NOD-Like Receptor Activation by Outer Membrane Vesicles from *Vibrio cholerae* Non-O1 Non-O139 Strains Is Modulated by the Quorum-Sensing Regulator HapR[▽]

H. Bielig,^{1¶} P. K. Rompikuntal,^{2¶} M. Dongre,² B. Zurek,¹ B. Lindmark,² M. Ramstedt,³
S. N. Wai,^{2*} and T. A. Kufer^{1*}

Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Joseph-Stelzmann-Str. 9, Geb. 37, 50931 Cologne, Germany¹; Department of Molecular Biology and Umeå Centre for Microbial Research, Umeå University, S-90187 Umeå, Sweden²; and Department of Chemistry, Umeå University, SE-90187 Umeå, Sweden³

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Vibrio cholerae is an inhabitant of aquatic systems and one of the causative agents of severe dehydrating diarrhea in humans. It has also emerged as an important cause of different kinds of inflammatory responses, and in particular, *V. cholerae* strains of the non-O1 non-O139 serogroups (NOVC) have been associated with such infections in human. We analyzed the potential of outer membrane vesicles (OMVs) derived from the NOVC strain V:5/04 to induce inflammatory responses in human host cells. V:5/04 OMVs were taken up by human epithelial cells and induced inflammatory responses. Small interfering RNA (siRNA)-mediated gene knockdown revealed that the inflammatory potential of NOVC OMVs was partially mediated by the nucleotide-binding domain-, leucine-rich repeat-containing family member NOD1. Physicochemical analysis of the content of these OMVs, in conjunction with NOD1 and NOD2 reporter assays in HEK293T cells, confirmed the presence of both NOD1 and NOD2 active peptidoglycan in the OMVs. Furthermore, we show that deletion of the quorum-sensing regulator HapR, which mimics an infective life style, specifically reduced the inflammatory potential of the V:5/04 OMVs and their ability to activate NOD1 and NOD2. In conclusion, our study shows that NOVC OMVs elicit immune responses mediated by NOD1 and NOD2 in mammalian host cells. Moreover, we provide evidence that the quorum-sensing machinery plays an important regulatory role in this process by attenuating the inflammatory potential of OMVs under infective conditions. This work thus identifies a new facet of how *Vibrio* affects host immune responses and defines a role for the quorum-sensing machinery in this process.

Vibrio cholerae is an inhabitant of aquatic systems and one of the causative agents of severe dehydrating diarrhea in humans. *V. cholerae* bacteria belonging to the O1 and O139 serogroups cause cholera epidemics in many developing countries (10, 23), whereas strains belonging to non-O1 non-O139 *V. cholerae* (NOVC) serogroups have been associated with endemic gastroenteritis and extraintestinal infections in humans (10, 23). Unlike the case for the O1 and O139 strains of *V. cholerae*, little is known about the virulence and pathogenicity of NOVC strains. Identification and characterization of the NOVC strains carrying virulence genes is important, as these serogroups may emerge as potential epidemic strains in the future. Recently, we found one of these strains, V:5/04, a clinical isolate that caused a severe sporadic outbreak in Sweden in 2004, to express virulence factors such as the type VI secretion system component Hcp (20). Here we further characterized this strain and found that it produced outer membrane vesicles with intrinsic inflammatory potential. Outer membrane vesicles (OMVs) are spherical fragments of bacterial membrane that are produced by a wide variety of Gram-negative bacteria during normal growth (5). These vesicles are formed by protrusions of bacterial outer membrane that are released into the environment. As these vesicles are released from the surface, they can also entrap parts of the underlying bacterial periplasm. OMVs have important functions in host-pathogen interactions, such as the delivery to host cells of active bacterial toxins, such as ClyA cytotoxin, α -hemolysin, and CNF1 of *Escherichia coli* (3, 25, 44) and hemolysin (Hly) of enterohemorrhagic *E. coli* (EHEC) (2). Moreover, biologically active *H. pylori* vacuolating cytotoxin A is associated with OMVs that bind to and are internalized by epithelial cells, as shown by the detection of OMVs in human gastric mucosa from *Helicobacter pylori*-infected individuals (11, 24). Furthermore, different studies provided evidence that OMVs influence inflammation and disease *in vivo*. In one example, it was shown that epithelial cells produce interleukin-8 (IL-8), a cytokine that is pivotal for neutrophil and monocyte recruitment, in response to *H. pylori*- and *Pseudomonas aeruginosa*-derived OMVs (4, 21). It was recently revealed that this effect is, at least partly, dependent on the delivery of nucleotide-binding domain-containing protein 1 (NOD1) active peptidoglycan (PGN) (22). Moreover, during meningococcal septicemia, meningococci are known to release OMVs in the circulation, which contributes to the high endotoxin levels characteristic of these infections (34). OMVs are thus expected to contain several physiologically relevant pathogen-associated molecular patterns (PAMPs) that can be recog-

* Corresponding author. Mailing address for T. A. Kufer: Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, Joseph-Stelzmann-Str. 9, Geb. 37, 50931 Cologne, Germany. Phone: 49 221 478 7279. Fax: 49 221 478 7288. E-mail: thomas.kufer@uk-koeln.de. Mailing address for S. N. Wai: Department of Molecular Biology and Umeå Centre for Microbial Research (UCMR), Umeå University, S-90187 Umeå, Sweden. Phone: 46 90 785 6704. Fax: 46 90 772630. E-mail: sun.nyunt.wai@molbiol.umu.se.

¶ These authors contributed equally to this work.

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nized by host pattern recognition receptors (PRRs) *in vivo* (12, 32). Indeed, *Salmonella* OMVs possess important proinflammatory and antigenic properties and have the intrinsic combination of antigens and adjuvant properties required of an effective non-replicating complex vaccine to stimulate immunity against *Salmonella* (1). Understanding the details of how OMVs trigger inflammatory responses in the host will help to evaluate the potential of OMVs as new vaccine candidates.

Several classes of PRRs refer sensing of invading microbes in humans. Recently, it was reported that *H. pylori* OMVs are actively taken up by epithelial cells (18, 22). We thus reasoned that intracellular PRRs would also be biologically important receptors for *Vibrio* OMVs. Recently, NOD1 and NOD2, two cytosolic expressed members of the nucleotide-binding domain-, leucine-rich repeat-containing family (NLR) of pattern recognition proteins, emerged as pivotal intracellular sensors for bacterial infection in mammals (for a review, see reference 12). Both proteins detect PGN fragments: NOD1 confers reactivity to peptides containing diaminopimelic acid (DAP) (7, 14), whereas NOD2 responds to muramyl-dipeptide (MDP) (15, 19). Initial studies showed that these proteins detect invasive bacteria such as *Shigella flexneri* (16); however, evidence suggests that NOD1 and NOD2 also respond to extracellularly presented bacteria and peptidoglycan (PGN). For *H. pylori*, *P. aeruginosa*, and *Neisseria gonorrhoeae* it is known that OMVs are involved in delivering PGN to host cells (22). However, it should be noted that membrane channels in the plasma membrane and the vesicular compartment also contribute to extracellular PGN sensing (35). We asked whether NOVC strains also shed OMVs that can deliver PGN to host cells in order to modulate innate immune responses and whether the quorum-sensing machinery that controls the expression of virulence genes in *V. cholerae* (33, 37, 41, 42, 45) might be able to influence the immunogenicity of NOVC-derived OMVs.

Our data revealed that the NOVC strain V:5/04 produces OMVs that are taken up by human epithelial cells and are able to induce inflammatory responses that are mediated partially by NOD1 and NOD2. Furthermore, we identified a role for the quorum-sensing switch as a regulator for OMV composition that affects the potential of the OMVs to trigger NOD1- and NOD2-mediated cellular responses in the host.

MATERIALS AND METHODS

Cultivation of *Vibrio* strains. The non-O1 non-O139 strain V:5/04 (20) and its Δ *hapR* mutant derivative were grown overnight at 37°C with shaking in Luria-Bertani (LB) broth supplemented, as appropriate, with kanamycin (50 µg/ml) or carbenicillin (100 µg/ml). The Δ *hapR* mutant was constructed by deleting of the entire *hapR* reading frame in non-O1 non-O139 strain V:5/04 as previously described (41, 45).

Isolation of OMVs. OMVs were isolated from culture supernatants as described in reference 43. Briefly, bacteria were inoculated in a 250-ml flask containing 100 ml of LB and incubated under shaking condition for 12 h. Bacterial cells were removed from the culture supernatant by centrifugation at 5,000 × *g* for 30 min. The supernatants were filtered through a 0.20-µm-pore-size syringe filter (Sartorius). The cell-free supernatants were centrifuged at 100,000 × *g* for 2 h at 4°C in a 45Ti rotor (Beckman Instruments Inc.) to pellet the vesicles, resuspended in 250 µl of phosphate-buffered saline (PBS), and adjusted to equal total protein concentrations as measured by the Bradford assay (Bio-Rad).

EM. Samples from the vesicle preparation were negatively stained with a solution of 0.1% uranyl acetate on carbon-coated Formvar grids and examined under an electron microscope (EM). Micrographs were taken with a JEOL

2000EX electron microscope (JEOL Co., Ltd., Akishima, Japan) operated at an accelerating voltage of 100 kV. The magnification used was ×40,000.

SDS-PAGE and immunoblot analysis. The isolated vesicles were subjected to polyacrylamide gel electrophoresis using the standard SDS-PAGE procedure (28). Gels were stained with Coomassie blue. Immunoblot analysis was performed as described previously (39) using polyclonal anti-OmpU antiserum (a kind gift from Masaaki Iwanaga, University of the Ryukyus, Japan) or rabbit polyclonal anti-RpoS antiserum (a kind gift from Joachim Reidl, Karl-Franzens-University Graz, Austria). The ECL⁺ chemiluminescence system was used to detect the level of chemiluminescence, which was then monitored using a Fluor-S Multilimager (Bio-Rad).

Cell culture. HEK293T and HeLa cells were cultivated at 37°C with 5% CO₂ in Dulbecco's modified Eagle's medium (Biochrom AG) supplemented with 10% heat-inactivated fetal calf serum (BioWest) and penicillin-streptomycin (100 IU/ml and 100 mg/ml, respectively). THP1 and THP1-Blue cells (a reporter line expressing the secreted embryonic alkaline phosphatase [SEAP] under control of a NF-κB promoter) (InvivoGen) were maintained in RPMI 1640 medium (Biochrom AG) supplemented with 10% heat-inactivated fetal calf serum (BioWest), penicillin-streptomycin (100 IU/ml and 100 mg/ml, respectively), and 200 µg/ml of Zeocin (InvivoGen) in the case of THP1-Blue. Cells were continuously tested for absence of mycoplasma infection by PCR.

Imaging. For indirect immunofluorescence microscopy, HeLa cells were seeded on sterile coverslips and subsequently incubated with the OMVs. To 100 µl of cell culture medium, 10 µl of vesicle samples (130 µg/ml) was added to give a total of 1.3 µg of OMVs per sample. Cells were fixed after the indicated times in 3% paraformaldehyde in phosphate-buffered saline and permeabilized with 0.5% Triton X-100 for 5 min. Cells were incubated in 3% bovine serum albumin in phosphate-buffered saline for 20 min. Staining was performed by subsequent incubation of primary and secondary antibodies in 3% bovine serum albumin. The primary antibody used was rabbit anti-OmpU (39) (1:250). The primary antibody was detected with Alexa 488-conjugated goat anti-rabbit IgG (1:500) (Invitrogen Molecular Probes) secondary antibody. DNA was stained with DAPI (4',6'-diamidino-2-phenylindole) (0.1 µg/ml; Invitrogen Molecular Probes) and actin with rhodamine-conjugated phalloidin (Sigma-Aldrich).

Reporter assays. Activation of NF-κB was measured using a modification of the luciferase reporter assay described previously (26). Briefly, HEK293T cells were seeded in 96-well plates and transfected using Fugene6 (Roche). Per well, 8.6 ng β-galactosidase plasmid, 13 ng luciferase reporter plasmid, and 0.1 ng NOD2 or 0.5 ng NOD1 expression plasmid, adjusted with pcDNA to 51 ng DNA total, were transfected. Cells were directly stimulated with 50 nM MDP, 500 nM TriDAP, or 0.01 µg/ml tumor necrosis factor (TNF) (all from InvivoGen), as indicated. After 16 h of incubation, the cells were lysed and luciferase activity was measured. Luciferase activity was normalized as a ratio to β-galactosidase activity, and the means and standard deviations (SDs) were calculated from triplicates.

NF-κB activity in the THP1-Blue cells was measured according to the supplier's protocol. Briefly, 1.5 × 10⁶ cells/ml were seeded into a 96-well plate, and cells were stimulated with the indicated PAMP and incubated for 16 h in 100 µl medium. Subsequently, secreted embryonic alkaline phosphatase (SEAP) activity was determined using the Quanti-blue reagent (InvivoGen). Photometric measurements were conducted at 620 nm at the appropriate time. Measurements were conducted in triplicates. To block Toll-like receptor 4 (TLR4) activation, OMVs were preincubated for 30 min with 25 µg/ml polymyxin B solution (92283; Sigma) and added in the presence of polymyxin B to the cells.

To determine cell viability, at 16 h after incubation of the cells under the given conditions, XTT assays (Cell Proliferation Kit II [XTT]; Roche) were performed according to the manufacturer's manual.

siRNA knockdown and qPCR from human cells. Gene silencing of NOD1 and NOD2 by small interfering RNA (siRNA) was performed by transfection of siRNA (Qiagen S100084483 and S100133049) using HiPerfect (Qiagen). THP1-Blue cells (4 × 10⁵/ml) were differentiated with 100 nM phorbol 12-myristate 13-acetate (PMA) in 500 µl of medium in 24-well plates, at 24 h prior siRNA transfection. At 72 h after siRNA treatment (100 nM siRNA/well), cells were stimulated as indicated. After an additional 16 h, supernatant and cells were collected. For quantitative reverse transcription-PCR (qRT-PCR), RNA was prepared from combined triplicates using the RNeasy kit (Qiagen) according to the manufacturer's instructions. For gene expression analysis, 1 µg total RNA was transcribed into cDNA using the first-strand cDNA synthesis kit with an oligo(dT) primer (Fermentas). Quantitative PCR (qPCR) reactions for measuring NOD1 and NOD2 expression were performed on an IQ-5 cyclor (Bio-Rad) using SYBR green master mix (Bio-Rad) with the following primer pairs: GAPDHfw, GGTATCGTGGAAGGACTCATGAC; GAPDHrev, ATGCCAGTGAGCTTCCCGTTTCAG; NOD1_fw, TCCAAAGCCAACAGAACTC;

NOD1_rev, CAGCATCCAGATGAACGTG, NOD2_fwd, GAAGTACATCCG CACCGAG, and NOD2_rev, GACACCATCCATGAGAAGACAG. Data from triplicate measurements were analyzed using the $\Delta\Delta CT$ method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression as a reference (Bio-Rad IQ5 software package).

ELISA. IL-8 was measured by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer's instructions (OptEIA human IL-8, no. 555244; Becton Dickinson) from supernatants of HeLa and THP1 cells treated for 8 h with OMVs or PAMPs. All measurements were conducted in triplicates, and cytokine concentrations were calculated to a recombinant human IL-8 standard.

XPS. Vesicles from 250 ml of bacterial culture were washed with PBS and resuspended in 250 μ l of PBS. After centrifugation, a volume of 20 μ l of the vesicle pellet was quickly frozen using liquid nitrogen inside the loading chamber of the X-ray photoelectron spectroscopy (XPS) spectrometer. XPS spectra were collected on the frozen sample using a Kratos Axis Ultra DLD spectrometer with a monochromated Al K α source operated at 150 W. The C 1s spectra were acquired using a pass energy of 20 eV, and the spectrometer charge-neutralizing system was used to compensate for sample charging. The binding energy scale was referenced to the C 1s aliphatic carbon peak at 285.0 eV and the spectral intensity normalized for the multivariate analysis. The analysis method gives the near-surface (<10 nm) composition of polysaccharide, lipid, and protein/peptidoglycan for each sample (M. Ramstedt et al., unpublished).

Statistical analysis. The results of all quantitative assays are reported as means and SDs from at least three independent experiments, if not stated otherwise. Comparison between groups for statistical significance was performed using a two-tailed Student *t* test. Differences were regarded as significant (*) when the *P* value was <0.05 or as highly significant (**) when the *P* value was <0.005. A *P* value of <0.0005 is represented by ***.

RESULTS

Characterization of OMVs released from the NOVC strain V:5/04. We recently characterized NOVC isolates from environmental and clinical sources. Although none of these NOVC isolates carried the cholera toxin gene (*ctx*) or the toxin-co-regulated pilus gene (*tcpA*), they contained genes encoding the putative virulence factors OmpU, cytolysin (VCC), and RTX toxin. We asked whether outer membrane vesicles might also be an important virulence factor or means to deliver virulence factors to host cells for these bacteria. Among these isolates, the NOVC strain V:5/04 (20) was chosen because the culture supernatant from these cells caused cytotoxicity in HeLa cells, suggesting the presence of soluble virulence factors or OMVs (discussed below). Quorum sensing is well known to control the expression of virulence factors in O1 serotypes of *V. cholerae* (33, 37, 41, 42, 45); however, the role of HapR in virulence gene regulation in NOVC is currently not well established. In order to investigate a putative function of quorum sensing in the regulation of OMV-mediated responses, we constructed a mutant of V:5/04 with an in-frame deletion of the *hapR* gene and analyzed it in comparison to wild-type (WT) NOVC V:5/04. We isolated OMVs from both strains, and the composition and structure of the OMVs were analyzed by SDS-PAGE, immunoblot analysis, and electron microscopic examination (Fig. 1). Although deletion of HapR led to slight changes in the appearance of the vesicles in EM analysis, yielding a more uniform size (Fig. 1A), the protein compositions of OMVs from V:5/04 Δ *hapR* and the parent wild-type strain were highly similar (Fig. 1B). The amounts of vesicles released from these two strains also were comparable as shown by immunoblot analysis detecting a major outer membrane protein, OmpU, as a marker protein for *Vibrio* OMVs (39) (Fig. 1B). Silver-stained SDS-PAGE of preparations of these OMVs also revealed similar amounts and patterns of lipopolysaccharide (LPS) in both samples (Fig. 1C). In conclusion, NOVC V:5/04

FIG. 1. Structure and composition of OMVs from the NOVC strains used. (A) EM pictures of preparations of OMVs derived from NOVC V:5/04 and V:5/04 Δ *hapR* bacteria. Bars, 200 nm. (B) Coomassie blue-stained polyacrylamide gel showing the protein contents of V:5/04 and V:5/04 Δ *hapR* OMVs. Detection of OmpU by immunoblot analysis is presented in the lower panel; 1.5 μ g total protein was loaded for each sample. (C) Silver-stained SDS-polyacrylamide gel showing the LPS contents of V:5/04 and V:5/04 Δ *hapR* OMVs.

produces OMVs, whereas the amounts of OMVs and the overall protein and LPS compositions in V:5/04 WT cells and V:5/04 Δ *hapR* mutant cells, which are locked in a “virulence-like” state, were similar.

NOVC OMVs are taken up by epithelial cells and induce inflammatory responses in host cells. In order to gain insight into the mode of interaction of *V. cholerae* OMVs with the mammalian cell, we incubated HeLa cells, which are epithelial cells derived from a cervix carcinoma, with OMVs isolated from V:5/04 for 8 h. HeLa cells were chosen as a well-accepted model for human epithelial cells that are known to physiologically respond to a variety of pathogenic bacteria by the production of inflammatory cytokines. OMVs were visualized by indirect immunofluorescence analysis with an antibody specific for the outer membrane protein OmpU. This revealed a specific localization pattern for the OMVs, which appeared to accumulate in the vicinity of the nucleus, whereas the antibody did not stain untreated cells (Fig. 2). Having shown that *V. cholerae* OMVs can be taken up by human cells, we next wanted to elucidate whether OMVs from V:5/04 and V:5/04 Δ *hapR*, which were both equally taken up by HeLa cells (data

FIG. 2. NOVC-derived OMVs are taken up by epithelial cells. HeLa cells were inoculated with V:5/04 OMVs or medium as a control. At 8 h after inoculation, cells were fixed and subjected to indirect immunofluorescence analysis. OMVs were stained using an OmpU-specific antibody (red). A merge image with actin stained in green and DNA in blue is shown (upper panel), as well as the OmpU signal alone in gray scale (lower panel). Bar = 10 μ m.

not shown), had different inflammatory potentials. OMVs were prepared from these strains and adjusted to equal total protein concentrations. In HeLa cells, V:5/04 OMVs induced a significant release of the proinflammatory cytokine IL-8 at 8 h after incubation (Fig. 3A). Interestingly, OMVs from the V:5/04 strain deficient in HapR induced a significantly lower IL-8 response (Fig. 3A). Similarly, V:5/04 OMVs also led to a significant release of IL-8 from human myeloid-like THP1 cells, which are highly responsive to PAMPs (Fig. 3B), and induced NF- κ B activation in the THP1 cell line derivative THP1-Blue, which expresses secreted embryonic alkaline phosphatase (SEAP) under control of an NF- κ B-responsive promoter (Fig. 3C). In contrast, V:5/04 Δ hapR OMVs induced significantly lower NF- κ B and IL-8 responses than wild-type V:5/04 in these cells (Fig. 3B and C). Importantly, the OMVs did not induce cell death in the cells during the incubation time used as monitored by a cytotoxicity assay (XTT) from the same experiments (data not shown). In Fig. 1C we showed that both OMV preparations contained LPS. We reasoned that this will largely contribute to the high inflammatory potential of the OMVs in THP1 cells that do express active TLR4. To address whether V:5/04 OMVs also elicited non-TLR4 responses, we quenched the activity of the LPS by incubation of the vesicles and cells with the LPS-sequestering drug polymyxin B. This showed that LPS contributed to most of the inflammatory activity. However, a highly significant response was still seen in the presence

of polymyxin B, suggesting that PRRs other than TLR4 are involved in the recognition of the OMVs (Fig. 3D).

In conclusion, we provide evidence that V:5/04 OMVs have a particularly high intrinsic inflammatory potential in different human cells. This is determined by the quorum-sensing regulatory system and is only partly dependent on OMV-associated LPS.

NOVC OMVs elicit NOD1- and NOD2-mediated responses.

As shown above, OMVs contain a mixture of different PAMPs. One relevant and ubiquitous bacterial PAMP is peptidoglycan (PGN), and its importance in the host immune response is well exemplified by the fact that the peptidoglycan subunit muramyl-dipeptide (MDP) is the active compound of complete Freund's adjuvant (9). Although initially TLR2 was proposed as a PGN sensor, recent evidence suggests that PGN is sensed solely by intracellularly localized proteins of the NLR family (17, 40). NOD1 and NOD2 are well-characterized PGN sensors that mount inflammatory responses mediated by activation of NF- κ B and mitogen-activated protein kinase (MAPK) signaling cascades in the host. To address whether NOVC OMVs contained PGN, we assayed activation of NOD1 and NOD2 by OMVs derived from *V. cholerae* V:5/04 and V:5/04 Δ hapR. To this end, we applied a widely used cell-based NF- κ B reporter assay to analyze NOD1- and NOD2-mediated activation in human embryonic kidney (HEK293T) cells (14–16). HEK293T cells were chosen because, in contrast to HeLa cells,

FIG. 3. OMVs activate inflammatory responses in different human host cells. (A) HeLa cells were treated with vesicles derived from NOVC V:5/04 or V:5/04 Δ hapR (1 μ g/ml). Stimulation with 0.1 μ g/ml LPS served as control for reactivity of the cells. At 8 h after inoculation, the IL-8 release was measured in the supernatant by ELISA. Means and SDs ($n = 3$) are shown. (B) THP1 cells were treated for 16 h with NOVC V:5/04 or V:5/04 Δ hapR OMVs (0.05 μ g/ml) or with MDP (1 μ M) or LPS (0.1 μ g/ml) as controls. IL-8 release was measured in the supernatant by ELISA. Means and SDs ($n = 3$) are shown. (C) THP1-Blue NF- κ B reporter cells were treated for 16 h with NOVC V:5/04 or V:5/04 Δ hapR OMVs (0.5 μ g/ml) or with MDP (10 μ M) or LPS (0.1 μ g/ml) as controls. NF- κ B activation was determined by measuring SEAP activity of the supernatant. Mean fold activation relative to the untreated control (set to 1) and SDs ($n = 3$) are shown. n.s., not stimulated. (D) Assay as for panel C, showing MDP, TriDAP, and LPS as controls. NOVC V:5/04 OMVs (0.5 μ g/ml) were applied in the absence or presence of polymyxin B (25 μ g/ml).

they do not express most PRRs such as the Toll-like receptors (TLRs) (14, 27). Indeed, when OMVs were applied to HEK293T cells in the absence of cotransfected NOD1 or NOD2, none of the tested OMVs significantly induced NF- κ B or IL-8 responses (Fig. 4, white bars), although this was not due to cytotoxicity as measured by the XTT assay (data not shown). However, upon transient transfection of the HEK293T cells with a NOD1 or NOD2 expression plasmid, V:5/04 OMVs significantly induced NOD1 (Fig. 4A)- and NOD2 (Fig. 4B)-dependent NF- κ B activation. Deletion of HapR significantly decreased the potential of the OMVs to activate NOD1 and NOD2 (Fig. 4A and B). Similar results were obtained when using an IL-8 reporter, confirming the NF- κ B results (Fig. 4C and D). To substantiate these results, we used the myeloid cell line THP1, which is known to express high levels of active endogenous NOD1 and NOD2. Although

this cell type is not likely to be infected by *Vibrio* *in vivo* in the first place, we used these cells as a model system for endogenous NOD1/2 activation as in our hands endogenous NOD1/2 responses of cultured epithelial cells were not robust enough to allow for a detailed analysis. Depletion of endogenous NOD1 and NOD2 by specific siRNA in THP1-Blue cells, a derivative of THP1 cells that stably express an NF- κ B driven reporter, led to the specific reduction of the NOD1 and NOD2 mRNA levels, respectively (Fig. 5B). This translated into a significant decrease in the NOD1/2-mediated NF- κ B activation induced by their cognate elicitors, TriDAP and MDP, respectively (Fig. 5A). As expected, V:5/04 OMVs induced a robust NF- κ B activation in cells treated with a nontargeting control siRNA, whereas knockdown of NOD1 led to a significant reduction of the OMV-induced NF- κ B response (Fig. 5A). Surprisingly, depletion of NOD2 had no significant effect, suggesting that

FIG. 4. OMVs trigger NOD1- and NOD2-dependent NF- κ B activation. HEK293T cells were transiently transfected with plasmids encoding NOD1 (left panel) or NOD2 (right panel) together with a NF- κ B (A and B) or IL-8 (C and D) luciferase reporter system. Cells were stimulated with the indicated OMVs (0.5 μ g/ml) or MDP (50 nM) and TriDAP (500 nM) as controls. RPS, NF- κ B activity of OMVs (0.5 μ g/ml) in cells transfected only with the reporter system. NF- κ B activity was determined by measuring luciferase activity of the cell lysates after 16 h of incubation. Means and SDs ($n = 3$) are shown. n.s., not stimulated.

NOD1 might be the physiologically relevant sensor of OMV-delivered peptidoglycan in this setting. Collectively, these data suggest that V5:04 OMVs contain NOD1/2 active peptidoglycan. To obtain additional evidence for the presence of PGN in the V5:04 OMVs and in order to analyze whether deletion of HapR might affect the peptidoglycan content of the OMVs, we analyzed the chemical composition of the *V. cholerae* OMVs by X-ray photoelectron spectroscopy (XPS). This revealed a significant difference in the chemical surface compositions of the OMVs from the two strains. The wild-type vesicle contained 55% ($\pm 4\%$) peptidoglycan/protein and 18% ($\pm 1\%$) polysaccharide, whereas the V5:04 Δ hapR OMVs, contained 29% ($\pm 10\%$) peptidoglycan/protein and 16% ($\pm 2\%$) polysaccharide (Fig. 5C). Of note also, the lipid content was different and V5:04 Δ hapR OMVs contained more lipids than WT OMVs (Fig. 5C). Since the amount of protein in the two vesicles samples was equal, the XPS data correlate with reduced peptidoglycan content in the V5:04 Δ hapR OMVs compared to V5:04 OMVs. In conclusion, we established that NOVC

OMVs contain PGN capable of activating both NOD1- and NOD2-mediated inflammatory responses (i.e., likely DAP-type peptidoglycan). Of note, this is in line with recent findings indicating that *H. pylori* can deliver NOD1-active peptidoglycan into the host by OMVs to modulate the immune response (22).

Quorum sensing specifically alters the inflammatory potential of OMVs but not of the whole bacteria. We were intrigued by the finding that quorum sensing affected NOD1/2 activation mediated by the OMVs. This suggested that the levels of NOD1/2 active PGN are actively controlled by gene products under the control of HapR during the *V. cholerae* infective lifestyle (33, 45). HapR is an important repressor of virulence gene expression, and many *Vibrio* virulence factors have been associated with the induction of cell death in host cells. Accordingly, we found that OMV-free supernatants of V5:04 Δ hapR induced more cell death than those of V5:04 in HeLa cells (Fig. 6A). Of note, this was opposite to the effect of HapR on the OMV-mediated inflammatory response. We next asked

FIG. 5. OMVs contain NOD1/2 active peptidoglycan. (A) The NOD1 or NOD2 gene was silenced in THP1-Blue cells by transfecting specific siRNA duplexes for 72 h. Cells were subsequently stimulated with NOVC V:5/04 OMVs (1 μ g/ml) or with TriDAP (10 μ g/ml) or MDP (10 μ M) as controls. NF- κ B activity was determined by measuring SEAP activity in the supernatant after 16 h and is given as mean fold over background from two independent experiments. Error bars indicate standard errors of the means ($n = 4$). n.s., not stimulated. (B) Quantitative PCR of NOD1 and NOD2 mRNAs prepared from samples used for panel A. Mean expression relative to GAPDH is shown normalized to NOD1/2 expression in cells treated with nontargeting siRNA (siCTRL) (set to 1). Error bars indicate SDs ($n = 3$). (C) The chemical composition of vesicle samples was determined using multivariate analysis of X-ray photoelectron spectroscopy (XPS) as described in Materials and Methods. The near-surface (<10 nm) composition of polysaccharide, lipid, and protein/peptidoglycan for each sample is shown (means and SDs; $n = 3$).

whether the inflammatory activity of whole bacterial lysates, generated by boiling of the bacteria, might also be differently affected by HapR compared to OMVs; that is, does *Vibrio* change the overall PGN/PAMP composition or selectively omit PGN or PAMPs from OMVs during infection? To this end, the inflammatory potential of equal amounts of lysates from V:5/04 and V:5/04 $\Delta hapR$ bacteria was tested. Cell lysates induced an NF- κ B response in THP1-Blue cells (Fig. 6D) as well as in the NOD2-HEK293T cell assay (Fig. 6E). Strikingly, in contrast to our observations with the OMVs, lysates from V:5/04 lacking HapR did not have a diminished potential to activate NF- κ B (Fig. 6B, D, and E). In contrast, lysates from V:5/04 $\Delta hapR$ bacteria induced even slightly stronger responses. Probing of equal amounts (total protein) of the two bacterial lysates for the RNA polymerase subunit sigma S (RpoS) showed equal lysis of the cells (Fig. 6C). However, the lysates did not induce NF- κ B activity in HEK293T cells with-

out transfection of NOD2, demonstrating a dependency of the response on NOD2 in this setting (Fig. 6E, RPS). Similar results were obtained when measuring IL-8 release from HeLa cells by ELISA (Fig. 6B). Taken together, these results suggested that the quorum-sensing system specifically influences the PGN content of OMV but has little impact on the overall composition of the bacterial cell wall in terms of changes in NOD1/2 active components.

DISCUSSION

We revealed that OMVs from *V. cholerae* induce NF- κ B-mediated innate immune responses in different human cell lines. Indeed, we observed that OMV production is a common feature in different *V. cholerae* strains (data not shown). In the present study, we show that OMVs from the NOVC strain V:5/04 induce a significant inflammatory response in the host

FIG. 6. Differential NOD1/2 response to bacterial cell lysate and OMVs. (A) Cell viability assay (XTT) of HeLa cells exposed to the indicated culture supernatants. The mean percentage of viable cells compared to the control (set to 100%) and SD ($n = 3$) are shown. (B) HeLa cells were treated with bacterial lysate or LPS (0.2 $\mu\text{g/ml}$) as a control. IL-8 release was measured in the supernatant by ELISA at 8 h postexposure. (C) Western blot of equal amounts of bacterial lysates probed with an RpoS-specific antibody. (D) THP1-Blue cells were treated for 16 h with the indicated bacterial lysate (0.05 $\mu\text{g/ml}$) or with MDP (10 μM), LPS (0.1 $\mu\text{g/ml}$), or a combination of both as controls. NF- κ B activity was determined by measuring SEAP activity in the supernatant. Activity is given as mean fold over background. Error bars indicate SDs ($n = 3$). (E) HEK293T cells were transiently transfected with a plasmid encoding NOD2 together with an NF- κ B luciferase reporter system. Cells were stimulated with bacterial lysate (0.5 $\mu\text{g/ml}$) or with MDP (50 nM) as a control. NF- κ B activity was determined by measuring luciferase activity in the cell lysates after 16 h of incubation. Means and SDs ($n = 3$) are shown. n.s., not stimulated.

cell, where V:5/04 OMVs robustly activated the intracellular PRRs NOD1 and NOD2. Intriguingly, this activity was dependent on the quorum-sensing master regulator HapR, a known control switch for virulence gene expression in *V. cholerae* O1 strains (33, 45), although HapR did not influence the amount of OMVs produced, arguing that a natural *hapR* mutant also might not show different OMV production. The quorum-sensing system might thus be a mechanism used by NOVC strains to control the immune response mediated by NOD1/2. Activation of NOD1 and NOD2 in selective assays by V:5/04 OMVs indirectly showed that PGN is associated with OMVs, which we confirmed by physicochemical characterization of the *V. cholerae* OMV preparations. Of note, deletion of *hapR* significantly attenuated the NOD1/2 activation potential of the V:5/04 OMVs but at the same time did not reduce the immunogenicity of whole bacterial lysates, indicating that genes under the control of HapR either change the composition of the PGN delivered by OMVs or lead to the exclusion of PGN from

the OMVs. One possible candidate gene involved in the peptidoglycan biosynthesis process is VCA0981, which encodes a protein with homology to the peptidoglycan-specific endopeptidase M23, a member of the family of periplasmic binding proteins (PBPs) which binds substrate and interacts with a membrane-bound complex. We found that the expression of VCA0981 was decreased in the *hapR* mutant in comparison with the wild-type strain V:5/04 (data not shown), suggesting that this gene product is involved in the observed difference between WT- and $\Delta hapR$ -derived vesicles in activation of NOD1 and NOD2. However, overexpression of the protein in HapR-deficient cells did not yield complementation of this phenotype, suggesting either that overexpression is not suitable for complementation or that additional factors under the control of HapR are involved in this process. Of note, other bacterial pathogens such as *Listeria monocytogenes* and *H. pylori* also employ strategies to modulate NOD1 and NOD2 responses when colonizing the host. *L. monocytogenes* uses N

deacetylation of PGN by the N-deacetylase PgdA (6), whereas the coccoid form of *H. pylori* escapes NOD1 detection by the AmiA PGN hydrolase (8). This suggests that PGN modeling and subsequent coupled activation of NOD1 and NOD2 constitute a more general theme for bacterial manipulation of the host innate immune response.

Sensing of PGN by NOD1 and NOD2 requires its translocation to the cytoplasmic compartment (14, 15). However, how this is achieved remains somewhat fragmentary. It has been shown for *H. pylori* that PGN can be introduced into the host cell by its type IV secretion system (43), whereas macrophages and some epithelial cell lines can directly take up MDP by a dynamin-dependent process (30, 31). Furthermore, pore-forming toxins were reported to mediate PGN uptake and stimulation of NLRs (36). Our data suggest that OMVs represent an alternative route to deliver bacterial PGN to the host. Indeed, delivery of bacterial PGN was also reported for *H. pylori* OMVs (21). A recent study revealed that also *Pseudomonas aeruginosa*- and *Neisseria gonorrhoeae*-derived OMVs contain PGN and activate NOD1 upon contact with host cells (22). Collectively, this indicates that PGN delivery by OMVs is a conserved and common bacterial strategy to modulate host immune responses. Further studies will help us to understand which mechanism is relevant under different physiological conditions. Of note, to the best of our knowledge, our work is the first to provide a role for the quorum-sensing machinery in OMV-mediated activation of host PRRs.

Recently, *V. cholerae* OMVs were shown to be good candidates for vaccines, as they induce strong immunity in mice (38). Furthermore, NOD1 and NOD2 activation has been linked to the onset of adaptive immune responses (13, 29). Thus, it is likely that PGN is one of the active adjuvant components accounting for the high immunogenicity of OMVs. Further studies will elucidate the immunogenic potential of V:5/04 and V:5/04Δ*hapR* OMVs in mice, ultimately helping to open new avenues for the development of more efficient vaccine strategies.

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