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Hemolytic Membrane Vesicles of Group B Streptococcus Promote Infection

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Background. Group B streptococci (GBS) are β-hemolytic, Gram-positive bacteria associated with fetal injury, preterm birth, spontaneous abortion, and neonatal infections. A key factor promoting GBS virulence is the β-hemolysin/cytolysin, a pigmented ornithine rhamnolipid (also known as granadaene) associated with the bacterial surface.

Methods. A previous study indicated that GBS produce small structures known as membrane vesicles (MVs), which contain virulence-associated proteins. In this study, we show that GBS MVs are pigmented and hemolytic, indicating that granadaene is functionally active in MVs.

Results. In addition, MVs from hyperhemolytic GBS induced greater cell death of neutrophils, T cells, and B cells compared with MVs from isogenic nonhemolytic GBS, implicating MVs as a potential mechanism for granadaene-mediated virulence. Finally, hemolytic MVs reduced oxidative killing of GBS and aggravated morbidity and mortality of neonatal mice infected with GBS.

Conclusions. These studies, taken together, reveal a novel mechanism by which GBS deploy a crucial virulence factor to promote bacterial dissemination and pathogenesis.

Keywords. granadaene; group B streptococcus; hemolysin; immune evasion; membrane vesicles.

Annually, at least 4 million preterm births or stillbirths and over 300 000 neonatal infections are attributable to group B streptococcus ([GBS] or *Streptococcus agalactiae*), a β-hemolytic, Gram-positive bacterium that commonly colonizes the female lower genital tract [1, 2]. Group B streptococcus is typically transmitted to the fetus via ascending infection, in which the bacteria traffic from the lower genital tract into the amniotic cavity, greatly increasing the risk of preterm birth, fetal injury, and stillbirth. In addition, neonates can acquire GBS through the aspiration of infected vaginal fluids during birth, leading to severe infections including pneumonia, meningitis, or sepsis. A major determinant promoting invasive GBS infection is the β-hemolysin/cytolysin, which is a pigmented ornithine rhamnolipid [3] also known as granadaene [4]. Several studies have shown that granadaene facilitates GBS dissemination by weakening host barriers at the maternal-fetal interface [3, 5], lung [6–8], and brain [9, 10]. Furthermore, the hemolytic pigment is cytotoxic to several host immune cells, including macrophages [11], neutrophils [5], mast cells [12], T cells, and B cells [13].

Hemolytic activity of GBS is associated with the bacterial cell surface, and previous studies have shown that direct contact between GBS and red blood cells (RBCs) is required for hemolysis [14] and that GBS membrane fragments are pigmented [15]. Beyond this, little experimental evidence exists on whether or how granadaene may be released from the bacterial cell. A recent study demonstrated that GBS produce membrane vesicles (MVs), which are small spherical buds originating from the bacterial cell membrane [16]. Group B streptococcus MVs were found to contain several GBS surface-associated virulence proteins, including hyaluronidase and metalloproteinases, and intra-amniotic injection caused weakening of choriodecidual membranes and fetal injury in mice [16]. In other pathogens, MVs have been shown to act as vehicles for toxins and effector molecules, delivering these factors to host cell targets [2, 17, 18]. We hypothesized that granadaene via MVs may exacerbate GBS infection. Our studies indicate that MVs isolated from hyperhemolytic (HH) GBS are pigmented, hemolytic, and cytotoxic to several host cells. Furthermore, we show that hemolytic MVs aggravate morbidity and mortality in neonatal mice infected with nonhemolytic (NH) GBS. These findings, taken together, reveal a novel mechanism of granadaene delivery during GBS infection and further elucidate the function of this key virulence factor.

MATERIALS AND METHODS

Ethics Statement

Written informed patient consent for donation of human blood was obtained with approval from the Seattle Children's Research

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Institute Institutional Review Board (protocol no. 11117) per the Principles in the WMA Declaration of Helsinki and Department of Health and Human Services Belmont Report. Children under the age of 18 were not recruited for blood donation.

All animal experiments were approved by the Seattle Children's Research Institutional Animal Care and Use Committee (protocol IACUC00036) and performed in strict accordance with the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health (Eighth Edition).

Bacterial Strains

Group B streptococcus Δ*covR* and GBSΔ*covR*Δ*cylE* were derived from the wild-type strain (WT) A909, as previously described [9, 19]. A909 is a clinical isolate obtained from a human neonate and is classified as serotype Ia [20]. All GBS liquid cultures were grown in tryptic soy broth ([TSB] Difco Laboratories) at 37°C, 5% CO_2. *Lactococcus lactis* pcylX-K and *L lactis* pEmpty generated previously [21] were grown in TSB at 37°C, 5% CO_2 with 5 µg/mL chloramphenicol (Sigma-Aldrich).

Isolation of Membrane Vesicles

Membrane vesicles MVs were isolated from WT and mutant strains of GBS using methods previously described [16], with slight modifications. Cultures (15 mL) of WT GBS, GBSΔ*covR*, GBSΔ*covR*Δ*cylE*, *L lactis* p*cylX-K*, and *L lactis* pEmpty were grown to $OD_{600\ nm}$ of 1. Bacterial cultures were centrifuged (2000 ×*g*) for 30 minutes at 4°C. Supernatants were collected and passed through a 0.22-µm syringe-driven filter (EMD Millipore) to remove residual bacterial cells. Then, the filtrate was added to a 10-kDa Amicon Ultra-15 filter device (EMD Millipore), which was centrifuged at 4000 ×*g* for 15 minutes. The concentrated solute was recovered, and the MVs were pelleted by ultracentrifugation (150 000 ×*g* for 3 hours at 4°C). The supernatant was removed without disturbing the pellet. The pellets containing MVs were resuspended in sterile phosphate-buffered saline (PBS) and were normalized among all strains to 5 mg/mL. For detailed methods regarding scanning electron microscopy of MVs and bacteria, see Supplementary Materials.

Hemolysis Assay

Membrane vesicle resuspension (10 µL) was sonicated (10 minutes) and then spotted on a blood agar plate (Remel) and allowed to dry for approximately 10 minutes. The plate was incubated at 37°C in 5% CO_2 overnight and then examined for a zone of hemolysis. The activity of proteinase K (PK) was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) before hemolysis assays (see Supplementary Materials). To test the effect of PK on MV hemolysis, 13.5 µL sonicated MV resuspension was mixed with 1.5 µL PK (2.5 mg/mL, [final] = 0.25 mg/mL) or PBS and incubated for 1 hour. Then, 10 µL was spotted and allowed to dry for 10 minutes. The plate was incubated at 37°C in 5% CO_2 overnight then analyzed for hemolysis. Plates were placed on a light box, and photographs were captured with the digital SLR camera and processed using Photoshop CC (Adobe).

To quantify hemolytic activity of MVs, a hemolytic assay was performed as described previously with purified pigment/control extracts [3]. In brief, human RBCs in PBS were coincubated with 10 µL sonicated MV resuspensions (5 mg/mL) from GBSΔ*covR* (or NH control MVs) in the presence of PK (0.25 mg/mL) or PBS for 1 hour at 37°C. Hemoglobin release in cell supernatants was measured, and percentage hemolysis was determined relative to Triton X-100 (0.1%)-treated positive controls and PBS-treated negative controls.

Cytotoxicity Assay

Primary human neutrophils, $CD4^+$ T cells, $CD8^+$ T cells, and B cells were isolated as described previously [5, 13]. Neutrophils were seeded into 96-well plates at 2.5×10^5 cells/well in 90 µL Roswell Park Memorial Institute (RPMI)-G medium, and T and B cells were seeded at 2.5×10^6 cells/well in 90 µL RPMI-G medium. Membrane vesicles (10 µL, 5 mg/mL) isolated from GBSΔ*covR* and GBSΔ*covR*ΔcylE were added to seeded cells and allowed to incubate at 37°C (neutrophils = 3 hours, T cells and B cells = 1 hour). As positive and negative controls, neutrophils were incubated in 0.1% Triton X-100 (Sigma-Aldrich) or sterile PBS, respectively. Cells were analyzed for cytotoxicity by the presence of cytoplasmic lactate dehydrogenase (LDH) in cell supernatants using the colorimetric LDH kit (Clontech), per the manufacturer's instructions. Percentage cytotoxicity was calculated by normalizing to PBS-treated cells (0% cell death) and Triton X-100-treated cells (100% cell death), as described previously [3, 5, 11].

Oxidative Killing Assay

Membrane vesicles isolated from GBSΔ*covR* or GBSΔ*covR*Δ*cylE* were resuspended in sterile PBS at 5 mg/mL, and 1.23 mg of each MV type was coincubated with 0.06% H_2O_2 (Sigma-Aldrich) for 45 minutes while rocking (500 µL total volume). Phosphate-buffered saline (no MVs) + 0.06% H_2O_2 and PBS-only (no MVs, no H_2O_2) conditions were included as positive and negative controls, respectively. Meanwhile, GBSΔ*covR*Δ*cylE* overnight cultures were subcultured in TSB, grown to mid-log phase (OD_{600} 0.3), washed twice in sterile PBS, and normalized to approximately 2×10^8 colony-forming units (CFU)/mL in PBS. Then, approximately 1×10^8 CFU (500 µL) was added to the preincubated MVs (and controls), bringing the final H_2O_2 concentration to 0.03%, as previously described for oxidative killing assays with GBS [22]. The mixtures incubated at 37°C, 5% CO_2 for 1 hour, and then 1000 units of catalase (from bovine liver; Sigma-Aldrich) were added to each reaction condition to quench remaining H_2O_2 (as previously described [22]), and surviving CFU were enumerated by dilution plating onto TSA.

Mouse Model

C57BL/6J mice between 12 and 36 hours of age were pooled and randomly assigned to dams. Each group of neonates was then designated to an experimental group (HH MVs + NH GBS, PBS + NH GBS, HH MVs + PBS, NH MVs + NH GBS, or PBS + PBS). According to the assigned experimental group, neonates were injected (intraperitoneal [I.P.]) with 50 µL HH MVs (5 mg/mL from GBSΔ*covR*) or PBS and 10 µL NH GBS (GBSΔ*covR*Δ*cylE*, 10^8 CFU/mL) or PBS and returned to their assigned dam. For the survival study, mice were monitored twice daily for 7 days for signs of morbidity and mortality. Moribund neonates were euthanized. Sample sizes for the survival study are as follows: n = 6 HH GBS MVs + NH GBS; n = 6 PBS + NH GBS; n = 5 HH GBS MVs + PBS; n = 9 NH MVs + NH GBS; n = 7 PBS + PBS. Randomly selected neonates from each experimental group (n = 1 NH GBS MVs + NH GBS; n = 1 PBS + NH GBS; n = 4 HH GBS MVs + NH GBS; n = 2 HH GBS MVs + PBS; and n = 1 PBS + PBS) were euthanized via decapitation at 24 hours postinoculation, and lungs were analyzed by hematoxylin and eosin (H&E) staining. For detailed methods of tissue preparation, H&E staining, and analysis, see Supplementary Materials. In addition, mice from the HH GBS MVs + NH GBS (n = 6) and NH GBS MVs + NH GBS (n = 7) groups were euthanized by decapitation 24 hours after inoculation, and lungs were processed into single-cell suspension and analyzed by flow cytometry. For detailed methods on cell preparation, antibody staining, analysis, and gating, see Supplementary Materials.

Statistical Analysis

A $P < .05$ was considered significant. Unless otherwise noted, an unpaired *t* test or one-way analysis of variance with Tukey's posttest was used to compare groups in in vitro assays. Survival data was plotted on a Kaplan-Meier curve and analyzed using the log-rank test. GraphPad Prism (version 7.03) was used for all statistical tests.

RESULTS

Group B Streptococci Hemolytic Pigment, Granadaene, Is Released in Membrane Vesicles

To test the hypothesis that GBS hemolytic pigment associates with MVs, we isolated MVs from GBS strains that overexpress the hemolytic pigment, namely, GBSA909Δ*covR* (lacks the hemolysin repressor CovR/S) and its isogenic NH strain GBSΔ*covR*Δ*cylE*. It is notable that HH GBS strains, including those containing mutations in the *covR/S* 2 component system exhibit increased virulence [3, 9, 23] and have been isolated from women in preterm labor [3] as well as from patients with other manifestations of GBS infections [23–26]. Using scanning electron microscopy, we confirmed that these GBS strains (Δ*covR* and Δ*covR*Δ*cylE*) produce MVs; these are seen as spherical structures proximal to and arising from GBS (Figure 1A, Supplementary Figure 1A and 1B), similar to those previously identified as MVs [16]. Ultracentrifugation of MVs from GBSA909Δ*covR* yielded a small pellet that was red/orange in color, unlike MVs from GBSΔ*covR*Δ*cylE*, indicating the presence of pigment in the MVs (Figure 1B, Supplementary Figure 1A). Membrane vesicle pellets were resuspended in PBS and then analyzed for hemolysis by spotting on red blood agar. We found that MVs from HH GBS were indeed hemolytic, whereas MVs from NH GBS were not (Figure 1C). Of note, significant hemolysis was not observed in MVs isolated from mildly hemolytic WT GBS (Supplementary Figure 2).

We then examined whether MVs from HH GBS are cytolytic to host immune cells similar to live bacteria and purified granadaene. Accordingly, MVs from HH GBSΔ*covR* (HH GBS MVs) or NH GBSΔ*covR*Δ*cylE* (NH GBS MVs) were coincubated with primary human neutrophils, $CD4^+$ T cells, $CD8^+$ T cells, or B cells, and cell death was measured by LDH release in cell supernatants. We found that MVs from HH GBS caused significantly greater cell death in all cell types compared with MVs from isogenic, NH GBS (Figure 1D). These data, taken together, demonstrate for the first time that GBS pigment is released from the bacterial cell surface with MVs, which induce hemolysis and cytolysis to host cells.

Next, we asked whether the release of granadaene in MVs was dependent on a GBS-specific factor. To test this, we used the Gram-positive bacterium *L lactis* expressing the GBS *cyl* operon (strain *L lactis* p*cylX-K*), which heterologously expresses granadaene as described recently [21]. We also included the NH/nonpigmented *L lactis* strain with only the vector (*L lactis* pEmpty), which does not produce granadaene [21]. After isolating MVs from these strains as described above, we noted that MVs from *L lactis* p*cylX-K* were pigmented and hemolytic on red blood agar (Figure 2, Supplementary Figure 1C), whereas MVs from *L lactis* pEmpty were neither pigmented nor hemolytic (Supplementary Figure 1C). Taken together, these findings demonstrate that granadaene is released in MVs from Gram-positive bacteria, and its association with MVs did not require any GBS-specific factor.

Hemolytic Activity of Membrane Vesicles Containing Granadaene Is Independent of Proteins

To confirm that protein or peptides do not contribute to the hemolytic activity observed in MVs, we tested hemolysis of MVs in the presence of PK. Membrane vesicles isolated from HH GBS and *L lactis* p*cylX-K* were subjected to PK (0.25 mg/mL) treatment for 1 hour at 37°C. As a control, the activity of the PK used in these studies was verified by incubating it (at 0.25 mg/mL) with 100 µg of bovine serum albumin. All samples were analyzed for protein content SDS-PAGE, and the data shown in Supplementary Figure 3 confirm that proteins present in the MVs are susceptible to degradation by PK. Next, MVs isolated from HH GBS or *L lactis* p*cylX-K*,

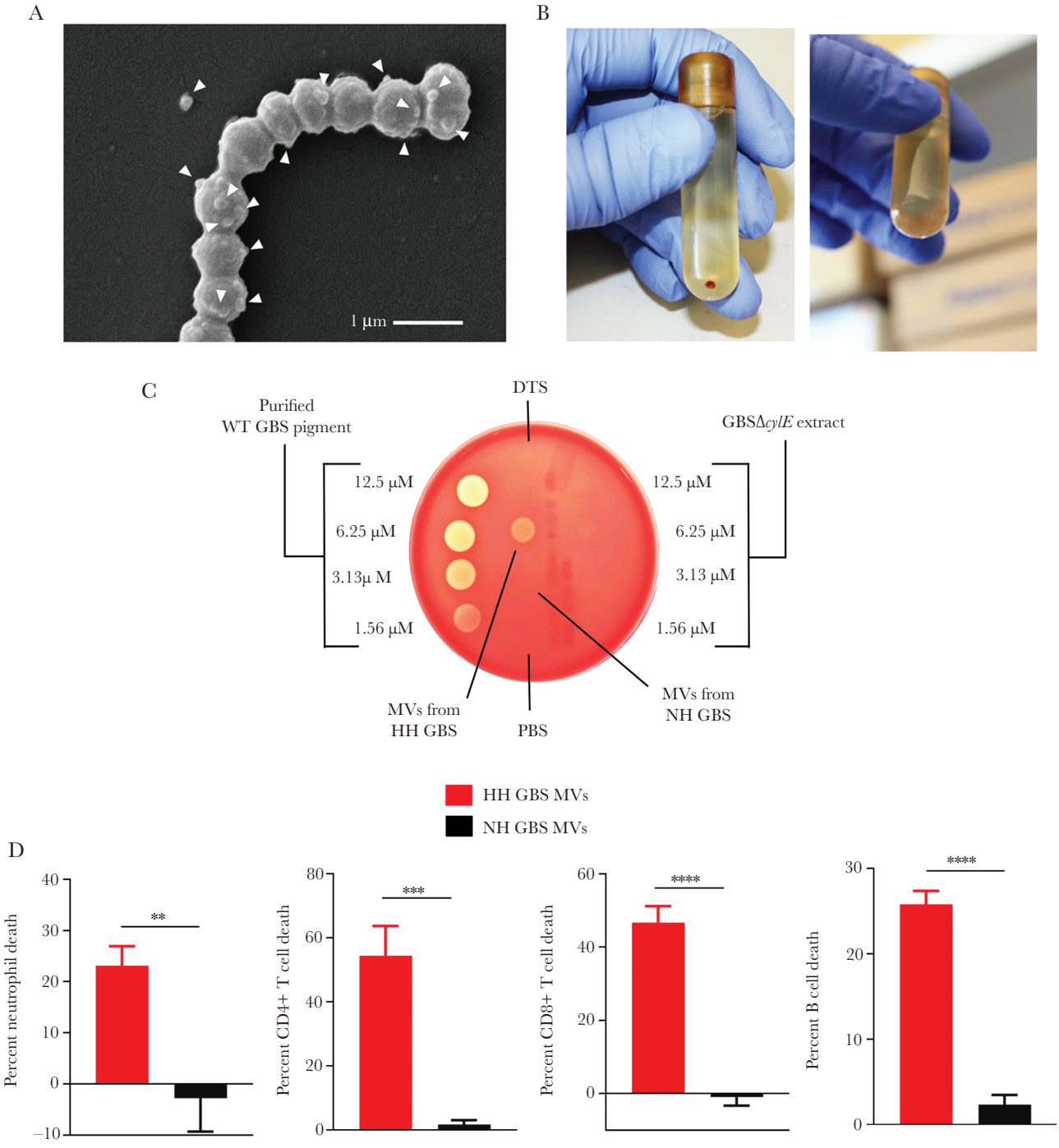

Figure 1. Membrane vesicles (MVs) isolated from hemolytic group B streptococci (GBS) are hemolytic and cytolytic. (A) Hyperhemolytic (HH) GBS (GBSΔ*covR*) were centrifuged, fixed, and analyzed by scanning electron microscopy. Arrowheads indicate MVs, which are seen as spherical structures emerging from the surface of bacterial cells. (B) Pelleted MVs from HH GBS (GBSΔ*covR*) or nonhemolytic (NH) GBS (GBSΔ*covR*Δ*cylE*) are shown. (C) Membrane vesicles from HH GBS (GBSΔ*covR*) or NH GBS (GBSΔ*covR*Δ*cylE*) were resuspended in phosphate-buffered saline (PBS), sonicated, and 10 µL was spotted onto red blood agar. Purified GBS pigment and equivalent amount of GBSΔ*cylE* extract in DTS at various dilutions were spotted (10 µL) for comparison. (D) Primary human neutrophils, $CD4^+$ T cells, $CD8^+$ T cells, or B cells were incubated with MVs from HH GBS or NH GBS (final concentration = 0.5 mg/mL), and cell death was measured by LDH release into the supernatant relative to Triton X-100 (0.1%)- and PBS-treated controls. Mean and standard error of the mean are shown from 3 experiments performed in technical triplicate. Groups were compared with unpaired, 2-tailed Student's *t* test. Neutrophils, $P = .0042$; $CD4^+$ T cells, $P = .0005$; $CD8^+$ T cells, $P < .0001$; B cells, $P < .0001$. **, $P < .01$; ***, $P < .001$; ****, $P < .0001$.

with and without PK treatment, were spotted onto a red blood agar plate or mixed with human RBCs for hemolysis assays. Nonhemolytic GBS MVs and *L lactis* pEmpty were included as controls. We observed that treatment with PK did not diminish hemolytic activity in MVs isolated from HH GBS or *L lactis* p*cylX-K* (Figure 2B and 2C). Taken together, these data indicate

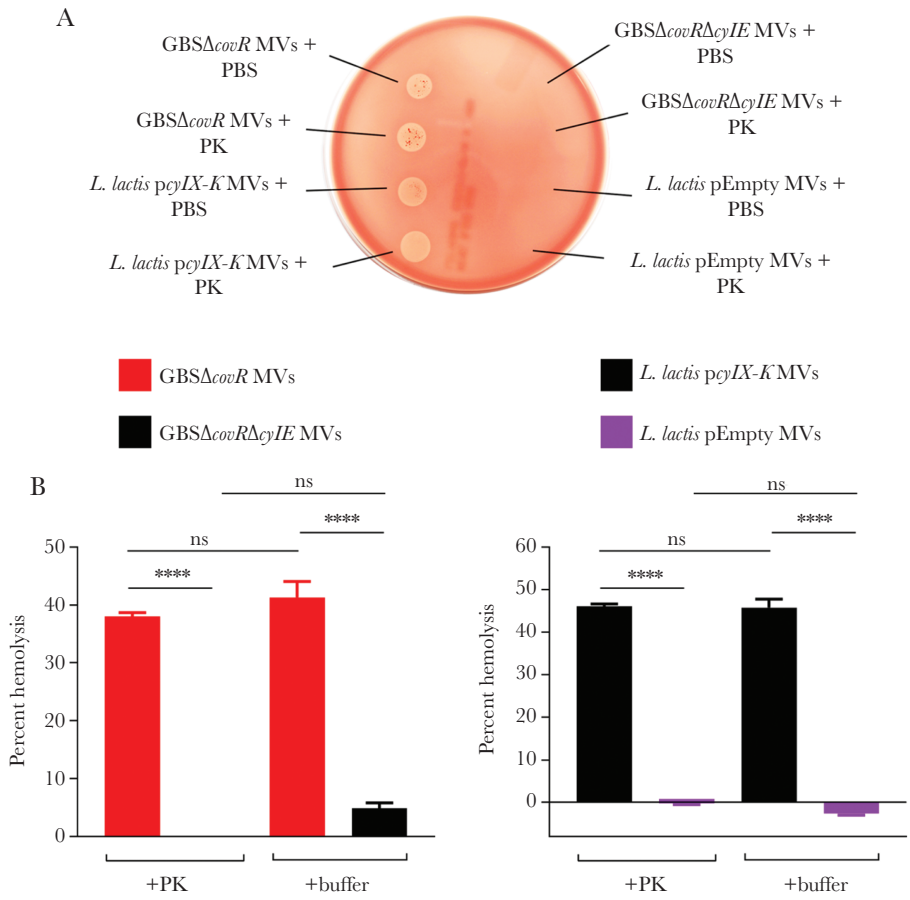

Figure 2. Membrane vesicles (MVs) are hemolytic after treatment with protease. (A) The MVs from GBSΔ*covR*, GBSΔ*covR*Δ*cylE*, *Lactococcus lactis* p*cylX-K*, or *L lactis* pEmpty were treated with proteinase K ([PK] final concentration 0.25 mg/mL) or an equivalent volume of phosphate-buffered saline (PBS) for 1 hour at 37°C, and 10 µL of each was spotted on blood agar. (B) The MVs from GBSΔ*covR*, GBSΔ*covR*Δ*cylE*, *L lactis* p*cylX-K*, or *L lactis* pEmpty were mixed with human erythrocytes in the presence of PBS or PK (0.25 mg/mL) for 1 hour, and percentage hemolysis was determined relative to Triton X-100 (0.1%)-treated positive controls. One-way analysis of variance with Tukey's posttest was used. Mean and standard error of the mean from 3 experiments performed in triplicate are shown. ****, $P < .0001$; ns, $P \geq .05$.

that hemolytic activity observed with MVs did not require any MV-associated proteins.

Hemolytic Membrane Vesicles Prevent Oxidative Killing of Group B Streptococci

Next, we asked whether hemolytic MVs protect GBS against antimicrobial host defenses encountered during infection, such as reactive oxygen species (ROS) that are often produced by macrophages and neutrophils. Based on previous observations that the hemolytic pigment of GBS has antioxidant properties [22], and that MVs from *Helicobacter pylori* promote bacterial survival against ROS [27], we hypothesized that MVs derived from HH GBS may protect GBS against oxidative killing. To test this hypothesis, we treated NH GBS (GBSΔ*covR*Δ*cylE*) with H_2O_2 that was preincubated with either HH GBS MVs or NH GBS MVs. As controls, GBS was exposed to H_2O_2 alone (ie, without MVs) or PBS only. After 1 hour, we quenched residual ROS with catalase and then enumerated surviving CFU. We observed that significantly more GBS survived H_2O_2 that had been pretreated with HH MVs compared with H_2O_2 pretreated with NH MVs (Figure 3A). These findings show that hemolytic pigment in MVs can dampen killing of GBS by H_2O_2, a major constituent of the oxidative burst [28].

Hemolytic Membrane Vesicles Exacerbate Group B Streptococci Pathogenesis in Neonatal Mice

Because our in vitro data indicated that hemolytic MVs promote GBS survival against ROS, we hypothesized that hemolytic MVs contribute to GBS pathogenesis in vivo. To test this, neonatal mice between 12 and 36 hours of age were inoculated (I.P.) with either (1) HH MVs alone, (2) HH MVs in the presence of NH GBS, (3) NH MVs with NH GBS, (4) NH GBS alone, or (5) control saline. The mice were observed for morbidity and mortality symptoms for up to 7 days postinoculation. We found that neonatal mice inoculated with HH MVs alone did not succumb to the challenge (Figure 3B). However, the presence of HH MVs aggravated morbidity and mortality of neonatal mice treated with NH GBS when compared with mice inoculated with NH GBS alone or NH MVs + NH GBS (Figure 3B). Because previous work has shown that I.P. infection of GBS can

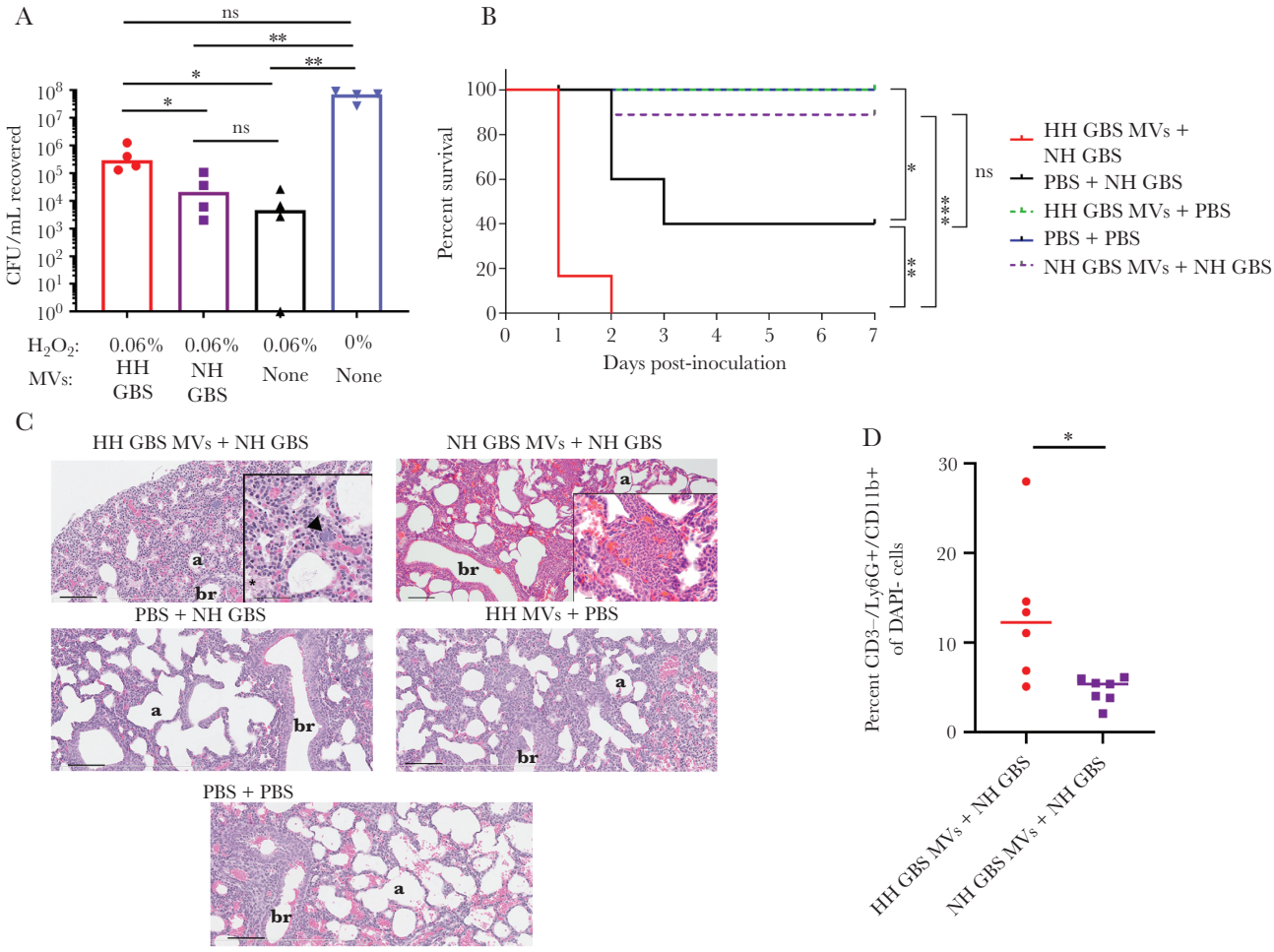

Figure 3. Hemolytic membrane vesicles (MVs) dampen oxidative killing and exacerbate group B streptococci (GBS) pathogenesis in neonatal mice. (A) Membrane vesicles isolated from hyperhemolytic (HH) GBSΔ*covR* or nonhemolytic (NH) GBSΔ*covR*Δ*cylE* were preincubated with 0.06% H_2O_2 for 45 minutes and then incubated with NH GBS at 37°C for 1 hour. After incubation, remaining H_2O_2 was quenched with 1000 units of catalase, and surviving colony-forming units (CFU) were enumerated by dilution plating onto TSA. A group consisting of no MVs + H_2O_2 and no MVs + no H_2O_2 were included as positive and negative controls, respectively. Treatment groups were compared using a Kruskal-Wallis test with multiple comparisons corrected for false discovery. Hyperhemolytic GBS MVs + H_2O_2 vs no MVs + H_2O_2, $P = .0170$; HH GBS MVs + H_2O_2 vs NH GBS MVs + H_2O_2, $P = .0395$. Nonhemolytic GBS MVs + H_2O_2 vs no MVs + H_2O_2, $P > .9999$; HH GBS MVs + H_2O_2 vs phosphate-buffered saline (PBS) only, $P = .0822$; NH GBS MVs + H_2O_2 vs PBS only, $P = .0032$; no MVs + H_2O_2 + PBS, $P = .0015$. Line at median. (B) Kaplain-Meier plot depicts survival of neonatal mice inoculated with MVs alone or GBS strains alone or GBS strains with MVs, as indicated. Survival between groups was compared using the log-rank test. PBS + PBS (blue) vs PBS + NH GBS (black), $P = .0316$; PBS + NH GBS (black) vs HH GBS MVs + NH GBS (red), $P = .0033$; NH GBS MVs + NH GBS (purple) vs HH GBS MVs + NH GBS MVs (red), $P = .0002$; NH GBS MVs + NH GBS (purple) vs PBS + NH GBS MVs (black), $P = .0610$. (C) Lungs from neonatal mice in each treatment group were harvested at 24 hours posttreatment, fixed, sectioned, and stained for hematoxylin and eosin. In the HH GBS MVs + NH GBS group, the arrow represents an aggregate of small basophilic circular structures (consistent with bacteria), and the asterisk indicates eosinophilic material within the alveoli (fibrin). Scale bar is 100 μm for all images except for insets. Scale bar for inset in HH GBS MVs + NH GBS is 50 μm, and scale bar for inset in NH GBS MVs + NH GBS is 10 μm. "b" indicates bronchiole (letter is placed in the lumen) and "a" indicates alveolar air space. (D) Lungs from neonatal mice inoculated with HH GBS MVs + NH GBS and NH GBS MVs + NH GBS were harvested 24 hours after inoculation, processed into single-cell suspensions, stained, and analyzed by flow cytometry. Percentage neutrophils ($CD3^-/Ly6G^+/CD11b^+$) of viable ($DAPI^-$) cells were compared using a Mann-Whitney test ($P = .0140$). Line at median. For all statistical comparisons, ns indicates $P > .05$, * indicates $P < .05$, ** indicates $P < .01$, and *** indicates $P < .001$.

cause lung infection in neonatal rats [29], histological examination of H&E-stained sections was performed on the lungs from mice obtained 24 hours postinoculation. Of the 4 mice inoculated with HH MVs + NH GBS, one set of lungs had a few clusters of small basophilic structures consistent with bacteria, which were not observed in the other groups. In addition, there was mild eosinophilic acellular material within the alveoli (consistent with fibrin) in the lungs of this neonate, which was generally minimal to absent in the lungs of neonates from the other groups (Figure 3C). Two other mice in this group had minimal focal neutrophilic inflammation, in the lung in one mouse and in the mediastinal tissues of another mouse. Although focal mild neutrophilic inflammation was observed in the lung of a mouse treated with NH MVs + NH GBS, flow cytometric analysis indicated that there were more neutrophils in the lungs of neonatal mice treated with HH MVs + NH GBS (Figure 4D). These findings show that MVs from HH GBS reduced survival and potentially exacerbated lung injury and/

or bacteremia in neonates infected with NH GBS. These data, taken together, indicate that hemolytic GBS MVs can promote GBS pathogenesis and neonatal morbidity and mortality.

DISCUSSION

Group B streptococci remain a leading etiological agent of infection in human newborns and are associated with preterm birth, stillbirth, and neonatal sepsis [1, 30–41]. A major barrier to the development of new prevention strategies is the lack of understanding of virulence factors important for GBS pathogenesis. In this work, we add new insight into a critical GBS virulence factor, the hemolytic pigment, and show how GBS may package and deploy this toxin to overcome host defenses and promote infection.

In 2013, Whidbey et al [3] showed that the GBS pigment (granadaene) is hemolytic in the presence of starch, demonstrating that the pigment and β-hemolysin of GBS were one in the same. Although this work represented a major advance in our understanding of GBS pathogenesis, the requirement for starch in purified pigment for hemolytic activity remained a mystery, because GBS does not produce starch per se and yet are hemolytic. In the present study, we observed that MVs isolated from hemolytic GBS or *L lactis* expressing the GBS *cyl* genes [21] are hemolytic and cytolytic, even when MV-associated proteins are degraded by PK (Figures 1 and 2). These findings demonstrate that exogenous, high-molecular-weight stabilizers such as starch are not essential for hemolysis and cytolysis when the pigment is associated with the bacterial membrane or membrane components. In addition, these data show that proteinaceous components of the bacterial membrane are also themselves not necessary for hemolytic activity, suggesting that positioning within the membrane may provide sufficient stabilization for pigment activity.

Our findings in MVs support previous data indicating that the hemolytic pigment is localized to the bacterial surface [14, 15] and reveal a novel mechanism by which GBS may release this toxin from the bacterial cell. Previous work showed that virulence-associated proteins such as extracellular matrix-degrading enzymes are packaged in GBS MVs and likely contribute to placental membrane disruption, fetal injury, and preterm birth [16]. Our in vitro studies showing that hemolytic MVs cause cell death in several host immune cells suggested that hemolytic MVs alone may be pathogenic. However, because HH GBS MVs without bacteria did not induce death or lung injury in the neonatal mouse model, hemolytic MVs on their own may be insufficient to cause morbidity in complex host systems. On the other hand, our in vitro and in vivo findings suggest that the hemolytic pigment in MVs promotes GBS survival and pathogenesis, even for a NH strain (Figure 3). Furthermore, our in vivo data indicate that increased neutrophil recruitment by HH GBS MVs in addition to bacterial infection may intensify morbidity and mortality in neonates. It is notable that simultaneous isolation of hemolytic and NH GBS from human cases has been reported [23, 25, 42] including in neonates [42].

Membrane vesicles have recently gained appreciation for their ability to promote bacterial survival by interfering with host defenses. For instance, *Staphylococcus aureus* MVs promoted bacterial survival by via neutrophil cytotoxicity [43], MVs from *Streptococcus pneumoniae* inhibited opsonophagocytic killing by sequestering complement components [43], and catalase-containing MVs from *H pylori* decreased ROS-mediated killing [27]. Our data indicate that similar to *H pylori*, MVs from GBS facilitate bacterial survival from oxidative killing, although protection is dependent on the presence of the hemolytic pigment in MVs (Figure 3A). It is likely that release of hemolytic MVs by GBS quenches ROS produced by recruited neutrophils, thereby attenuating host defenses against GBS.

CONCLUSIONS

In summary, we identify a heretofore undescribed mechanism of pigment toxin-mediated virulence during GBS infection. Our results suggest that GBS releases the hemolytic pigment via MVs, which quench microbicidal oxidants and enable bacteria to survive. Reactive oxygen species production by neutrophils is critical to clearance of GBS by the host [5, 22, 44], and our findings provide new insight into how GBS overcomes hostile host environments and also lay a foundation for future studies examining the role of GBS MVs in immune evasion.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. B. A. and L. R. designed experiments. B. A., P. Q., V. S.-U., A. F., and A. B. performed experiments. B. A., J. M. S., and L. R. analyzed data. B. A. and L. R. wrote the manuscript.

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