Bacterial Hyaluronidase Promotes Ascending GBS Infection and Preterm Birth

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ABSTRACT Preterm birth increases the risk of adverse birth outcomes and is the leading cause of neonatal mortality. A significant cause of preterm birth is in utero infection with vaginal microorganisms. These vaginal microorganisms are often recovered from the amniotic fluid of preterm birth cases. A vaginal microorganism frequently associated with preterm birth is group B streptococcus (GBS), or Streptococcus agalactiae. However, the molecular mechanisms underlying GBS ascension are poorly understood. Here, we describe the role of the GBS hyaluronidase in ascending infection and preterm birth. We show that clinical GBS strains associated with preterm labor or neonatal infections have increased hyaluronidase activity compared to commensal strains obtained from rectovaginal swabs of healthy women. Using a murine model of ascending infection, we show that hyaluronidase activity was associated with increased ascending GBS infection, preterm birth, and fetal demise. Interestingly, hyaluronidase activity reduced uterine inflammation but did not impact placental or fetal inflammation. Our study shows that hyaluronidase activity enables GBS to subvert uterine immune responses, leading to increased rates of ascending infection and preterm birth. These findings have important implications for the development of therapies to prevent in utero infection and preterm birth.

IMPORTANCE GBS are a family of bacteria that frequently colonize the vagina of pregnant women. In some cases, GBS ascend from the vagina into the uterine space, leading to fetal injury and preterm birth. Unfortunately, little is known about the mechanisms underlying ascending GBS infection. In this study, we show that a GBS virulence factor, HylB, shows higher activity in strains isolated from cases of preterm birth than those isolates from rectovaginal swabs of healthy women. We discovered that GBS rely on HylB to avoid immune detection in uterine tissue, but not placental tissue, which leads to increased rates of fetal injury and preterm birth. These studies provide novel insight into the underlying mechanisms of ascending infection.

Preterm birth is a major indicator for neonatal morbidity and mortality (1, 2). Approximately 6 to 15% of all deliveries are preterm, resulting in an estimated 4 million neonatal deaths per year, making preterm birth the leading cause of mortality in neonates and in children under 5 years of age (3–7). The largest burden of neonatal and under-5 mortality due to preterm birth is concentrated in sub-Saharan Africa and southern Asia, where health care systems are often too weak to effectively manage high preterm birth rates (8). Preterm birth rates are also alarmingly high in the developed world, including North America, where preterm birth has an annual health care cost in the tens of billions of dollars (2, 9). In order to reduce the burden and subsequent cost of preterm birth, we need a better understanding of the causes and physiology of its biological processes.

Although the clinical events associated with preterm birth have been well studied, its underlying causes remain ill defined. An estimated 25 to 40% of preterm births are a result of in utero bacterial infection (10). Bacteria can be recovered from the amniotic fluid of preterm birth cases (11–13). Bacteria reach the amniotic fluid by means of ascending infection, which occurs when bacteria penetrate the cervical barrier and enter the uterus (2). Once in the uterine space, bacteria cause multiple physiological events associated with preterm birth, including increased levels of proinflammatory cytokines, chorioamniotic membrane rupture, cervical ripening, and uterine contraction (2, 11, 14–16). One group of bacteria associated with these physiological events that has been recovered from amniotic fluid is group B streptococcus (GBS), or Streptococcus agalactiae (12, 14, 15, 17, 18). GBS are a leading cause of neonatal morbidity and mortality, and approximately 30% of healthy women are rectovaginally colonized with GBS (3–5, 7). Heavy vaginal GBS colonization is the primary risk factor for GBS-associated preterm birth (19, 20). Despite the large number of women at risk for GBS-associated preterm birth, little is known about the bacterial and host factors involved in GBS colonization and ascending infection.

Multiple host and bacterial factors play a role in ascending infection and preterm birth. One such factor is the high-molecular-weight polymer hyaluronic acid, which is cleaved by...
hyaluronidases. Hyaluronic acid polymers have multiple roles, including a structural role in extracellular matrix (ECM) formation, aiding in cell migration, cell-cell signaling, and induction of ECM remodeling enzymes and inflammation (21). Recently, it has been shown that cervical hyaluronic acid protects against ascending infection and preterm birth due to its role in epithelial barrier function and that lipopolysaccharide-induced murine cervical hyaluronidase expression increases preterm birth rates (22–24). These studies highlight the importance of hyaluronic acid and hyaluronidases during pathogen colonization and preterm birth, but a mechanistic connection between pathogen hyaluronidase activity and preterm birth is not known.

Interestingly, GBS produces a hyaluronidase (here referred to as "HylB"), encoded by the hylB gene. HylB was first identified in the 1950s and is well characterized as a specific exolytic enzyme (25–27). It was recently determined that HylB plays an important role in GBS evasion of the host immune system (28). These studies show that GBS degrades hyaluronic acid into disaccharide fragments, which blocks Toll-like receptors (TLRs) 2 and 4, preventing GBS ligands from activating proinflammatory signaling cascades (28). Despite these exciting advances, it is unknown if HylB is important for ascending GBS infection and/or preterm birth.

Here, we show that clinical GBS strains isolated from women in preterm labor had increased levels of HylB activity compared to commensal strains isolated from rectovaginal swabs of pregnant women not in labor. Using a mouse model of ascending GBS infection, we observed that genetic ablation of hylB in GBS leads to decreased rates of bacterial ascension and fetal demise. Finally, we show that maternal uterine cells infected with wild-type (WT) GBS exhibit a lower level of inflammation compared to cells infected with GBS lacking HylB. These data are the first to describe a role for the GBS hyaluronidase in ascending infection and preterm birth.

RESULTS
Clinical GBS isolates associated with invasive disease exhibit increased hyaluronidase activity. We hypothesized that GBS-encoded hyaluronidase activity may be critical for ascending infection and preterm birth. To measure HylB activity in various GBS isolates, we adapted a hyaluronidase activity assay that was previously described (29). Among our laboratory collection of GBS strains representing each capsular serotype, we observed that strains showed various levels of hyaluronidase activity. GBS strains A909 (serotype Ia) and NEM316 (serotype III) showed little to no activity, whereas strains NCTC 01/82 (serotype IV), CBJ111 (serotype V), and JM9 (serotype XIII) showed the highest levels of activity (Fig. 1A). Interestingly, strains belonging to the same capsular serotype exhibited various levels of hyaluronidase activity (compare COH1 to NEM316 and NCTC 10/84 to CBJ111). These data suggest that while hyaluronidase activity varies among GBS strains, it is not correlated to capsular serotype.

We next sought to compare hyaluronidase activities in clinical GBS strains isolated from amniotic fluid or neonatal blood (invasive infection, n = 23) to those obtained from women who were rectovaginally colonized with GBS without symptoms of invasive infection (n = 48). Overall hyaluronidase activity was higher in GBS strains obtained from invasive settings compared to that of commensal settings (Fig. 1B). Finally, we stratified invasive GBS strains into those isolated from neonatal disease without preterm birth (n = 15) and GBS-associated preterm birth (n = 8). Interestingly, isolates from cases of GBS-associated preterm birth displayed modestly higher levels of hyaluronidase activity compared to isolates from neonatal disease and much higher levels of activity compared to isolates from vaginal swabs (Fig. 1C). Taken to-
together, these data suggest a role for HylB in invasive GBS disease and preterm birth.

**HylB promotes ascending GBS infection and adverse birth outcomes.** Given the higher levels of hyaluronidase activity in GBS strains isolated from women in preterm labor, we examined if HylB activity is important for ascending infection and preterm birth. We adapted a pregnant murine model of ascending GBS infection, recently developed by Randis and colleagues (30), with the modification that we omitted the use of gelatin during vaginal inoculation of pregnant mice. We constructed a hylB mutant (here referred to as GBSΔhylB) in WT GBS COH1 (serotype III, ST17 clone associated with increased virulence [31]) and confirmed the loss of hyaluronidase activity in the GBSΔhylB mutant by gel electrophoresis. The results shown in Fig. 2 indicate that

**FIGURE 2** HylB activity leads to increased rates of ascending infection and adverse pregnancy outcomes, including preterm birth. Hyaluronic acid incubated with or without GBS was analyzed by agarose gel electrophoresis. The WT GBS strain COH1 shows increased hyaluronidase activity (lane 2, WT GBS) compared to the isogenic hylB mutant (lane 3, GBSΔhylB), which shows no hyaluronidase activity (compare to lane 1, input [A]). Pregnant female C57BL/6J mice (a schematic can be found in panel B) were inoculated with approximately 10^8 CFU of WT GBS (n = 12) or GBSΔhylB (n = 11). Contingency analyses were performed on observed outcomes of these mice. Observed outcomes were ascending bacterial infection (C), pups with adverse outcome (either born prematurely or with in utero fetal death [D]), and pups born prematurely (E). Fisher’s exact test was used to assess statistical significance between groups (A to C [*, P > 0.05; ****, P > 0.00005]).
WT GBS COH1 displays high hyaluronidase activity (indicated by the lower hyaluronic acid band in Fig. 2A, lane 2), whereas the GBSΔhylB strain displays no hyaluronidase activity (Fig. 2A, lane 3).

Next, we vaginally inoculated pregnant C57BL/6j mice on day 15 of pregnancy (E15) with approximately 10⁶ CFU of WT GBS or isogenic GBSΔhylB (Fig. 2B). The mice were then monitored for signs of preterm birth (defined as vaginal bleeding or pups in cage) for up to 72 h postinoculation. Upon signs of preterm birth or at 72 h postinoculation (whichever occurred first), the mice were euthanized, a midline laparotomy was performed, and 72 h postinfection (whichever occurred first), the mice were euthanized, a midline laparotomy was performed, and distal and proximal placentas, and distal and proximal pups of these mice were removed and assessed for bacterial load by serial dilution plating. Data are displayed as individual data points with median bars. Mann-Whitney U test was used to assess statistical significance between groups (*, P < 0.05; **, P < 0.01).

Hyaluronidase (HylB) activity dampens uterine immune responses during ascending GBS infection. Previous studies by Kolak et al. have shown that GBS utilizes the hylB-encoded hyaluronidase to degrade hyaluronic acid into disaccharide fragments, which blocks TLR2/4 signaling to prevent inflammation (28). Uterine epithelial cells express high levels of TLRs 1, 2, and 6, which are more responsive to Gram-positive bacteria than to other types of pathogens (32). Additionally, uterine TLR2 expression in mice increases as gestation progresses, whereas placental and fetal TLR2 expression decreases during gestational progression (33). To determine if the GBS hyaluronidase mediates uterine immune responses during ascending infection, we performed Lumex assays to measure levels of inflammatory cytokines in uterine tissues from pregnant mice vaginally inoculated with WT GBS or GBSΔhylB (Fig. 4Ai to Di). We found that inflammatory cytokines from mice inoculated with GBSΔhylB clustered into discrete low and high groups that correlated with the presence or absence of bacteria. Therefore, we stratified the uterine samples to those with bacteria and noted significantly higher levels of tumor necrosis factor alpha (TNF-α) and macrophage inflammatory protein 2 (MIP2) (Fig. 4Aii and Bii) and modestly higher levels of interleukin-6 (IL-6) and MIP1β (Fig. 4Ci and Di) in the uterine tissues of mice inoculated with GBSΔhylB compared to that of mice inoculated with WT GBS. Levels of inflammatory markers in the bacterium-free uterine tissues (all from mice inoculated with GBSΔhylB) were similar to that of mice inoculated with phosphate-buffered saline (PBS) (Fig. 4Aiii to Diii). No differences were observed in levels of IL-10, IL-1β, or GROα in uterine tissues (see Fig. S2 in the supplemental material), nor were there differences in the levels of TNF-α, MIP2, IL-6, MIP1β, IL-10, IL-1β, or GROα in placental or fetal tissues (see Fig. S3 in the supplemental material). These data suggest that immune responses in uterine tissues, rather than placental or fetal tissues, are responsible for decreasing the incidence of ascending GBS infection and that GBS circumvents this response by blocking TLRs through HylB activity.

Hyaluronidase activity dampens uterine immune responses, but not placental immune responses, in human tissues. Given our in vivo findings, we set out to determine if HylB modulates inflammatory responses in human tissues. We first used an ex vivo model of GBS infection of chorioamniotic membranes. Intact chorioamniotic membranes were collected from healthy women not in labor undergoing caesarean sections at term and were mounted on Transwells as previously described (15). Membranes were then inoculated with approximately 10⁶ CFU on the choriodecidual/maternal side, which was supplemented with 1.25 mg/ml hyaluronic acid. At 4 and 24 h postinoculation, medium was collected from both the chorionic and amniotic sides of the membranes. TNF-α, IL-6, and IL-8 concentrations were measured by enzyme-linked immunosorbent assay (ELISA). As was observed in vivo, GBS induced significant cytokine expression from the gestational membranes, but this inflammation was not decreased by HylB (see Fig. S4 in the supplemental material). These data indicate that HylB does not play a role in dampening the inflammatory response from the placenta and suggest that another tissue is responsible for preventing ascending infection.

We then tested the impact of HylB in uterine tissue using an immortalized human endometrial cell (HEC-1-B) model of infection. HEC-1-B cells were infected with GBS at a multiplicity of infection (MOI) of 0.01 in media supplemented with 1.25 mg/ml

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FIGURE 3  HylB activity leads to increased bacterial ascension. Pregnant female C57BL/6j mice were inoculated with approximately 10⁶ CFU of WT GBS (n = 12) or GBSΔhylB (n = 11). The vaginal tract, uterine horns, distal and proximal placentas, and distal and proximal pups of these mice were assessed for bacterial load by serial dilution plating. Data are displayed as individual data points with median bars. Mann-Whitney U test was used to assess statistical significance between groups (*, P < 0.05; **, P < 0.01).
FIGURE 4  GBS HylB activity blocks uterine inflammation in vivo. Luminex assays were used to assess the levels of the inflammatory markers TNF-α (A), MIP2 (B), IL-6 (C), and MIP1β (D) in the uterine tissues of pregnant female C57BL/6J mice inoculated with approximately 10^8 CFU of WT GBS strain COH1 (n = 12), GBSΔhylB (n = 11), or PBS (n = 3). Data are represented as immune responses in uterine samples irrespective of bacteria (Ai to Di) or as uterine samples with GBS to those without GBS (either inoculated with GBS or PBS [Aii to Dii]). Unpaired Student’s t test was used to assess statistical significance between groups (A to D [* P > 0.05]). Data bars display the mean.
hyaluronic acid. Cell culture supernatant was collected at 48 h postinfection, and levels of TNF-α, IL-6, and IL-8 were measured by ELISA. Interestingly, HEC-1-B cells infected with GBS lacking hylB display significantly higher levels of inflammatory cytokines TNF-α, IL-6, and IL-8 than WT GBS, which corroborates our in vivo findings (Fig. 5). These data suggest a role for uterine immune responses in preventing ascending infection by clearing GBS that have entered the uterine space. GBS that are able to block uterine immune responses through HylB appear to be able to disseminate into deeper tissues, such as placental and fetal tissues, leading to adverse pregnancy outcomes such as in utero fetal demise and/or preterm birth. Collectively, our studies indicate the importance of GBS-encoded hyaluronidase in suppression of uterine immune responses during ascending infection and preterm birth.

DISCUSSION

The work presented in this article establishes a novel role for the GBS hyaluronidase, HylB, as a critical virulence factor in ascending GBS infection and resulting preterm birth. We have shown that GBS strains isolated from women undergoing preterm labor show higher levels of hyaluronidase activity and that this activity permits ascending infection by reducing antibacterial inflammation in uterine tissue. Recent work has described the role of HylB in tissue dissemination and immune evasion during septic GBS infection, but the role of HylB in ascending infection has heretofore been unstudied (28, 34). Our data indicate that HylB is important for pathogenesis during ascending infection. GBSΔhylB showed decreased bacterial ascension and fetal demise and induced more inflammation in both human and murine uterine tissues. Moreover, as hyaluronidasases encoded by other bacterial pathogens, such as Streptococcus pyogenes and Staphylococcus aureus, are critical for virulence (35, 36), our work will be relevant to understanding the role of microbial hyaluronidasases during disease pathogenesis.

Hyaluronic acid plays a pivotal role in the progression of pregnancy and labor (22–24, 37, 38). During parturition, hyaluronic acid production drastically increases in cervical tissue until it comprises approximately 1.0% of the dry weight of the cervix (23). For a successful vaginal birth, the cervix must undergo a softening process (referred to as cervical ripening) in order to allow cervical distention (39). Cleavage of hyaluronic acid is vital for proper cervical ripening. Humans and mice produce multiple hyaluronidasases to cleave the abundance of hyaluronic acid present in the cervix, and application of exogenous hyaluronidase is one method used to induce cervical ripening and labor (40). Interestingly, vaginal inoculation with hyaluronidase-encoding Escherichia coli leads to increased preterm birth rates in mice (22). These data suggest that microbial hyaluronidasases can induce cervical ripening and labor, and our data support this hypothesis. While we do not know that the GBS hyaluronidase specifically induces cervical ripening, we show that it is crucial in increasing GBS infection-associated preterm birth (Fig. 2D and E). The ability of the GBS hyaluronidase, and other microbial hyaluronidasases, to induce cervical ripening needs to be explored further. Inhibition of microbial hyaluronidasases may be a possible avenue for developing therapeutics to reduce preterm birthrates.

Traditionally, inflammation is thought to be a driver of preterm labor, so it is counterintuitive that genetic ablation of hylB leads to more uterine inflammation but less preterm birth. We speculate that this is due to the temporal nature of uterine inflam-

![FIGURE 5](mbio.asm.org) HyalB activity dampens immune responses in immortalized endometrial cells. ELISAs were used to assess the levels of TNF-α (A), IL-8 (B), and IL-6 (C) in immortalized endometrial HEC-1-B cells that were infected for 48 h with WT GBS or GBSΔhylB at an MOI of 0.01 in media supplemented with 1.25 mg/ml hyaluronic acid (HA). Experiments were performed in triplicate and repeated six times. One-way ANOVA with Tukey’s multiple correction test was used to assess statistical significance between groups (A to C). Asterisks over single bars indicate significant compared to all other groups (**, P < 0.005; ****, P < 0.00005).
that the uterus is the primary immunological barrier that prevents Gram-positive bacterial invasion of gestational tissues. If this immunological barrier fails due to inhibited TLR signaling, ascended bacteria may avoid detection by the host. This may allow dissemination into placental and fetal tissues and preterm birth, as was observed in pregnant mice inoculated with WT GBS (Fig. 2C and 3). Conversely, if TLR signaling is not inhibited, an increase in uterine inflammation may lead to bacterial clearance, such as what we observed in pregnant mice inoculated with GBS/H9004/hylB (Fig. 2C, 3A, and 4A to D). The timing and magnitude of inflammation may also play important roles in preterm labor as an outcome of ascending bacterial infection. If the increase in uterine inflammation is a transient event, rather than a sustained event, the host may be able to clear the infection without the induction of labor. It is interesting that mice inoculated with GBS/H9004 that did not show ascending infection also had lower levels of inflammation than those that did show ascending infection (Fig. 4). We hypothesize that ascended bacteria caused a large spike in inflammation early in infection, leading to their clearance from the uterine space. Once the bacteria are cleared, the inflammation may rapidly dissipate, promoting healthy pregnancy (Fig. 6). These results imply a need for earlier GBS screening during pregnancy, but additional research is needed to fully address this hypothesis.

Preterm birth is the leading cause of adverse pregnancy outcomes for both the mother and fetus, and a significant number of preterm births are due to in utero infection with vaginal flora, such as GBS (1, 2, 4, 6–8, 14). Understanding the virulence factors that allow vaginal flora to switch from commensal colonizers to ascending pathogens is crucial for developing successful interventions to prevent in utero infection and resulting preterm birth. While studies have characterized the role of the GBS hemolytic pigment in vaginal colonization, ascending infection, induction of inflammation, and fetal damage (30, 41, 42), we know little about the role of other GBS virulence factors involved in these processes. By defining the mechanism underlying these different virulence factors, it may be possible to develop strategies for intervention. Interestingly, multiple compounds are able to inhibit microbial hyaluronidase activity (36, 43). To our knowledge, little attention has been given to finding a hyaluronidase inhibitor specific to GBS, but such an inhibitor may be an effective means of combating multiple types of GBS infections.

We have described a novel role for the GBS hyaluronidase in ascending infection and preterm birth. First, we draw a strong association between GBS hyaluronidase activity and serious disease outcomes in clinical isolates derived from human samples. Next, we show that the presence of the GBS hyaluronidase permits increased bacterial ascension from the vaginal tract to the uterine space, which is associated with a reduction in uterine inflammation and leukocyte invasion compared to GBS lacking the hyaluronidase. Finally, we are suggesting a more refined role for uterine inflammation during pregnancy, wherein inflammation can potentially lead to the elimination of ascended bacteria without inducing preterm labor. Future research should focus on further defining the role of the GBS hyaluronidase in these processes and developing a hyaluronidase inhibitor to prevent ascending GBS infections.

MATERIALS AND METHODS

Ethics statement. All animal experiments were approved by the Seattle Children’s Research Institutional Animal Care and Use Committee (protocol 13907) and performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, 8th ed. (44).

Bacterial strains. The chemicals in this study were purchased from Sigma Aldrich unless stated otherwise. The WT GBS strain COH1 used in these studies is a serotype III (sequence type 17 [ST17] clone) clinical isolate from an infected human newborn (45). GBS cultures were grown in tryptic soy broth (TSB) or on tryptic soy agar (TSA) (Difco Laborato-
ries) at 30 or 37°C in 5% CO₂ and monitored at 600 nm. The hybB allelic replacement mutant was generated using methods previously described (46). Briefly, 1.0-kb regions flanking either side of the hybB gene (hybB 3' forward, CCA AGG AGT GTA AAC; hybB 3' reverse, TTT AGC CAT TTT TAC TCC TTA GGT TTT AAA ATT GTA AAC; hybB 5' forward, ATT GTT TTA GCT AAC CGT ACA TAA AAA ACC TAT C; hybB 5' reverse, GTA GCC GCT AGG GAC AAC TGT CCT TGA TAA ATT GAC) and a kanamycin resistance cassette (Kan’r forward, GGA GTA AAA AAT GCC TAA AAT GAG AAT ATC AC; Kan’r reverse, ACG GTT AGC TAA AAA AAT TCA TCT AGC AAA ATA TAA TAT TTT ATT TCC) were PCR amplified from genomic DNA (for flanking regions) or from the plasmid pCIV2 (for the Kan’r cassette). Each primer contained a short (15- to 25-bp) region of homology that corresponded to either the temperature-sensitive cloning vector pH304 or the next corresponding region. The pH304 cloning vector was PCR linearized (pHY304 forward, GTC CCT AGC GCC TAC GGG; pHY304 reverse, CTC TTT AGC TGC TGT GAA GCT GTC), and all four fragments (2 flanking regions, Kan’r cassette, and linear PHY304) were ligated using the Gibson Assembly cloning kit (New England Biolabs). This plasmid was then electroporated into the WT GBS strain COH1, and selection for allelic replacement was performed as previously described (46).

**Clinical isolates.** GBS clinical isolates were collected as previously described (15, 41). Briefly, rectovaginal swabs were obtained from women in their third trimester of pregnancy at the University of Washington Medical Center and Harborview Medical Center in 2007 under University of Washington IRB 30308; samples were collected without any identifiers or clinical information, and a waiver for written informed consent was obtained for testing anonymous samples. GBS clinical isolates from amniotic fluid, chorioamnion, and/or cord blood were obtained using the Gibson Assembly cloning kit (New England Biolabs). This plasmid was then electroporated into the WT GBS strain COH1, and selection for allelic replacement was performed as previously described (46). Six- to-8-week-old female C57BL/6j mice were obtained from The Jackson Laboratory and used for ascending infection studies. Female mice were individually paired with male C57BL/6J mice for 2 days and then separated and monitored for 14 days postseparation. Pregnancy was confirmed by observable weight gain and palpation for the presence of pups. Mice were inoculated on day 15 of pregnancy (E15). Overnight GBS cultures were subcultured 1:20, grown to optical density at 600 nm (OD600) of 0.3, pelleted at 5,000 rpm for 8 min, washed once with sterile PBS, and resuspended in sterile PBS to a final concentration of 10⁸ CFU/ml. Mice were anesthetized using 4% isoflurane, and 10⁶ (±10⁶ CFU) of inoculum was administered into the vaginal lumen using a micropipette. Mice were left inverted for 5 additional minutes under anesthesia and then returned to their cages and monitored until ambulatory. Mice were monitored twice daily up to 72 h postinoculation for signs of preterm birth (vaginal bleeding and/or pups in the cage). At 72 h postinfection, or earlier if preterm birth was observed, mothers were euthanized and a midline laparotomy was performed to identify fetal injury and loss of pregnancy and to collect maternal and fetal tissues. Tissues were excised, homogenized, and serially plated on TSA to determine the number of CFU associated with maternal and fetal tissues. All data were normalized to total tissue weight in grams. Homogenized tissue was then incubated overnight at 4°C in lysis buffer (0.15 M NaCl, 1 mM NaHCO₃ [pH 7.2]) and pelleted, and supernatants were collected for further analysis as described below.

**Luminex assays of murine tissues.** Tissue lysates from the mouse ascending infection model (see “Murine model of ascending GBS infection” above) were thawed and centrifuged at 10,000 × g for 5 min at 4°C to remove residual cell debris. Fifty microliters of the supernatants was then used for cytokine analysis (IL-10, IL-1β, IL-6, GROα, TNF-α, MIP2, and MIP1β) by Luminex assay (Procartaplex Multiplex immunoassay; eBioScience) as per the manufacturer’s instructions. Expected cytokine concentrations for the standard curve were translated into logarithmic scale and fit to a 5-parameter logistic curve, and median fluorescence intensity readings from unknown samples were then interpolated from this standard curve using GraphPad Prism 6 (La Jolla, CA). These data were translated out of logarithmic scale back into concentration values (picograms per milliliter) and were then normalized to total tissue weight in milligrams.

**Cytokine analysis in GBS-infected human chorioamnion.** Human chorioamniotic/placental membranes were collected from deidentified healthy term pregnancies undergoing scheduled cesarean sections at the University of Washington Hospital. Since identifiable information was not collected, this research is exempt from IRB review (see letter from University of Washington IRB, determination form 44282, “Use of discarded placentas”). Chorioamniotic membranes were cultured on Transwells as previously described (15). Briefly, membranes were dissected
from placenta immediately following delivery and transported on ice. Membranes were rinsed in PBS and mounted on Transwell inserts (12 mm) lacking the synthetic filter membrane. Gestational membranes were held in place with sterile latex bands. Transwells were placed in 12-well plates with 0.5 ml medium (Dulbecco’s minimal medium [DMEM] with 1-glutamine supplemented with 1% fetal bovine serum [FBS] and penicillin/streptomycin) in the upper chamber and 1.5 ml medium in the lower chamber at 37 C. Following overnight acclimation, membranes on Transwell inserts were washed and inoculated with approximately 10^7 CFU in the upper (choriocolic/adamantal) chamber in DMEM supplemented with or without 1.25 mg/ml hyaluronic acid. Medium was sampled at 4 and 24 h from both the apical and basal compartments and stored at 20°C until analysis. ELISAs for cytokine levels were purchased from R and D Systems and performed as per the manufacturer’s instruction. Medium from infected tissue was diluted 1:50 (TNF-α) or 1:100 (IL-6, IL-8, and CCL4), and medium from control tissue was diluted 1:4.

Statistical analysis. The Mann-Whitney test, Fisher’s exact test, or Tukey’s multiple comparison test following analysis of variance (ANOVA) was used to estimate differences as appropriate, and a P value of <0.05 was considered significant. Statistics were performed using GraphPad Prism version 5.0 for Windows (GraphPad Software, La Jolla, CA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00781-16/-/DCSupplemental. Figure S1, PDF file, 0.1 MB. Figure S2, PDF file, 0.2 MB. Figure S3, PDF file, 0.2 MB. Figure S4, PDF file, 0.3 MB.

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