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Elicitation of Epithelial Cell-Derived Immune Effectors by Outer Membrane Vesicles of Nontypeable *Haemophilus influenzae*▽†

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Outer membrane vesicles (OMVs) are produced by all Gram-negative microorganisms studied to date. The contributions of OMVs to biological processes are diverse and include mediation of bacterial stress responses, selective packaging and secretion of virulence determinants, modulation of the host immune response, and contributions to biofilm formation and stability. First characterized as transformasomes in *Haemophilus*, these membranous blebs facilitate transfer of DNA among bacteria. Nontypeable *Haemophilus influenzae* (NTHI), an opportunistic pathogen of the upper and lower respiratory tracts, produces OMVs *in vivo*, but there is a paucity of information regarding both the composition and role of OMVs during NTHI colonization and pathogenesis. We demonstrated that purified NTHI vesicles are 20 to 200 nm in diameter and contain DNA, adhesin P5, IgA endopeptidase, serine protease, and heme utilization protein, suggesting a multifaceted role in virulence. NTHI OMVs can bind to human pharyngeal epithelial cells, resulting in a time- and temperature-dependent aggregation on the host cell surface, with subsequent internalization. OMVs colocalize with the endocytosis protein caveolin, indicating that internalization is mediated by caveolae, which are cholesterol-rich lipid raft domains. Upon interaction with epithelial cells, NTHI OMVs stimulate significant release of the immunomodulatory cytokine interleukin-8 (IL-8) as well as the antimicrobial peptide LL-37. Thus, we demonstrated that NTHI OMVs contain virulence-associated proteins that dynamically interact with and invade host epithelial cells. Beyond their ability to mediate DNA transfer in *Haemophilus*, OMV stimulation of host immunomodulatory cytokine and antimicrobial peptide release supports a dynamic role for vesiculation in NTHI pathogenesis and clinically relevant disease progression.

Nontypeable *Haemophilus influenzae* (NTHI) is a Gram-negative commensal inhabitant of the human nasopharynx that causes diseases of the mammalian upper and lower airways. NTHI predominates in otitis media (OM) and other localized respiratory diseases, such as acute sinusitis and community-acquired pneumonia, and has important consequences in patients with chronic obstructive pulmonary disease (COPD) or cystic fibrosis, with direct and indirect costs of diagnosing and managing OM exceeding $5 billion annually in the United States alone (21, 22, 32, 35, 40, 41, 48). To gain a more comprehensive understanding of the dynamic interplay between microbe-expressed virulence factors and the host immune response, particularly during the transition from commensal to pathogen, the secretion of virulence-associated bacterial components must be investigated further. The blebbing of outer membrane vesicles (OMVs) is one common and recently appreciated mechanism of release of virulence-associated proteins by Gram-negative bacteria such as NTHI.

OMVs are ubiquitously shed among pathogenic and nonpathogenic Gram-negative bacteria (7, 23, 27). Vesicles are composed of luminal periplasmic material bound by a layer of bacterial outer membrane components (e.g., lipopolysaccharide, phospholipids, and outer membrane proteins) and play an active role in pathogenesis, as they are proposed to be vehicles for virulence factor delivery to host cells (3, 4, 15, 17, 19, 20, 51). The functions of bacterial OMVs are versatile, such as regulation of the bacterial stress response, reduction of toxic compounds in the environment, quorum sensing, and coaggregation of bacteria, thus enabling colonization and biofilm formation (7, 11, 24, 29, 34, 43, 53, 55). In addition to these functions, OMVs mediate an immunomodulatory role in bacterial pathogenesis (4). *Moraxella* OMVs contain the immunostimulatory superantigen molecule MID, which induces a nonspecific immune response that fails to target *Moraxella* whole bacterial cells, thus contributing to pathogenesis (50). Furthermore, OMVs stimulate Toll-like receptor 2 (TLR-2), TLR-4, and TLR-9 host cell signaling molecules and the release of immunomodulatory cytokines such as interleukin-6 (IL-6), IL-8, and IL-12 (7, 23, 49). The ability of OMVs to modulate robust host immune responses presents OMVs as attractive vaccine candidates; both natural and engineered OMVs have been shown to confer immunity to challenges with pathogenic bacteria (49, 50).

NTHI, a versatile opportunistic pathogen, has been reported to shed outer membrane vesicles both *in vitro* (6) and *in vivo* (14). Thus, NTHI OMVs are likely to be immunomodulatory, contributing to NTHI pathogenesis. Outer membrane vesicles produced by *Haemophilus* were described to have a role in natural competence (6) and were characterized as transformasomes due to their ability to transport DNA, both internally

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and on the surface, among bacterial cells (18). Importantly, *Haemophilus* vesicles could induce blood-brain barrier permeability during experimental meningitis (54). The biological role of outer membrane vesicles produced by clinically relevant NTHI strains remains unknown, and there is a paucity of comprehensive information regarding the components of vesicles and their role in clinically relevant *Haemophilus* disease states. Thus, we investigated OMV production, identified vesicle protein cargo, and monitored OMV interaction with and stimulation of host respiratory and middle ear epithelial cells. We further monitored this interaction with host epithelial cells by fluorescence microscopy and elucidated the mechanism of OMV internalization. The ability of OMVs to modulate host cell responses suggests that outer membrane vesicles are a potent contributor to NTHI pathogenesis and play a role in the delicate balance between the commensal and pathogenic lifestyles of NTHI.

MATERIALS AND METHODS

Bacterial strains and culture conditions. NTHI strain 86-028NP is a minimally passaged clinical isolate obtained at Nationwide Children's Hospital in Columbus, OH. This strain has been sequenced and characterized extensively (12, 31). Strain 86-028NP was grown on chocolate II agar (Becton Dickinson, Sparks, MD) or in brain heart infusion broth supplemented with 2 μg heme/ml and 1 μg NAD/ml (sBHI). Bacteria were cultured from overnight growth on chocolate II agar, resuspended in sBHI to an optical density at 490 nm (OD_{490}) of 0.65, diluted 1:100 in fresh sBHI medium, and grown overnight (16 h) at 37°C and 180 rpm.

Purification and imaging of OMVs. OMVs were isolated from strain 86-028NP cultured overnight (16 h) at 37°C and 180 rpm in sBHI broth. Whole cells were removed by centrifugation (10,000 × *g* for 10 min at 4°C). Supernatants were concentrated through a 100-kDa filter by use of a tangential-flow filtration system (Millipore, Billerica, MA) to a final volume that was one-sixth or less of the starting volume (approximately 250 ml for a 2-liter culture). The concentrated supernatant was filter sterilized (0.22 μm; Millipore), and OMVs were subsequently isolated from concentrated supernatants according to published methods (2, 19). Briefly, the purified retentate was centrifuged at 38,000 × *g* for 1 h (4°C) to pellet crude vesicles. Crude OMVs were resuspended in sterile 50 mM HEPES–150 mM NaCl. Crude vesicles were further purified by density gradient centrifugation (overnight, 150,000 × *g*, 4°C, SW41T.i rotor) on a discontinuous OptiPrep-iodixanol (Grenier Bio-One, Monroe, NC) gradient (3 ml 50%, 2 ml 45%, 2 ml 40%, 2 ml 35%, 2 ml 30%, and 1 ml 25% OptiPrep in 50 mM HEPES–150 mM NaCl). Gradient fractions (780 μl) were collected from top to bottom and resolved in a 12% acrylamide gel, and vesicle proteins were identified by silver nitrate staining. Vesicle-containing fractions were pooled, diluted 10× in 50 mM HEPES–150 mM NaCl, and pelleted (150,000 × *g*, 1 h, 4°C, 70Ti rotor). Vesicles were resuspended in 250 μl 50 mM HEPES–150 mM NaCl, stored at 4°C, and utilized within 4 weeks. Vesicle sterility was confirmed by plating 10 μl of each preparation on a chocolate II agar plate. No colonies were detected in any of the purified vesicle preparations. Bacterium-free OMVs were quantified for protein content by Bradford assay. By protein amount, 1 liter of culture yielded approximately 80 μg of purified OMVs.

Purified OMV samples (in 50 mM HEPES–150 mM NaCl) were applied to carbon-coated copper grids (Electron Microscopy Sciences), stained with 2% uranyl acetate, and air dried. Samples were visualized at 80 kV on a Hitachi H-7650 transmission electron microscope with AMtv542 software.

Purified OMVs were fluorescently labeled as previously described (20). Briefly, purified vesicles were pelleted (150,000 × *g* for 30 min, MTX 150 tabletop ultracentrifuge, S55-A2 fixed-angle microtube rotor) and resuspended in binding buffer (0.1 M sodium bicarbonate). Alexa Fluor 488 (Invitrogen, Carlsbad, CA) was added to the binding buffer suspension and incubated for 1 h at room temperature on a rotator. Vesicles were then pelleted and washed three times with Dulbecco's phosphate-buffered saline (D-PBS) to remove unbound dye. A final suspension was made in 50 mM HEPES–150 mM NaCl. The labeled OMVs were tested for sterility prior to use, stored at 4°C, and utilized within 4 weeks of labeling. Labeled OMVs were quantified by Bradford assay and did not contain aggregates as observed by fluorescence microscopy.

Proteinase K treatment of OMVs. Vesicles (50 μg) were incubated with 100 μg proteinase K/ml at 37°C on a rotator for 30 min. As a control, an equivalent amount of vesicles was incubated in the absence of proteinase K. Following incubation, vesicles were pelleted (150,000 × *g* for 30 min, MTX 150 tabletop ultracentrifuge, S55-A2 fixed-angle microtube rotor) and resuspended in D-PBS. This washing procedure was repeated 2 additional times to remove proteinase K. Washed vesicles were brought up in 50 mM HEPES–150 mM NaCl, stored at 4°C, and utilized within 1 week of proteinase K treatment.

DNA quantitation. Quantitation of DNA content in OMVs was performed as previously described (39), with the following modifications. Briefly, surface-associated and luminal DNAs were quantified by PicoGreen assay (Invitrogen). Ten micrograms of OMV protein was pelleted (150,000 × *g*, 30 min) and resuspended in 50 mM HEPES–150 mM NaCl or GES lysis reagent (5 M guanidinium thiocyanate, 100 mM EDTA, 0.5% Sarkosyl) to release DNA from OMVs. OMVs were treated with Ambion DNase according to the manufacturer's instructions to digest DNA bound to the outer surfaces of OMVs. Vesicles were pelleted (150,000 × *g*, 30 min) and resuspended in HEPES buffer, and supernatants were saved for DNA quantitation.

SDS-PAGE analysis of NTHI protein fractions. OMV samples were compared to NTHI 86-028NP cytoplasmic (CP), periplasmic (PP), and outer membrane (OMP) proteins prepared as previously described (9) and analyzed by SDS-PAGE. Enriched NTHI periplasmic and cytoplasmic protein fractions were obtained through whole-cell fractionation. Briefly, NTHI was grown to mid-log phase in sBHI. Cells were pelleted by centrifugation and resuspended in 2 mg polymyxin B sulfate/ml (Sigma) to permeabilize the outer membrane and release periplasmic proteins. Spheroplasts were pelleted by centrifugation. Periplasmic proteins were removed in the supernatant. The spheroplast pellet was lysed by freeze-thawing, membranes were pelleted by ultracentrifugation, and the supernatant (containing cytoplasm) was removed. All protein concentrations were determined by Bradford assay and normalized to 1 μg total protein. The proteins were resolved in a 12% acrylamide gel at 100 V for 120 min in 1× TGS buffer (Bio-Rad, Hercules, CA). Protein bands were visualized by silver nitrate staining.

Proteome analysis. Proteomic analysis was performed at the Institute for Genome Sciences and Policy Service Core at Duke University, Durham, NC. Briefly, purified OMVs were probe sonicated and resuspended in 50 μl of 50 mM Ambic buffer to a protein concentration of approximately 1.0 mg/ml (total of 50 μg of protein). Vesicles were resuspended in a volume of 500 μl for a protein concentration of 0.01 μg/μl. One hundred microliters of the vesicle suspension was digested, lyophilized, and resuspended in 20 μl of liquid chromatography (LC) buffer. A volume of 8 μl was used for mass spectrometry (MS) and analyzed using LC-MS/MS. Peptide masses were matched with the NCBI species-specific database for NTHI 86-028NP. There were approximately 1,830 entries in the database, which was downloaded from the NCBI website on 20 April 2011. There were approximately 100 proteins identified with 2 or more peptides and another approximately 50 proteins identified with reasonable confidence but only 1 peptide. The overall false discovery rate for the data set was ~4%; however, it was virtually 0% for the proteins with 2 or more peptides.

IL-8 and LL-37 detection. Primary chinchilla middle ear epithelial (CMEE) cells were isolated from adult chinchilla middle ear mucosa and cultured in CMEE growth medium at 37°C with 90% humidity and 5% CO_2 (33). Human pharyngeal (Detroit 562; CCL-138) epithelial cells were obtained from the ATCC (Manassas, VA) and cultured in Eagle's minimal essential medium (EMEM; ATCC, Manassas, VA) supplemented with 10% fetal bovine serum (FBS) at 37°C and incubated at a 90% humidity and 5% CO_2. The cells were passaged and grown in 96-well cell culture plates or an 8-well chamber slide system (Lab-Tek II; Thermo Fisher Scientific) to 80% confluence prior to utilization for IL-8 and LL-37 release and fluorescence microscopy.

CMEE and Detroit 562 cells were exposed to 1 μg of purified vesicles in a 120-μl total volume and were cultured at 37°C. Supernatants were collected at 24 and 48 h. IL-8 levels in the supernatants of both experimental and control (medium alone) wells were determined by enzyme-linked immunosorbent assay (ELISA), and absorbance values were compared to a standard curve prepared following the manufacturer's instructions (BD OptEIA human IL-8 ELISA kit). *P* values were determined by paired Student's *t* test.

Detroit 562 cells in a 96-well plate were exposed to 5 μl of proteinase K-treated vesicles, nontreated vesicles, or proteinase K alone. Supernatants were collected after 24 h, and IL-8 release was determined by comparison to a standard curve prepared according to the manufacturer's instructions (BD OptEIA human IL-8 ELISA kit).

Additionally, to determine whether OMVs stimulate components of host innate immunity, the 24-h supernatants were resolved by SDS-PAGE (Ready Gel Tris-HCl precast gels; Bio-Rad, Hercules, CA), transferred to polyvinylidene difluoride (PVDF; Bio-Rad, Hercules, CA) membranes, and blocked in 3% skim

milk. Membranes were incubated with rabbit anti-LL-37 (Phoenix Pharmaceuticals, Burlingame, CA) overnight at 4°C, washed, and incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG(H+L) (Invitrogen, Carlsbad, CA). Membranes were washed, and peroxidase activity was detected using SuperSignal West Femto chemiluminescent substrate (Thermo Scientific, Rockford, IL).

Fluorescence microscopy. Detroit 562 human pharynx epithelial cells were grown in an eight-well chamber slide system (Lab-Tek II) and exposed to fluorescently labeled vesicles. Cells were incubated at 37°C and 4°C (in parallel) for 30 min and then incubated, at their respective temperatures, for 2 h with 1 μg total Alexa Fluor-labeled OMVs suspended in EMEM. Alternately, cells were treated with 1 μg filipin/ml for 30 min prior to exposure to 1 μg Alexa Fluor-conjugated OMVs for 4 h. Following incubation with OMVs, cells were washed 3 times with PBS and stained for 10 min; plasma membranes were stained with 1 mg/ml of wheat germ agglutinin-Alexa Fluor 594 in PBS (Invitrogen, Carlsbad, CA), and DNA was stained with 0.5 mg/ml Hoechst 34580 (Invitrogen, Carlsbad, CA). Cells were fixed with 4% paraformaldehyde overnight at 4°C, and a coverslip was attached using ProLong Gold (Invitrogen). Slides were imaged with an Axiovert 200 M inverted epifluorescence microscope equipped with an Axiocam MRM charge-coupled device (CCD) camera (Carl Zeiss Inc., Thornwood, NY). Three-dimensional reconstructions of planar image stacks were processed using ImageJ computer software. Images shown are representative of three independent experiments.

Quantitative analysis of vesicle aggregation. Representative image data sets from three independent experiments were rendered into a 3-dimensional image. The image was flattened in the *z* dimension such that the vesicles in all planes remained in focus in a single image. The image of the green channel only (representing the Alexa 488-labeled vesicles) was inverted in color tone in black and white such that the vesicles appear black in Adobe Photoshop. The "threshold" algorithm was applied in NIH ImageJ (http://rsbweb.nih.gov/ij/) across the entire image until all of the smallest vesicles were observed. The "analyze particles" function of NIH ImageJ was applied, using the parameters of a particle size of 0 to 1,000 and a circularity of 0 to 1. Each image set analyzed contained 160 to 414 vesicles. There were a total of 677 vesicles in the no-filipin group and 934 vesicles in the filipin-treated group. There was a statistically significant difference between the sizes of the vesicles in the treatment groups ($P < 0.0258$) by unpaired Student's *t* test (Graphpad Prism).

Subcellular fractionation of OMV-treated epithelial cells. To determine whether NTHI OMVs are internalized by host epithelial cells via a caveola-dependent mechanism, we isolated detergent-insoluble epithelial lipid raft domains according to a protocol modified from a previously described method (47). Briefly, human pharyngeal Detroit 562 cells were grown to 85% confluence in a 25-cm^2 flask and were incubated with or without 50 μg NTHI OMVs for 24 h. Following incubation, the cells were washed three times with ice-cold D-PBS and solubilized in 1 ml 1% Triton X-100 and MBS (0.25 M NaCl and 25 mM morpholineethanesulfonic acid [MES], pH 6.8) supplemented with protease inhibitor cocktail (CalBioChem, La Jolla, CA) on ice for 1 h without agitation. Cells were removed with a cell scraper, and 1 ml cell lysate suspension was adjusted to 40% OptiPrep in MBS, overlaid with 2 ml each of 35, 25, 15, and 5% OptiPrep in MBS, and centrifuged overnight at 100,000 × *g*. Twelve equal fractions (1 ml) were collected from the top of the gradient and were assayed for NTHI outer membrane proteins, caveolin, and clathrin heavy chain by immunoblot analysis. Subcellular fractions were separated by SDS-PAGE, transferred to nitrocellulose (Bio-Rad, Hercules, CA), and blocked in 3% skim milk. Membranes were incubated with mouse anti-caveolin (BD Biosciences Bioimaging, Rockville, MD), mouse anti-clathrin heavy chain (BD Biosciences Bioimaging, Rockville, MD), or chinchilla anti-NTHI OMP serum overnight at 4°C, washed, and incubated with goat anti-mouse IgG(H+L)–HRP (Invitrogen, Carlsbad, CA) or anti-protein A–HRP (Invitrogen, Carlsbad, CA). Membranes were washed, and peroxidase activity was detected using ECL Plus Western blotting detection reagent (GE Healthcare Life Sciences, Piscataway, NJ). OMVs alone were subjected to OptiPrep gradient centrifugation and assayed for endocytosis markers. OMVs did not cross-react with either clathrin or caveolin antibodies. Additionally, cells alone were probed for NTHI OMPs and did not display a protein signal by immunoblotting, and endocytosis markers were found in high-density fractions.

RESULTS

NTHI produces outer membrane vesicles that contain lipo-oligosaccharide (LOS), DNA, and virulence-associated proteins. There has been little information regarding *Haemophilus* OMVs since their initial characterization as transformasomes 30 years ago. Given that OMV production by other Gram-negative bacteria is biologically relevant (7), we purified and characterized the production of NTHI OMVs. We determined that a clinical NTHI isolate, the prototypic strain 86-028NP, produced protein-rich OMVs from cells grown to stationary phase in rich medium. NTHI vesicle preparations were purified by density gradient purification as previously described (15). We observed that NTHI vesicles equilibrated to a density of 28% in OptiPrep solution, as determined by refractometry of gradient fractions separated by SDS-PAGE (Fig. 1A). Peak fractions containing OMVs were pooled and visualized by transmission electron microscopy. We observed spherical blebs of 20 to 200 nm in diameter which contained electron-dense luminal components and were encased in a spherical membrane (Fig. 1B).

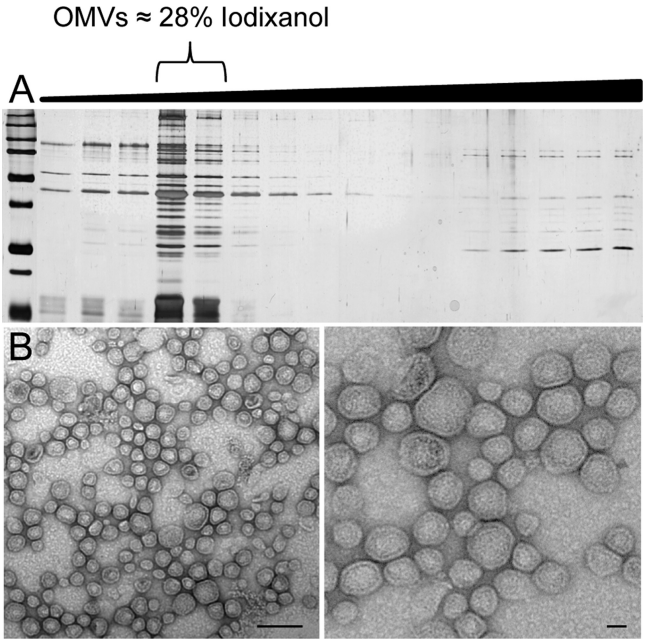

FIG. 1. Purification of NTHI OMVs. NTHI produces OMVs that are 20 to 200 nm in diameter and contain bacterial proteins. (A) Differential density centrifugation of NTHI OMVs. Crude vesicle preparations were layered on the bottom of an iodixanol density gradient solution and centrifuged, and equal-volume fractions were obtained from the top, low-density portion of the gradient (increases in fraction density are indicated by a black wedge). Fractions were resolved by SDS-PAGE and visualized by silver staining for OMV-associated proteins. OMVs floated to the density of a 28% iodixanol solution. LOS was observed in fractions containing OMVs. (B) Purified NTHI vesicles were placed on copper grids and stained with uranyl acetate for visualization by electron microscopy at increasing magnifications. Bar, 100 nm.

In order to characterize their composition and identify protein cargo of NTHI OMVs, we compared OMV proteins to enriched cytoplasmic, periplasmic, and outer membrane protein fractions from whole NTHI bacteria. Proteins were resolved by SDS-PAGE and visualized by silver staining (Fig. 2). OMVs contained proteins of similar size and mass to those found in the periplasm and outer membrane of NTHI, consistent with the current model of OMV biogenesis (5, 7, 26, 27, 52). NTHI OMVs contained proteins with masses of 32 or 37 kDa (P5 fimbrin) and 39 kDa (P2) that were identified as outer

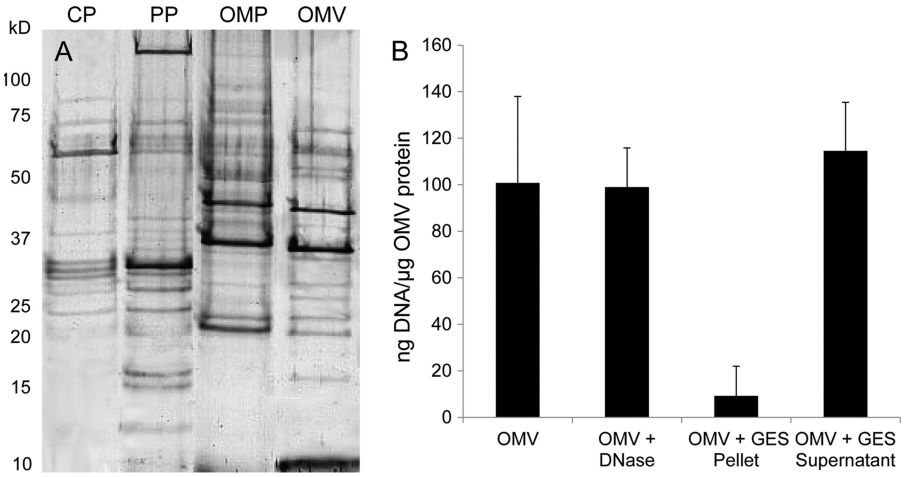

FIG. 2. OMVs package outer membrane and periplasmic proteins and contain surface and luminal DNAs. (A) The OMV protein profile was compared to NTHI strain 86-028NP cytoplasmic (CP), periplasmic (PP), and outer membrane (OMP) proteins isolated from whole cells. Protein preparations were separated by SDS-PAGE and visualized by silver staining. (B) Surface-associated and luminal DNAs were quantified for OMVs, OMVs treated with DNase, and the OMV pellet and supernatant following incubation with GES lysis reagent. Error bars represent the standard errors of the means for three independent assays repeated in triplicate ($n = 3$).

membrane proteins, as they were not detected in periplasmic or cytoplasmic fractions (data not shown). The outer membrane protein P5 has been shown previously to mediate NTHI pathogenicity and is currently targeted as an otitis media vaccine candidate (36). In addition to outer membrane proteins, NTHI OMVs contain lipooligosaccharide and DNA. Based on the previous results demonstrating that NTHI blebs mediate transformation, we also determined the DNA content of the purified OMVs and found that they contained approximately 100.8 ng DNA/1 μg OMV protein. DNA was packaged primarily within the vesicle lumen (94%) and was released upon vesicle lysis (Fig. 2B). Approximately 6% of the DNA, likely contained on the external surface, was susceptible to DNase. Surface-associated DNA may contribute to biofilm nucleation (43), as OMVs have been identified as a component of the biofilm matrix (13, 44, 55). Our initial characterization revealed that NTHI OMVs contain virulence-associated OMPs and DNA, yet we observed additional proteins compared to NTHI outer membrane components (Fig. 2). Protein selectivity or exclusion from NTHI OMVs may dictate their functional role and better define the mechanism of OMV formation.

To further define the proteomic profile of NTHI OMVs, vesicles were subjected to tryptic digestion and mass spectrometry. Proteomic analysis identified 142 *Haemophilus* proteins that were packaged in NTHI OMVs, including mainly periplasmic and outer membrane proteins (Table 1; see Table S1 in the supplemental material). Proteins found in vesicles from other bacterial species have been discovered to exhibit a wide range of functions. Concordant with these observations, the vast array of proteins found in NTHI OMVs includes ABC transporters, adhesins, virulence factors, and proteins known to contribute to bacterial survival. Therefore, NTHI OMVs contain not only DNA and LOS but also a vast array of proteins that could contribute to their physiological role in the host.

Outer membrane vesicles stimulate production of the immunomodulatory cytokine IL-8 and the antimicrobial propeptide hCAP18/LL-37. Since NTHI OMVs contain LOS and package proteins previously shown to contribute to NTHI pathogenesis, we exposed host epithelial cells to purified NTHI OMVs and assessed secretion of IL-8, an immunomodulatory cytokine. Human pharynx cells and primary chinchilla middle ear epithelial cells exposed to NTHI OMVs secreted significantly more IL-8 than cells exposed to medium alone (Fig. 3). In order to determine the contribution of OMV surface proteins to IL-8 stimulation and release, epithelial cells were exposed to OMVs pretreated with proteinase K to degrade surface-exposed proteins (see Fig. S1 in the supplemental material). We determined that proteinase K-treated OMVs stimulated the release of similar amounts of IL-8 to those for nontreated vesicles (Fig. 3C). These data suggest that OMV LOS and luminal proteins, and possibly other vesicle-associated molecules, such as DNA, contribute to IL-8 release from host cells (42). We hypothesize that LOS is the key modulator of cytokine stimulation in host cells, however, since proteinase K treatment did not alter the ability of vesicles to interact with and stimulate host epithelial cells. Furthermore, the majority of the DNA was contained within the vesicle lumen, not on the vesicle surface, and OMV failed to stimulate increased expression of TLR-9 (data not shown), suggesting that DNA is not a significant contributor to modulation of host cell responses. These data are consistent with the recent observation that DNA does not contribute significantly to the inflammatory response to vesicles (8).

Our observation of IL-8 elicitation led us to investigate the role of OMVs in stimulation of other host innate immune responses, such as antimicrobial peptides (AMPs). AMPs are small, cationic molecules that target and disrupt bacterial membranes. NTHI OMVs were found to induce the production of the antimicrobial propeptide hCAP18/LL-37 by host epithelial cells. We exposed human pharyngeal cells to purified NTHI OMVs and assessed LL-37 secretion by immunoblotting (Fig. 3D). We observed the presence of a protein with an apparent molecular mass of 18 kDa by immunoblotting after

TABLE 1. NTHI 86-028NP OMV proteins identified by LC-MS/MS

Identified protein (over 95% confidence)	Gene no.	GenBank accession no.	Molecular mass (kDa)	No. of protein hits[a]
Outer membrane protein P2 precursor	NTHI0225	68056946	40	135
Outer membrane protein P5	NTHI1332	68057911	38	46
Probable amino acid ABC transporter binding protein	NTHI1243	68057836	28	34
HMW1B (OMP-85-like protein required for secretion of HMW1A and HMW2A)	NTHI1984	68058015	61	34
HMW2B (OMP-85-like protein required for HMW1A and HMW2A secretion)	NTHI1449	68058015	61	34
IgA-specific serine endopeptidase	NTHI1164	68057774	197	32
Outer membrane protein P1 precursor	NTHI0522	68057203	50	30
Probable D-methionine-binding lipoprotein MetQ	NTHI0877	68057518	30	27
NAD nucleotidase	NTHI0303	68057010	66	26
Heme-binding protein A	NTHI1021	68057646	61	26
Spermidine/putrescine-binding periplasmic protein 1 precursor	NTHI1823	68058340	43	26
Probable periplasmic serine protease do/HhoA-like precursor	NTHI1905	68058410	49	25
Heme utilization protein	NTHI1390	68057962	103	25
L-Lactate dehydrogenase	NTHI2049	68058536	42	25
Hemoglobin-haptoglobin binding protein B	NTHI0782	68057439	114	24
Putative periplasmic chelated iron binding protein	NTHI0481	68057164	32	23
2′,3′-Cyclic-nucleotide 2′-phosphodiesterase	NTHI0741	68057402	73	22
Periplasmic oligopeptide-binding protein	NTHI1292	68057874	61	22
Phosphate-binding periplasmic protein precursor PstS	NTHI1774	68058299	37	18
Elongation factor Tu	NTHI0712	68057378	43	17
Putative lipoprotein	NTHI1957	68058456	63	16
Protective surface antigen D15	NTHI1084	68057702	88	16
Iron utilization periplasmic protein hFbpA	NTHI0177	68056907	36	15
Glycerol-3-phosphate transporter	NTHI0809	68057464	53	15
TRAP-type C_4-dicarboxylate transport system, periplasmic component	NTHI0232	68056953	36	14
HMW1A (high-molecular-weight adhesin 1)	NTHI1983	68058480	154	14
D-Galactose-binding periplasmic protein precursor	NTHI0987	68057616	38	10
Conserved hypothetical protein	NTHI1208	68057809	41	10
TolB	NTHI0502	68057184	45	10
Conserved hypothetical lipoprotein	NTHI0266	68056981	29	10
Glycerophosphoryl diester phosphodiesterase precursor	NTHI0811	68057465	42	10

[a] Purified OMVs isolated from 86-028NP were analyzed using LC-MS/MS and were matched with the NCBI *H. influenzae* 86-028NP database. Listed proteins include those with 10 or more protein hits, all of which have over 95% confidence. A full list of identified proteins (142) can be found in Table S1 in the supplemental material.

both 24 and 48 h of OMV exposure. This protein is consistent with the propeptide form of LL-37 that is stored in epithelial cells, released, and activated upon cleavage by an extracellular protease (46, 56). These data suggest that OMVs are capable of stimulating host cells to release host defense peptides and, taken together, demonstrate that NTHI OMVs elicit a multifaceted immunomodulatory response from host epithelial cells.

NTHI vesicles bind to and are internalized by epithelial host cells. The ability of NTHI OMVs to stimulate production and release of the immunomodulatory cytokine IL-8 suggests direct interaction with the host cell. In order to further elucidate the mechanism of OMV-host cell interaction, we exposed human pharyngeal cells to Alexa Fluor-labeled vesicles and visualized the OMV-host cell interaction by fluorescence microscopy (Fig. 4). Rendered three-dimensional reconstructions of images revealed that OMVs bind to and interact with the host cell surface (Fig. 4, left panels). OMVs were observed to bind in a time-dependent manner, with OMV aggregates forming after as little as 30 min of exposure. OMVs remained surface associated with the epithelial cells after a series of washes, indicating an intimate interaction between host cells and NTHI OMVs. Additionally, host cells appeared to internalize OMVs, and OMV-associated fluorescence was observed throughout the cell layer (Fig. 4, left panels, side view).

To confirm this observation, epithelial cells were incubated at 37°C or 4°C prior to and during exposure to labeled OMVs. Three-dimensional renderings revealed internalization in cells maintained at 37°C, represented by a robust OMV-associated signal throughout the epithelial cells (Fig. 4A, 37°C panel). In contrast, when cells were incubated at 4°C, OMVs were associated primarily with the cell surface and OMV-associated fluorescence did not appear throughout the cell layer, indicating a decreased ability of host cells to internalize vesicles (Fig. 4A, 4°C panel). These data suggest that NTHI outer membrane vesicles directly bind to, interact with, and are internalized by host epithelial cells in a temperature-dependent manner.

NTHI OMVs are internalized into epithelial cells via a caveola-dependent mechanism. Host cells have been shown to internalize bacterial OMVs through cholesterol-enriched lipid rafts or clathrin-mediated endocytosis (10, 20, 38). To assess the mechanism of NTHI OMV internalization, human pharyngeal cells were incubated with NTHI OMVs for 24 h, lysed, and separated into subcellular fractions by density gradient centrifugation as previously described (47). In this manner, we determined whether vesicle-associated proteins would cofractionate with either caveolin or clathrin, protein components of caveola- and clathrin-dependent endocytosis mechanisms, respectively. We demonstrated that OMVs cofractionated with caveolin protein and not with clathrin, as determined by immunoblot (Fig. 5). In parallel gradients, vesicles alone did not equilibrate to the same

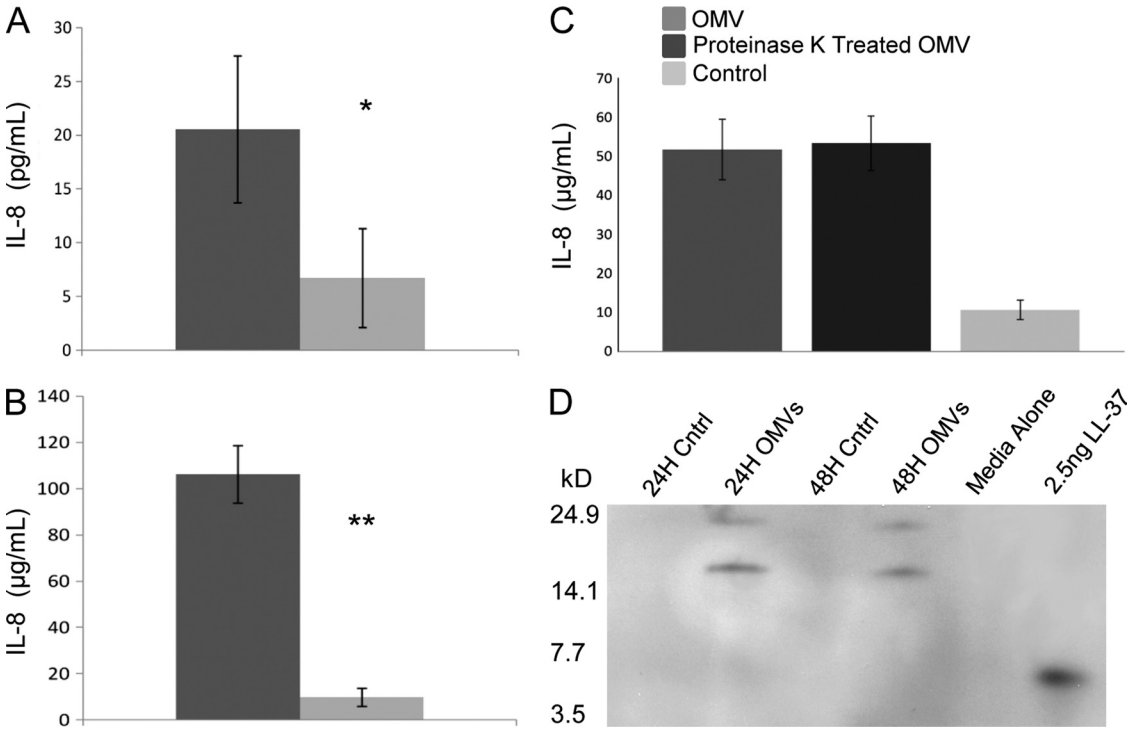

FIG. 3. NTHI OMVs stimulate IL-8 production and hCAP18/LL-37 release. Primary CMEE cells (A) and Detroit 562 human pharyngeal cells (B) release significant amounts of IL-8 upon OMV exposure compared to cells incubated in the absence of OMVs (medium alone). Data represent three independent experiments ($n = 3$) performed in triplicate. P values were determined by paired Student's t test. *, $P \leq 0.0153$; **, $P \leq 0.0001$. (C) Proteinase K treatment does not diminish OMV-induced IL-8 release. Detroit 562 cells release IL-8 in response to both OMVs and proteinase K-treated OMVs. Data represent one experiment performed in triplicate. (D) hCAP18/LL-37 propeptide (18 kDa) is released from epithelial cells in response to OMV exposure for 24 and 48 h. The cleaved product peptide, LL-37, is approximately 4.7 kDa.

density fraction as either endocytosis marker, nor did OMVs cofractionate with clathrin (see Fig. S2 in the supplemental material). Collectively, these data indicate that cell-associated OMVs colocalize with caveolin protein in the host cell, suggesting internalization via a caveola-dependent mechanism. In order to confirm this observation, we exposed host epithelial cells to OMVs in the presence or absence of filipin, an inhibitor of cholesterol biosynthesis which diminishes recycling of caveolae and cholesterol- and caveolin-enriched lipid rafts. We observed that filipin disrupted OMV colocalization with caveolin protein, as indicated by a shift in the density fraction localization of both OMPs and caveolin (Fig. 5). The shift in colocalization is represented graphically by measurement of the average pixel density of the immunoblots in relation to the background signal (graphs in Fig. 5). Additionally, internalization was not observed by fluorescence microscopy of filipin-treated cells exposed to OMVs (Fig. 4B, + Filipin panel). We monitored differences in vesicle size between the two groups as a measure of aggregation and internalization and calculated whether inhibition of caveola formation resulted in the diminished OMV aggregation. We demonstrated that there was a statistically significant reduction in overall vesicle size in filipin-treated cells compared to cells not exposed to the inhibitor ($P < 0.0258$). Collectively, these data support the hypothesis that host cells internalize NTHI OMVs by using a caveola-dependent mechanism.

DISCUSSION

Recent reports that describe the production, characterization, and functional roles of OMVs in bacterial virulence and their immunomodulatory effects on host cells have propelled an interest in further defining the contributions of OMVs to commensal and pathogenic bacterial behaviors (7). Although all Gram-negative organisms studied to date produce OMVs, there have been no structural or functional studies of OMVs released from clinically relevant NTHI strains. Thus, we investigated NTHI OMV release, protein composition, and modulation of host cell activity. NTHI OMVs contain proteins known to mediate NTHI virulence, elicit a potent response from epithelial cells, and are poised to play a significant role in NTHI infection and pathogenesis.

We identified 142 *Haemophilus* proteins packaged within these membranous structures, including virulence-associated outer membrane proteins, adhesins, endopeptidases, and nutrient utilization proteins (Table 1; see Table S1 in the supplemental material). This proteomic profile characterizes the distribution of these proteins outside the bacterial cell by vesicle secretion. Gradient-purified NTHI OMVs contained primarily outer membrane and periplasmic components and were mostly devoid of cytoplasm-associated proteins, in support of current models of OMV biogenesis (5, 7, 27).

The contribution of NTHI OMV pathogenesis remains undefined, yet the presence of NTHI adhesins and proteins es-

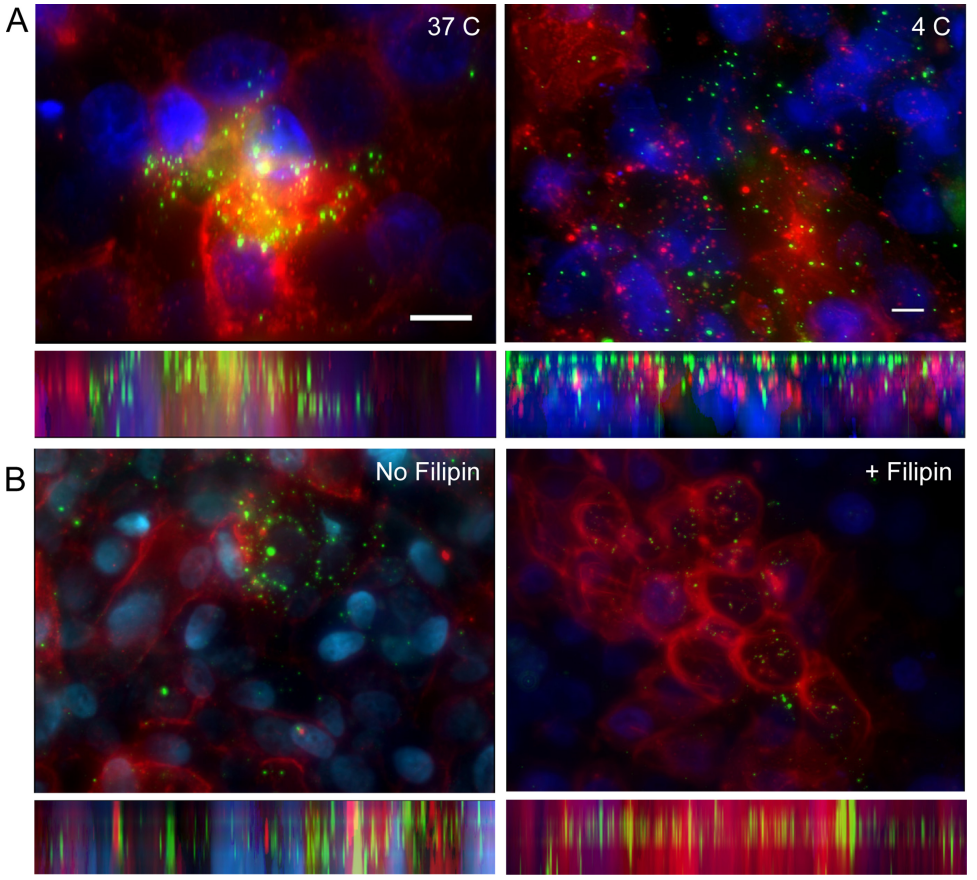

FIG. 4. NTHI vesicles interact with host cells and are internalized. Alexa Fluor 488-labeled OMVs (1 μg; green) were incubated with Detroit 562 cells for 2 h at 37°C, washed, and monitored for interaction with host epithelial cells. Cell membranes were labeled with wheat germ agglutinin (red; Molecular Probes), and cell nuclei (blue) were stained with Hoechst dye (Molecular Probes). All images are 3-dimensional renderings of planar optical sections with top-down views (above) and orthogonal side views (below). (A) Temperature-dependent uptake of OMVs by epithelial host cells. Detroit 562 human pharyngeal cells were exposed to labeled vesicles and incubated at 37°C and 4°C. Bar, 10 μm. (B) Detroit 562 cells were pretreated with filipin, an inhibitor of cholesterol recycling, and then exposed to labeled OMVs in the presence of filipin for 4 h. In parallel, cells were exposed to OMVs in the absence of filipin treatment and incubated for 4 h at 37°C. Images are representative of three independent experiments. There was a statistically significant difference ($P < 0.0258$) between the sizes of the vesicles in the filipin-treated group and the no-filipin group.

sential for NTHI survival may mediate biologically important functional roles. In fact, it has been well characterized that the outer membrane protein P5 (OMP P5), contained in OMVs, contributes to NTHI pathogenesis, and it is currently designated an attractive vaccine candidate for NTHI-mediated diseases (36). Interestingly, OMP P5 contains a "decoy" epitope that elicits a robust immune response which targets a surface-exposed loop of the P5 protein, yet this response does not protect from NTHI-mediated disease (37). The contribution of vesicle-associated P5 to this decoy response is currently unknown. The lack of other periplasmic binding proteins, such as SapA (28) and HitA (1), and of the outer membrane protein OMP P6 (32) may indicate selectivity of protein incorporation in vesicle biogenesis or an impact of environmental stressors on selective protein packaging.

The diverse array of protein cargo led us to investigate how NTHI OMVs interact with host cells. Using fluorescence microscopy, we observed vesicle interaction and internalization following exposure to host epithelial cells. We noted a decrease in surface-associated vesicles over time, presumably due to OMV internalization. This intimate vesicle-host cell interaction, characterized as vesicle internalization, was disrupted when cells were cultured at 4°C and also when cells were treated with filipin, suggesting that OMV internalization was mediated via caveolin-enriched endocytic uptake. Our observations are consistent with studies of OMV internalization in enterotoxigenic *Escherichia coli* (ETEC), *Pseudomonas*, and *Moraxella* species (8, 20, 42), all of which produce OMVs that are internalized via a caveola-dependent mechanism.

Vesicles interact dynamically with host cells and elicit a robust cellular inflammatory response which includes IL-6, IL-8, IL-12, NF-κB, and gamma interferon (IFN-γ) (49). Specifically, OMVs produced by *Pseudomonas* and ETEC have been shown to induce the immunomodulatory cytokine IL-8 (2, 8). We demonstrated that NTHI OMVs elicit release of significant amounts of IL-8 by human pharynx and primary chinchilla middle ear epithelial cells. IL-8 release was not dependent on surface proteins, as proteinase K treatment, which removed over 30% of total OMV protein, failed to significantly decrease the amount of IL-8 released by host cells (Fig. 3; see

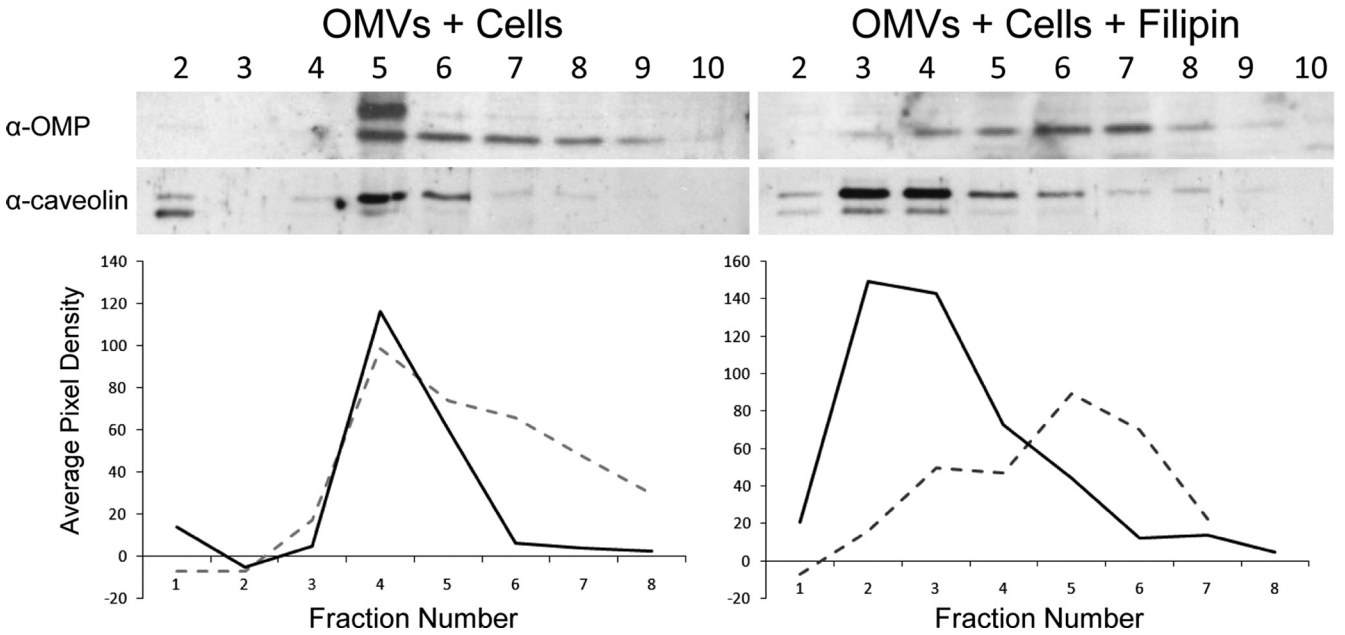

FIG. 5. NTHI OMVs are internalized via caveolae. Disruption of caveolae alters OMV association and uptake by epithelial cells. OMVs cofractionated with caveolin following exposure to Detroit 562 pharyngeal cells for 24 h at 37°C. The addition of filipin inhibited colocalization of OMV proteins and caveolin. Signal intensities of caveolin and OMV fractions were determined by densitometry (average pixel density relative to background signal). Dashed lines, OMV signals; solid lines, caveolin signals.

Fig. S1 in the supplemental material). The induction of IL-8 release by OMVs may be in response to other OMV components, such as LOS, luminal proteins, or DNA, as previously shown with *Moraxella catarrhalis* (42).

Yet why secrete OMVs that elicit an immunomodulatory cytokine release by the host epithelium? It is possible that OMVs act as a decoy, directing the immune response, in a nonspecific manner, away from the whole bacterium, thus providing opportunity for the commensal bacterium to establish colonization without intense bombardment from the immune system. *M. catarrhalis* OMVs act as a decoy to the immune system to enable the whole bacterium to evade immune detection (50). A redirected immune response may benefit NTHI survival and growth *in vivo*, as host inflammation serves to increase the availability of essential nutrients and thus provides a selective advantage for NTHI colonization. For instance, the inflammatory release of the iron-containing compound heme into the local environment would benefit the growth of NTHI, a heme auxotroph (25).

The role of OMVs is not limited to the stimulation of inflammation. The LOS and DNA that are present in OMVs are major components of bacterial biofilms (13, 16, 55), suggesting that OMVs may play a role in biofilm nucleation and maintenance. NTHI produces OMVs *in vivo* which have been observed in biofilms (14). Furthermore, vesicles are able to coaggregate bacteria, enabling colonization and biofilm formation (11, 24, 29, 43, 53). Taking the data together, we hypothesize that NTHI OMVs play a similar role in the establishment, architecture, and structural stability of NTHI biofilms. We have shown here that NTHI OMVs contain both LOS and DNA, integral components of the biofilm matrix and thus biofilm formation, a critical factor of NTHI pathogenesis (30).

Furthermore, NTHI OMVs stimulate the release of the host innate immune component LL-37, an AMP that is secreted from host epithelium in response to bacterial colonization (Fig. 3D) (45). We propose that vesiculation may provide a mechanism to dampen the host innate immune response to thus benefit NTHI evasion of host immunity. We are currently investigating whether AMP exposure will alter release of NTHI OMVs, a likely consequence of AMP-mediated stress on bacterial membranes.

Future studies will consider the many potential biological consequences of *Haemophilus* outer membrane vesicle release. These membranous blebs are complex packages of virulence proteins, DNA, and LOS, leading to abundant and diverse functional possibilities in the host. While we now know that OMVs interact with and are internalized by host cells, the breadth of their impact on the life and disease states of NTHI in the host remains unknown. We hypothesize that NTHI vesiculation contributes to numerous critical functions of NTHI pathogenesis, such as host interaction, innate immune resistance, and biofilm maintenance. It will be important to further define the contribution of OMVs and their interactions with the host to unravel their role in pathogenesis.

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