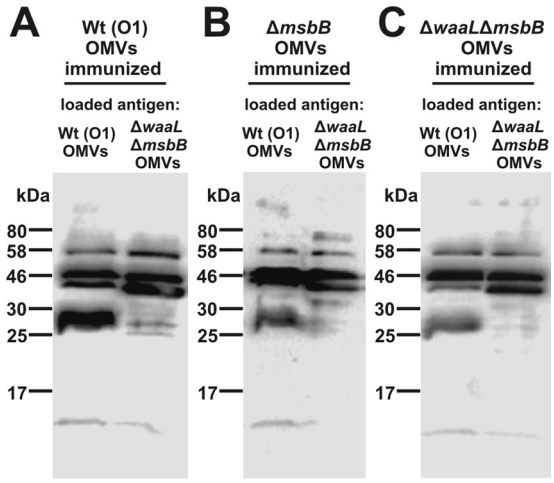

FIG 6 Immunoblot analysis of the IgG reactivity against *V. cholerae* surface proteins in sera from OMV-immunized mice. Representative immunoblots were loaded with WT (O1) OMVs as well as Δ*waaL* Δ*msbB* OMVs as indicated above each blot and incubated with sera collected at day 86 from mice immunized with WT (O1) OMVs (A), Δ*msbB* OMVs (B), or Δ*waaL* Δ*msbB* OMVs (C). Lines to the left indicate the molecular masses of the protein standards in kDa.

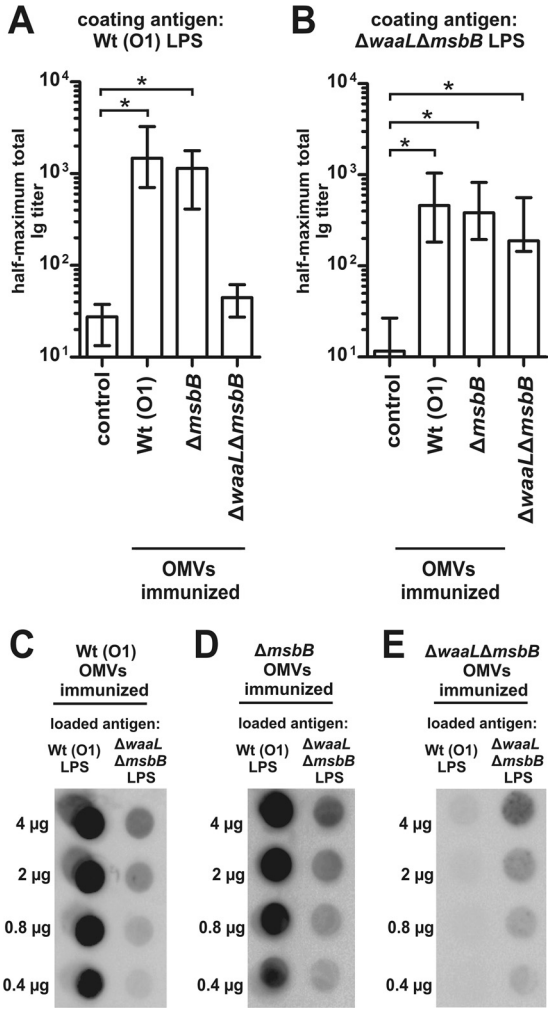

FIG 7 Characterization of the immune response against LPS in sera from OMV-immunized mice. (A and B) Half-maximum total Ig titers to LPS isolated from WT (O1) (A) or Δ*waaL* Δ*msbB* (B). Results are shown for sera collected at day 86 from mice intranasally immunized with WT (O1) OMVs, Δ*msbB* OMVs, and Δ*waaL* Δ*msbB* OMVs and from the nonvaccinated control group ($n \geq 6$ for each group). The error bars indicate the interquartile range of each data set. Significant differences between the data sets are indicated by asterisks ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons). (C, D, and E) Dot blot analysis of total Ig reactivity against LPS in sera from mice immunized with WT (O1) OMVs (C), Δ*msbB* OMVs (D), or Δ*waaL* Δ*msbB* OMVs (E). Representative dot blots were loaded with purified WT (O1) LPS as well as Δ*waaL* Δ*msbB* LPS as indicated above each blot and incubated with sera collected at day 86 from immunized mice of the respective immunization groups. Different amounts of the isolated LPS ranging from 0.4 to 4 μg were absorbed on a nitrocellulose membrane as indicated on the left side.

antibodies against antigens, which are either masked or less abundant in WT (O1) OMVs. Thus, analysis of the immune response using WT (O1) OMVs as coating antigen might not be the best approach to detect the overall induced immune response in Δ*waaL* Δ*msbB* OMV-immunized mice. Therefore, we reanalyzed the half-maximum total Ig titers of all groups using Δ*waaL* Δ*msbB* OMVs as coating material (Fig. 5B). This alternative analysis resulted in higher median half-maximum total Ig titers for the Δ*waaL* Δ*msbB* OMV-immunized mice, while the titers of the other groups remained at a similar level, independent of the coating antigen. Consequently, the half-maximum total Ig titers of the WT (O1) OMV and Δ*waaL* Δ*msbB* OMV-immunized mice were not significantly different when Δ*waaL* Δ*msbB* OMVs were used as coating material. In summary, all immunization groups displayed a robust immune response with similar levels of Ig titers, but the results obtained by ELISA suggest differences in the specificity of the immune response.

Immunization with OMVs derived from WT (O1) or Δ*waaL* Δ*msbB* elicits different antibody specificity to LPS. To investigate the specificity of antibodies against proteins present in the OMVs, immunoblot analysis was performed using WT (O1) and Δ*waaL* Δ*msbB* OMVs as antigens. Representative immunoblots were incubated with sera collected at day 86 from one mouse of each immunization group as well as from the nonvaccinated control group (Fig. 6). No bands were detected on immunoblots using sera from nonvaccinated control group mice (data not shown). In concordance with previous studies (33), multiple bands were detected in the OMV protein profile using sera of immunized mice. Similar band patterns appeared without obvious differences in any sera of the three immunization groups. Thus, the groups immunized with OMVs derived from WT (O1), Δ*msbB*, or Δ*waaL* Δ*msbB* OMVs induced a similar antibody specificity on the protein level. Furthermore, the most immunogenic proteins, relevant for the detected antibody response, have to be present in WT (O1), Δ*msbB*, or Δ*waaL* Δ*msbB* OMVs.

Next, we analyzed the immune response and antibody specificity raised against the LPS structure by ELISA and dot blot analysis. We determined the half-maximum total Ig titers in sera from all immunization groups using either purified WT (O1) or Δ*waaL* Δ*msbB* LPS as coating material (Fig. 7A and B, respectively). The WT (O1) LPS represents full-length LPS with O antigen attached, while the double mutant LPS consists only of the lipid A-core OS. Independent of the coating material, immunization with WT (O1) or Δ*msbB* OMVs induced a comparable anti-LPS immune response, which was significantly higher than the nonvaccinated

control group ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons). In contrast, the detected immune response against LPS in sera of the Δ*waaL* Δ*msbB* OMV immunization group depends on the coating material. Against purified WT (O1) LPS, this immunization group exhibits only low half-maximum total Ig titers, which were not significantly different from those of the nonvaccinated control group ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons). However, the half-maximum total Ig titers of the Δ*waaL* Δ*msbB* OMV immunization group were much higher when purified Δ*waaL* Δ*msbB* LPS was used and reached levels comparable to those of the groups immunized with WT (O1) or Δ*msbB* OMVs.

We performed dot blot analysis to confirm the differences in the specificity of the LPS antibody response observed by ELISA. Dot blots were loaded with serial dilutions of the purified full-length WT (O1) LPS or Δ*waaL* Δ*msbB* LPS and subsequently incubated with sera collected from immunized mice (Fig. 7C to E). Neither the full-length nor the precursor LPS was detected on dot blots using sera from nonvaccinated control group mice (data not shown). The lipid A-core OS precursor was detected at similar intensities by the sera of all immunization groups in a concentration-dependent manner (Fig. 7C to E, right lanes). This indicates that the lipid A-core OS serves as an immunogenic antigen in all OMV-immunized mice. In contrast, the full-length LPS could be detected only using sera of WT (O1) or Δ*msbB* OMV-immunized mice, but not using sera of Δ*waaL* Δ*msbB* OMV-immunized mice (Fig. 7C to E, left lanes). This suggests that only mice immunized with OMVs containing full-length LPS with attached O antigen raised antibodies that are capable of recognizing such full-length LPS. This indicates that only the mice immunized with WT (O1) or Δ*msbB* OMVs have raised antibodies against the O antigen and most of the immune response against the LPS in these immunization groups is indeed directed against the O antigen. In summary, the difference in the antibody specificity directed against the LPS with attached O antigen discriminates between the immune response induced in mice immunized with WT (O1) and Δ*msbB* OMVs and that induced in the group immunized with Δ*waaL* Δ*msbB* OMVs.

OMVs lacking the O antigen fail to induce a protective immune response. In order to investigate whether the induced humoral immune response also results in protection against *V. cholerae* infection, we used a modified version of the infant mouse model, wherein the offspring of immunized dams were challenged and the level of protection was measured by the degree of colonization in the small intestine. Since protection in our model correlates mainly with the access to breast milk from immunized dams, the infected neonatal mice were given back to their respective dams for the challenge period (32, 33). In order to investigate the protection against both clinically relevant serogroups, the offspring of each immunization group were divided into two subgroups and challenged orally with approximately 6×10^4 CFU/mouse with either the homologous *V. cholerae* strain O1 or the heterologous strain O139. These infection doses correspond to 40-fold (O139) or 300-fold (O1) the ID_{50} and have been used previously for challenge experiments (31–33). The results are presented in Fig. 8. In the case of a challenge with serogroup O1, neonatal mice of the nonvaccinated control group as well as of the Δ*waaL* Δ*msbB* immunization group were stably colonized with a median colonization level of around 10^5 to 10^6 CFU/small intestine (Fig. 8A). In contrast, neonatal mice from WT (O1) or Δ*msbB* OMV-immunized mice showed a significantly reduced colonization or were completely protected from colonization with serogroup O1. Challenging neonatal mice from dams immunized with any OMV derivatives with a heterologous *V. cholerae* strain (O139) resulted in colonization with similar numbers of recovered CFU of approximately 10^4 CFU/small intestine (Fig. 8B). Taken together, the results of the challenge experiments reveal a correlation between the presence of antibodies directed against the respective O antigen and the induction of a protective immune response. Consequently, all immunization groups showed no protection against O139, and the mice immunized with Δ*waaL* Δ*msbB* OMVs failed to demonstrate protection against O1 and O139.

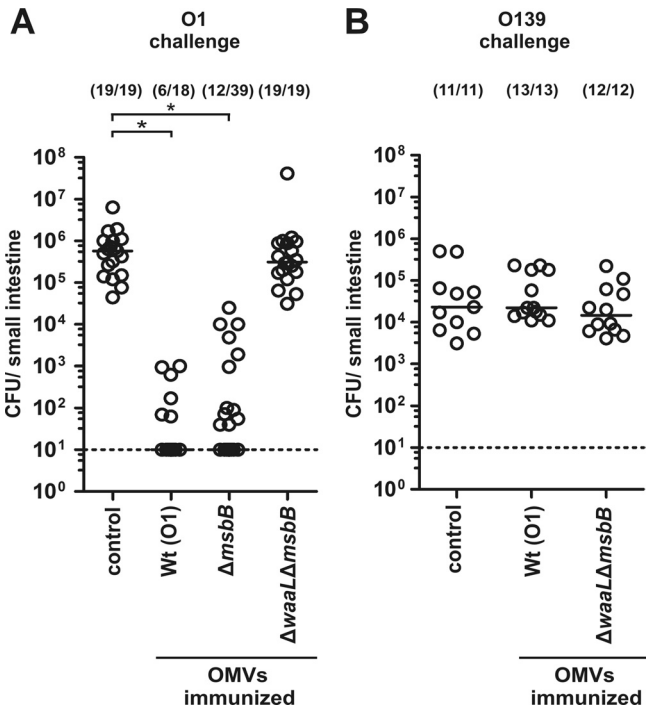

FIG 8 Homologous and heterologous *V. cholerae* challenge of neonates born to mice immunized with OMVs derived from WT (O1), Δ*msbB*, and Δ*waaL* Δ*msbB*. The numbers of recovered CFU/small intestine are shown for neonates born to immunized as well as nonvaccinated mice, which were challenged with WT serogroup O1 (A) or WT serogroup O139 (B). Each circle represents the number of CFU from one neonatal mouse, and the horizontal bar indicates the median of each data set. Neonatal mice were challenged with an infection dose approximately 300- or 40-fold greater than the ID_{50} for O1 or O139, respectively. When no bacteria were recovered, the number of CFU was set to the limit of detection of 10 CFU/small intestine (dotted line). The numbers of infected mice are given above the respective data set as the ratio of the number of mice with detectable colonization to the total number of challenged mice. Significant differences between the data sets are indicated by asterisks ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons).

The presence of antibodies directed against an abundant surface structure, such as the O antigen, is ostensibly crucial for a protective immune response. Interestingly, the toxin-coregulated pilus (TCP) of *V. cholerae*, which represents an important colonization factor, is also an associated surface structure and was reported to be a protective antigen in a passive immunization study using the infant mouse model (67, 80, 81). We wanted to test whether the lack of the O antigen can be compensated for by the presence of the TCP representing an alternative surface antigen.

Thus, OMVs derived from Δ*waaL* Δ*msbB* were purified under TCP-expressing conditions using AKI broth (58, 59). In the protein profile of the Δ*waaL* Δ*msbB* OMVs grown under AKI conditions, a band at approximately 18 kDa is more intense than the same band for OMVs derived from the isogenic strain grown in LB broth (Fig. 3, lane 4). Analysis by mass spectrometry of this band yielded a mass that was consistent with TcpA, which is the major subunit of the pilus. Thus, we confirmed the expression of TCP under AKI and verified the association of the pilus with the OMVs. These TCP-decorated OMVs were used for an immunization study as described above. Again, LPS quantification using a Purpald assay revealed amounts of LPS in Δ*waaL* Δ*msbB* OMVs derived from an AKI culture that were similar to those of OMVs isolated from LB grown cultures (see Table S2 in the supplemental material). In general, characterization of the qualitative and quantitative humoral immune response directed against proteins or LPS revealed no differences between the mice immunized with Δ*waaL* Δ*msbB* OMVs derived from an LB culture or the AKI culture, respectively (data not shown). Additionally, we determined the immune response against TcpA in sera of mice immunized with both versions of the Δ*waaL* Δ*msbB* OMVs by ELISA (Fig. 9A). The mice immunized with AKI-derived Δ*waaL* Δ*msbB* OMVs exhibited a small but significant 3-fold increase of antibodies against TcpA compared to the group immunized with the LB-derived OMVs. However, in comparison to the pronounced immune response against other proteins or LPS, the titers against TcpA are quite low. Thus, either the amount of TcpA associated with the OMVs was too low or TcpA is not highly immunogenic in the context of *V. cholerae* OMVs. Neonatal mice born to dams immunized with AKI-derived Δ*waaL* Δ*msbB* OMVs were split into two groups and challenged with *V. cholerae* serogroup O1 or serogroup O139. The O1 challenge revealed a stable colonization of around 10^5 to 10^6 CFU/small intestine and consequently no protection (Fig. 9B). In the case of the O139 challenge, the median colonization rate was 5×10^3 CFU/small intestine and therefore slightly reduced compared to nonvaccinated control mice or mice immunized with LB-derived OMVs (Fig. 8B and 9C). Furthermore, one neonatal mouse had no recoverable CFU and two neonatal mice exhibited a very low colonization (Fig. 9C). Nevertheless, a significant reduction of the colonization level, with the majority of mice not colonized, could not be observed. Thus, isolation of OMVs derived from AKI cultures results in the presence of TcpA, but this surface antigen is insufficient to induce a protective immune response and therefore cannot compensate for the lack of the O antigen.

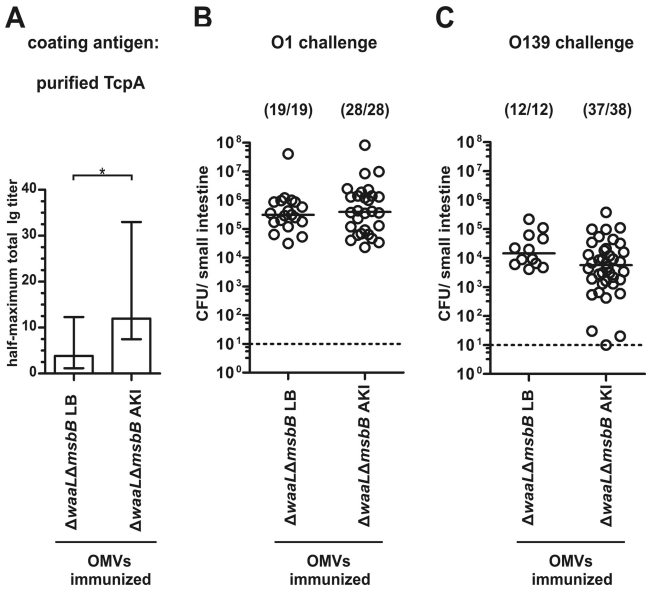

FIG 9 Analysis of the immune response induced by intranasal immunization of mice with Δ*waaL* Δ*msbB* OMVs grown under AKI conditions. (A) Determination of the immune response against purified TcpA in sera of mice immunized with Δ*waaL* Δ*msbB* OMVs derived from an LB culture (Δ*waaL* Δ*msbB* LB) or an AKI culture (Δ*waaL* Δ*msbB* AKI) ($n \geq 6$ for each group). Shown are the median half-maximum total Ig titers to purified TcpA. The error bars indicate the interquartile range of each data set. Significant differences between the data sets are marked by asterisks ($P < 0.05$; Mann-Whitney U test). (B and C) Homologous and heterologous *V. cholerae* challenge of neonates born to mice immunized with Δ*waaL* Δ*msbB* OMVs derived from an AKI culture (Δ*waaL* Δ*msbB* AKI). Although already presented in Fig. 8, the respective results obtained by the *V. cholerae* challenge of neonates born to mice immunized with Δ*waaL* Δ*msbB* OMVs derived from an LB culture (Δ*waaL* Δ*msbB* LB) are included in these graphs to allow a direct comparison. The numbers of recovered CFU/small intestine are shown for neonates born to mice immunized with Δ*waaL* Δ*msbB* OMVs derived from an AKI or LB culture, which were challenged with serogroup O1 (B) or O139 (C). Each circle represents the number of CFU from one neonatal mouse, and the horizontal bar indicates the median of each data set. Neonatal mice were challenged with an infection dose approximately 300- or 40-fold greater than the ID_{50} for O1 or O139, respectively. When no bacteria were recovered, the number of CFU was set to the limit of detection of 10 CFU/small intestine (dotted line). The numbers of infected mice are given above the respective data set as the ratio of the number of mice with detectable colonization to the total number of challenged mice.

O antigen antibodies are highly effective in inhibition of motility *in vitro* and essential for protection *in vivo*. Previous results suggested that protection of neonatal mice from *V. cholerae* infection occurs through the inhibition of bacterial motility by antibodies from OMV-immunized dams (31). We hypothesized that the blockage occurs via antibodies directed against the O antigen, which might bind to the LPS present in the outer membrane sheath that covers the single polar flagellum of *V. cholerae*. We performed an established *in vitro* motility assay in which pooled sera of OMV-immunized or nonvaccinated mice as well as specific antibodies were mixed 1:2 with a bacterial suspension of a *V. cholerae* strain serogroup O1 and examined by dark-field microscopy using a long exposure (Fig. 10A) (31). Motile bacteria appear as swirls or lines, whereas nonmotile bacteria appear as bright dots. Furthermore, we quantified the number of motile bacteria per field in each image (Fig. 10B). Sera from nonvaccinated control mice were used as a control and did not result in any inhibition of motility. Only the use of sera from WT (O1) OMV-immunized mice, which contain O antigen antibodies, as well as the use of commercially available antisera directed against the O1 antigen, results in complete blockage of motility. Neither serum from Δ*waaL* Δ*msbB* OMV-immunized mice, nor FlaA antiserum, targeting the major flagellin subunit of the *V. cholerae* flagellum, nor OmpU antiserum, targeting the most abundant outer membrane porin of *V. cholerae*, caused a reduction of the motility. For serum blocking motility, viability of *V. cholerae* was determined by plating for CFU counts after 30 min of incubation. Sera from WT (O1) OMV-immunized mice and commercially available O1 antigen antisera showed no reduction in CFU compared to the PBS control (see Fig. S5 in the supplemental material). Thus, the observed effects cannot be explained by reduced viability upon contact with these sera.

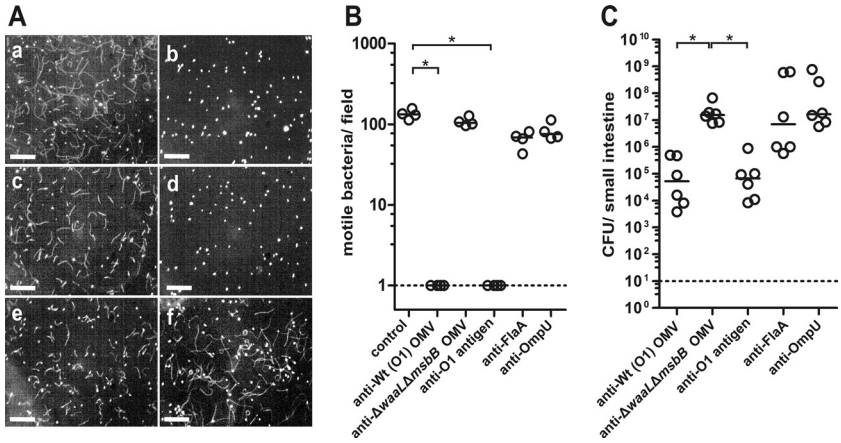

FIG 10 Protective immune response correlates with the presence of O antigen antibodies, which inhibit the motility of *V. cholerae*. (A) Shown are representative images using *V. cholerae* serogroup O1 (WT) (OD_{600} = 0.16) mixed with nonvaccinated control sera (a), sera of WT (O1) OMV-immunized mice (b), sera of Δ*waaL* Δ*msbB* OMV-immunized mice (c), anti-O1 antigen antibodies (d), anti-FlaA antibodies (e), and anti-OmpU antibodies (f). After 10 min of incubation, the bacterial motility was visualized by dark-field microscopy using a 456-ms exposure. Motile bacteria appear as lines or swirls and nonmotile bacteria as dots. Bars, 50 μm. (B) Quantification of motility in the presence of nonvaccinated control sera, sera of WT (O1) OMV-immunized mice, sera of Δ*waaL* Δ*msbB* OMV-immunized mice, anti-O1 antigen antibodies, anti-FlaA antibodies, or anti-OmpU antibodies. Each symbol represents an independent experiment, and the horizontal bar indicates the median of each data set. When no motile bacteria were visible, the number was set to the limit of detection of 1 bacterium/field (dotted line). Significant differences between the data sets are indicated by asterisks ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons). (C) Passive immunization of neonates born to naive dams using *V. cholerae* serogroup O1 (WT) mixed with sera of WT (O1) OMV-immunized mice, sera of Δ*waaL* Δ*msbB* OMV-immunized mice, anti-O1 antigen antibodies, anti-FlaA antibodies, or anti-OmpU antibodies. Significant differences between the data sets are indicated by asterisks ($P < 0.05$; Kruskal-Wallis test and *post hoc* Dunn's multiple comparisons).

To prove that antibodies directed against the O antigen are sufficient for protection against *V. cholerae* infection, we performed a passive immunization study (Fig. 10C). Naive infant mice were subdivided into five groups and challenged with a defined infection dose of *V. cholerae* serogroup O1 mixed with one of the five antisera also used in the motility assay, separately. The level of protection was measured by the degree of colonization in the small intestine after 24 h. Sera from WT (O1) OMV- or Δ*waaL* Δ*msbB* OMV-immunized mice served as internal controls for the passive immunization assay, since the respective immunized mice showed either a high or no protective immune response against challenge with *V. cholerae* O1 (Fig. 8A). As expected, mice passively immunized with sera from Δ*waaL* Δ*msbB* OMV-immunized mice exhibited a high colonization of 10^7 CFU/small intestine, while mice passively immunized with sera from WT (O1) OMV-immunized mice showed only a median colonization of 10^5 CFU. The groups that received anti-O1 antigen antibodies showed a significant 100-fold reduction in their colonization level, whereas the other groups receiving either anti-FlaA or anti-OmpU antibodies were stably colonized, with a median colonization level of around 10^7 CFU/small intestine. Thus, the observed colonization rates in the passive immunization assay reflect the results obtained from the *in vitro* motility assay.

DISCUSSION

In previous studies, we introduced OMVs of *V. cholerae* as an alternative vaccine candidate against cholera infections and demonstrated the induction of a robust protective immune response upon immunization with OMVs using the mouse model. So far, we primarily used unmodified *V. cholerae* OMVs with substantial amounts of lipid A as part of the LPS. Such a hexa-acylated lipid A moiety is a very potent activator of TLR-4, which could result in adverse vaccine reactogenicity and safety limitations for human applications. The LPS content can be reduced by detergent extraction, which is applied for production of classical *N. meningitidis* OMV vaccines used to control epidemics of serogroup B disease in Norway, Cuba, Chile, Brazil, and New Zealand. As demonstrated in this study, the O antigen is the dominant protective antigen of the *V. cholerae* OMV vaccine. Thus, we focused on a different approach to minimize the potential toxicity based on the use of *V. cholerae* strains with genetically detoxified LPS.

An important step toward this goal was the recent characterization of a secondary acyltransferase, MsbB, of *V. cholerae* (70, 71). Based on these results, we analyzed OMVs derived from an *msbB* deletion mutant as a detoxified vaccine candidate. OMVs derived from a Δ*msbB* strain demonstrated a significantly lower induction of inflammatory markers compared to WT (O1) OMVs in macrophages, which is consistent with the published literature on underacylated LPS (72, 82, 83). The beneficial effects of mutations resulting in OMV donor strains with underacylated LPS are under investigation for a second generation of OMV-based *N. meningitidis* vaccines (54, 84, 85). The remaining induction of an inflammatory response of detoxified Δ*msbB* OMVs might be caused by the residual activity of the underacylated LPS as well as additional components, i.e., lipoproteins, which are activators of the TLR-2 cascade (86). It is noteworthy that the biggest reduction in response to detoxified Δ*msbB* OMVs was seen for the pyrogenic cytokine IL-6, which is one of the most important markers for determination of clinical toxicity used by the pharmaceutical industry and national control authorities (87).

Throughout the study, we could not detect any differences in the specificity of the humoral immune response on the protein or on the LPS level between WT (O1) OMV- and Δ*msbB* OMV-immunized mice. Naturally, the OMVs with underacylated LPS could have lost some adjuvant activity compared to the WT (O1) OMVs. Indeed, we observed a slight tendency toward a lower im-

mune response in the mice immunized with Δ*msbB* OMVs than in those immunized with WT (O1) OMVs. One could speculate that this might have a negative impact on the efficacy of the OMV vaccine candidate. However, this difference did not affect the induction of a protective immune response in Δ*msbB* OMV-immunized mice. Thus, Δ*msbB* OMVs still induce a robust immune response, and other components in the OMVs might compensate for the loss of the hexa-acylated lipid A as an internal adjuvant. Based on these results, a complex formulation with accessory buffer solutions and additional adjuvants is unlikely to be required for the Δ*msbB* OMVs, which minimizes the production costs of an OMV-based cholera vaccine. Hence, the detoxified OMVs derived from a Δ*msbB* strain with attenuated LPS improve the safety of the vaccine candidate and open up the possibility toward a parenteral administration of the vaccine.

An ideal cholera vaccine should protect against both clinically relevant serogroups O1 and O139. We previously reported that immunization with O1 OMVs could not protect against challenge with O139 and vice versa. However, a mixture of O1 OMVs and O139 OMVs provided protection from colonization upon challenge with both serogroups (31). The same report demonstrated that most of the immune response is directed against LPS, indicating that LPS is the most immunogenic antigen in the OMVs (31). In order to avoid the use of an OMV mixture derived from different donors, we wanted to create a single donor strain for the cholera OMV vaccine production. We hypothesized that elimination of the O antigen as the discrimination marker between the serogroups O1 and O139 might result in a serogroup-independent OMV vaccine candidate. Ideally, the lack of the O antigen should redirect the immune response to alternative OMV antigens present in both serogroups.

To analyze such a potential serogroup-independent vaccine candidate, we used OMVs derived from a Δ*waaL* Δ*msbB* strain providing an underacylated LPS without O antigen. In general, the ELISA studies indicate that Δ*waaL* Δ*msbB* OMV-immunized mice induced a humoral immune response at levels comparable to those of WT (O1) OMV-immunized mice. Thus, Δ*waaL* Δ*msbB* OMVs seem to be as immunogenic as WT (O1) OMVs under the tested conditions. However, some distinct differences in the specificity have been observed. Intriguingly, the highest Ig titers in sera of Δ*waaL* Δ*msbB* OMV-immunized mice were revealed only if Δ*waaL* Δ*msbB* OMVs were used as coating material, while a lower immune response was detected if WT (O1) OMVs were used as coating antigen. In contrast, WT (O1) OMV-immunized mice showed comparable high titers independent of the OMV origin. This might indicate that immunization with Δ*waaL* Δ*msbB* OMVs induces a specific subset of antibodies directed against structures that either are not present or are masked in WT (O1) OMVs. Further analysis of the antibody specificity revealed that all three immunization groups exhibited similar protein-directed immune responses, whereas the Δ*waaL* Δ*msbB* OMV-immunized mice differ in their LPS-directed immune response compared to WT (O1) OMV-immunized mice. Naturally, the full-length LPS with substituted O antigen was present only in WT (O1) and Δ*msbB* OMVs. Thus, only mice receiving these OMVs produced a substantial amount of anti-O antigen antibodies, whereas such antibodies are absent in mice immunized with Δ*waaL* Δ*msbB* OMVs. Nevertheless, all three immunization groups produced a similar response against the lipid A-core OS.

Immunization with Δ*waaL* Δ*msbB* OMVs failed to induce a protective immune response against both *V. cholerae* serogroups O1 and O139. Thus, we did not generate a serogroup-independent vaccine candidate but indirectly revealed that antibodies directed against the O antigen are crucial for the protection from colonization upon challenge with *V. cholerae*. We tested whether the presence of TcpA as an alternative surface antigen might compensate for the lack of the O antigen. TcpA is the major subunit of the main colonization factor TCP and has been suggested as a good target for recombinant subunit vaccines (88–90). Fortunately, expression of TCP can be simply achieved by growing the bacteria in AKI broth (58). Indeed, we found TcpA to be present in the isolated OMVs derived from an AKI culture. However, mice immunized with these OMVs induced only a low immune response against TcpA, and no protection against a challenge using *V. cholerae* O1 or O139 was observed. Thus, the presence of TcpA, expressed from its natural promoter, in OMVs is not sufficient to compensate for the lack of the O antigen. In order to keep the production costs for an OMV-based cholera vaccine low, we favored a relatively simple isolation protocol based on TCP-inducing culture conditions rather than an expression of TcpA using inducible promoter systems. Hence, we cannot exclude that a larger amount of TcpA in the OMVs would have resulted in higher antibody titers against TcpA and might have induced a protective immune response. If necessary, an alternative approach resulting in high TcpA expression can be revisited, but it does not seem to be an essential requirement for an OMV-based vaccine to include TcpA as an antigen.

Recently, we revealed a correlation between the *in vivo* protection upon immunization with OMVs and an inhibition of motility of *V. cholerae* by sera of OMV-immunized mice (31). It was speculated that the anti-LPS antibodies present in the breast milk of the immunized dams block motility of *V. cholerae* in the neonates and thereby prevent a successful colonization. In concordance with this hypothesis, the sera from WT (O1) OMV-immunized mice blocked motility, while sera from Δ*waaL* Δ*msbB* OMV-immunized mice did not. As demonstrated in this study, Δ*waaL* Δ*msbB* OMV-immunized mice induced a similar immune response against the lipid A-core OS, but not against the O antigen. Thus, we hypothesized that anti-O antigen antibodies are crucial for the inhibition of motility. Using defined antisera, we were able to confirm this hypothesis, as only the antisera directed against the O antigen could block motility. Furthermore, a passive immunization experiment using these antisera revealed that only the anti-O antigen antibodies are capable of reducing the colonization at levels comparable to those of sera from WT (O1) OMV-immunized mice. Thus, we can refine our hypothesis and state that anti-O antigen antibodies are essential to block motility by binding to the sheathed flagellum. Interestingly, neither antiserum against the major flagellin, FlaA, nor antiserum against the most abundant outer membrane protein, OmpU, could block motility. Thus, these antigens are either masked by the sheath or not highly abundant in the sheath of the flagellum. Consequently, the O antigen is the dominant protective antigen of the OMV-based cholera vaccine candidate.

Interestingly, the importance of anti-LPS antibodies has been also suggested for other cholera vaccine candidates, including whole-cell killed vaccines, live attenuated vaccines, and subunit vaccines (81, 91–95). It has been speculated that the protective efficacy of anti-LPS antibodies is mediated through either vibriocidal activity or inhibition of adhesion to the epithelial surface (25,

96–98). We herein present an alternative mechanism by which anti-O antigen antibodies effectively block motility and thereby prevent effective colonization. Recent epidemiological data revealed a correlation between the presence of LPS-specific IgG memory B cells and protection against infection with *V. cholerae* O1 (99). Furthermore, a recent analysis of the immune response in cholera patients indicates that antibodies predominantly targeting the O antigen of *V. cholerae* correlate with protection against cholera (98). Thus, the induction of an anti-O antigen response seems also to be associated with protection against cholera in humans. These epidemiological correlations and the findings from our immunization study using the mouse model strengthen the idea that the O antigen might be the dominant protective antigen against cholera infections. Hence, a future direction in cholera vaccine design might be to focus on the development of a vaccine specifically inducing long-lasting, high-titer immune responses against the clinically relevant *V. cholerae* O antigens.

Future comparative studies have to prove whether the OMV vaccine candidate is the best delivery vehicle for LPS. Mucosal immunization with purified LPS does not induce a strong antibody response, probably since LPS is a well-established T-cell-independent antigen (100, 101). Thus, a simple vaccine based on purified LPS is not applicable. Obviously, OMVs provide a balanced blend of LPS, lipids, and proteins to overcome mucosal tolerance and allow the induction of a robust LPS-directed immune response upon mucosal immunization. Such beneficial properties of OMVs, bacterial ghosts, or artificially engineered liposomes, are currently being addressed by a number of research groups (102). Ongoing studies are focused on comparing the effectiveness of the OMV-based vaccine candidate with that of the whole-cell killed vaccine. So far, we can summarize that the OMV vaccine candidate is temperature stable and can be easily administered (probably without trained health care staff), a mixture of OMVs derived from O1 and O139 induces a protective immune response against both clinically relevant serogroups, detoxification of the OMVs by LPS modifications is possible, and OMVs provide most likely a higher concentration of the relevant O antigen than does the whole-cell killed vaccine (31–33). We can only speculate on the production costs, but feasible large-scale production is demonstrated by the *N. meningitidis* OMV vaccine, and isolation of the OMVs should be cheaper than the production of conjugate subunit vaccines. Immunization with OMVs induces an immune response against a variety of surface structures, although probably only the population of anti-O antigen antibodies is responsible for a protective immune response. However, one cannot exclude that presence of antibodies directed against other surface components than LPS might have additive effects on the protective efficacy.

ACKNOWLEDGMENTS

This work was supported by the Austrian FWF grants P25691 to S.S. and W901-B12 (DK Molecular Enzymology) to D. R. L., S. F., J. R., and S. S.

We are grateful to Chamel Khoury for critically reading the manuscript and to Thierry Claudel for assistance with the design of the oligonucleotides used for qRT-PCR as well as to J. Kaper and R. Taylor for kindly providing the plasmids pSSH2188 and pET15b-cI-TcpA, respectively.

REFERENCES

1. **Koch R.** 1884. An address on cholera and its bacillus. Br. Med. J. **2:**403–407.
2. **Enserink M.** 2010. Infectious diseases. Haiti's outbreak is latest in cholera's new global assault. Science **330:**738–739.
3. **CDC.** 2010. Cholera outbreak—Haiti, October 2010. MMWR Morb. Mortal. Wkly. Rep. **59:**1411.
4. **Harris JB, LaRocque RC, Qadri F, Ryan ET, Calderwood SB.** 2012. Cholera. Lancet **379:**2466–2476.
5. **Sack DA, Sack RB, Nair GB, Siddique AK.** 2004. Cholera. Lancet **363:**223–233.
6. **Levine MM.** 2006. Enteric infections and the vaccines to counter them: future directions. Vaccine **24:**3865–3873.
7. **Bryce J, Boschi-Pinto C, Shibuya K, Black RE.** 2005. WHO estimates of the causes of death in children. Lancet **365:**1147–1152.
8. **WHO.** 2010. Cholera vaccines: WHO position paper. Wkly. Epidemiol. Rec. **85:**117–128.
9. **Zuckerman JN, Rombo L, Fisch A.** 2007. The true burden and risk of cholera: implications for prevention and control. Lancet Infect. Dis. **7:**521–530.
10. **Kaper JB, Morris JG, Jr, Levine MM.** 1995. Cholera. Clin. Microbiol. Rev. **8:**48–86.
11. **Clemens JD, Sack DA, Chakraborty J, Rao MR, Ahmed F, Harris JR, van Loon F, Khan MR, Yunis M, Huda S, Bradford AK, Svennerholm AM, Holmgren J.** 1990. Field trial of oral cholera vaccines in Bangladesh: evaluation of anti-bacterial and anti-toxic breast-milk immunity in response to ingestion of the vaccines. Vaccine **8:**469–472.
12. **Jertborn M, Svennerholm AM, Holmgren J.** 1992. Safety and immunogenicity of an oral recombinant cholera B subunit-whole cell vaccine in Swedish volunteers. Vaccine **10:**130–132.
13. **Jertborn M, Svennerholm AM, Holmgren J.** 1993. Evaluation of different immunization schedules for oral cholera B subunit-whole cell vaccine in Swedish volunteers. Vaccine **11:**1007–1012.
14. **Jertborn M, Svennerholm AM, Holmgren J.** 1994. Immunological memory after immunization with oral cholera B subunit–whole-cell vaccine in Swedish volunteers. Vaccine **12:**1078–1082.
15. **Bishop AL, Camilli A.** 2011. *Vibrio cholerae*: lessons for mucosal vaccine design. Expert Rev. Vaccines **10:**79–94.
16. **Cumberland S.** 2009. An old enemy returns. Bull. World Health Organ. **87:**85–86.
17. **Shin S, Desai SN, Sah BK, Clemens JD.** 2011. Oral vaccines against cholera. Clin. Infect. Dis. **52:**1343–1349.
18. **Trach DD, Cam PD, Ke NT, Rao MR, Dinh D, Hang PV, Hung NV, Canh DG, Thiem VD, Naficy A, Ivanoff B, Svennerholm AM, Holmgren J, Clemens JD.** 2002. Investigations into the safety and immunogenicity of a killed oral cholera vaccine developed in Viet Nam. Bull. World Health Organ. **80:**2–8.
19. **Desai SN, Clemens JD.** 2012. An overview of cholera vaccines and their public health implications. Curr. Opin. Pediatr. **24:**85–91.
20. **Trach DD, Clemens JD, Ke NT, Thuy HT, Son ND, Canh DG, Hang PV, Rao MR.** 1997. Field trial of a locally produced, killed, oral cholera vaccine in Vietnam. Lancet **349:**231–235.
21. **Ryan ET, Calderwood SB, Qadri F.** 2006. Live attenuated oral cholera vaccines. Expert Rev. Vaccines **5:**483–494.
22. **Asaduzzaman M, Ryan ET, John M, Hang L, Khan AI, Faruque AS, Taylor RK, Calderwood SB, Qadri F.** 2004. The major subunit of the toxin-coregulated pilus TcpA induces mucosal and systemic immunoglobulin A immune responses in patients with cholera caused by *Vibrio cholerae* O1 and O139. Infect. Immun. **72:**4448–4454.
23. **Kossaczka Z, Shiloach J, Johnson V, Taylor DN, Finkelstein RA, Robbins JB, Szu SC.** 2000. *Vibrio cholerae* O139 conjugate vaccines: synthesis and immunogenicity of *V. cholerae* O139 capsular polysaccharide conjugates with recombinant diphtheria toxin mutant in mice. Infect. Immun. **68:**5037–5043.
24. **Cabrera O, Martinez ME, Cuello M, Soto CR, Valmaseda T, Cedre B, Gonzalez GS.** 2006. Preparation and evaluation of *Vibrio cholerae* O1 EL Tor Ogawa lipopolysaccharide-tetanus toxoid conjugates. Vaccine **24**(Suppl 2):S74–S75.
25. **Gupta RK, Szu SC, Finkelstein RA, Robbins JB.** 1992. Synthesis, characterization, and some immunological properties of conjugates composed of the detoxified lipopolysaccharide of *Vibrio cholerae* O1 serotype Inaba bound to cholera toxin. Infect. Immun. **60:**3201–3208.

26. **Garcia L, Jidy MD, Garcia H, Rodriguez BL, Fernandez R, Ano G, Cedre B, Valmaseda T, Suzarte E, Ramirez M, Pino Y, Campos J, Menendez J, Valera R, Gonzalez D, Gonzalez I, Perez O, Serrano T, Lastre M, Miralles F, Del Campo J, Maestre JL, Perez JL, Talavera A, Perez A, Marrero K, Ledon T, Fando R.** 2005. The vaccine candidate *Vibrio cholerae* 638 is protective against cholera in healthy volunteers. Infect. Immun. **73:**3018–3024.
27. **Qadri F, Chowdhury MI, Faruque SM, Salam MA, Ahmed T, Begum YA, Saha A, Alam MS, Zaman K, Seidlein LV, Park E, Killeen KP, Mekalanos JJ, Clemens JD, Sack DA.** 2005. Randomized, controlled study of the safety and immunogenicity of Peru-15, a live attenuated oral vaccine candidate for cholera, in adult volunteers in Bangladesh. J. Infect. Dis. **192:**573–579.
28. **Qadri F, Chowdhury MI, Faruque SM, Salam MA, Ahmed T, Begum YA, Saha A, Al Tarique A, Seidlein LV, Park E, Killeen KP, Mekalanos JJ, Clemens JD, Sack DA.** 2007. Peru-15, a live attenuated oral cholera vaccine, is safe and immunogenic in Bangladeshi toddlers and infants. Vaccine **25:**231–238.
29. **Tacket CO, Losonsky G, Nataro JP, Comstock L, Michalski J, Edelman R, Kaper JB, Levine MM.** 1995. Initial clinical studies of CVD 112 *Vibrio cholerae* O139 live oral vaccine: safety and efficacy against experimental challenge. J. Infect. Dis. **172:**883–886.
30. **Coster TS, Kileen KP, Waldor MK, Beattie D, Spriggs D, Kenner JR, Trofa A, Sadoff J, Mekalanos JJ, Taylor DN.** 1995. Safety, immunogenicity and efficacy of a live attenuated *Vibrio cholerae* O139 vaccine prototype, Bengal-15. Lancet **345:**949–952.
31. **Bishop AL, Schild S, Patimalla B, Klein B, Camilli A.** 2010. Mucosal immunization with *Vibrio cholerae* outer membrane vesicles provides maternal protection mediated by antilipopolysaccharide antibodies that inhibit bacterial motility. Infect. Immun. **78:**4402–4420.
32. **Schild S, Nelson EJ, Bishop AL, Camilli A.** 2009. Characterization of *Vibrio cholerae* outer membrane vesicles as a candidate vaccine for cholera. Infect. Immun. **77:**472–484.
33. **Schild S, Nelson EJ, Camilli A.** 2008. Immunization with *Vibrio cholerae* outer membrane vesicles induces protective immunity in mice. Infect. Immun. **76:**4554–4563.
34. **Mashburn-Warren L, McLean RJ, Whiteley M.** 2008. Gram-negative outer membrane vesicles: beyond the cell surface. Geobiology **6:**214–219.
35. **Kulp A, Kuehn MJ.** 2010. Biological functions and biogenesis of secreted bacterial outer membrane vesicles. Annu. Rev. Microbiol. **64:**163–184.
36. **Chatterjee SN, Chaudhuri K.** 2003. Lipopolysaccharides of *Vibrio cholerae*. I. Physical and chemical characterization. Biochim. Biophys. Acta **1639:**65–79.
37. **Follett EA, Gordon J.** 1963. An electron microscope study of *Vibrio* flagella. J. Gen. Microbiol. **32:**235–239.
38. **Freter R, O'Brian PCM, Macsai MS.** 1981. Role of chemotaxis in the association of motile bacteria with intestinal mucosa: *in vivo* studies. Infect. Immun. **34:**234–240.
39. **Freter R, Jones GW.** 1976. Adhesive properties of *Vibrio cholerae*: nature of the interaction with intact mucosal surfaces. Infect. Immun. **14:**246–256.
40. **Lee SH, Butler SM, Camilli A.** 2001. Selection for *in vivo* regulators of bacterial virulence. Proc. Natl. Acad. Sci. U. S. A. **98:**6889–6894.
41. **Liu Z, Miyashiro T, Tsou A, Hsiao A, Goulian M, Zhu J.** 2008. Mucosal penetration primes *Vibrio cholerae* for host colonization by repressing quorum sensing. Proc. Natl. Acad. Sci. U. S. A. **105:**9769–9774.
42. **Morris DC, Peng F, Barker JR, Klose KE.** 2008. Lipidation of an FlrC-dependent protein is required for enhanced intestinal colonization by *Vibrio cholerae*. J. Bacteriol. **190:**231–239.
43. **Raetz CR, Whitfield C.** 2002. Lipopolysaccharide endotoxins. Annu. Rev. Biochem. **71:**635–700.
44. **Mukaida N, Ishikawa Y, Ikeda N, Fujioka N, Watanabe S, Kuno K, Matsushima K.** 1996. Novel insight into molecular mechanism of endotoxin shock: biochemical analysis of LPS receptor signaling in a cell-free system targeting NF-kappaB and regulation of cytokine production/action through beta2 integrin in vivo. J. Leukoc. Biol. **59:**145–151.
45. **Engelhardt R, Mackensen A, Galanos C, Andreesen R.** 1990. Biological response to intravenously administered endotoxin in patients with advanced cancer. J. Biol. Response Mod. **9:**480–491.
46. **Inagawa H, Kohchi C, Soma G.** 2011. Oral administration of lipopolysaccharides for the prevention of various diseases: benefit and usefulness. Anticancer Res. **31:**2431–2436.
47. **Cross AS, Opal SM, Palardy JE, Drabick JJ, Warren HS, Huber C, Cook P, Bhattacharjee AK.** 2003. Phase I study of detoxified *Escherichia coli* J5 lipopolysaccharide (J5dLPS)/group B meningococcal outer membrane protein (OMP) complex vaccine in human subjects. Vaccine **21:**4576–4587.
48. **Rioux S, Girard C, Dubreuil JD, Jacques M.** 1998. Evaluation of the protective efficacy of *Actinobacillus pleuropneumoniae* serotype 1 detoxified lipopolysaccharides or O-polysaccharide-protein conjugate in pigs. Res. Vet. Sci. **65:**165–167.
49. **Bhattacharjee AK, Opal SM, Taylor R, Naso R, Semenuk M, Zollinger WD, Moran EE, Young L, Hammack C, Sadoff JC, Cross AS.** 1996. A noncovalent complex vaccine prepared with detoxified *Escherichia coli* J5 (Rc chemotype) lipopolysaccharide and *Neisseria meningitidis* group B outer membrane protein produces protective antibodies against gram-negative bacteremia. J. Infect. Dis. **173:**1157–1163.
50. **Konadu E, Shiloach J, Bryla DA, Robbins JB, Szu SC.** 1996. Synthesis, characterization, and immunological properties in mice of conjugates composed of detoxified lipopolysaccharide of *Salmonella paratyphi* A bound to tetanus toxoid with emphasis on the role of O acetyls. Infect. Immun. **64:**2709–2715.
51. **Ding HF, Nakoneczna I, Hsu HS.** 1990. Protective immunity induced in mice by detoxified *Salmonella* lipopolysaccharide. J. Med. Microbiol. **31:**95–102.
52. **Sadarangani M, Pollard AJ.** 2010. Serogroup B meningococcal vaccines—an unfinished story. Lancet Infect. Dis. **10:**112–124.
53. **Holst J, Martin D, Arnold R, Huergo CC, Oster P, O'Hallahan J, Rosenqvist E.** 2009. Properties and clinical performance of vaccines containing outer membrane vesicles from *Neisseria meningitidis*. Vaccine **27**(Suppl 2):B3–B12.
54. **van der Ley P, van den Dobbelsteen G.** 2011. Next-generation outer membrane vesicle vaccines against *Neisseria meningitidis* based on nontoxic LPS mutants. Hum. Vaccin. **7:**886–890.
55. **Keiser PB, Biggs-Cicatelli S, Moran EE, Schmiel DH, Pinto VB, Burden RE, Miller LB, Moon JE, Bowden RA, Cummings JF, Zollinger WD.** 2011. A phase 1 study of a meningococcal native outer membrane vesicle vaccine made from a group B strain with deleted lpxL1 and synX, over-expressed factor H binding protein, two PorAs and stabilized OpcA expression. Vaccine **29:**1413–1420.
56. **Oster P, Lennon D, O'Hallahan J, Mulholland K, Reid S, Martin D.** 2005. MeNZB: a safe and highly immunogenic tailor-made vaccine against the New Zealand *Neisseria meningitidis* serogroup B disease epidemic strain. Vaccine **23:**2191–2196.
57. **Fredriksen JH, Rosenqvist E, Wedege E, Bryn K, Bjune G, Froholm LO, Lindbak AK, Mogster B, Namork E, Rye U, Stabbetorp G, Winsnes R, Aase B, Closs O.** 1991. Production, characterization and control of MenB-vaccine "Folkehelsa": an outer membrane vesicle vaccine against group B meningococcal disease. NIPH Ann. **14:**67–79, discussion 79–80.
58. **Iwanaga M, Yamamoto K.** 1985. New medium for the production of cholera toxin by *Vibrio cholerae* O1 biotype El Tor. J. Clin. Microbiol. **22:**405–408.
59. **Iwanaga M, Yamamoto K, Higa N, Ichinose Y, Nakasone N, Tanabe M.** 1986. Culture conditions for stimulating cholera toxin production by *Vibrio cholerae* O1 El Tor. Microbiol. Immunol. **30:**1075–1083.
60. **Seper A, Fengler VH, Roier S, Wolinski H, Kohlwein SD, Bishop AL, Camilli A, Reidl J, Schild S.** 2011. Extracellular nucleases and extracellular DNA play important roles in *Vibrio cholerae* biofilm formation. Mol. Microbiol. **82:**1015–1037.
61. **Donnenberg MS, Kaper JB.** 1991. Construction of an *eae* deletion mutant of enteropathogenic *Escherichia coli* by using a positive-selection suicide vector. Infect. Immun. **59:**4310–4317.
62. **Tsai CM, Frasch CE.** 1982. A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels. Anal. Biochem. **119:**115–119.
63. **Lee CH, Tsai CM.** 1999. Quantification of bacterial lipopolysaccharides by the Purpald assay: measuring formaldehyde generated from 2-keto-3-deoxyoctonate and heptose at the inner core by periodate oxidation. Anal. Biochem. **267:**161–168.
64. **Shevchenko A, Wilm M, Vorm O, Mann M.** 1996. Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68:**850–858.
65. **Folch J, Lees M, Sloane Stanley GH.** 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226:**497–509.

66. **Harrison LM, Rallabhandi P, Michalski J, Zhou X, Steyert SR, Vogel SN, Kaper JB.** 2008. *Vibrio cholerae* flagellins induce Toll-like receptor 5-mediated interleukin-8 production through mitogen-activated protein kinase and NF-kappaB activation. Infect. Immun. **76:**5524–5534.
67. **Taylor RK, Kirn TJ, Meeks MD, Wade TK, Wade WF.** 2004. A *Vibrio cholerae* classical TcpA amino acid sequence induces protective antibody that binds an area hypothesized to be important for toxin-coregulated pilus structure. Infect. Immun. **72:**6050–6060.
68. **Kang D, Gho YS, Suh M, Kang C.** 2002. Highly sensitive and fast protein detection with coomassie brilliant blue in sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bull. Kor. Chem. Soc. **23:**1511–1512.
69. **Roier S, Leitner DR, Iwashkiw J, Schild-Prufert K, Feldman MF, Krohne G, Reidl J, Schild S.** 2012. Intranasal immunization with nontypeable *Haemophilus influenzae* outer membrane vesicles induces cross-protective immunity in mice. PLoS One **7:**e42664. doi:10.1371/journal.pone.0042664.
70. **Hankins JV, Madsen JA, Giles DK, Childers BM, Klose KE, Brodbelt JS, Trent MS.** 2011. Elucidation of a novel *Vibrio cholerae* lipid A secondary hydroxy-acyltransferase and its role in innate immune recognition. Mol. Microbiol. **81:**1313–1329.
71. **Matson JS, Yoo HJ, Hakansson K, Dirita VJ.** 2010. Polymyxin B resistance in El Tor *Vibrio cholerae* requires lipid acylation catalyzed by MsbB. J. Bacteriol. **192:**2044–2052.
72. **Somerville JE, Jr, Cassiano L, Bainbridge B, Cunningham MD, Darveau RP.** 1996. A novel *Escherichia coli* lipid A mutant that produces an antiinflammatory lipopolysaccharide. J. Clin. Invest. **97:**359–365.
73. **Ranallo RT, Kaminski RW, George T, Kordis AA, Chen Q, Szabo K, Venkatesan MM.** 2012. Virulence, inflammatory potential, and adaptive immunity induced by *Shigella flexneri msbB* mutants. Infect. Immun. **78:**400–412.
74. **Teghanemt A, Zhang D, Levis EN, Weiss JP, Gioannini TL.** 2005. Molecular basis of reduced potency of underacylated endotoxins. J. Immunol. **175:**4669–4676.
75. **Hankins JV, Madsen JA, Giles DK, Brodbelt JS, Trent MS.** 2012. Amino acid addition to *Vibrio cholerae* LPS establishes a link between surface remodeling in gram-positive and gram-negative bacteria. Proc. Natl. Acad. Sci. U. S. A. **109:**8722–8727.
76. **Nesper J, Schild S, Lauriano CM, Kraiss A, Klose KE, Reidl J.** 2002. Role of *Vibrio cholerae* O139 surface polysaccharides in intestinal colonization. Infect. Immun. **70:**5990–5996.
77. **Marolda CL, Vicarioli J, Valvano MA.** 2004. Wzx proteins involved in biosynthesis of O antigen function in association with the first sugar of the O-specific lipopolysaccharide subunit. Microbiology **150:**4095–4105.
78. **Feldman MF, Marolda CL, Monteiro MA, Perry MB, Parodi AJ, Valvano MA.** 1999. The activity of a putative polyisoprenol-linked sugar translocase (Wzx) involved in *Escherichia coli* O antigen assembly is independent of the chemical structure of the O repeat. J. Biol. Chem. **274:**35129–35138.
79. **Schild S, Lamprecht AK, Reidl J.** 2005. Molecular and functional characterization of O antigen transfer in *Vibrio cholerae*. J. Biol. Chem. **280:**25936–25947.
80. **Sharma DP, Stroeher UH, Thomas CJ, Manning PA, Attridge SR.** 1989. The toxin-coregulated pilus (TCP) of *Vibrio cholerae*: molecular cloning of genes involved in pilus biosynthesis and evaluation of TCP as a protective antigen in the infant mouse model. Microb. Pathog. **7:**437–448.
81. **Taylor RK, Kirn TJ, Bose N, Stonehouse E, Tripathi SA, Kovac P, Wade WF.** 2004. Progress towards development of a cholera subunit vaccine. Chem. Biodivers. **1:**1036–1057.
82. **Hone DM, Powell J, Crowley RW, Maneval D, Lewis GK.** 1998. Lipopolysaccharide from an *Escherichia coli htrB msbB* mutant induces high levels of MIP-1 alpha and MIP-1 beta secretion without inducing TNF-alpha and IL-1 beta. J. Hum. Virol. **1:**251–256.
83. **Kim SH, Kim KS, Lee SR, Kim E, Kim MS, Lee EY, Gho YS, Kim JW, Bishop RE, Chang KT.** 2009. Structural modifications of outer membrane vesicles to refine them as vaccine delivery vehicles. Biochim. Biophys. Acta **1788:**2150–2159.
84. **Fisseha M, Chen P, Brandt B, Kijek T, Moran E, Zollinger W.** 2005. Characterization of native outer membrane vesicles from lpxL mutant strains of *Neisseria meningitidis* for use in parenteral vaccination. Infect. Immun. **73:**4070–4080.
85. **van der Ley P, Steeghs L, Hamstra HJ, ten Hove J, Zomer B, van Alphen L.** 2001. Modification of lipid A biosynthesis in *Neisseria meningitidis lpxL* mutants: influence on lipopolysaccharide structure, toxicity, and adjuvant activity. Infect. Immun. **69:**5981–5990.
86. **Pridmore AC, Wyllie DH, Abdillahi F, Steeghs L, van der Ley P, Dower SK, Read RC.** 2001. A lipopolysaccharide-deficient mutant of *Neisseria meningitidis* elicits attenuated cytokine release by human macrophages and signals via toll-like receptor (TLR) 2 but not via TLR4/MD2. J. Infect. Dis. **183:**89–96.
87. **Gaines Das RE, Brugger P, Patel M, Mistry Y, Poole S.** 2004. Monocyte activation test for pro-inflammatory and pyrogenic contaminants of parenteral drugs: test design and data analysis. J. Immunol. Methods **288:**165–177.
88. **Voss E, Manning PA, Attridge SR.** 1996. The toxin-coregulated pilus is a colonization factor and protective antigen of *Vibrio cholerae* El Tor. Microb. Pathog. **20:**141–153.
89. **Sun DX, Mekalanos JJ, Taylor RK.** 1990. Antibodies directed against the toxin-coregulated pilus isolated from *Vibrio cholerae* provide protection in the infant mouse experimental cholera model. J. Infect. Dis. **161:**1231–1236.
90. **Sharma DP, Thomas C, Hall RH, Levine MM, Attridge SR.** 1989. Significance of toxin-coregulated pili as protective antigens of *Vibrio cholerae* in the infant mouse model. Vaccine **7:**451–456.
91. **Albert MJ, Alam K, Ansaruzzaman M, Qadri F, Sack RB.** 1994. Lack of cross-protection against diarrhea due to *Vibrio cholerae* O139 (Bengal strain) after oral immunization of rabbits with V. cholerae O1 vaccine strain CVD103-HgR. J. Infect. Dis. **169:**230–231.
92. **Albert MJ, Alam K, Rahman AS, Huda S, Sack RB.** 1994. Lack of cross-protection against diarrhea due to *Vibrio cholerae* O1 after oral immunization of rabbits with V. cholerae O139 Bengal. J. Infect. Dis. **169:**709–710.
93. **Svennerholm AM.** 1975. Experimental studies on cholera immunization. 4. The antibody response to formalinized *Vibrio cholerae* and purified endotoxin with special reference to protective capacity. Int. Arch. Allergy Appl. Immunol. **49:**434–452.
94. **Svennerholm AM, Holmgren J.** 1976. Synergistic protective effect in rabbits of immunization with *Vibrio cholerae* lipopolysaccharide and toxin/toxoid. Infect. Immun. **13:**735–740.
95. **Provenzano D, Kovac P, Wade WF.** 2006. The ABCs (Antibody, B cells, and Carbohydrate epitopes) of cholera immunity: considerations for an improved vaccine. Microbiol. Immunol. **50:**899–927.
96. **Mukhopadhyay S, Nandi B, Ghose AC.** 2000. Antibodies (IgG) to lipopolysaccharide of *Vibrio cholerae* O1 mediate protection through inhibition of intestinal adherence and colonisation in a mouse model. FEMS Microbiol. Lett. **185:**29–35.
97. **Vijayashree S, Nayak N, Panigrahi D, Sehgal S.** 2003. Role of cell surface antigens of *Vibrio cholerae* 01 and non 01 serovars in intestinal adhesion. Indian J. Pathol. Microbiol. **46:**259–260.
98. **Johnson RA, Uddin T, Aktar A, Mohasin M, Alam MM, Chowdhury F, Harris JB, Larocque RC, Kelly Bufano M, Yu Y, Wu-Freeman Y, Leung DT, Sarracino D, Krastins B, Charles RC, Xu P, Kovac P, Calderwood SB, Qadri F, Ryan ET.** 2012. Comparison of immune responses to the O-specific polysaccharide and lipopolysaccharide of *Vibrio cholerae* O1 in Bangladeshi adult patients with cholera. Clin. Vaccine Immunol. **19:**1712–1721.
99. **Patel SM, Rahman MA, Mohasin M, Riyadh MA, Leung DT, Alam MM, Chowdhury F, Khan AI, Weil AA, Aktar A, Nazim M, LaRocque RC, Ryan ET, Calderwood SB, Qadri F, Harris JB.** 2012. Memory B cell responses to *Vibrio cholerae* O1 lipopolysaccharide are associated with protection against infection from household contacts of patients with cholera in Bangladesh. Clin. Vaccine Immunol. **19:**842–848.
100. **Stein KE.** 1992. Thymus-independent and thymus-dependent responses to polysaccharide antigens. J. Infect. Dis. **165**(Suppl 1):S49–S52.
101. **Orr N, Robin G, Cohen D, Arnon R, Lowell GH.** 1993. Immunogenicity and efficacy of oral or intranasal *Shigella flexneri* 2a and *Shigella sonnei* proteosome-lipopolysaccharide vaccines in animal models. Infect. Immun. **61:**2390–2395.
102. **Mann JF, Acevedo R, Campo JD, Perez O, Ferro VA.** 2009. Delivery systems: a vaccine strategy for overcoming mucosal tolerance? Expert Rev. Vaccines **8:**103–112.
103. **Hanahan D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.

104. **Miller VL, Mekalanos JJ.** 1988. A novel suicide vector and its use in construction of insertion mutations: osmoregulation of outer membrane proteins and virulence determinants in *Vibrio cholerae* requires *toxR*. J. Bacteriol. **170:**2575–2583.
105. **Bishop AL, Tarique AA, Patimalla B, Calderwood SB, Qadri F, Camilli A.** 2012. Immunization of mice with *Vibrio cholerae* outer-membrane vesicles protects against hyperinfectious challenge and blocks transmission. J. Infect. Dis. **205:**412–421.
106. **Miller VL, DiRita VJ, Mekalanos JJ.** 1989. Identification of *toxS*, a regulatory gene whose product enhances *toxR*-mediated activation of the cholera toxin promoter. J. Bacteriol. **171:**1288–1293.
107. **Waldor MK, Mekalanos JJ.** 1994. Emergence of a new cholera pandemic: molecular analysis of virulence determinants in *Vibrio cholerae* O139 and development of a live vaccine prototype. J. Infect. Dis. **170:** 278–283.
108. **Guzman L-M, Beblin D, Carson MJ, Beckwith J.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose pBAD promotor. J. Bacteriol. **177:**4121–4130.
109. **Nesper J, Kraiss A, Schild S, Blass J, Klose KE, Bockemuhl J, Reidl J.** 2002. Comparative and genetic analyses of the putative *Vibrio cholerae* lipopolysaccharide core oligosaccharide biosynthesis (*wav*) gene cluster. Infect. Immun. **70:**2419–2433.