COMPOSITION AND FUNCTION: UNRAVELING THE ROLE OF GROUP B STREPTOCOCCAL MEMBRANE VESICLES

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ABSTRACT

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Group B *Streptococcus* (GBS) is an opportunistic pathogen that asymptomatically colonizes the vaginal tract of approximately 30% of women. In individuals with an altered immune state such as the elderly, pregnant women, and neonates, GBS can cause invasive infections. During pregnancy, GBS frequently contributes to infections *in utero* that can result in chorioamnionitis, preterm birth, or miscarriage. The bacterial factors that promote these adverse outcomes, however, are under studied. For instance, previous reports have demonstrated that GBS produces membrane vesicles (MVs), which have been linked to adverse pregnancy outcomes in a murine model. Nonetheless, little is known about the composition of GBS MVs and their role in pathogenesis and impact on the immune response. Therefore, using genotypically diverse strains of GBS, I sought to examine MV production and composition, as well as their impact on ascending infection *in vivo* to better understand their role in GBS disease. While MVs contain a variety of immunomodulatory virulence factors, including hyaluronidase, C5a peptidase, and sialidase, the relative abundance of these virulence factors varies in a lineage dependent manner. With this information in hand, I then assessed the host response to GBS derived MVs using human THP-1 derived macrophages. I found that regardless of strain, MVs induce a potent proinflammatory immune response characterized by the production of proinflammatory cytokines and chemokines. Notably, many neutrophil chemokines were highly upregulated in response to MVs, which is consistent with observations seen during murine challenge with MVs. I went on to demonstrate that MVs, in the absence of t, induce a potent IL-

1ß response, which is dependent on caspase-1 and NLRP3, suggesting that the NLRP3 inflammasome is a sensor of GBS derived MVs. Previous data had suggested that MVs induce inflammation at the maternal-fetal interface. We have confirmed these findings and identified a potential mechanism for this inflammatory response. Furthermore, others utilized models of intra-amniotic injection that bypasses many immune defenses such as the extraplacental membranes and the cervix, which may prevent this response, while we assessed the role of MVs during vaginal challenge with GBS. However, we found that the addition of exogenous MVs does not promote ascending infection *in vivo.* While bacteria were able to ascend from the vaginal tract into gestational tissues, similar levels of bacteria were found regardless of the addition of exogenous MVs. Consistent with this observation, using flow cytometry we showed that neither exogenous MVs supplemented at the time of infection nor vaginal supplementation of MVs alone alter inflammatory responses at the maternal-fetal interface, suggesting that these innate barriers are capable of blocking MV mediated effects. The work presented here dramatically alters our understanding of the composition and host-pathogen interactions of GBS MVs, by substantially increasing our knowledge of both their composition and function during infection.

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KEY TO ABBREVIATIONS

GBS	Group B Streptococcus
EOD	Early Onset Disease
LOD	Late Onset Disease
CDC	Centers for Disease Control
CPS	Capsular polysaccharide
RFLP	Restriction fragment length polymorphism
PCR	Polymerase Chain Reaction
NT	Non-typable
STs	Sequence Types
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
HylB	Hyaluronidase
TLR	Toll-Like Receptor
ScpB	C5a Peptidase
PG	Prostaglandin
Siglecs	Sialic-acid-binding immunoglobulin-like lectins
PAMPs	Pathogen Associated Molecular Patterns
NLRs	Nod-Like Receptors
PRRs	Pattern Recognition Receptors
NLRP	Nucleotide-binding oligomerization domain, Leucine rich Repeat and-Pyrin Domain

DAMPs	Danger Associated Molecular Patterns
NK	Natural Killer
NKT	Natural Killer T-cell
MVs	Membrane vesicles
IAP	Intrapartum Antibiotic Prophylaxis
THB	Todd-Hewitt Broth
THA	Todd-Hewitt Agar
CFUs	Colony Forming Units
PBS	Phosphate Buffered Saline
TEM	Transmission Electron Microscopy
SEM	Scanning Electron Microscopy
ANOVA	Analysis of Variance
PCA	Principal Component Analysis
OD	Optical Density
IL	Interleukin
PMA	Phorbol 12-myristate 13-acetate
MOI	Multiplicity of Infection
LPS	Lipopolysaccharide
LDH	Lactate Dehydrogenase
RNA	Ribonucleic acid
cDNA	Complementary Deoxyribose nucleic acid

DNA	Deoxyribose nucleic acid
RT-qPCR	Reverse Transcriptase-quantitative polymerase chain reaction
RLU	Relative Light Units
GD	Gestational Day

CHAPTER 1

Literature Review: Group B *Streptococcus* and Its Effects on Pregnancy Outcomes

GROUP B STREPTOCOCCAL DISEASE AND BURDEN

Group B *Streptococcus* (GBS), also known as *Streptococcus agalactiae*, is a Gram-positive opportunistic pathogen, that asymptomatically colonizes the vaginal tract and gastrointestinal tract of ~30% of individuals (1). Although a large proportion of the population is colonized with GBS, only a small number develop invasive disease. Indeed, invasive GBS disease is primarily observed in immunocompromised individuals such as neonates, the elderly, and those with preexisting conditions such as diabetes mellitus (1-3). While invasive GBS disease in the elderly is an emerging threat, the highest burden of disease occurs in neonates and pregnant women (4). Neonatal GBS disease can come in two forms: early-onset disease (EOD) or late-onset disease (LOD). While EOD occurs within 7 days post birth and is commonly associated with pneumonia, respiratory failure, and sepsis, LOD can occur up to 7 months post-birth and is commonly associated with bacteremia and meningitis (5). Similarly, during pregnancy, GBS infections can result in numerous clinical presentations including chorioamnionitis, miscarriage, and stillbirth (3, 5). Several epidemiologic studies have also demonstrated that GBS colonization is a risk factor for preterm birth, an observation which would shift our understanding of global GBS disease burden (6-8).

Globally, 1 in 10 live births are preterm (<37 weeks gestation), with roughly 40% of these preterm births being caused by infection (9). Similarly, 25-50% of stillbirths are attributable to *in utero* infections (9, 10). Importantly, it has been estimated that GBS causes over 3.5 million preterm births and 104,000 stillbirths annually (6). These numbers, however, likely underestimate the frequency of *in utero* infections as 5% of healthy, term, cesarean delivered placentas test positive for GBS, indicating that these subclinical infections persist (11). Additionally, testing for GBS is limited in under-developed nations such as South Africa, which

uses a risk-based approach rather than a universal testing strategy to identify mothers with enhanced risk of delivering a neonate with GBS disease (12). Further, subclinical infections *in utero* may also contribute to underreporting of these GBS-associated infections (11).

GBS colonization and infection during pregnancy can lead to a wide array of consequences. Mild infections, for instance, can present as subclinical chorioamnionitis, whereas severe infections can result in preterm birth, miscarriage, or stillbirth (6, 10, 13-15). Currently, the top cause of death for children under 5 years of age is due to complications arising from preterm birth (16). Several studies have also established a link between chorioamnionitis and atypical neurologic development, which increases the risk of developing autism spectrum disorders and highlights the broader implications of subclinical infections (17, 18). These severe and lasting consequences represent a significant burden for the healthcare system and for families, who often experience mental health challenges for months after a preterm birth (19).

PROPHYLAXIS AND TREATMENTS FOR GBS

In the US and other developed countries, pregnant women are routinely screened for GBS in the third trimester between 36-37 weeks of gestation using standard culture-based methods (20). For women testing positive, the Centers for Disease Control and Prevention (CDC) recommends intrapartum antibiotic prophylaxis, typically involving intravenous penicillin, to which GBS is highly susceptible (20). While this treatment strategy is effective at preventing early onset GBS disease in neonates, it does not prevent late onset disease, chorioamnionitis, preterm birth, or miscarriage (21). Furthermore, antibiotic usage has been tied to alterations in the neonatal microbiome, which can result in long-term consequences including increased risk of obesity if the perturbation is minor or increased risk of being underweight if the perturbation is

substantial (22, 23). Improving treatment strategies to prevent these consequences and adverse outcomes is therefore of the utmost importance.

The persistence of adverse outcomes in pregnancy has led many researchers to explore the possibility of a GBS-specific vaccine. Many vaccines have been developed, with the most promising candidate being a poly-conjugated vaccine targeting the capsular polysaccharide (24). While this vaccine has shown promise in clinical trials, it has yet to be approved by any governmental agency (25). Further, it is unclear how well this vaccine will prevent adverse pregnancy outcomes associated with GBS since these studies have yet to assess vaccine efficacy during *in utero* GBS infection. Therefore, developing an understanding of how GBS contributes to adverse pregnancy outcomes such as intrauterine infections, chorioamnionitis, preterm birth, and miscarriage, is critical.

GBS STRAIN VARIATION AND TYPING METHODS

A leading explanation for the discrepancy between colonization and invasive disease frequencies is strain variation. Early attempts to classify GBS into subtypes relied heavily on characterizing the type of capsular polysaccharide (CPS), the primary GBS virulence factor that dictates the serotype (26). To date, 10 distinct GBS serotypes have been identified (27, 28). Typing of the GBS CPS can be performed using various techniques including latex agglutination, antisera-based approaches, or via sequence analysis of the CPS genes (29). Molecular assays utilizing PCR-based restriction fragment length polymorphism (RFLP) analysis have also been developed to further classify the CPS types (30-32). These assays are useful for screening large strain collections and examining associations between CPS types and other molecular traits. Indeed, CPS typing demonstrated that serotype III GBS strains were more

strongly associated with invasive neonatal EOD, accounting for ~65% of all neonatal infections in some studies (33, 34). Alternatively, serotype V strains were more strongly associated with disease in the elderly, as well as increased frequencies of antibiotic resistance (35-37). Nonetheless, limitations with CPS typing, including the identification of non-typable (NT) strains and misclassification due to genomic rearrangements, have increased the need for more sensitive typing methods (34, 38, 39).

With the advent of DNA sequencing, the field rapidly adopted multilocus sequence typing (MLST) as a reliable method for segregating groups of clinical isolates into sequence types (STs) based on phylogenetic relationships (40). In multiple studies of clinical GBS strains from different geographic locations, the application of MLST demonstrated that specific lineages are associated with different clinical outcomes. For instance, several studies have demonstrated that ST-12 strains were strongly associated with asymptomatic colonization, whereas ST-17 and ST-1 strains were more commonly associated with neonatal and elderly disease, respectively (40-43). Although MLST provides excellent discriminatory power, the use of whole-genome sequencing can further differentiate strains, as was demonstrated in a comparative genomics study and via the use of clustered regularly interspaced short palindromic repeats (CRISPR) typing (44, 45), which used CRISPR spacers and terminal direct repeat regions for differentiation. Using various combinations of spacer regions, CRISPR typing can discriminate between clusters containing multiple STs, which allows for more targeted identification of sub-lineages associated with adverse clinical outcomes.

Several groups have independently shown that GBS virulence gene profiles and virulence gene diversity vary across lineages. The ST-17 strains, which have been linked to neonatal infections in multiple studies, possess unique alleles in key genes that are important for

attachment and invasion (46-48). Some of these variable proteins have been used in vaccine trials; however, these surface protein antigens are often masked by capsular polysaccharide, limiting vaccine effectivity across various isolates (46-49). Although this gene level variation is useful for molecular subtyping, it also demonstrates how the virulence potential differs across GBS lineages. For instance, studies have shown that allelic variation in the genes encoding the two-component system, CsrRS, as well as the C5a peptidase directly impacts protein functionality (50, 51). Additionally, enhanced survival of a ST-17 strain inside macrophages was attributable to the differential expression of key virulence genes *in vitro* (52, 53). Korir et al. also demonstrated that the ST-12 strains were more tolerant to ampicillin relative to ST-17 strains (53). Together, these findings suggest that both virulence gene variation at both the gene and expressional level affects the pathogenic potential of clinical isolates with distinct genetic backgrounds.

MECHANISMS PROMOTING GBS IN UTERO INFECTIONS

Current research indicates that GBS can cause infections via distinct routes. Neonatal infections were historically believed to be acquired through a vertical route of transmission, i.e., by aspiration of GBS during passage through the birth canal of colonized mothers or from contaminated breastmilk (54, 55). While vertical transmission likely occurs to some extent, growing evidence suggests that infections can occur prior to birth. Several studies have demonstrated that *in utero* infections occur following ascension of GBS from the vaginal tract to the gestational tissues (56, 57). The symptoms and severity of these *in utero* infections have been linked to the tissues that have been infected, the degree of tissue invasion, and bacterial density (13, 14, 58). Although the burden and severity of these pregnancy-associated infections is

apparent, it is not clear why some women develop invasive GBS disease and others remain asymptomatically colonized.

Ascending GBS infections start with colonization of the vaginal tract, where GBS readily attaches to and invades the vaginal epithelium (57, 59, 60). GBS can also associate with the cervical epithelium where it has been shown to colonize both the ecto and endocervix at high levels (61). During pregnancy, the presence of the cervical mucus plug typically serves as an antimicrobial barrier containing antimicrobial peptides and leukocytes; however, GBS can overcome these defenses and ascend past the cervical mucus plug (62, 63). Following ascension into the uterus, GBS encounters the extraplacental membranes, which are composed of three layers: the decidua, the chorion, and the amnion (64). GBS can readily attach to all three layers and induce weakening of the chorion and amnion layers, either directly via microbial virulence factors or indirectly through immune activation, leaving this tissue susceptible to rupture (65-67). During ascending infection, the first layer of the extraplacental membranes that is breached is the decidua, followed by the chorion and amnion. Once these layers are breached, GBS can gain access to the amniotic fluid, which supports growth of the bacterium (68). Contaminated amniotic fluid can then be rapidly aspirated or ingested by the developing fetus causing a systemic infection (69).

The mechanisms that promote GBS ascension *in vivo*, however, are poorly understood as only a few factors have been linked to ascension previously. One example of a bacterial factor linked to ascension is hyaluronidase (HylB), an enzyme that degrades the hyaluronic acid that is present in connective tissues, particularly those lining the reproductive tract (70, 71). During the normal course of infection, hyaluronic acid is broken down into proinflammatory fragments that can be sensed by toll-like receptor (TLR)-2 and TLR4 found on both phagocytic and non-

immune cells (72). The GBS hyaluronidase breaks down these proinflammatory hyaluronic acid fragments into smaller inactive forms, which act as receptor antagonists (72), to subvert the host immune response. Strains of GBS lacking *hylB* have a reduced ability to ascend into gestational tissues, and their presence induces increased levels of inflammatory cytokines because of the lack of receptor antagonists (56). While it is unclear exactly how HylB promotes ascending infection, it appears to skew cytokine, prostaglandin and matrix metalloprotease production in non-human primate and murine models of ascending infection (56, 73).

A similar effect on ascending infection was also demonstrated through studies of *cpsE*, which encodes a bacterial protein important for capsular polysaccharide biosynthesis. Indeed, mutants containing deletions in *cpsE* displayed reduced ascension in a mouse model, thereby highlighting its potential role in ascending infection (74). Furthermore, *cpsE* mutants had reduced capsular polysaccharide thickness and biofilm production, which correlated with a decreased level of placental inflammation (74). Together, these data indicate that *cspE*-mediated effects are likely due to a diminished ability to evade the host immune response.

An example of a host factor linked to GBS ascension is the induction of vaginal exfoliation. When in the vaginal tract, GBS promotes an epithelial to mesenchymal transition in vaginal epithelial cells, which ultimately reduces barrier integrity (57). This transition allows GBS to invade deeper into the vaginal tissues. When integrin-dependent vaginal exfoliation was blocked, however, GBS ascension into the uterus was reduced, indicating that vaginal barrier integrity plays an important role in the establishment of *in utero* infections (57).

Although only three factors have been shown to directly impact ascending infection, several known bacterial virulence factors may promote the adverse pathology associated with GBS *in utero* infections. Examples include the C5a peptidase (*scpB*) and hemolytic pigment

(*cylE*) as well as the Srr1, BsaB, and FbsC. The C5a peptidase, for instance, facilitates tissue invasion and immune evasion by binding to fibronectin and cleaving the C5a complement component, respectively. Similarly, the adhesion molecules, BsaB, FbsC and Srr1/2, were demonstrated to facilitate attachment and invasion of GBS to host extracellular matrix components, which is critical for pathogenesis (60, 75, 76). Conversely, the ß-hemolysin has been shown to promote pyroptosis and weakening of human amnion epithelial cells, while deletion mutants were highly attenuated at causing this weakening (77, 78). Given the lack of animal model systems available to study ascending infections, however, it is likely that other factors are also important but have yet to be characterized.

GBS AND PRETERM BIRTH

Normal term parturition is a highly coordinated process that occurs at approximately 39 weeks of gestation. The first steps take place in the weeks leading up to parturition when the cervix undergoes a process of softening, whereby collagen becomes less abundant and more disorganized (79). This process is followed by cervical ripening, which is characterized by a swift increase in hyaluronic acid, tissue hydration, and an influx of eosinophils and monocytes, processes which are partly dependent on prostaglandin signaling (80-82). These changes allow for the cervix to become pliable for cervical effacement and dilation with the onset of uterine contractions (64). Additionally, as the cervix is remodeled, there is often a loss of the cervical mucus plug, which primarily serves as an antimicrobial barrier between the vaginal tract and the *in utero* environment (63). At term, membrane rupture is typically followed by an induction of uterine contractions. These contractions are stimulated by a functional withdrawal of progesterone signaling in the myometrium (83, 84), which triggers proinflammatory signaling

that culminates in myometrial contractions (84). Uterine contractions, which originate at the uterine fundus, pull at the lateral sides of the uterus and stretch the cervix, leading to cervical effacement and dilation (64). Once the cervix is fully dilated, the fetus is delivered followed by the placenta (64).

It is evident that preterm birth is a complex condition with many root causes. Generally, the main causes of preterm labor are cervical insufficiency, myometrial activation, infection, placentation disorders, or a breakdown of maternal-fetal tolerance (85). Clinically, it is believed that a large cause of preterm birth is associated with cervical insufficiency. The early induction of cervical ripening leads to preterm cervical effacement and dilation, which is characterized by a rapid influx of neutrophils and was suggested to be mediated by prostaglandins (86, 87). Prostaglandins (PG) also play a role in preterm myometrial activation, with higher levels of amniotic PGF2a being observed in women with spontaneous preterm labor (88).

Preterm birth can also occur due to defects at the maternal-fetal interface (85) or from placentation disorders such as preeclampsia (89). Spiral arteries found at the placental bed have decreased remodeling in both spontaneous preterm births and preeclamptic pregnancies, suggesting that there may be a common link between these disorders (90). Abnormal immune responses at the maternal fetal interface have also been associated with preterm birth. Notably, one study demonstrated that a portion of preterm births are associated with elevated fetal T-cell activation, suggesting a breakdown in maternal-fetal tolerance (91). While controversial, there is growing evidence that maternal-fetal rejection may play a role in some preterm births (91-93).

Infections during pregnancy impact various stages of parturition to trigger preterm birth. Viral infections, for instance, have been shown to promote cervical remodeling and subsequent cervical insufficiency leading to increased risk of infection and preterm labor (94, 95). Similarly,

bacterial pathogens can weaken gestational membranes and lead to preterm rupture of the extraplacental membranes (66, 67). Additionally, bacterial signals can trigger myometrial activation via the stimulation of functional progesterone withdrawal (96). Together, these data suggest that bacterial infections can promote preterm birth by inducing various mechanisms that impact parturition prior to term gestation.

Numerous groups have demonstrated that GBS can trigger preterm birth via various mechanisms. In both murine and non-human primate models, GBS can trigger rapid induction of preterm labor (97-99). In non-human primates, GBS inoculation into the choriodecidual space triggers myometrial contractions and proinflammatory cytokine signaling in the fetal compartment (97). Various models of ascending infection have also demonstrated that GBS induces rapid recruitment of neutrophils and lymphocytes to the gestational membranes. These responders trigger cell death pathways such as NET-osis and MET-osis, which promote tissue weakening and potentially membrane rupture (99, 100). This response is supported by epidemiologic data showing that GBS promotes preterm rupture of membranes in pregnant women (8). While no association has been observed between GBS colonization and cervical insufficiency, high levels of vaginal colonization with GBS was associated with preterm birth in a prior study (7). As a potential reservoir of GBS colonization, the cervix appears to play a critical role in facilitating these adverse pathologies (59).

MODELING HOST INTERACTIONS TO GBS

Several models have been used to assess host-microbe interactions during GBS infections with varying degrees of manipulability, complexity, and relevance to human disease. These models range from relatively simple *in vitro* or *ex vivo* culture systems to highly complex *in vivo*

infections. Taking a multifaceted approach, i.e., using a combination of these models, allows for scientific rigor and optimizes the translatability to humans.

Several *in vitro* models have been established to assess the host response to GBS infections during pregnancy. Immortalized cell lines derived from human gestational tissues, for example, are readily used and include decidualized stromal cells, amnion epithelial cells, and trophoblast cells (65, 77, 101). Furthermore, human monocyte cell lines have been used to examine cytokine and signaling responses from leukocytes (53, 101, 102). These systems are highly manipulatable, amenable to chemical/protein inhibitors, and genetic editing, which makes them highly useful for determining the impact of GBS on different cell types. While assessing responses from these cell types in monoculture is of clear importance, researchers are starting to determine how these cells interact during infection, with the goal of creating a better model for studying complex host-microbe interactions (101). While more complex organoid and organ-on-a-chip models have been developed to model these interactions and recapitulate human responses (103-105), to date, no studies using these models have been conducted with GBS.

In vitro models to assess host-microbe interactions include primary cell culture and explant-based systems. Studies in both primary placental macrophages and peripheral blood monocyte cultures appear to recapitulate findings from macrophage cell lines (100, 106, 107). Characterization of placental and extraplacental membrane responses has also had some success, with relatively easy isolation and culture protocols (108, 109). Similarly, term human extraplacental membranes and placentas can be treated with GBS to assess multiple responses including tissue weakening, cytokine responses, and PG production (109, 110). While these models are translatable to human infections, they are difficult to manipulate and are limited in the number of experimental replicates that can be conducted. Consequently, they are typically

used to validate findings from cell culture experiments and only assess responses in one tissue type rather than systemically in multiple tissues, which can limit interpretation.

Unlike *in vitro* models, live animal models are highly complex with a similar physiology to humans. The two primary model organisms used to evaluate GBS host-pathogen interactions are non-human primate and murine models (56, 57, 66, 74, 97, 98, 111-113). Murine models have been used more frequently due to their lower cost and the increased number of tools available for their manipulation. For *in utero* infections, the two primary models used include the direct injection model and the vaginal infection model (56, 57, 74, 111, 113). Both models require timed matings of mice to be performed prior to inoculation. While various timepoints have been used, most murine models infect at gestational day 13.5-15.5 (56, 57, 74, 99). With an average murine gestational length of 19-21 days, this corresponds to late 2nd trimester or early 3rd trimester in human pregnancies.

In the vaginal infection model, GBS is injected into the vaginal lumen at a dose of 10^3 – 10^9 colony forming units (CFUs) (56, 57, 74, 99). Injection will result in infection *in utero* or preterm birth within 48-72 hours post inoculation. With the direct injection model, however, the GBS inoculum is directly injected into the amniotic cavity or is delivered via intra-uterine injection, either surgically or ultrasound guided. In this model, the advantage is that infection is immediate with symptoms developing by 72 hours post infection (113, 114). The disadvantages of this model are that direct injection enables GBS to bypass important host defenses found in the cervix and the extraplacental membranes that may impact infection. Consequently, direct injection less accurately mimics the true host environment encountered by the bacterium during a natural infection. The vaginal inoculation model is therefore the preferred model for GBS

infection due to its physiologic translatability and ease of use for researchers with expertise in animal model systems.

Although there are clear strengths for murine models of infection, a major concern is that mice are distantly related to humans. As a result, non-human primates have also been used to study GBS infections due to their similar physiology to humans (66, 73, 97, 98, 111). The most prominent non-human primate model involves chronically catheterizing pregnant rhesus macaques (early 3rd trimester; approximately gestational day 120 in a 172-day gestation), which is performed between the uterine muscle and the choriodecidual membranes, allowing for direct infection at the extraplacental membranes (66, 73, 97, 98, 111). This model results in rapid induction of preterm labor and inflammatory cytokines at the maternal fetal interface, suggesting it recapitulates GBS-induced preterm birth (97). Nonetheless, it does not recapitulate the normal progression of infection observed in pregnant women because it bypasses the cervix. Furthermore, due to the high expense, limited availability, and ethical burden of using these models, they are rarely used for comprehensive studies regarding GBS pathogenesis and host-pathogen interactions.

HOST RESPONSES TO GBS

During GBS infections the host has been shown to mount a potent immune response, which is evidenced by high levels of leukocyte infiltration and potent cytokine induction (13, 14, 58). Acute immune responses elicited toward GBS include increased neutrophil infiltration and macrophage activation, coupled with a high level of proinflammatory cytokine and chemokine production (58, 100, 102, 106, 115). Unsurprisingly, a growing body of literature has been focused on understanding how the immune system senses and responds to GBS (Figure 1.1).

Recognition of GBS

Several pattern recognition receptors (PRRs) have been shown to sense GBS broadly falling into 3 main classes: Toll-Like Receptors (TLRs), Nod-Like Receptors (NLRs), and Sialic acid-binding immunoglobulin-type lectins (Siglecs) (Summarized in Figure 1.1). TLRs are surface or endosomal receptors that recognize pathogen associated molecular patterns (PAMPs) (116, 117). In humans, there are 10 known TLRs, named TLR1-10, whereas 13 TLRs, named TLR1-13, have been classified in mice (116, 117). Currently, five major TLRs are capable of recognizing GBS. Four of these TLRs are found in both mice and humans (TLR2, TLR6, TLR7, and TLR9), while one is exclusively found in mice (TLR13). TLR2, for instance, was shown to recognize GBS lipoproteins, though recognition depended on the maturation of secretion bacterial lipoproteins (118). TLR2 and its' heterodimer TLR6 is also known to recognize GBS lipoteichoic acid; however, the inflammatory response to GBS lipoteichoic acid is diminished relative to lipoteichoic acid from other pathogens including *Staphylococcus aureus* (119).

GBS RNAs can also be recognized by TLRs. Conventional dendritic cells produce IFN-ß in response to GBS, which was shown to be dependent on the interaction between TLR7 and ssRNA (120). While other immune populations such as monocytes and macrophages respond to GBS-derived ssRNA, TLR7 does not appear to play a role, suggesting the presence of redundant ssRNA recognizing receptors (121). Similarly, TLR13 was found to play an important role in the recognition of GBS in murine macrophages; however, blood monocytes ($CD45^{high}$, $CD11b^{high}$, $Ly6G^{low}$) were non-responsive (122). While TLR13 typically responds to methylated RNAs, removing RNA methylation in GBS does not impact TLR13-dependent recognition, suggesting the presence of additional ligands (123). Lastly, TLR9 was shown to be important for the recognition of GBS DNA (124). Mice deficient in TLR9, for instance, had decreased IL-6 and

IL-12 responses as well as decreased production of reactive nitrogen species, suggesting a critical role in cellular activation (124).

Less information is known about the role of NLR signaling in response to GBS. NOD2 knockout mice display minor alterations in splenic cytokine expression during GBS challenge; however, no systemic alterations were observed (125). Similarly, NOD2 plays little to no role in dendritic cell mediated recognition of GBS, suggesting that GBS is not recognized in a NOD2 dependent fashion (126). While conventional NOD receptors have not been shown to recognize GBS, the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin domain containing receptors (NLRPs) have also been implicated in sensing the pathogen (127, 128). Unlike TLRs and NOD receptors, which conventionally recognize PAMPs, NLRPs can sense either PAMPs or danger associated molecular patterns (DAMPs) (129). The only receptor of this class known to recognize GBS is NLRP3, which is critical for the induction of IL-1ß production via activation of inflammasome signaling (130). In numerous studies, NLRP3 has been shown to recognize infection via the GBS hemolysin, which is an ornithine rhamnolipid toxin (78, 130, 131). Indeed, it was suggested that the hemolysin serves as a DAMP that can induce membrane permeability and K+ efflux, triggering activation of the NLRP3 inflammasome, culminating in caspase-1 activation and IL-1ß release (130). NLRP3 sensing was shown to be critical in systemic infection as well as bone marrow derived dendritic cell and neutrophil responses towards GBS (130, 131). Interestingly, similar studies have shown that GBS RNA can also trigger NLRP3 activation, suggesting redundant mechanisms of sensing infection (78).

Siglecs are a family of cell surface receptors that recognize sialic residues on various glycans, which are traditionally found on host cells (132, 133). While Siglecs are typically found on the surface of various immune cells, these receptors are also expressed in the female

reproductive tract (134-136). Unlike TLRs and NLRs, Siglecs can be broken down into activating and inhibiting receptors, and function by tuning up or down immune responses in the presence of inflammation (132, 133). For example, natural killer cells express various Siglec receptors on the host cell surface, such as Siglec 7, which can dampen natural killer cell-dependent killing of host cells (137). Similarly, blockage of Siglec 9 signaling has been shown to enhance NK cell mediated killing of HIV infected cells (138). While Siglecs typically do not directly recognize pathogens, they can recognize GBS (139). GBS, like some other pathogenic members of the *Streptococcus* genus, have evolved a capsular polysaccharide with a terminal sialic acid residue, which mimics host cell residues (140). This molecular mimicry enables Siglecs to directly bind to GBS (139); Siglecs 5, 6, 7, 8, 9, 11, and 14 have been shown to directly bind to GBS (134). This interaction, however, is dependent not only on terminal sialic acid residues, but also on the level of capsular glycosylation (139). Due to the diverse nature of GBS capsular polysaccharides, it is not surprising that this interaction is dependent on capsule type. Several of these Siglecs can dampen the immune response to GBS, enabling evasion of the host immune response (140, 141). Siglec 9 dampens neutrophil activation and promotes GBS survival (141), and interaction with neutrophil localized Siglec 9 decreases neutrophil oxidative burst and extracellular trap formation (140).

Siglec receptors can have opposing roles in inflammatory responses; Siglec 14 and Siglec 5, for example, have contradictory roles during GBS infections (142). While Siglec 5 binding dampens neutrophil responses and NLRP3 activation, Siglec 14 increases NLRP3 signaling (127, 143). This signaling is still dependent on expression of hemolysin, though Siglecs 14 and 5 can tune the level of NLRP3 activation to allow for enhanced pathogen survival or induction of a more potent inflammatory response (127, 143). These paired receptors, however,

are polymorphic in humans, with some individuals displaying Siglec 14 “null” phenotypes. Cells expressing Siglec 14 “null” alleles display lowered immune responses to GBS infection and increased GBS proliferation (142). Interestingly, GBS positive individuals displaying Siglec 14 “null” genotypes had an increased risk of preterm birth relative to individuals with a wildtype Siglec 14 allele in a prior study (142). This finding suggests this Siglec5/14 axis is a critical determinant of GBS associated disease (142).

Cytokine Responses to Group B Streptococcus

Once engaged, pattern recognition receptors induce the production of potent cytokines in response to GBS (102, 126), which results in an immune response capable of clearing the infection. The cytokine response elicited towards GBS fall into four functional categories: chemokines, proinflammatory cytokines, anti-inflammatory cytokines, and pyrogens.

Chemokines are a class of cytokines whose primary function is to recruit immune cells to the site of infection (144). GBS induces the production of several chemokines, oftentimes with target cell redundancy. When tissues become infected with GBS, it is common to see an influx of leukocytes into infected tissues (58). Neutrophils, which are normally restricted to the bloodstream in healthy patients, are dramatically increased in GBS infected tissues (14, 58). When tissues become infected with GBS, both immune and non-immune cells release high levels of neutrophil-associated chemokines including IL-8, CXCL1, and CCL3 as well as the monocyte chemokine CCL8 (102, 145-149). Many chemokines with varying host cell affinities are also produced in response to GBS. One example is CXCL9, which can recruit cytotoxic lymphocytes, natural killer (NK) cells, natural killer T (NKT) cells, and monocytes to the site of infection (102). Similarly, CCL5 and CCL8 can induce the recruitment of T-cells, mast cells, monocytes,

NK cells, and dendritic cells to the site (102, 150). While CCL3 is important for neutrophil chemotaxis, it can also recruit macrophages and monocytes into infected tissues (145, 148). This target cell redundancy, whereby the immune system recruits the same cell type using multiple chemokine signaling pathways, ensures that a potent immune response is stimulated in response to GBS.

The second and third groups of cytokines induced in response to GBS have opposing functions. Proinflammatory cytokines, for instance, primarily act by activating cytokine production in target cells or by directly activating a target immune cell function (151). Comparatively, anti-inflammatory cytokines dampen the response by shutting down cytokine production or preventing cellular activation to avoid an overwhelming immune response (152). Several "activating" cytokines are also upregulated in response to GBS; these include IL-12, IL-18, and IFN-y (149, 153-156), which function in concert to promote macrophage activation. Depletion of IL-12 or IL-18 during GBS challenge reduces IFN-y production and promotes host demise (155). This indicates that IL-12 and IL-18 serve to promote IFN-y production. Subsequently, IFNy in concert with TLR stimulation can then promote macrophage activation (157). It is likely that IL-12 and IL-18 act primarily by promoting IFN-y production by helper T-cells, which are known to be critical sources of IFN-y during GBS infection (156). Conversely, only one anti-inflammatory cytokine, IL-10, has been shown to be produced in response to GBS. IL-10 predominately functions by altering STAT3 signaling, which limits proinflammatory cytokine and chemokine expression (102, 149, 152, 158). It is also known as cytokine synthesis inhibitory factor, and likely acts by preventing excessive inflammation that can lead to adverse outcomes for the host (159).

The final group of cytokines that are induced in response to GBS are the pyrogens, which are critical for the induction of fever during infection (160). IL-6, TNF-a, and IL-1ß have been shown repeatedly to be induced in response to GBS, and are critical for an effective host response (102, 115, 146, 149, 153, 161). While all three cytokines function as pyrogens, they also have additional functions including regulation of cellular proliferation, apoptosis, and the acute phase response (162-164). In particularly, IL-1ß also functions in immune circuits to affect production of other cytokines and effector molecules such as CXCL1 (147). Additionally, IL-1ß has been shown to play a critical role during GBS infections, with knockout mice displaying increased mortality due to an increased bacterial burden (147). Understanding how GBS elicits the production of this cytokine is currently a critical question within the field.

Potent induction of IL-1ß requires two conserved signals (128). The first "priming signal" is in the form of a PAMP that activates TLR or NLR signaling, culminating in proinflammatory gene expression, typically via the transcription factor NF-kB (117, 165). This results in the production of pro-IL-1ß, which is stored in the cytoplasm in an inactive form (165). The second signal, which is typically a DAMP or PAMP, will then activate the nucleotide-binding oligomerization domain, leucine rich repeat and pyrin (NLRP) domain containing receptors, which then oligomerize and trigger the formation of the inflammasome (166-169). The inflammasome cleaves the protease pro-caspase-1 into its mature, active form (168, 169). Mature caspase-1 can then rapidly cleave pro-IL-1ß into its active form, which is then secreted into the extracellular space (170). Both GBS associated RNA and the ß-hemolytic pigment have been shown to activate the induction of IL-1ß, suggesting these are critical PAMPs and DAMPs; however, only NLRP3 has been directly shown to sense these signals indicating that other NLRPs likely do not contribute to IL-1ß production (78, 114, 130). Interestingly, we have shown

strain variation in the ability to activate this system, with ST-17 strains inducing a more potent IL-1ß response than ST-12 strains, suggesting that these PAMPs or DAMPs may be variably present across isolates (102). Alternatively, this could suggest that strains possess other factors that modulate NLRP3 inflammasome activation.

Host Response Diversity

A growing number of studies have shown that while GBS infections generally induce the production of the cytokines mentioned above, the level of cytokine production has some variability between infections (102, 171, 172). Several factors can contribute to this varied host response including host variability and strain variation. Host variation in Siglec 14 has been directly shown to impact the production of IL-1ß via modulation of the NLRP3 inflammasome (127, 142). Patients with a defective Siglec 14 display decreased cytokine responses to GBS, increased bacterial survival, and increased risk of preterm birth in GBS positive women (142). Similarly, neonates are particularly susceptible to infection due to their immature immune state (173). Neonatal phagocytes have decreased cytokine responses, decreased ability to form NETs, and altered phagocyte functionality, which likely contributes to their increased risk of infection (173-175).

Our group and others have similarly demonstrated that strain variation is a critical determinant of cytokine responses. In particular, ST-17 strains elicit strong proinflammatory immune responses and have been shown to elicit more potent IL-1ß, IL-6, IL-10, CCL8, CXCL9, and CCL5 responses compared to other lineages (102, 176). Conversely, ST-12 strains generally stimulate lower immune responses relative to other lineages, suggesting that this facilitates asymptomatic colonization (102). While the CPS has been shown to be an important

modulator of these divergent inflammatory responses, it remains unclear whether other factors may also play a role (177).

GROUP B STREPTOCOCCAL MEMBRANE VESICLES

Membrane vesicles are spherical, lipid bilayers that originate from the bacterial membrane. First identified as structures originating from the outer membrane of Gram-negative bacterial species, they were originally termed outer membrane vesicles. Preliminary studies demonstrated that outer membrane vesicles contain active virulence factors that can promote infection (178-181). They have also been shown to be proinflammatory and in some species a promising vaccine candidate (182-184).

In recent years a growing body of evidence has suggested that Gram positive bacteria can also produce membrane vesicles (MVs) (185). MVs are critical to the virulence of many clinically relevant pathogens including *Staphylococcus aureus, Bacillus anthracis,* and *Listeria monocytogenes* (186-191). These MVs have varied functions including toxin delivery, cell death induction, and the bacterial response to antibiotics (189-193). In many cases these MVs also elicit a potent inflammatory response making them a promising vaccine target (194, 195).

Several studies have attempted to characterize the underlying mechanisms driving MV production in Gram positives (185, 196). While some believe their production could simply be an artifact of division, growing evidence suggests that alternative processes also contribute to their formation (186). Studies in *Bacillus anthracis* demonstrated that MVs can be formed during by the induction of phage holin and endolysin expression (197). While clearly largescale induction of lysogenic phage activation would have detrimental effects on the bacterial population, inducing a small level of phage activation within a bacterial population allows for

MV production, while still allowing for survival (197). Further, in Group A *Streptococcus* the CsrRS two component system has been shown to regulate MV production, however, the exact mechanism underlying this regulation is unclear (198). Yet other studies have also shown that many additionally factors can regulate MV biogenesis including antibiotic exposure, osmotic stress, and pH stress (199, 200). Taken together these data suggest that MV biogenesis is likely a varied mechanism across Gram positive species, with multiple redundant biogenesis mechanisms occurring within a single species. Interestingly, although we don't know the exact mechanism of their biogenesis, MVs are increasingly being identified in many Gram positive species (201).

GBS produces MVs that can induce adverse pregnancy outcomes in murine models of infection (112, 113). When injected into the amniotic cavity MVs are capable of inducing both fetal death and preterm birth (113). Similarly, models of neonatal infection have demonstrated that MVs can promote neonatal death (112). In both studies, researchers noted that adverse outcomes were accompanied by a high degree of inflammatory infiltrate or cytokine production, indicating that MVs may induce their effects in part due to the induction of inflammatory responses (112, 113). Challenge with GBS MVs is characterized by a high degree of neutrophilic and lymphocytic infiltrate, the induction of the neutrophil chemokine KC, and membrane weakening (112, 113). Additionally, evidence suggests that these MVs may be able to ascend into the uterus of mice, suggesting a potential role in shaping ascending infections.

MVs appear to induce some direct effects as well, in part due to their composition. Current evidence suggests that MVs from some isolates display hemolytic activity and some ability to weaken tissue integrity (112, 113). Studies to determine how MVs promote these functions have found that MVs can in some cases contain ß-hemolysin and some proteins (112, 113). Only 10 proteins have been shown to be contained within MVs, most of which do not give

insight into the reasons MVs elicit these responses (113). The GBS hyaluronidase, which can dampen immune responses to GBS, was found in GBS MVs; however, its functionality in MVs is unknown (72, 113). While interesting this is likely a gross underestimate of the total number of MV associated proteins. Additionally, it is unclear if these properties are widespread across clinical isolates since both studies to date have used a singular type-strain of GBS.

CURRENT CHALLENGES AND GAPS IN KNOWLEDGE

Despite major progress in reducing the burden of neonatal GBS infections, less has been done to decrease the burden of GBS-associated *in utero* infections and preterm birth, which represents a major public health challenge (6). Although we are still attempting to determine the exact burden of these infections, we do understand the severe consequences (14, 17). Understanding how GBS can induce these adverse pregnancy outcomes is therefore of the utmost importance.

GBS MVs from one clinical strain have been implicated as a contributor of preterm birth, fetal demise in murine models of intra-amniotic injection (113). Similarly, MVs have been shown to promote neonatal mortality during systemic neonatal challenge (112). A growing body of evidence suggests that these effects are due to both the composition and inflammatory response elicited towards GBS MVs. While the virulence factor hyaluronidase (HylB) is present within GBS MVs, the protein composition of MVs has been limited to 10 total peptides, most of which have no characterized function in GBS (113). Additionally, only one type-strain of GBS, A909, has been shown to produce MVs (112, 113). It remains unclear whether MVs are produced by other clinical isolates. Furthermore, a comprehensive, unbiased analysis of GBS MV protein composition is warranted to gain insight into the potential function of GBS MVs.

Additionally, a comparative proteomics approach will allow us to determine the factors driving MV composition. By using a comparative proteomics approach, we will also be able to identify proteins conserved across strains, which could serve as potential biomarkers or therapeutic targets.

Although MVs may elicit their effects by inducing inflammation, there is limited data about how host cells sense and respond to GBS MVs. Neutrophilic and lymphocytic infiltrate is seen during *in utero* and neonatal challenge (112, 113). It appears that the neutrophil chemokine KC, known as CXCL10 in humans, is partially responsible for this neutrophil influx as it is upregulated in the choriodecidua of infected mice (112). Investigators are now attempting to use immunotherapy to prevent infection-mediated preterm birth; therefore, understanding the host response to GBS MVs can aide in the development of immunotherapeutics (98). By assessing the host response to GBS MVs from diverse lineages we can further identify which responses are universal, while also identifying whether strain specific differences in the host response to MVs persist. Additionally, by elucidating upstream sensors of GBS, we can hopefully identify receptor antagonists to prevent inflammatory signaling, thereby preventing the subsequent inflammatory mediated complications.

Although current evidence suggests that MVs play a role during *in utero* infections, no study to date has examined the role of GBS derived MVs during ascending infection. MVs are known to contain hyaluronidase, which can contribute to ascending infection (56, 113). Additionally, their tissue weakening properties in theory could aid in tissue weakening and bacterial dissemination, suggesting they could promote ascending infection (113). While some evidence exists suggesting that MVs can ascend past the cervix, these mice were treated vaginally with detergents prior to challenge, which could confound conclusions in this study

(113). Furthermore, by injecting MVs directly into the amniotic infection, several barriers to infection are bypassed, which could limit MV mediated effects; therefore, it is unclear whether vaginally administered MVs elicit the same effects (113).

This dissertation aims to address three main questions that persist within the GBS field relating to MVs. 1) What is the protein composition of GBS MVs, and does that composition vary across isolates? 2) What pattern recognition receptors are engaged by MVs, and what cytokines are released in response to MVs? 3) Do GBS MVs promote ascending infection? Data from this dissertation will not only greatly expand our knowledge of GBS MVs, but also inform future studies into their functions. Our studies will also identify therapeutic and diagnostic markers of MVs that can inform studies aimed at preventing MV mediated complications.

In chapter 2 we aimed to determine how GBS strain diversity impacts both the production and protein composition of MVs. We hypothesized that both production and composition would correlate with specific phylogenetic lineages. In chapter 3 we aimed to characterize the host response to GBS derived MVs, similarly using diverse clinical isolates. We hypothesized that GBS MVs would induce a proinflammatory immune response from human macrophages. We further hypothesized that specific host receptors would recognize GBS and contribute to this proinflammatory immune response. Lastly, in chapter 4 we aimed to characterize the role of GBS MVs in shaping ascending infection by using an *in vivo* model of *in utero* infection. We hypothesized that similarly to previous studies, MVs would promote adverse pregnancy outcomes and promote ascending infection.

FIGURES

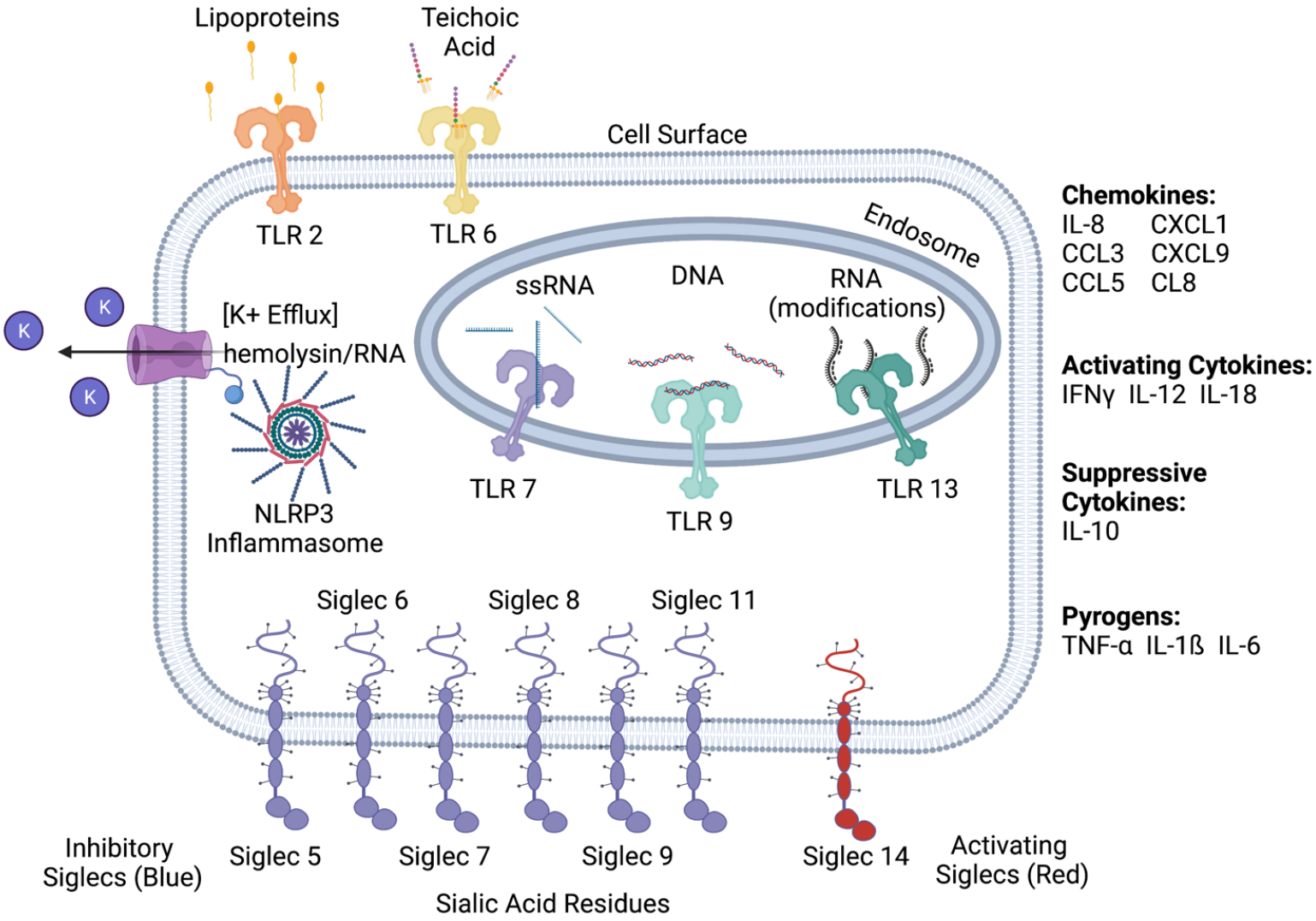

Figure 1.1 Summary of the host response to GBS

Figure 1.1 (cont'd)

A variety of receptors are known to bind GBS, including Toll like receptors (TLRs), Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin Domains (NLRPs), and Sialic-acid-binding immunoglobulin-like lectins (Siglecs). These receptors have a variety of ligands, all of which recognize features of a microbial infection (including RNA modfications, cell wall components, and polysaccharide modifications). These receptors, notably NLRPs, are also able to sense cellular damage signals, which is often associated with infection. While several of these receptors are surface associated, certain receptors can be cytosolic or endosomally localized allowing for recognition of intracellular infections. Engagement of these receptors can induce the production of various effectors, such as chemokines, activating/suppressive cytokines, or pyrogens, which are oftentimes induced by redundant receptors. By having a plethora of receptors that are able to recognize GBS, the host can ensure an appropriate response is formed in response to infection

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CHAPTER 2

Production and Composition of Group B Streptococcal Membrane Vesicles Vary Across Diverse Lineages

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ABSTRACT

Although the neonatal and fetal pathogen Group B *Streptococcus* (GBS) asymptomatically colonizes the vaginal tract of ~30% of pregnant women, only a fraction of their offspring develops invasive disease. We and others have postulated that these dimorphic clinical phenotypes are driven by strain variability; however, the bacterial factors that promote these divergent clinical phenotypes remain unclear. It was previously shown that GBS produces membrane vesicles (MVs) that contain active virulence factors capable of inducing adverse pregnancy outcomes. Because the relationship between strain variation and vesicle composition or production is unknown, we sought to quantify MV production and examine the protein composition, using label-free proteomics on MVs produced by diverse clinical GBS strains representing three phylogenetically distinct lineages. We found that MV production varied across strains, with certain strains displaying nearly two-fold increases in production relative to others. Hierarchical clustering and principal component analysis of the proteomes revealed that MV composition is lineage-dependent but independent of clinical phenotype. Multiple proteins that contribute to virulence or immunomodulation, including hyaluronidase, C5a peptidase, and sialidases, were differentially abundant in MVs, and were partially responsible for this divergence. Together, these data indicate that production and composition of GBS MVs vary in a strain-dependent manner, suggesting that MVs have lineage-specific functions relating to virulence. Such differences may contribute to variation in clinical phenotypes observed among individuals infected with GBS strains representing distinct lineages.

INTRODUCTION

Group B *Streptococcus* (GBS) is an opportunistic pathogen that asymptomatically colonizes ~30% of women either vaginally or rectally (1). In individuals with a compromised or altered immune state, including pregnant women, neonates, the elderly, and people living with diabetes mellitus, GBS can cause severe infections (1). Presentation of disease is variable between individuals: in elderly patients and neonates, GBS infection typically presents as septicemia, whereas in pregnant women it more commonly causes chorioamnionitis, preterm birth, or stillbirth (2, 3).

Despite the high prevalence of GBS colonization during pregnancy, only a fraction of babies born to colonized mothers develop an infection. In the United States pregnant individuals colonized with GBS are given antibiotics to reduce the risk of neonatal GBS infection, but even without such prophylaxis most neonates born to GBS-colonized mothers remain infection-free (4). The factors that determine whether a neonate develops GBS sepsis or not are incompletely understood, but evidence implicates bacterial strain variation as a key factor. For example, certain polysaccharide capsular serotypes of GBS are much more common at causing perinatal infections than others (5).

Application of multilocus sequence typing (MLST) has also demonstrated that GBS isolates comprise multiple sequence types (STs) that are differentially correlated with disease outcomes (6). While ST-12 strains have been associated with asymptomatic colonization (7), ST-1 and ST-17 strains have been linked to invasive disease in adults and neonates, respectively (6, 8-10). Moreover, our group has previously shown that different STs interact variably with host cells. ST-17 strains, for instance, had an enhanced ability to attach to gestational tissues, elicited stronger proinflammatory responses, and could persist longer inside macrophages than other STs

(11-13). Conversely, ST-12 strains were found to display increased tolerance to ampicillin relative to ST-17 strains (13), highlighting the divergence of these lineages and variation in the ability to withstand different stressors. The mechanisms underlying these strain-dependent differences, however, are poorly understood.

Many bacteria produce membrane vesicles (MVs) of varying sizes (20-500 nm) containing toxins and other virulence factors that can modulate immune responses and influence pathogenesis (14). In addition, GBS can produce MVs that have been implicated in driving infection risk, though this remains an area in need of more research (15, 16). While the exact role of GBS MVs in pathogenesis is not clear, intra-amniotic injection of GBS MVs produced by an invasive ST-7 strain induced preterm birth and intrauterine fetal death in mice (15). GBS MVs were also found to contain active virulence factors that could weaken murine gestational membranes, stimulate immune cell recruitment, and lyse host cells (15, 16). Hence, an important, unanswered question is whether MVs derived from strains belonging to distinct phylogenetic lineages and clinical sources vary in composition and pathogenic potential.

In this study, we sought to compare the quantity and protein composition of MVs produced by genetically distinct GBS strains and evaluate the relationships between proteomic profiles, strain characteristics, and clinical presentation. To accomplish these goals, we isolated MVs from six clinical strains representing three phylogenetic lineages (ST-1, ST-12, and ST-17), and used label-free proteomics to define the protein composition. Using this approach, we report that MV production and composition varies in a strain and ST-dependent manner, highlighting the importance of strain diversity on pathogenic potential.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and growth curves

Six GBS strains, GB00020, GB00037, GB00112, GB00411, GB00653, and GB01455, were isolated as described (17, 18); the strain names have been abbreviated for clarity. The six strains were selected for inclusion based on the isolation source and epidemiological data as well as the ST and capsular (cps) serotype designations. Three strains, GB37, GB411, and GB1455, were classified as "invasive" because they were isolated from septic neonates with early onset GBS disease or were cultured from a stillbirth (GB1455) (17). The three "colonizing" isolates, GB20, GB112, and GB653, were isolated from vaginal/rectal swabs from mothers before or after childbirth (18). All six strains were previously characterized by MLST and cps typing (7, 9) and represent the following common ST/serotype combinations: ST-1, cpsV (GB20, GB37), ST-12, cpsII (GB653, GB1455), and ST-17, cpsIII (GB112, GB411). One colonizing and one invasive strain were included in each of the ST/cps groups.

Because we had no prior knowledge of MV production across lineages, these strains were selected based on molecular data as well as epidemiological and clinical associations described previously. The ST-17 strains, for instance, have consistently been associated with invasive neonatal disease (6, 7, 10) and were more likely to persist in mothers following childbirth and intrapartum antibiotic prophylaxis (IAP) (7). ST-12 strains, however, were more common during pregnancy and more readily lost following IAP (7, 9). Although ST-1 strains have been linked to invasive disease in adults (8), they were more commonly recovered from women during pregnancy than neonates in our studies (7, 9). It is also important to note that the ST-1 neonatal GB37 strain has unique traits in that it is non-pigmented and non-hemolytic (19). This wide range of strains

with varying characteristics and epidemiological associations was chosen to maximize our ability to detect differences in MV production across strains.

Strains were cultured using Todd-Hewitt Broth (THB) or Todd-Hewitt Agar (THA) (BD Diagnostics, Franklin Lakes, New Jersey, USA) overnight at 37°C with 5% CO_2. For enumeration of colony forming units (CFUs), bacteria were serially diluted in Phosphate Buffered Saline (PBS) and plated onto THA. Plates with 20-200 colonies were counted and the number of colonies per mL was determined. Growth curves were performed by diluting overnight THB cultures 1:50 into fresh THB. Cultures were grown for six hours with OD_{600} measurements taken hourly. Growth curves were performed in triplicate for each isolate.

Membrane vesicle (MV) isolation and purification

The isolation and purification of MVs was performed as described (15, 20-22), with some modifications. Briefly, overnight THB cultures were diluted 1:50 into fresh broth and grown to late logarithmic phase (optical density at 600 nm, $OD_{600} = 0.9 \pm 0.05$). Aliquots of culture were serially diluted and plated on THA for bacterial enumeration. Cultures were centrifuged at 2000 x g for 20 minutes at 4°C. Supernatants were collected and re-centrifuged at 8500 x g for 15 minutes at 4°C, followed by filtration through a 0.22 μm filter and concentration using Amicon Ultra-15 centrifugal filters (10k Da cutoff) (Millipore Sigma, Burlington, MA, USA). Concentrated supernatants were subjected to ultracentrifugation for 2 hours at 150,000 x g at 4°C. For quantification, pellets were washed by resuspending in PBS, re-pelleting at 150,000 x g at 4°C, and resuspending in PBS; pellets were stored at -80°C until usage.

For proteomics, pellets were resuspended in PBS and purified using qEV Single size exclusion columns (IZON Science, Christchurch, New Zealand) per the manufacturer's instructions. MV fractions were collected and re-concentrated using the Amicon Ultra-4 centrifugal filters (10 kDa cutoff) (MilliporeSigma, Burlington, Massachusetts, USA) and brought to a final volume of 100 μL in PBS. To preserve the integrity of vesicle proteins, ProBlock Gold Bacterial Protease Inhibitor Cocktail (GoldBio, St. Louis, Missouri, USA) was added. MVs were stored at -80°C until usage.

Electron microscopy

To visualize GBS and the MVs associated with each strain, scanning electron microscopy (SEM) was performed on bacterial cultures grown to stationary phase in THB. Culture aliquots were fixed in equal volumes of 4% glutaraldehyde in 0.1 M phosphate buffered saline (pH 7.4), placed on poly-L-lysine coated 12 mm coverslips, and incubated for 5 minutes. The coverslips were washed with water and dehydrated through increasing concentrations of ethanol (25%, 50%, 75%, 95%) for five minutes in each followed by three 5-minute changes in 100% ethanol. Samples were dried in a Leica Microsystems (model EM CPD300) critical point drier using liquid carbon dioxide as the transitional field. Lastly, samples were mounted on aluminum stubs using epoxy glue (System Three Quick Cure 5, System Three Resins, Inc, Lacey, Washington, USA) and coated with osmium (~10 mm thickness) using a NEOC-AT osmium coater (Meiwafosis Co., Ltd, Tokyo, Japan). Imaging was performed using a JEOL 7500F scanning electron microscope.

To evaluate MV morphology and purity without contaminating extracellular components, transmission electron microscopy (TEM) was performed on purified vesicles as described (21). MVs were fixed in 4% paraformaldehyde, loaded onto formvar-carbon coated grids, and

counterstained with 2.5% glutaraldehyde and 0.1% uranyl acetate in PBS. Samples were imaged using a JEOL 1400 Flash transmission electron microscope. During proteomics experiments, preparations with high concentration of MVs and minimal extravesicular contamination were included for downstream analyses. Each proteomics preparation was imaged with TEM prior to analysis to confirm the presence of spherical MVs.

Quantification of vesicle production

Nanoparticle tracking analysis was performed to quantify MVs produced by each strain (n=8-9 replicates per strain) using a NanoSight NS300 (Malvern Panalytical Westborough, MA, USA) equipped with an automated syringe sampler as described previously (21, 23). For each sample, MVs were diluted in phosphate buffered saline (1:100 – 1:1000) and injected with a flow rate of 50. Once loaded, five 20-second videos were recorded at a screen gain of 1 and camera level of 13. After capture, videos were analyzed at a screen gain of 10 and a detection threshold of 4 and data were subsequently exported to a CSV file for analysis using the R package tidyNano (accessed via: https://nguyens7.github.io/tidyNano) (23). Total MV counts were normalized by dividing by the colony forming units (CFUs) of each original bacterial culture following growth to an OD_{600} of 0.9 ± 0.05. Differences in MV quantities were assessed using the Kruskal Wallis test followed by a *posthoc* Dunn's Test with a Benjamini-Hochberg correction. Outliers were identified by multiplying the interquartile range by 1.5, which was used to extend the upper and lower quartiles.

Proteomics and genomics

Proteomic LC-MS/MS analysis of MVs was performed in duplicate or triplicate by the Proteomics Core at the Michigan State University Research Technology Support Facility (RTSF). Protein concentrations of purified MVs were determined using the Pierce Bicinchoninic Acid Assay (ThermoFisher Scientific, Waltham, Massachusetts) supplemented with 2% SDS in water to reduce the background signal from excess lipids contained within the vesicles. MVs (1.5 μg) were concentrated into a single band in a 4-20% Tris-Glycine SDS-PAGE gel (BioRad, Hercules, CA) that was fixed and stained using colloidal Coomassie blue (24).

Protein bands were excised from the gels and stored in 5% acetic acid at 4°C. Prior to analysis, in-gel trypsin digest and peptide extraction were performed. Briefly, gel bands were dehydrated twice using 100% acetonitrile and incubated with 10 mM dithiothreitol in 100 mM ammonium bicarbonate (pH~8.0) at 56ºC for 45 minutes. Bands were incubated in the dark with 50 mM iodoacetamide in 100 mM ammonium bicarbonate for 20 minutes followed by another dehydration. Sequencing grade modified trypsin (0.01 μg/uL in 50 mM ammonium bicarbonate) was added to each gel band and incubated at 37ºC overnight. Peptides extracted by bath sonication (in 60% acetonitrile, 1% trichloroacetic acid solution) were vacuum dried and re-suspended (in 2% acetonitrile/0.1% trifluoroacetic) prior to separation using a Thermo ACCLAIM C18 trapping column. Peptides were sprayed onto a ThermoFisher Q-Exactive HFX mass spectrometer for 90 minutes; the top 30 ions per survey were analyzed further using high energy induced dissociation. MS/MS spectra were converted into peak lists using Mascot Distiller v2.7.0 and searched against a SwissProt database containing all GBS sequences available through the National Center for Biotechnology Information (NCBI; accessed 2/08/2019). Contaminants were identified and removed using Mascot searching algorithm v2.7.0, while protein identities were validated using

Scaffold v4.11.1. Raw proteomic data was submitted to the MassIVE database and can be accessed via ftp://massive.ucsd.edu/MSV000087985/ or at https://doi.org/doi:10.25345/C5RC1H.

Whole-genome sequencing was performed previously on GB00020 (25) and GB00037 (19). These genomes were examined more comprehensively to confirm the presence of specific genes found to be absent in the proteomics analysis. Raw reads were trimmed using Trimmomatic 0.39 (26) followed by an assessment of sequence quality using FastQC (Barbraham Bioinformatics). *De novo* genome assembly was performed on high-quality paired-end reads using SPAdes 3.13.1 (27). Assembly quality was assessed using QUAST 5.0.2. Protein sequences were downloaded from GenBank and aligned to assembled contigs using tblastn. Proteins with 90% identity or higher were considered present.

Data analysis

To compare MV proteins between strains, proteomic data from all strains were compiled and normalized for inter-experimental variability using Scaffold. Only proteins with a minimum of two identified peptides falling above a 1% false discovery rate and 95% protein threshold, were considered for downstream analysis. Proteins identified as contaminants (via the Mascot searching algorithm v 2.6.0) were removed, whereas proteins identified in both replicates for at least one strain were classified as MV-associated. Subcellular localization analysis was performed using pSORTdb (https://db.psort.org) with protein localization data for GBS strain 2603VR (downloaded from pSORTdb on 3/6/2021). Data visualization and statistical analyses were performed using R version 4.1.0 (https://www.R-project.org). Principle component analysis (PCA) was performed and visualized using the prcomp and fviz_pca functions, respectively. Hierarchical clustering was performed using the pheatmap function and clustered using Euclidean distances.

Shapiro tests were used to determine whether data followed a normal distribution and Student t-test (two-sided) or Kruskal-Wallis one-way analysis of variance (ANOVA), in combination with the Dunn's *posthoc* test, were utilized to test for differences between groups. Multiple hypothesis testing was corrected using Benjamini-Hochberg or Bonferroni correction when necessary.

RESULTS

MVs are produced by different GBS strains representing common STs.

Prior to MV isolation, each strain was monitored for growth, which did not differ significantly throughout the logarithmic phase (Figure S 2.1). Although a slight decrease in OD_{600} was observed for GB1455 in early stationary phase, all strains reached late logarithmic/early stationary phase at an OD_{600} of 0.9. In addition, all strains displayed a similar length of each growth phase, suggesting minimal differences in growth dynamics.

To determine whether each of the six GBS strains could produce MVs, we first used SEM to examine bacterial cultures grown overnight to stationary phase (Figure 2.1). Visualization using SEM revealed abundant production of MVs by all six strains and showed that some MVs were closely associated with bacterial cells as was described in prior studies (14). Because these overnight cultures likely contain cellular debris as well as MVs, further confirmation was necessary to rule out extra-vesicular contamination. To limit the possibility of detecting debris in the MV preparations, we grew each of the six strains to late logarithmic phase at an OD_{600} of 0.9 ± 0.05 prior to MV isolation and purification. Imaging by TEM revealed that MVs were produced by all six strains. On average, they ranged in diameter between ~50-100 nm and displayed a spherical morphology with a lipid bilayer and slightly electron dense interior (noted by arrows in

Figure 2.2). The MVs appeared similar to other bacterial-derived MVs described in the literature (14) and for GBS strain A909 (15).

MV production differs across GBS strains.

Because electron microscopy suggested differences in MV production, we used NanoSight analysis to quantify MV size and production. MVs from each of the six strains displayed a uniform size distribution, ranging between 100 and 200 nm (Figure 2.3A). Similar size distributions were also observed by ST. For MV quantification, total MV counts were normalized to the number of CFUs in the original bacterial cultures. Among the six strains, the average number of MVs/CFU was 0.108 with a range of 0.048-0.206 MVs/CFU; however, there was considerable variation between strains (Figure 2.3B). Although no difference in MV quantity was observed in colonizing versus invasive strains belonging to ST-1 or ST-17, the ST-1 strains produced significantly fewer MVs relative to the ST-17 strains (Figure S 2.2; $p < 0.0001$). While the colonizing ST-12 (cpsII) GB653 strain produced similar vesicle quantities as the two ST-17 (cpsIII) strains, the invasive ST-12 (cpsII) isolate, GB1455, produced significantly more MVs than all other strains examined ($p < 0.05$). By contrast, the colonizing ST-1 (cpsV) isolate, GB20, produced significantly fewer MVs compared to the strains representing all other STs ($p < 0.05$) except for the other ST-1 (GB37) strain ($p = 0.55$).

Variation in MV protein abundance and identification of a shared proteome.

Proteomics of purified MVs identified 643 total proteins among the six isolates with an average of 458 proteins per strain and range of 239-614 proteins per strain (Data not shown). Of note, the number of unique proteins varied by strain. MVs from ST-1 strains, for instance, had

fewer unique proteins relative to the other STs with an average of 281 proteins compared to 601 and 493 for the ST-12 and ST-17 strains, respectively. Regardless of ST, however, pSORTdb predicted numerous proteins to be membrane (12-17%) and cell wall (2-11%) localized, while 22-52% were predicted to be localized in the cytoplasm (Figure 2.4A). Although many proteins had a predicted subcellular localization, a large proportion of proteins had unidentified or unpredicted subcellular localization.

Among the total proteins detected, 62 were found in all biological replicates for the six strains (Data not shown). These proteins did not vary in spectral abundance between STs and represent the shared MV proteome. Of these 62 proteins, 11 were highly abundant with a mean spectral count greater than 50 (Data not shown). Putative, uncharacterized transporters constituted many of these shared proteins, accounting for 39-44% of membrane protein spectral counts. In addition, 19-25% of spectral counts were predicted to have a membrane associated subcellular localization (Figure 2.4B).

In other species, studies have demonstrated that MV composition can vary across strains, which could confer strain specific functionality (28, 29). Therefore, we sought to determine how many of these proteins were strain-specific or shared among the six strains examined. Of all 643 proteins detected, 192 (29.9%) were detected in at least one biological replicate for all six strains regardless of the clinical phenotype or ST (Figure 2.5). This analysis enhanced our certainty that a protein was present in a given strain, while permitting us to compare its abundance across strains even if it was not detected. Notably, 124 (19.3%) proteins were shared by the four ST-12 and ST-17 strains but were absent in the ST-1 strains, suggesting that the ST-1 MVs have a unique protein composition. To determine whether these compositional differences were due to genome divergence, analysis of whole-genome sequencing data revealed that 122 of the 124 corresponding

protein genes were present in the ST-1 genomes. Interestingly, the two proteins absent from these genomes were ARC24477.1 and ARC24478.1 encoding a CHAP-domain containing protein and an abortive phage resistance protein, respectively, both of which are located within a putative phage. Although a minor proportion of proteins were ST- or strain specific, none were shared by all invasive or all colonizing strains.

Compositional protein profiles differ across GBS MVs.

Given that differences in protein abundance were observed, we next considered the relationship between protein composition and strain characteristics. Rather than differential protein abundance analysis, we assessed whole proteome composition using PCA (Figure 2.6). This method takes into consideration the spectral abundance of all proteins simultaneously, giving a more thorough evaluation of population level changes in composition. In our analysis we found that the first two principal components accounted for a high proportion of the total variation (50.1%). Although the protein composition of MVs from invasive and colonizing strains overlapped, it was segregated by ST. Some overlap, however, was observed between the ST-12 confidence ellipse and those for other STs. No overlap was seen between the ST-1 and ST-17 strains, highlighting their distinct proteomes. This distinct clustering was not observed when the relationship between protein composition and clinical phenotype was analyzed (Figure S 2.3). Specifically, invasive and colonizing samples displayed a high degree of overlap with little to no separation of their respective confidence ellipses.

To confirm the PCA results, we then applied a hierarchical clustering algorithm to our dataset, which utilizes a different statistical assessment to evaluate the relationship between MV composition and various strain characteristics. Indeed, hierarchical clustering of the protein data

further demonstrated that MVs from strains belonging to the same ST had similar protein profiles, forming distinct clusters by ST regardless of the clinical phenotype (Figure 2.7). For instance, proteins from the ST-12 and ST-17 strains formed a distinct branch in the phylogeny that was separate from the ST-1 proteins, thereby indicating that their protein composition was more similar to each other than to ST-1 strains. This observation supports the PCA, showing a higher degree of overlap between ST-12 and ST-17 strains compared to ST-1 strains. Nonetheless, ST-12 and ST-17 strains were still distinguishable, with distinct nodes forming based on protein composition, indicating their divergent composition. This analysis provided additional confirmation that ST-1 strains lacked several proteins that were highly abundant in both the ST-12 and ST-17 strains. To a lesser degree than the ST-1 MVs, several highly abundant proteins found among the ST-17 strains were also absent in the ST-12 strains.

Differential abundance of key virulence factors in MVs from distinct GBS strains.

To determine which proteins contributed most to the segregation observed in the PCA as well as the hierarchical clustering analysis, we more thoroughly examined the 335 proteins that were significantly enriched in at least one ST (Data not shown). Notably, several purported virulence factors including the C5a peptidase, hyaluronidase, and sialidase were highly enriched in a ST-dependent manner (Figure 2.8). Both the hyaluronidase and C5a peptidase were significantly more abundant in the two ST-17 strains compared to the ST-1 and ST-12 strains, whereas the sialidase was detected at significantly higher levels in ST-1 versus ST-12 strains.

Several proteins of unknown function were also among the most highly abundant and differentially enriched proteins detected. One hypothetical protein, for instance, was significantly more abundant in the ST-1 strains relative to strains representing the other two lineages (Figure

2.8). Similarly, another hypothetical protein was more abundant in the ST-12 strains (Figure S 2.4); however, considerable variation was observed across replicates. Numerous phage-associated proteins including a holin and capsid protein, were also detected and found to be more abundant in the ST-17 strains along with several proteins associated with cell division (Figure S 2.4-2.5**)**. For example, the average abundance of cell division proteins FtsE, FtsQ, FtsZ,and FtsY, was significantly greater in the two ST-17 strains compared to those from other lineages. Differences in proteins linked to cell wall modification such as penicillin-binding proteins and capsule biosynthesis proteins, were also detected (Figure S 2.6).

DISCUSSION

Current knowledge regarding GBS derived MVs is restricted to one clinical strain (15, 16) and hence, we sought to examine MV production and composition in a set of clinical strains with different traits. While no clear association was observed between clinical phenotype and the production or composition of MVs, we have demonstrated that the GBS MV proteome is ST-dependent. The same was observed for MV production, though some variation was noted between strains of the same ST. Together, these data indicate that GBS MVs have strain-dependent functions that could impact survival in hosts, immunomodulation, and virulence.

This study expands our current knowledge of GBS MVs by highlighting their potential impact on virulence. Specifically, we demonstrated that GBS MVs have a high abundance of immunomodulatory virulence factors including C5a peptidase, hyaluronidase, and sialidase (30-32). The bifunctional C5a peptidase has been shown to interact with fibronectin and degrade the proinflammatory complement component (C5a) while simultaneously promoting bacterial invasion into host cells (30, 31). MVs from both ST-17 (cpsIII) strains examined herein contained high levels of C5a peptidase, whereas ST-1 and ST-12 strains lacked this protein. Intriguingly, ST-17 strains were previously shown to possess distinct virulence gene profiles as well as unique alleles of *scpB* encoding the C5a peptidase (33, 34), suggesting that ST-17 strains may be primed to cause invasive infections. This suggestion is in line with epidemiological data showing that ST-17 strains are important for invasive disease in adults and neonates (6, 8, 9) as well as mechanistic studies showing an enhanced ability to attach to gestational tissues, induce stronger proinflammatory responses, and persist inside macrophages (11-13). Nonetheless, it is important to note that our clinical definitions of “invasive” versus “colonizing” strain types may not be representative of each strain population. Although strains isolated from an active infection clearly

demonstrate “invasive” potential, it is possible that strains designated as “colonizing” could also cause an infection in specific circumstances and host environments.

Although sialidases have no known role in GBS pathogenesis (32), these proteins were shown to be immunomodulatory in other bacterial species (35, 36) while simultaneously promoting biofilm production and metabolism of host sugars (37, 38). The presence and abundance of sialidase was variable: the ST-1 and ST-17 MVs all contained sialidase, but the ST-12 MVs lacked it. In two prior studies examining GBS MVs produced by a ST-7 strain, A909, neither C5a peptidase nor sialidase were identified (15, 16), further highlighting differences across strains. However, we cannot rule out the possibility that the abundance of these virulence factors was beneath the detection limit in those studies. Similarly, the previous analysis of GBS MVs highlighted the importance of hyaluronidase (15). This immunomodulatory factor has previously been shown to promote ascending infection, degrade host extracellular matrix components, and dampen the host immune response (31). While we also found high levels of hyaluronidase in ST-17 MVs, our results further show that the ST-12 and ST-1 MVs contained significantly lower amounts of this protein. Additionally, the ST-1 strains lacked 124 proteins found in MVs from other lineages. Analysis of the ST-1 genomes detected the presence of the genes encoding 122 of these proteins, suggesting that lineage-specific composition is not due to genome divergence. Because we have previously shown that virulence gene expression in clinical isolates varies during infection of host cells (11), variation in gene expression profiles could drive MV compositional differences. Alternatively, in the absence of varied gene expression, it is possible that there is strain specific packaging of proteins within MVs. Further studies, however, are required to determine the mechanisms driving this altered composition.

It is also important to note that multiple uncharacterized and hypothetical proteins were detected. Previous reports have demonstrated that in Gram positive species, roughly 30-60% of all MV proteins map to the cytoplasm (39, 40). While our results are consistent with this observation showing ~22-52% of all proteins mapping to the cytoplasm, roughly 25-41% of the GBS MV proteins had an unidentifiable subcellular localization. Similar trends of ST-dependent enrichment of several hypothetical proteins were observed, with these representing some of the most highly abundant proteins. Although some uncharacterized proteins, such as those classified as putative ABC transporters, have predicted functions, their role in vesicle function or virulence is currently unknown. Future analyses must be undertaken to identify which proteins play a role in MV associated pathogenesis.

Through this study, we have also identified a shared proteome among MVs from phylogenetically distinct GBS strains. In total, 62 proteins were consistently found within GBS MVs regardless of the ST. Indeed, over 17% of these shared proteins were highly abundant, indicating that they may be important for MV functionality. Even though many of these proteins have yet to be characterized, we identified an abundance of transporter proteins in MVs suggesting a potential role in MV function. Consequently, some these shared proteins may be of value as potential MV markers in future studies.

While various mechanisms have been proposed for the biogenesis of Gram positive MVs, those mechanisms important for GBS MV biogenesis are unclear (14, 41). Our data demonstrate that diverse GBS strains produce MVs with consistent size distributions, indicating that GBS MV production is ubiquitous. Purported mechanisms of MV biogenesis in other pathogens include phage mediated biogenesis (42, 43), membrane budding during division (44), and cell wall remodeling (14, 45). Consistent with these mechanisms, our proteomics analysis revealed the

presence of phage associated proteins, division septum-associated proteins and cell wall-modifying enzymes. Several of these proteins were also differentially abundant, with some proteins being more highly enriched in certain STs than others. For instance, phage proteins were enriched in ST-17 strains but were nearly absent in ST-12 and ST-1 strains. Although we observed similar enrichment of cell division proteins in ST-12 and ST-17 strains relative to the ST-1 strains, cell wall modifying proteins were most abundant in the ST-17 strains. Taken together, these data indicate that MVs are produced by diverse strains with varying traits; however, it is possible that the mechanisms for MV biogenesis are strain dependent. Additional studies are needed to test this hypothesis.

Although our study has enhanced our understanding of the proteomic composition of GBS MVs, it has a few limitations. Because strains of each GBS lineage possess the same capsule (cps) type, it is difficult to differentiate between ST versus cps effects. Another concern when dealing with MVs is the presence of non-vesicular contaminants. In some eukaryotic and prokaryotic systems where the composition of MVs is well defined, markers are used to assess purity (46-48). Due to the relatively unknown composition of GBS MVs, however, we were unable to target specific markers to evaluate the purity. Rather, we relied on size exclusion chromatography followed by TEM to further remove non-vesicular proteins from each MV preparation. While we likely have some contaminant proteins, the purity of our preparations exceeds those performed in prior GBS studies (15, 16) and mimics protocols optimized for removing extravesicular macromolecules from Gram positive MVs (15, 16, 49, 50). Indeed, studies in *Staphylococcus aureus* and *Streptococcus mutans* have confirmed the presence of similar proportions of cytoplasmic and extracellular proteins within MVs (39, 40). The MV isolation method used herein

is standard for the field, however, it is important to note that the isolation is not complete, as a small proportion of MVs can remain associated with the bacterial surface post-isolation. Current protocols to isolate surface-associated MVs remain limited. Because our protocol was consistent across all production and proteomics experiments, the data could be directly compared across strains, thereby greatly enhancing our understanding of GBS MV composition. Although other macromolecules have also been detected within GBS MVs (15), it is not clear whether these macromolecules have a ST dependent composition and hence, further studies are warranted.

In summary, this analysis of GBS MVs from strains representing three phylogenetically distinct lineages demonstrates strain dependent composition and production of MVs. Our data further show that MVs carry known virulence factors as well as proteins of unknown function that vary in abundance between strains, suggesting they may have an altered functionality or ability to promote virulence. Follow up studies elucidating virulence and immunomodulatory properties of GBS MVs isolated from a larger and more diverse strain collection are therefore warranted, particularly given the high level of variation in protein composition observed among only these six strains. Taken together, these findings further highlight the importance of strain variation in GBS pathogenesis and shed light on the potential role of MVs in virulence.

FIGURES

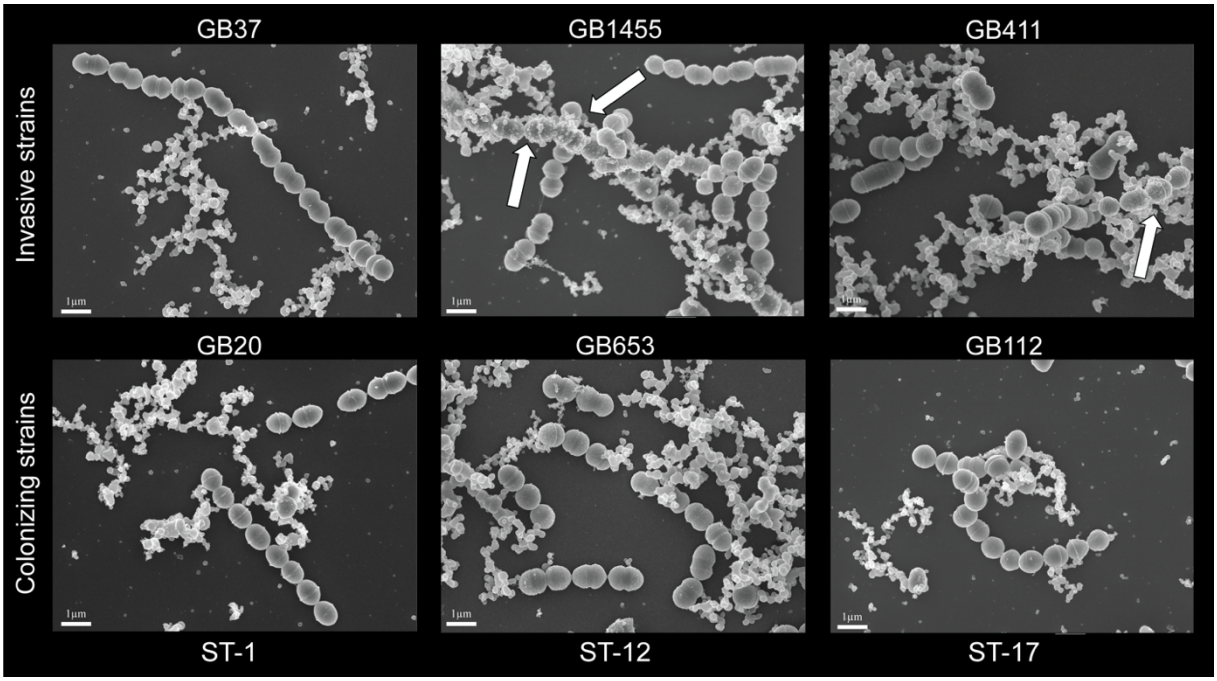

Figure 2.1: Scanning electron microscopy (SEM) of membrane vesicles (MVs) from overnight cultures.

Overnight cultures of six group B *Streptococcus* (GBS) strains were visualized by SEM at 10,000x magnification following growth to stationary phase. White arrows show MVs that are closely associated with bacterial cells. A minimum of 2 replicates per strain were examined and the SEM scale bars represent 1 μm length.

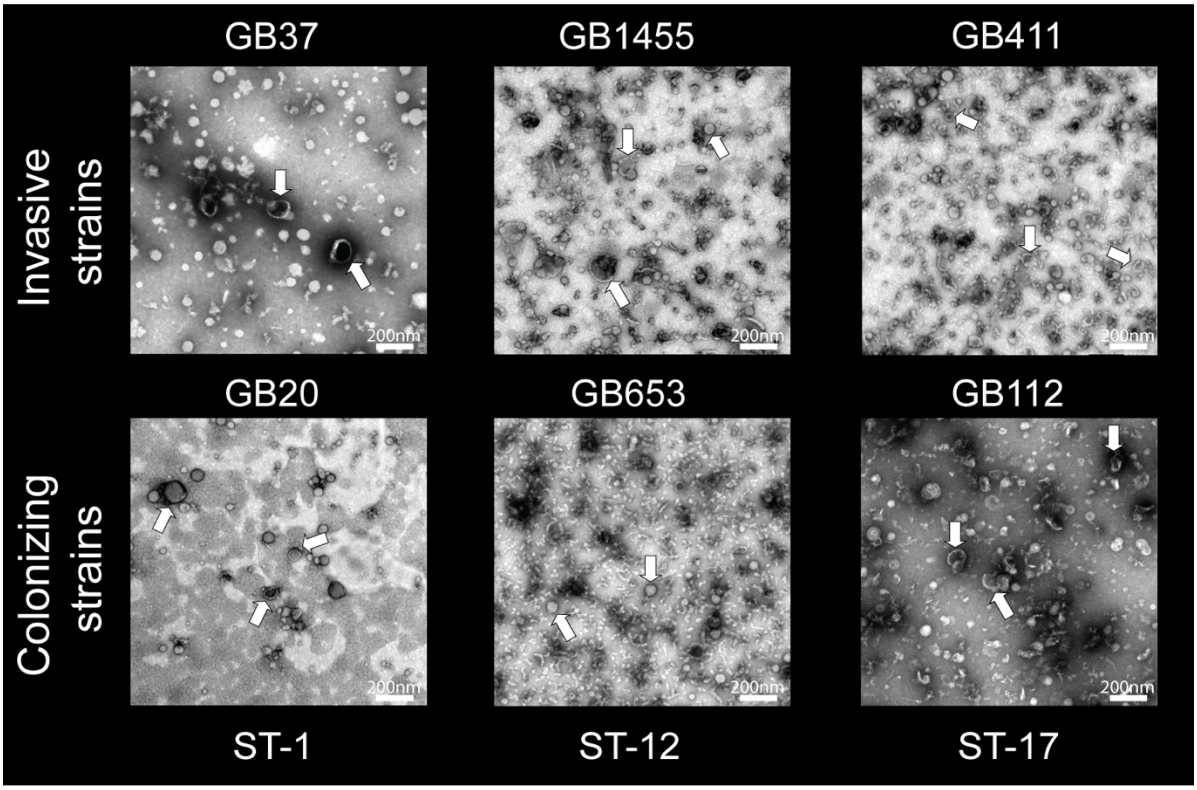

Figure 2.2: Transmission electron microscopy (TEM) of membrane vesicles (MVs) isolated from the six group B streptococcal strains.

An abundance of spherical structures with a bright membrane bilayer and slightly electron dense interior was observed ranging in size between ~50-100 nm. The MV population was isolated from each group B *Streptococcus* (GBS) strain following late logarithmic growth. MVs were purified using ultracentrifugation and size exclusion chromatography (2-3 replicates per strain). TEM images were taken at a magnification of 20,000x; the scale bars indicate a length of 200 nm.

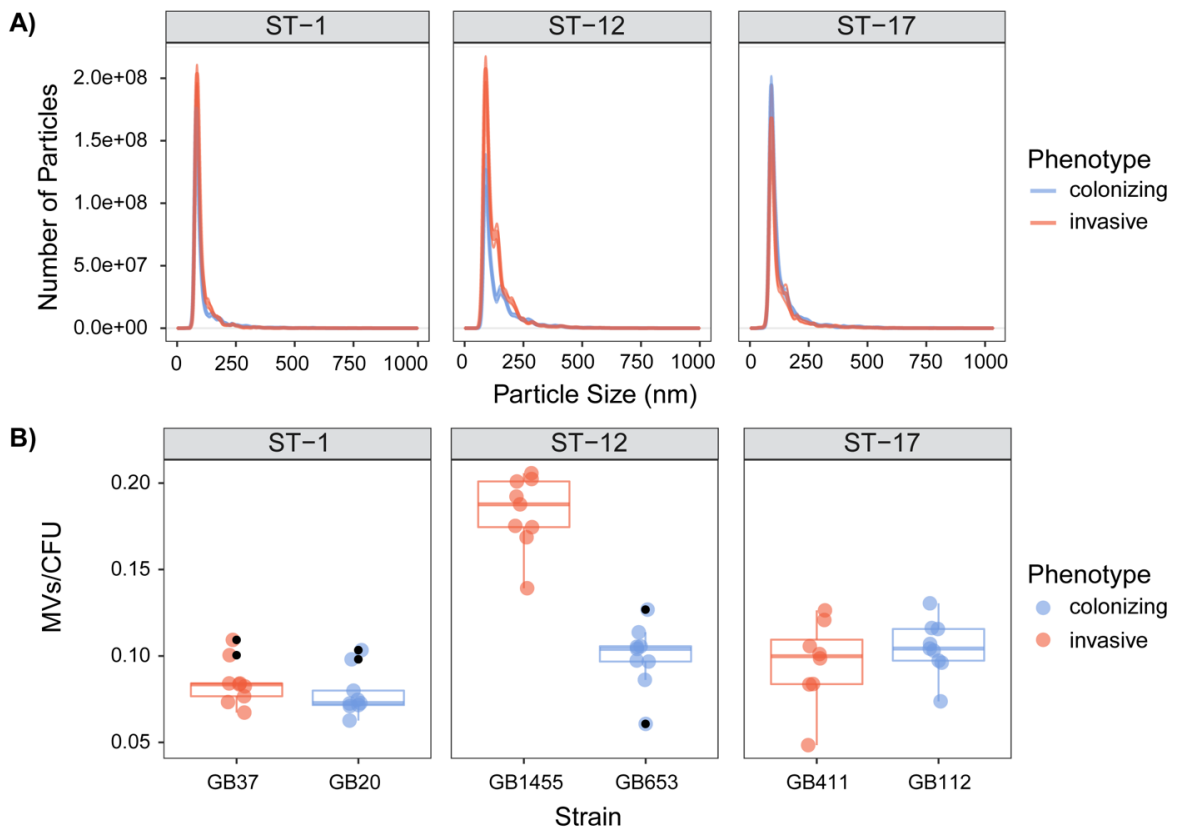

Figure 2.3: Quantitative assessment of membrane vesicle (MV) production across strains. MVs were isolated by differential centrifugation and quantified using NanoSight analysis. The vesicle A) size distribution and B) number per bacterial colony forming units (CFUs) are shown for the invasive and colonizing strains by sequence type (ST). Total MV counts were normalized to the number of CFUs in the original bacterial cultures. For panel B, the lines show the mean across 8-9 biological replicates (indicated by colored dots). Shaded regions surrounding the lines are the standard error of the mean and the black dots are outliers identified by multiplying the interquartile range by 1.5, which was used to extend the upper and lower quartiles. Outliers were observed for three of the six strains and were excluded prior to statistical analysis.

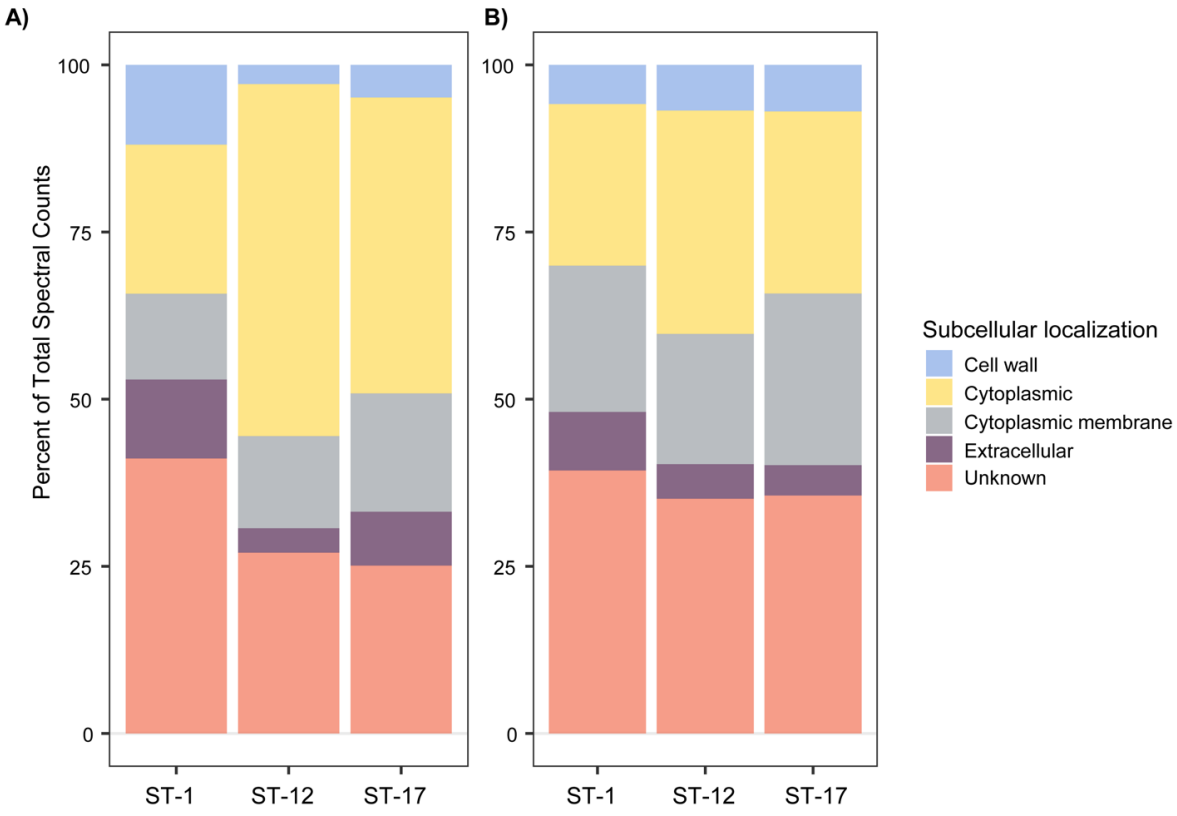

Figure 2.4: Subcellular localization analysis of membrane vesicle (MV) proteomes.

The subcellular localization of **A**) all 643 MV proteins identified, and **B**) a subset of 62 shared MV proteins identified using a pSORTdb database for published *Streptococcus agalactiae* sequences (accessed 3/3/21). Percentages were determined from mean spectral counts for a given sequence type (ST).

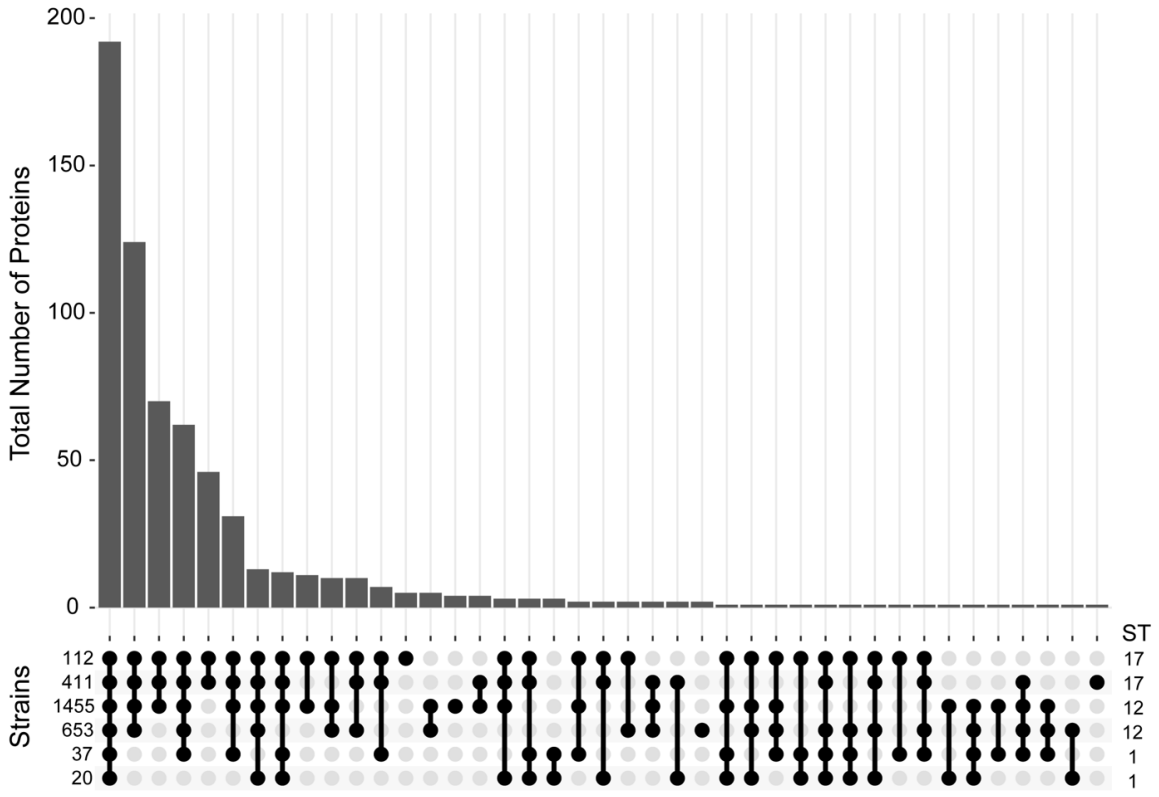

Figure 2.5: Distribution of proteins detected in membrane vesicles (MVs) among six strains.

An Upset plot was generated to show the distribution of all 643 proteins detected across the six GBS strains examined. The y-axis indicates the total number of proteins detected for a given set of strains. Protein presence is defined as having a non-zero spectral count for a given protein in at least one biological replicate for a specific strain. The matrix at the base of the plot shows the strains ordered vertically by sequence type (ST) with filled bubbles indicating which strains are positive for the number of proteins detected, and overlaid bars representing number of shared proteins.

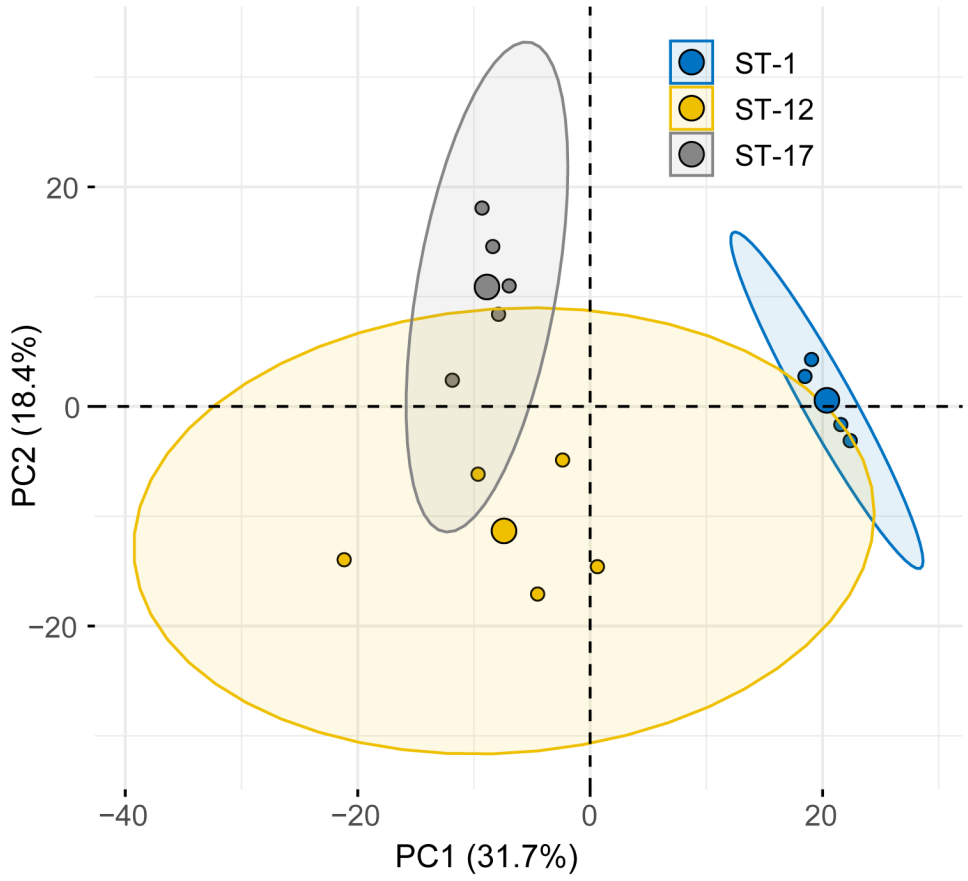

Figure 2.6: Principal component analysis (PCA) reveals lineage-specific clustering of membrane vesicle (MV) proteomes.

PCA of the MV proteomes produced by six strains stratified by sequence type (ST). The large central dot of each ellipse represents the mean point of the corresponding 95% confidence ellipse, while the smaller points represent individual proteomic samples. Confidence ellipses comprise 95% of the samples based on the underlying distribution. Axes percentages represent the amount of variation accounted for by each principal component (PC).

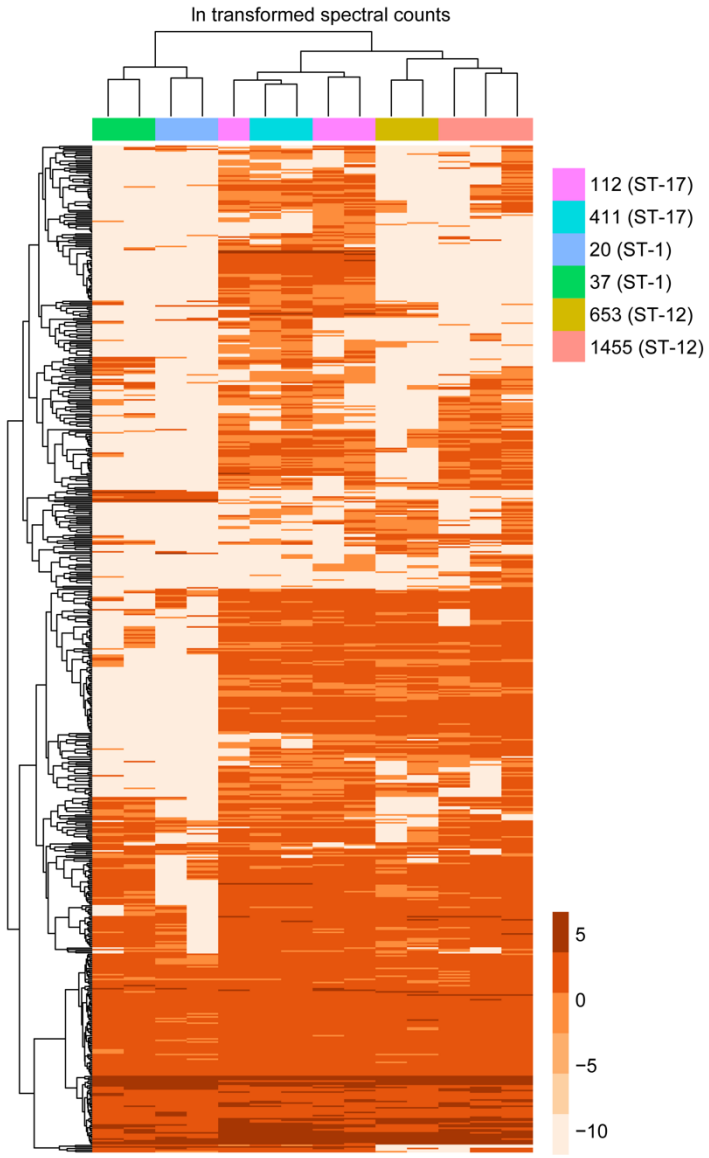

Figure 2.7: Hierarchical clustering of membrane vesicle (MV) proteomes shows sequence type (ST) specific clustering.

A heatmap was generated using hierarchical clustering with the pheatmap function in R, which uses Euclidean distance to cluster rows and columns with similar profiles. Individual rows represent a single accession number for an identified protein, with the color gradient of individual boxes corresponding to the natural log (Ln) transformation of spectral counts for a given protein of interest. Columns represent a single proteomic sample, which are color coded by strain.

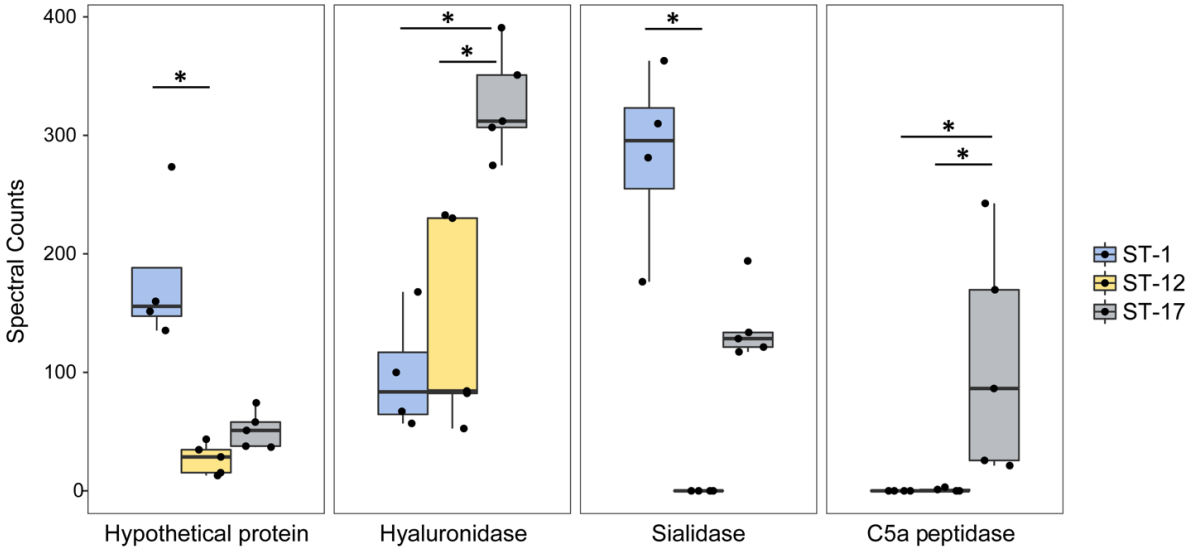

Figure 2.8: Highly abundant proteins are present at variable levels in group B streptococcal membrane vesicles (MVs).

The spectral counts of specific proteins were plotted after stratifying by the sequence type (ST). The median spectral count associated with each ST is represented within each box. The black dots represent a single biological replicate for a given strain. Statistical comparison was performed using a Kruskal Wallis test. Multiple pairwise comparisons were then made using the pairw.kw function in R, which uses a conservative Bonferroni correction method to correct for multiple hypothesis testing. Comparisons with p-values < 0.05 are denoted with an asterisk.

SUPPLEMENTARY FIGURES

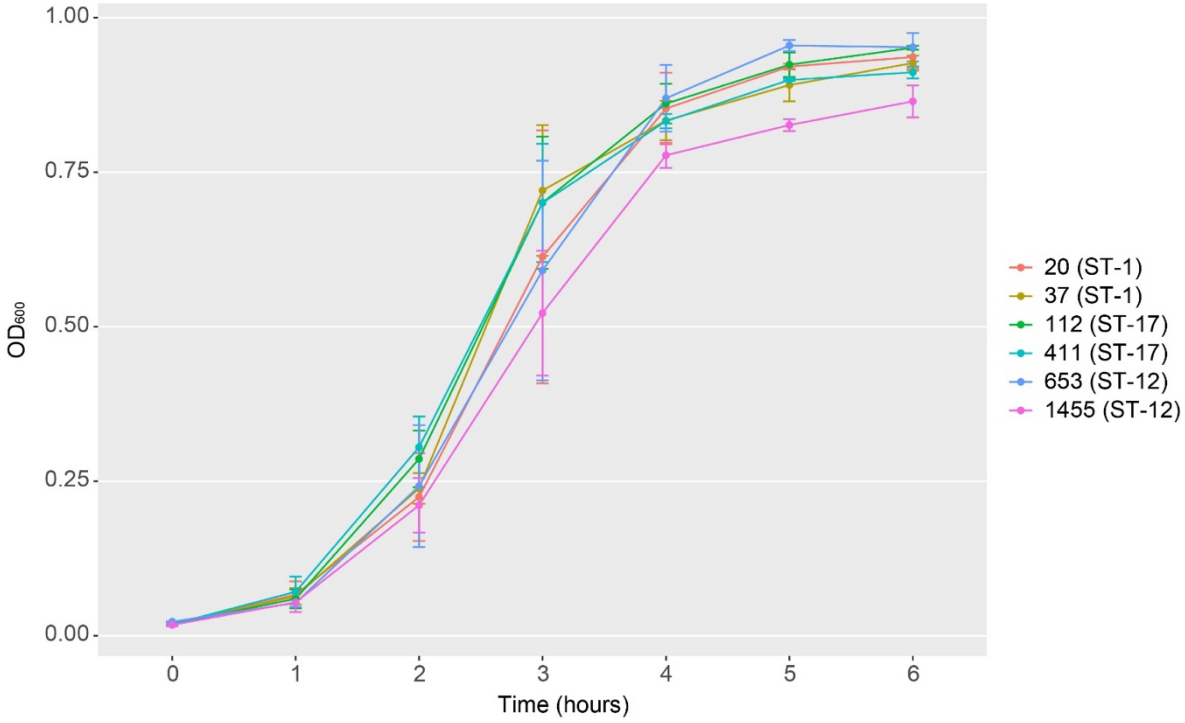

Figure S 2.9: Growth of six group B streptococcal strains used for membrane vesicle (MV) isolation.

Bacterial overnight cultures were back diluted 1:50 into fresh media and allowed to grow for 6 hours at 37°C with 5% CO_2. Optical Density $(OD)_{600}$ measurements were obtained hourly. Growth curves for each strain were performed in biological triplicate and analyzed and plotted using R. Hourly means were compared between strains using a Kruskal Wallis test followed by a Dunn's Test with a conservative Benjamini-Hochberg correction.

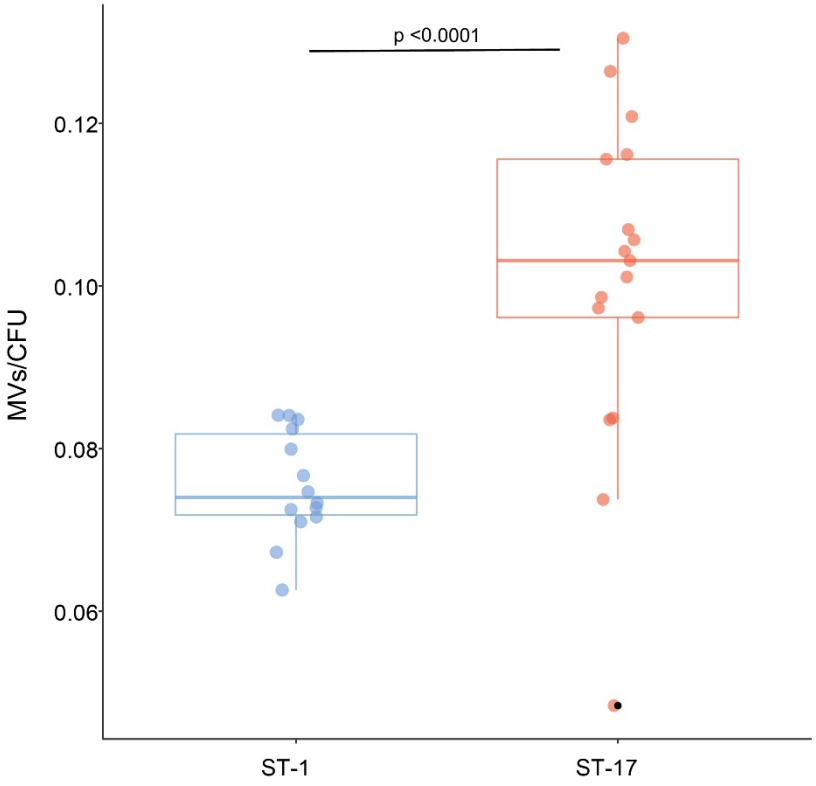

Figure S 2.10: Membrane vesicle (MV) production varies by sequence type (ST).

Vesicles were isolated using differential centrifugation and quantified by NanoSight analysis and plotted per bacterial colony forming units (CFUs). Data from each strain sharing the same ST were combined regardless of the clinical phenotype. Each colored dot represents a biological replicate. Black dots represent outliers that were identified by multiplying the interquartile range by 1.5, which was used to extend the upper and lower quartiles. When comparing production between ST-1 and ST-17, a two-sided students t-test was performed with significance defined as $p<0.05$.

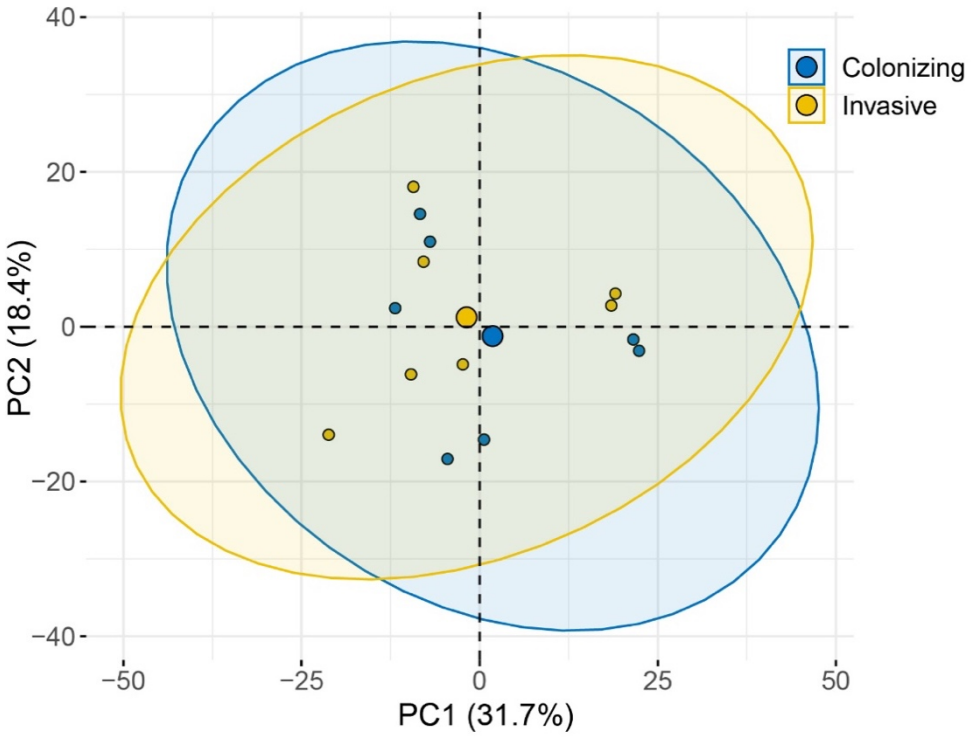

Figure S 2.11: Principal component analysis (PCA) shows lack of association between clinical phenotype and membrane vesicle (MV) proteome composition.

A PCA of the MV proteomes produced by six strains are shown after stratifying by clinical phenotype. The large central dot of each ellipse represents the mean point of the corresponding 95% confidence ellipse, while the smaller points represent individual proteomic samples. Axes percentages represent the amount of variation accounted for by the given principal component (PC).

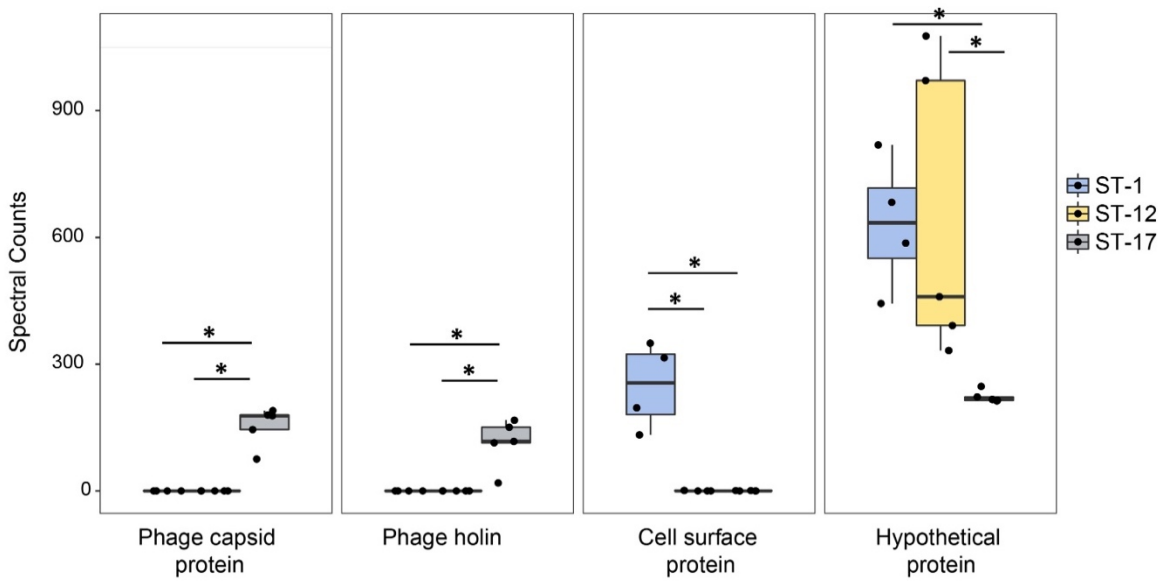

Figure S 2.12: Differentially abundant membrane vesicle (MV) proteins associated with phages or unknown functions.

The spectral counts of specific proteins were plotted after stratifying by the sequence type (ST). The median spectral count associated with each ST is represented within each box. The black dots represent a single biological replicate for a given strain. Statistical comparisons were performed using a Kruskal Wallis test. Multiple pairwise comparisons were made using the pairw.kw function in R, which uses a conservative Bonferroni correction method to correct for multiple hypothesis testing. Comparisons with p-values < 0.05 are denoted with an asterisk.

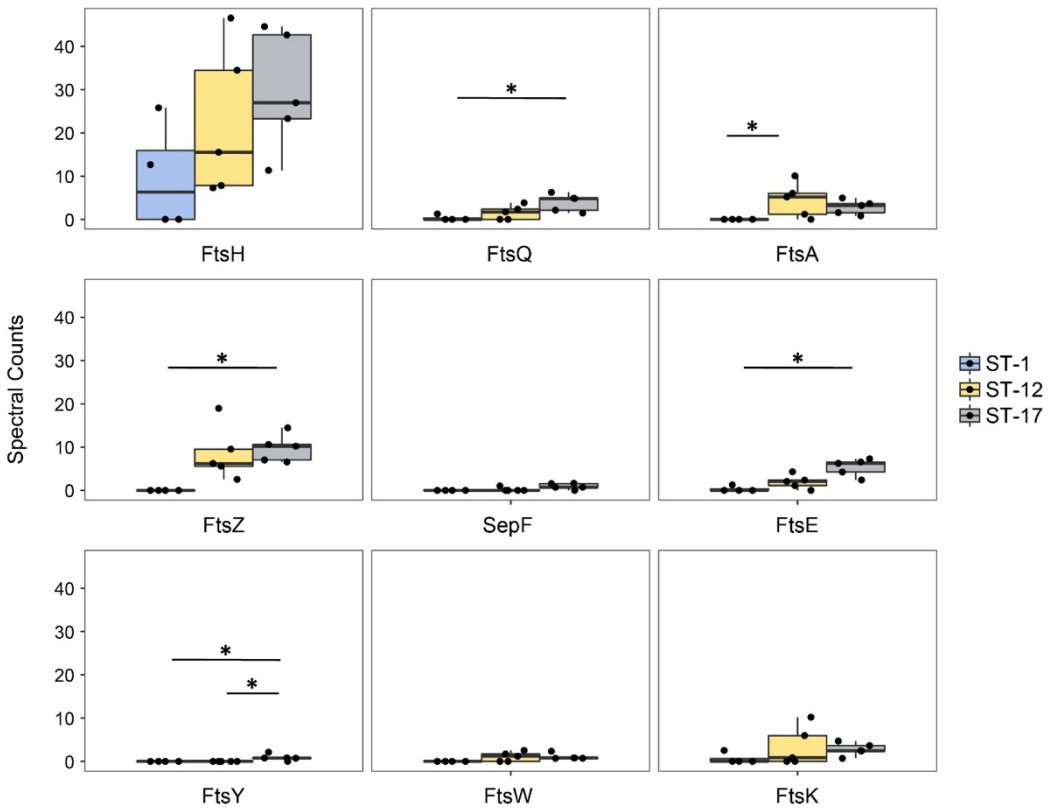

Figure S 2.13: Membrane vesicle proteins associated with cellular division.

The spectral counts of specific proteins linked to cellular division were plotted by sequence type (ST). The median spectral count associated with each ST is represented within each box. The black dots represent a single biological replicate for a given strain. Statistical comparison was performed using a Kruskal Wallis test. Multiple pairwise comparisons were made using the pairw.kw function in R, which uses a conservative Bonferroni correction method to correct for multiple hypothesis testing. Comparisons with p-values < 0.05 are denoted with an asterisk.

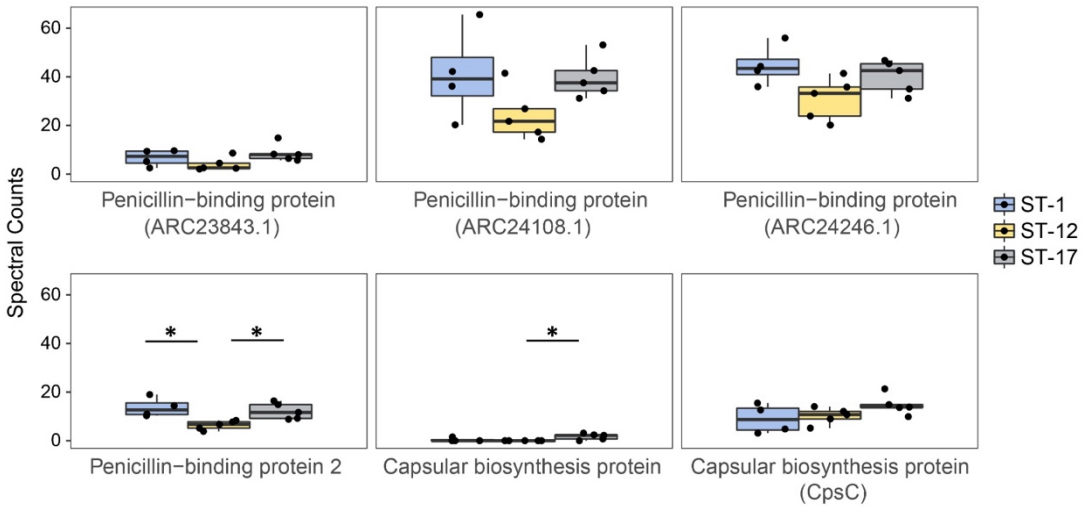

Figure S 2.14: Membrane vesicle proteins associated with cell wall modification.

The spectral counts of specific cell wall modification proteins were plotted by sequence type (ST). The median spectral count associated with each ST is represented within each box. The black dots represent a single biological replicate for a given strain. Statistical comparison was performed using a Kruskal Wallis test. Multiple pairwise comparisons were then made using the pairw.kw function in R, which uses a conservative Bonferroni correction method to correct for multiple hypothesis testing. Comparisons with p-values < 0.05 are denoted with an asterisk.

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CHAPTER 3

Group B Streptococcal Membrane Vesicles Induce Proinflammatory Cytokine Production and Are Sensed in an NLRP3 Inflammasome Dependent Mechanism in Human Macrophages

ABSTRACT

Group B *Streptococcus* (GBS) is a major cause of fetal and neonatal mortality worldwide. Many of the adverse effects associated with GBS are associated with inflammation that leads to chorioamnionitis, preterm birth, sepsis, and meningitis; therefore, understanding bacterial factors that promote inflammation is of critical importance. Membrane vesicles (MVs), which are produced by many pathogenic and non-pathogenic bacteria, may modulate inflammatory effects of some organisms. In mice, GBS MVs injected intra-amniotically can induce preterm birth and fetal death. Although it is known that GBS MVs induce large-scale leukocyte recruitment into infected tissues, the immune effectors driving these responses are unclear. Here, we hypothesized that macrophages respond to GBS-derived MVs by producing proinflammatory cytokines and are recognized through one or more pattern recognition receptors. We show that THP-1 macrophages produce high levels of neutrophil- and monocyte-specific chemokines in response to MVs derived from different clinical isolates of GBS. Interleukin (IL)-1ß was significantly upregulated in response to MVs, which was independent of NF-kB signaling but dependent on both caspase-1 and NLRP3. Together these data indicate that MVs contain one or more pathogen-associated molecular patterns that can be sensed by the immune system. Furthermore, this study has identified the NLRP3 inflammasome as a novel sensor of GBS MVs. Our data additionally indicate that MVs may serve as immune effectors that can be targeted for immunotherapeutics, particularly given that similar responses were observed across this subset of GBS isolates.

INTRODUCTION

Group B *Streptococcus* (GBS) is an opportunistic pathogen that colonizes the vaginal or rectal tract of ~30% of women (1). While colonization is often asymptomatic, GBS can cause severe disease in pregnant women and neonates (1). Pregnancy and neonatal associated GBS infections are often characterized by pathologies exhibiting a high degree of inflammation. During pregnancy, this can present as placental villitis, and preterm birth, whereas in neonates, GBS can cause meningitis and sepsis (2-4). Despite the high colonization frequencies in mothers, only a fraction of women and their neonates develop these threatening infections. The reasons for this discrepancy, however, are unknown.

We and others postulate strain variation contributes to the discrepancy in disease outcome. Indeed, specific phylogenetic lineages of GBS, which are defined by multilocus sequence typing (MLST), are more likely to cause neonatal infections (5-7). Notably, sequence type (ST)-17 strains are more commonly associated with invasive neonatal infections (5, 8, 9), whereas ST-1 strains are associated with invasive disease in adults (10). Conversely, ST-12 strains have been linked to asymptomatic maternal colonization (11). While the reasons for these associations are unclear, growing evidence suggests that ST-17 strains are more likely to cause invasive infections. We demonstrated that ST-17 strains elicit stronger proinflammatory immune responses and persist longer inside macrophages than other strains (12, 13). Interestingly, ST-1 and ST-17 strains also induce stronger NF-kB activation compared to ST-12 strains (13). While ST-17 strains have unique virulence gene profiles relative to other lineages, the specific bacterial factors that promote altered inflammatory responses remain poorly understood (14-16).

We recently showed that GBS produces membrane vesicles (MVs) that vary in abundance and protein composition across STs (17-19). More specifically, several

immunomodulatory virulence factors, including hyaluronidase, C5a peptidase, and sialidase, were highly and differentially abundant across STs (19). In, addition, GBS MVs were shown to induce production of the chemokine CXCL1 in a murine model of *in utero* infection (17), which has been shown in other GBS infection models (20, 21). Furthermore, MVs induce substantial recruitment of neutrophils and lymphocytes into murine extraplacental membranes, which mimics GBS-associated chorioamnionitis in humans (17, 22). Taken together, these data indicate that MVs promote proinflammatory immune responses; however, no prior studies have comprehensively examined how human leukocytes respond to GBS MVs.

As sentinel leukocytes at the maternal fetal interface, macrophages play an important role in shaping immune responses. At the maternal-fetal interface, macrophages make up 20-30% of leukocytes (23) and play pivotal roles in fertility (24), placental function (25), and host-pathogen interactions at the maternal-fetal interface (26-28). The THP-1 monocyte-derived macrophage cell line serves as a model system to evaluate host responses to GBS (12, 29). Using this model, we previously showed that THP-1 cells produce high levels of proinflammatory cytokines in response to GBS. Interestingly, several cytokines display lineage-specific inflammatory responses, with ST-17 strains eliciting a more potent inflammatory response compared to other lineages (13). Here, we examined macrophage responses to GBS MVs isolated from a diverse set of strains and found that these MVs induce the production of proinflammatory cytokines and chemokines. We also identified NLRP3 as a sensor of GBS derived MVs. In all, this study has expanded our current understanding of how host cells respond to GBS MVs. Additionally, by identifying the pathways upregulated by MVs, we have identified the proinflammatory pathways and receptors that could be used as potential immunotherapeutic targets.

METHODS

Bacterial Strains and Culture

GBS strains GB0037 (GB37), GB0411 (GB411), GB0653 (GB653), and GB1455 were isolated as described previously (30, 31). The invasive strains GB37, GB411, and GB1455, were isolated from the blood or cerebrospinal fluid of infants with early onset GBS disease (30), while the colonizing strain GB653 was isolated from vaginal/rectal swabs collected from an asymptomatically colonized mother before or after childbirth (31). These isolates were previously characterized by MLST and capsular serotyping (9, 11). The GBS strains analyzed here represent colonizing and invasive isolates belonging to each of three common STs: ST-1 (GB37), ST-12 (GB1455 and GB653), and ST-17 (GB411). Strains were cultured using Todd-Hewitt Broth (THB) or Todd-Hewitt Agar (THA) (BD Diagnostics, Franklin Lakes, New Jersey, USA) overnight at 37°C with 5% CO_2.

Membrane vesicle (MV) isolation

MVs were isolated as previously described (19). Briefly, overnight THB cultures were diluted 1:50 into fresh broth and grown to late logarithmic phase (optical density $(OD)_{600} = 0.9$). Cultures were centrifuged at 2000 x g for 20 minutes at 4°C. Supernatants were collected and re-centrifuged at 8500 x g for 15 minutes at 4°C, followed by filtration through a 0.22µm filter and concentration using Amicon Ultra-15 centrifugal filters (10kDa cutoff) (MilliporeSigma, Burlington, MA, USA). Concentrated supernatants were subjected to ultracentrifugation for 2 hours at 150,000 x g at 4°C. Pellets were resuspended in PBS and purified using qEV Single size exclusion columns (IZON Science, Christchurch, New Zealand) per the manufacturer's instructions. MV fractions were collected and re-concentrated using the Amicon Ultra-4

centrifugal filters (10 kDa cutoff) (MilliporeSigma, Burlington, Massachusetts, USA) and brought to a final volume of 100µL in PBS. MVs were aliquoted and stored at -80°C until further use.

Nanoparticle Tracking Analysis

MVs were quantified via nanoparticle tracking analysis using a NanoSight NS300 (Malvern Panalytical Westborough, MA, USA) equipped with an automated syringe sampler, as described previously (19, 32, 33). For each sample, MVs were diluted in PBS (1:100 – 1:1000) and injected with a flow rate of 50. Once loaded, five 20-second videos were recorded at a screen gain of 1 and camera level of 13, which were analyzed at a screen gain of 10 and a detection threshold of 4 after capture. Data were subsequently exported to a CSV file for analysis using the R package tidyNano (32).

THP-1 Cell Culture

THP-1 cells (TIB-202) were obtained through ATCC (Manassas, VA) and stored according to vendor guidelines (34). Briefly, cells were cultured in RPMI 1640 (Gibco, ThermoFisher, Waltham, MA) supplemented with L-Glutamine, 10% fetal bovine serum (FBS), and 1% antibiotic-antimycotic (100 ug/mL Streptomycin, 0.25 ug/mL Amphotericin B, & 100 U/mL Penicillin; Gibco, ThermoFisher, Waltham, MA) as previously described (12, 13). For experiments, THP-1 cells were only used until passage 10. When indicated, THP-1 monocytes were differentiated into macrophages with phorbol 12-myristate 13-acetate (PMA) as previously described (12, 13). Cells were differentiated in RPMI (without phenol red) supplemented with L-Glutamine, 2% FBS and 100nM PMA for 24 hours prior to experimentation (12, 13).

For experiments using GBS treated cells, THP-1 cells were washed twice with PBS prior to infection. The bacteria were resuspended in RPMI and added to the THP-1 cells at a multiplicity of infection (MOI) of 10 bacteria per cell. Cells were incubated for 1 hour and the media was subsequently aspirated. Cells were washed thrice with PBS and fresh RPMI with L-Glutamine (no phenol red) containing 2% FBS, 100nM PMA, penicillin (5ug/mL) and gentamicin (100ug/mL) was added (termed RPMI 2/0). Cells were incubated for an additional 24 hours. For MV treatment, cells were washed twice, and fresh RPMI 2/0 containing MVs at an MOI of 100 MVs per differentiated macrophage was added and incubated for 25 hours. Cells were treated with LPS (1µg/ml, clone L2654, Millipore Sigma, Burlington, MA) to serve as positive controls. At the end of each treatment period, supernatants were collected, centrifuged for 10 minutes at 4000 rev/min at 4°C and aliquoted. Samples were stored at -80°C until used for downstream analysis.

Cytokine and Cytotoxicity Analysis

For semiquantitative analysis of cytokines in supernatants from THP-1 cultures, we employed a human cytokine antibody microarray (ab133998, Abcam, Cambridge, UK) which was performed according to manufacturer's instructions as previously described (13). Cells were seeded into 6 well plates at a density of 4 x 10^6 and treated as described above. Membranes were imaged using an Amersham Imager 600 (GE Life Sciences), and densitometry was performed using ImageJ software. Cytokines falling above a fold change of 2 relative to mock treated were considered upregulated and further analyzed. For subsequent analyses of cytokine production, caspase-1 activation, and cell death, cells were seeded into 12 well plates at a density of 2 x 10^6 cells per well and treated as described above. Cytokines with more than a 2-fold change relative

to mock-treated cells were verified using a custom ProcartaPlex bead assay (ThermoFisher, Waltham, MA) as described by the manufacturer. These assays were read and analyzed using a Luminex 200 and Luminex xPONENT v3.1 software, respectively (Luminex Corp., Austin, Texas). Cellular cytotoxicity was assessed using a CyQuant lactate dehytrogenase (LDH) assay (Invitrogen, Waltham, MA) per the manufacturer's instructions.

Immunofluorescence Staining

THP-1 cells were seeded into 4-well Nunc Lab-Tek II Chamber slides (ThermoFisher, Waltham, MA) at a density of 10^5 cells per well and differentiated as described above. Cells were treated with either MVs (MOI 100) or LPS (1ug/mL) for 0.5, 3, or 24 hours and analyzed for the NF-kB subunit p65 by immunofluorescence as described (35). Briefly, the cells were fixed using 4% paraformaldehyde in PBS for 10 minutes, washed three times with ice cold PBS, and permeabilized for 10 minutes in 0.2% Triton-X in PBS. Cells were washed three more times in PBS and blocked in 10% goat serum/1% BSA/0.3% Tween in PBS for 20 minutes. Rabbit anti-NF-kB antibody (1:1600; clone D14E12; Cell Signaling Technology, Danvers, MA) was added to cells and incubated overnight at 4°C. Cells were washed three times and incubated with Alexa Fluor Goat anti-rabbit 546nm secondary antibody (10µg/mL; Invitrogen, Waltham, MA) for 1 hour, and washed again in PBS. Coverslips were mounted using Vectashield DAPI (Vector Laboratories, Inc., Burlingame, CA), and representative images were obtained using a Nikon Eclipse Ti outfitted with a 20x plan fluor objective. Immunofluorescent microscopy was performed in biological triplicate for each timepoint and treatment.

Caspase-1 Activity, Responses, and Inflammasome Inhibition

After treatment of THP-1 cells, caspase-1 activity was quantified in supernatants using a commercially available assay (Caspase-GLO 1 Assay; Promega, Madison, WI) according to the manufacturer's instructions. Caspase-1 activity in supernatants was quantified using a GloMax Navigator (Promega, Madison, WI). To assess the impact of caspase-1 on MV-induced IL-1β production, PMA-differentiated THP-1 cells were seeded into 12-well plates and pretreated with 50μM of the caspase-1 inhibitor, Ac-YVAD-CHO (Cayman Chemical Company, Ann Arbor, MI), or 10 μM of the NLRP3 inhibitor, MCC950 (Invitrogen, Waltham, MA), for 30 minutes. Cells were treated with either LPS or GBS as described above, and IL-1β concentrations were measured using a ProcartaPlex simplex assay (ThermoFisher, Waltham, MA).

qPCR Analysis

PMA-differentiated THP-1 cells, which were seeded in 12-well plates at a density of 2 x 10^6 cells per well, were left untreated or treated with either GBS bacteria, LPS, or MVs as described above. After 2 or 4 hours, supernatants were aspirated and cells were lysed by adding 1mL Trizol reagent (Invitrogen, Waltham, MA) and gentle scraping. Samples were stored at -20ºC until RNA extraction was performed using Phase Lock gel heavy tubes per manufacturer's instructions (Quanta Bio, Beverly, MA). RNA was quantified using a Nanodrop 8000 spectrophotometer (Thermo Scientific, Waltham, MA) and stored at -20 ºC until usage. Reverse transcription was performed on 0.5μg of total RNA using Quantitect Reverse Transcription Kit (Qiagen, Hilden, Germany), and 2μL of the resulting cDNA was amplified by PCR using TaqMan Universal PCR Master Mix (Applied Biosystems, Waltham, MA) with Taqman probes specific for pro-IL-1β (Assay ID: Hs00174097_m1) and GAPDH (Assay ID: Hs99999905_m1).

PCR was performed in a QuantStudio 5 real time thermal cycler for 35 cycles (Applied Biosystems, Waltham, MA).

Data analysis

Data analysis was performed using R studio. Shapiro-wilk tests were used to determine whether data followed a normal distribution. Normally distributed data were analyzed for significance using a two-way analysis of variance (ANOVA), in combination with a Tukey HSD *post hoc* test. Alternatively, non-parametric data were analyzed using a Kruskal-Wallis test, in combination with the Dunn's *posthoc* test to test for differences between groups. Multiple hypothesis testing was corrected using Benjamini-Hochberg or Bonferroni correction when necessary. The analyses used for individual experiments are denoted in the figure legends for clarity.

RESULTS

GBS MVs elicit proinflammatory cytokine responses

We first sought to characterize the cytokine response elicited by MVs from a diverse set of GBS strains representing major sequence types (STs) in clinical circulation. Specifically, we characterized the cytokine response to MVs from an ST-1 strain (GB37), two ST-12 strains (GB653 and GB1455), and one ST-17 strain (GB411). Of note, GB37, GB1455, and GB411 were all isolated from infants with invasive infections, whereas GB653 was isolated from an asymptomatically colonized mother. Human cytokine antibody microarrays revealed that MVs from GB411 and GB653 induced cytokine production from THP-1 macrophages. Of the 80 cytokines and chemokines assayed, 7 were upregulated at least 2-fold in comparison to the untreated cells (Figure S 3.1). Cytokines upregulated in responses to MVs included the monocyte and neutrophil chemokines, CCL1, CCL2, CXCL1, CCL20, the pyrogen IL-1β, and the proinflammatory cytokine IL-6 (Figure 3.1, Figure S 3.1). Several cytokines were also induced differentially between the two isolates: MVs from GB411 induced CXCL1, CCL1, and IL-1ß more strongly than MVs from GB653 (Figure 3.1). However, the same trend was not observed when comparing cytokines between bacteria-treated THP-1 cells since GB411 and GB653 elicited similar cytokine responses for each of these targets (Figure 3.1).

To validate these differences in cytokine production, we used quantitative Luminex-based assays. Consistent with our previous results (13), GBS induced a potent proinflammatory response relative to untreated controls (Figure S 3.2-3.3), though IL-6 production remained unchanged by MV exposures (Figure S 3.3). Moreover, the MVs induced CCL1, CCL20, CXCL10, CXCL1, and IL-1ß, with no differences between the strains from which the MVs were

derived (Figure 3.2). While CCL2 displayed an elevated response relative to mock treatment, this induction was only significant for MVs produced by GB37, GB411, and GB1455.

Next, we assessed cytotoxicity for all strains examined above using a lactate dehydrogenase activity assay to ensure that these responses were not biased due to differential cell death. In these analyses, we found that bacteria induced moderate cytotoxicity that varied slightly across bacterial strains (Figure S 3.4). Notably, GB37 induced significantly more cytotoxicity than GB1455; however, this cytotoxicity was modest. Although low levels of cytotoxicity were observed during MV treatments, with an average of ~6%, the cytotoxicity levels did not vary across MVs produced by the four different GBS strains.

MVs induce caspase-1 activation

Since IL-1ß was significantly increased in response to all GBS MVs regardless of the strain, we sought to classify the inflammatory pathways that impact its production. Using the Caspase-GLO 1 assay, we detected caspase-1 activity in our untreated controls as well as our LPS-stimulated control, albeit at a substantially higher magnitude in our LPS control (Figure S 3.5). Detectable caspase-1 activity was also observed in response to MVs and the GBS strains, though some differences were noted. Compared to untreated controls, MVs from GB37, GB411, and GB1455 induced the most potent caspase-1 responses, providing confirmation that MVs were capable of inducing caspase-1 activity (Figure 3.3A, Figure S 3.5). Similarly, GB411 bacteria induced a higher degree of caspase-1 activation compared to untreated controls, which is consistent with our previous findings (Figure S 3.6).

Next, we sought to determine if alternative pathways may be contributing to the conversion of pro-IL-1β to mature active IL-1β. To assess this, we pretreated THP-1 cells with

the capsase-1 inhibitor Ac-YVAD-CHO prior to treatment with MVs or LPS for 25 hours. We found that LPS and untreated controls both produced lower amounts of IL-1β when pretreated with Ac-YVAD-CHO compared to the vehicle controls (83% and 90% reduction, respectively); Figure 3.3B). Furthermore, inhibition of caspase-1 by Ac-YVAD-CHO resulted in almost complete abrogation of MV-stimulated IL-1β secretion (91% reduction) compared to the vehicle control. Importantly, alterations in IL-1β production were not associated with cell death (Figure S 3.7). This finding therefore demonstrates that caspase-1 activation is necessary for the maturation of pro-IL-1β to mature IL-1β in response to GBS MVs, regardless of the strain type (Figure 3.3B).

NLRP3 is essential for MV-mediated IL-1β secretion

Having established that caspase-1 is required for IL-1β maturation, we next investigated the upstream sensor of MVs. Because GBS triggers inflammasome activation via a NLRP3-dependent mechanism, we assessed whether inhibition of NLRP3 could impact caspase-1 activation in response to GBS MVs (36). Notably, inhibition of NLRP3 with the MCC950 inhibitor prevented both MV- and GBS-induced caspase-1 activity (Figure 3.4A and Figure S 3.8). We observed a similar trend in our control cells, demonstrating some baseline inflammasome activity in THP-1 cells; however, the magnitude of inflammasome activation was lower in control groups (Figure 3.4A). Inhibition of NLRP3 also reduced cytotoxicity for both the GBS bacteria- and MV-treated cells; however, this result was not observed for our untreated controls (Figure 3.4B).

Using a similar approach, we also assessed whether NLRP3 impacted secretion of IL-1β from THP-1 cells. In these experiments, we found that inhibition of NLRP3 signaling

significantly decreased IL-1β secretion in both the media and LPS controls relative to the vehicle controls (Figure 3.4C). While the decrease was significant in both groups, the effect was lower for the untreated controls. Moreover, NLRP3 inhibition reduced IL-1β secretion in response to both GBS and the MVs demonstrating that MV-induced IL-1β requires NLRP3 (Figure 3.4C).

MVs do not trigger transcriptional activation of pro-IL-1β

We next assessed whether the high levels of IL-1ß produced in response to GBS MVs were due to the release of existing pools of pro-IL-1ß, or if MVs could directly induce transcription of pro-IL-1ß. Using RT-qPCR analysis, we observed no significant increase in pro-IL-1ß gene expression relative to untreated cells for LPS, MV, or bacteria treated THP-1 cells at 2 hours post infection (Figure 3.5B). At 4 hours post infection, however, LPS induced a significant increase in pro-IL-1ß gene expression relative to untreated cells, but no similar increases were observed in response to MVs or GBS (Figure 3.5B). Using immunofluorescence, we similarly found that while LPS rapidly induced the translocation of the NF-kB subunit p65, neither untreated nor MV treated THP-1s induced NF-kB translocation. Notably, MVs induced no NF-kB translocation in response to MVs after a 2-hour exposure (Figure 3.5A). Similar results were observed at 30 minutes and 24 hours post exposure (Data not shown). Together, these data indicate that MVs do not induce a largescale alteration in pro-IL-1ß gene expression or NF-kB activation, suggesting that elevated IL-1β secretion is likely due to post-translational regulation.

DISCUSSION

Previous studies demonstrated that *in utero* exposure to GBS MVs induced recruitment of neutrophils and lymphocytes into the gestational membranes (17), while MVs induced neutrophil recruitment to the lung in a neonatal sepsis model (18); however, the signals that perpetuate the influx of leukocytes remains unclear. Herein, we have demonstrated that MVs induce expression of proinflammatory cytokines and chemokines in human macrophages *in vitro*, which are likely responsible for the inflammatory infiltrate observed *in vivo* (17, 18). Additionally, we found that MVs induce the production of IL-1β by activating pro-IL-1β maturation in an NLRP3 and caspase-1 dependent manner, but independently of NF-kB signaling.

By expanding our current understanding of the cytokine responses towards GBS derived MVs, we have identified the modulators that likely impact the adverse pathologies observed *in vivo.* A previous study demonstrated that the murine chemokine KC, known as CXCL1 in humans, was shown to be significantly upregulated in response to MVs (17). In support of these findings, we have demonstrated that production of CXCL1 and many additional chemokines are significantly upregulated in human macrophages following challenge with GBS MVs. Notably, CCL1, CCL20, CXCL1, and CXCL10 were all significantly upregulated in response to MVs from four clinical strains. Similarly, the chemokine CCL2 was significantly elevated in response to three of the clinical strains we examined. These chemokines were previously shown to be critical for the recruitment of leukocytes to the site of infection, with varying target cell specificities. CXCL1 and CCL20, for example, attract neutrophils (37-39), whereas CCL1 and CCL2 attract monocytes and macrophages (40, 41). Additionally, CCL20 and CXCL10 recruit lymphocytes (39, 42). Unsurprisingly, many of the cytokines have been implicated in GBS-associated disease. For example, CCL20 is upregulated during infection at the blood brain barrier

(44). Similarly, CCL2 has been shown to be strongly upregulated during GBS sepsis cases, suggesting it may be important for the host response to GBS infections (45). Taken together these data indicate that GBS MVs serve as a critical initiator of disease associated cytokine responses.

Another cytokine that was significantly upregulated in response to MVs was the pyrogen IL-1ß, which plays a critical role in the host defense to GBS infections by promoting production of additional neutrophil specific chemokines (20, 46). Although IL-1ß does not directly have any chemoattractant activity, IL-1ß signaling does impact the production of CXCL1 in GBS infections (20). In fact, IL1R knockout mice display reduced neutrophil recruitment and significant increases in mortality when challenged with GBS (44). Given the abundant recruitment of neutrophils and lymphocytes into MV challenged tissues (17), these data provide critical insights into the mechanisms driving this leukocyte infiltration. Although we and others have previously shown strain variation in IL-1ß production in response to whole bacteria (13, 47) we found that MVs elicited a consistent level of IL-1ß from human macrophages, suggesting that it may serve as an important biomarker or possibly a therapeutic target.

Previous studies have highlighted the signaling pathways involved in producing mature IL-1ß. Notably, high levels of this cytokine were only produced when both the activation of TLR (toll-like receptor) signaling and inflammasome activation occurred (48). TLR signaling occurs when pathogen-associated molecular patterns (PAMPs) engage their cognate receptor (49, 50), which results in the induction of proinflammatory gene expression, including the inactive form of this cytokine, pro- IL-1ß (50). Canonically, the induction of pro-IL-1ß gene expression depends on translocation of the transcription factor NF-kB, into the nucleus (51).

For pro-IL-1ß to be secreted in its mature, active form, a second signal is required. This signal is typically in the form of a danger associated molecular pattern (DAMPs), such as a change in membrane potential due to membrane damage (52, 53). DAMPs are sensed by NLRPs (Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain-containing receptors) (52, 54). Once sensed, NLRPs oligomerize with other subunits, forming the inflammasome, (52, 55, 56) which cleaves pro-caspase-1 into its mature, active form (56, 57). Active caspase-1 then cleaves pro-IL-1ß, triggering its release (57). This concerted process results in release of stored pools of pro-IL-1ß, allowing for rapid immune activation.

Notably, we have demonstrated that GBS MVs trigger caspase-1 activation in human macrophages and that the secretion of IL-1ß is dependent on caspase-1 activation. Our findings further indicate that MVs do not trigger expression of pro-IL-1β or activation of NF-kB, suggesting that IL-1ß production in response to MVs is largely due to post-transcriptional regulatory mechanisms. Interestingly, we also found that caspase-1 activation is ablated in the absence of NLRP3, suggesting that NLRP3 is a sensor of GBS MVs. While previous reports have demonstrated that GBS induces IL-1β production in a NLRP3-dependent manner, this is the first study to demonstrate that GBS MVs contribute to this response (28, 36, 58). Furthermore, we are not aware of any other studies that have identified a pattern recognition receptor capable of sensing GBS MVs. This newfound information may allow for the development of receptor antagonist therapies targeting the NLRP3 dependent recognition of GBS MVs, which could prevent host inflammation and subsequent adverse pregnancy outcomes.

Our data also indicate that inhibition of the NLRP3 inflammasome reduces MV-induced cytotoxicity of macrophages *in vitro*. Several studies have shown that GBS virulence factors, such as hemolysin can induce NLRP3-dependent pyroptosis (36, 58-60). Other studies indicate

that GBS mediated pyroptosis is mediated by the activation of the pore forming mediator of pyroptosis, gasdermin D (61, 62). In our examination of THP-1 macrophages, both the MVs and bacteria mediated a modest amount of cell death, which was dependent on the NLRP3 inflammasome, suggesting that MVs can induce pyroptosis, which could suggest gasdermin D activation. While further studies are needed to confirm this hypothesis, the high levels of IL-1β production together with NLRP3 mediated cell death indicate that MVs may be partly responsible for GBS-mediated pyroptosis.

Our analyses also suggest that MVs do not induce pro- IL-1ß gene expression. Indeed, while LPS induced a potent upregulation of pro-IL-1ß by 4 hours post-exposure, we observed no upregulation of pro-IL-1ß in response to MVs or bacteria at either timepoint. Additionally, this lack of induction correlated with the activation of NF-kB signaling, which suggests the following: 1) MVs do not overwhelmingly induce the expression of pro-IL-1ß; and 2) the upregulation of IL-1ß signaling is likely due to the activation of inflammasome signaling in primed macrophages. Taken together, these data indicate that GBS MVs induce the production of IL-1ß in primed macrophages, which is likely a conserved feature of GBS MVs.

A prior study demonstrated that GBS MVs contain active hemolysin and that MV-associated hemolysin exacerbates neonatal sepsis *in vivo* (18). Although it was suggested that GBS-mediated caspase-1 induction requires GBS hemolysin (36), our data indicate that MVs from a non-hemolytic strain of GBS (GB37) still induce a robust IL-1ß response and activate caspase-1 (63). This finding indicates that other factors found inside MVs also induce caspase-1 activation. Indeed, use of proteomics in our prior study found that MVs of different genetic backgrounds contained multiple virulence factors that have been linked to inflammatory responses previously (19). Of note, several factors known to promote immune evasion, such as

hyaluronidase, sialidase, and C5a peptidase, were found to be present in GBS MVs at variable levels across diverse phylogenetic backgrounds (19). While these factors are known to diminish host sensing of GBS, other MV derived factors likely promote these inflammatory responses. Nonetheless, future studies are required to classify the role that these other factors play in activating these signaling cascades.

Despite advancing our current understanding of the host response elicited towards GBS MVs, it is important to recognize the limitations of our study. Although no strain-specific immune responses towards GBS MVs were observed, we only examined 4 distinct clinical isolates that could have limited our ability to detect differences. Furthermore, our cytokine analysis was limited to those included in the antibody microarrays; hence, it is likely that other responses may also be important. Although our results are consistent with previous reports regarding the host response to GBS MVs (17, 18), it is possible that our system lacks the appropriate complexity to fully model the host response to GBS MVs. Indeed, although THP-1 cells have been shown to largely recapitulate the responses elicited from peripheral blood mononuclear cells, the magnitude of their responses can vary between these two systems (64). Furthermore, the use of cells in monoculture does not capture the complexity of the host responses observed *in vivo.* Therefore, future studies using alternative model systems are warranted.

Overall, data from this study enhance our understanding of how GBS MVs promote both adverse pregnancy and neonatal infection outcomes (Figure 3.6). It has been established that GBS MVs promote adverse outcomes partly by enhancing neutrophil recruitment (17, 18). In conditions such as chorioamnionitis, we suggest that the sensing of MVs by macrophages may promote proinflammatory immune signaling. Consistent with these findings, we have

demonstrated that MVs promote the release of many neutrophil recruiting chemokines as well as the pyrogen IL-1β, which are important for neutrophil recruitment that promote tissue damage via net-osis (20, 39, 65, 66). We also demonstrate that the MV-mediated induction of IL-1β is dependent on caspase-1 activation that further promotes a proinflammatory environment. Through both direct and indirect tissue damage, MVs likely play a role in weakening gestational membranes, inducing chorioamnionitis, and promoting preterm labor due to enhanced induction of these inflammatory responses (Figure 3.6). Collectively, these findings expand our understanding of how the immune system respond to these bacterial components that contain important virulence factors capable of initiating an inflammatory response. While the specific PAMPs and DAMPs contained in MVs are not known, this study provides a foundation for future studies aiming to classify the specific factors within MVs that trigger these responses.

These data illustrate that GBS MVs are capable of inducing potent proinflammatory cytokine responses, which is due in part to the activation of the inflammasome. This study has furthered our understanding of how GBS MVs interact with the host, by identifying the cytokine response towards GBS MVs as well as by identifying NLRP3 as a sensor of MVs. Furthermore, because these cytokine responses are largely conserved across genetically distinct clinical GBS isolates, these responses may represent important targets for immunotherapy or as biomarkers for disease status. Taken together, this study has provided mechanistic insight into the immune response elicited towards GBS MVs.

FIGURES:

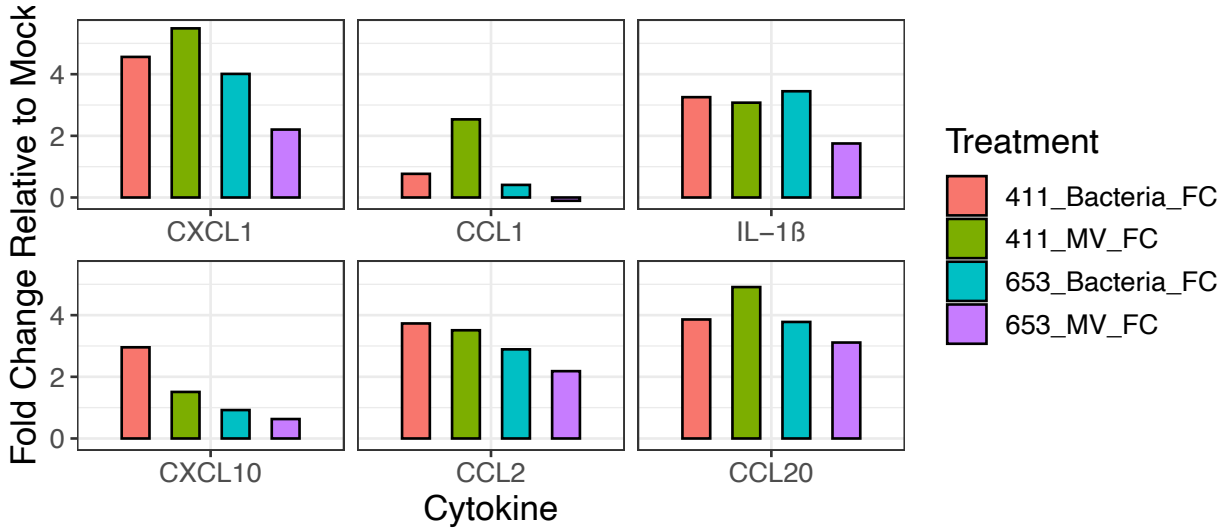

Figure 3.1: Human antibody cytokine microarray reveals highly upregulated cytokines in response to GBS and GBS MVs.

Human cytokine antibody microarrays (Abcam) were probed with supernatants from untreated, bacteria-treated, or MV-treated THP-1-derived macrophages. Membrane densitometry was assessed using ImageJ software. The bacterial strains used are an invasive ST-17 strain (GB411) and a colonizing ST-12 strain (GB653). Shown here are hits of interest that displayed greater than 2-fold change (FC) induction relative to untreated controls in at least one group. Bars indicate the mean fold change relative to mock treatment. n = 2/treatment.

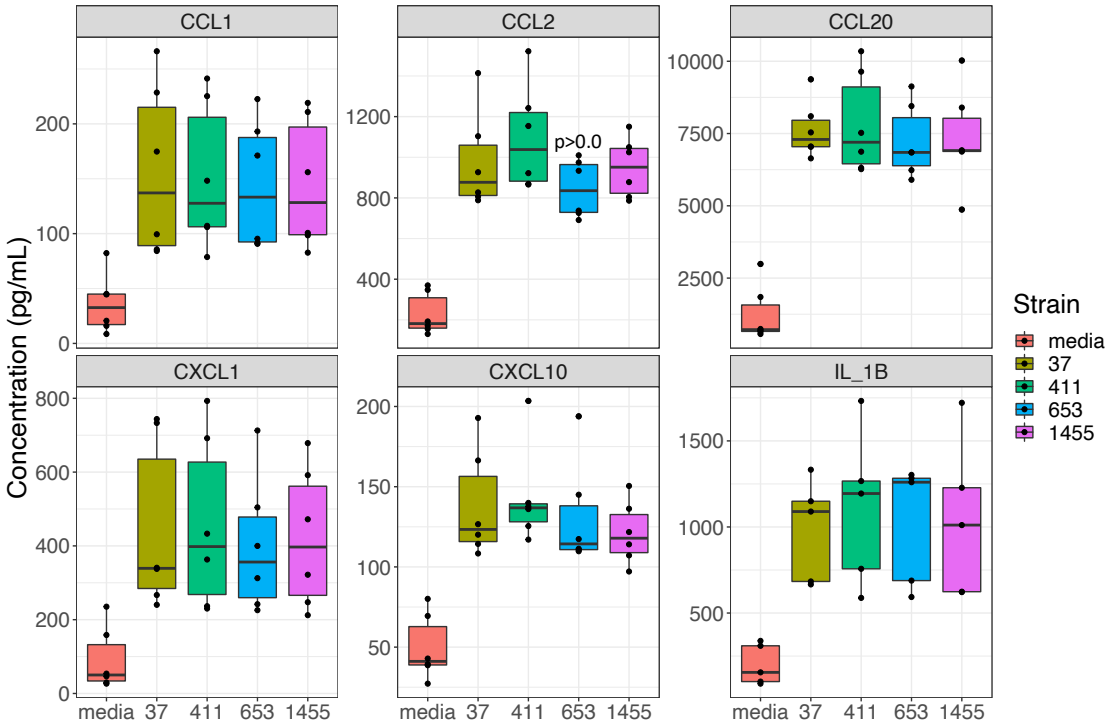

Figure 3.2: MVs induce proinflammatory cytokine and chemokine responses.

Supernatants from THP-1 derived macrophages which were untreated or treated with MVs (MOI 100) for 25 hours were assessed for cytokine production using ProcartaPlex multiplex or singleplex (IL-1B) bead-based assays. Individual black dots indicate a single biological replicate (n = 5-6 for each group). Statistics were determined using either an ANOVA with a Tukey HSD post hoc or a Kruskal Wallis test with a Dunn Test post hoc when appropriate. All comparisons to mock treatment were significant ($p<0.05$) unless noted with a specific p-value.

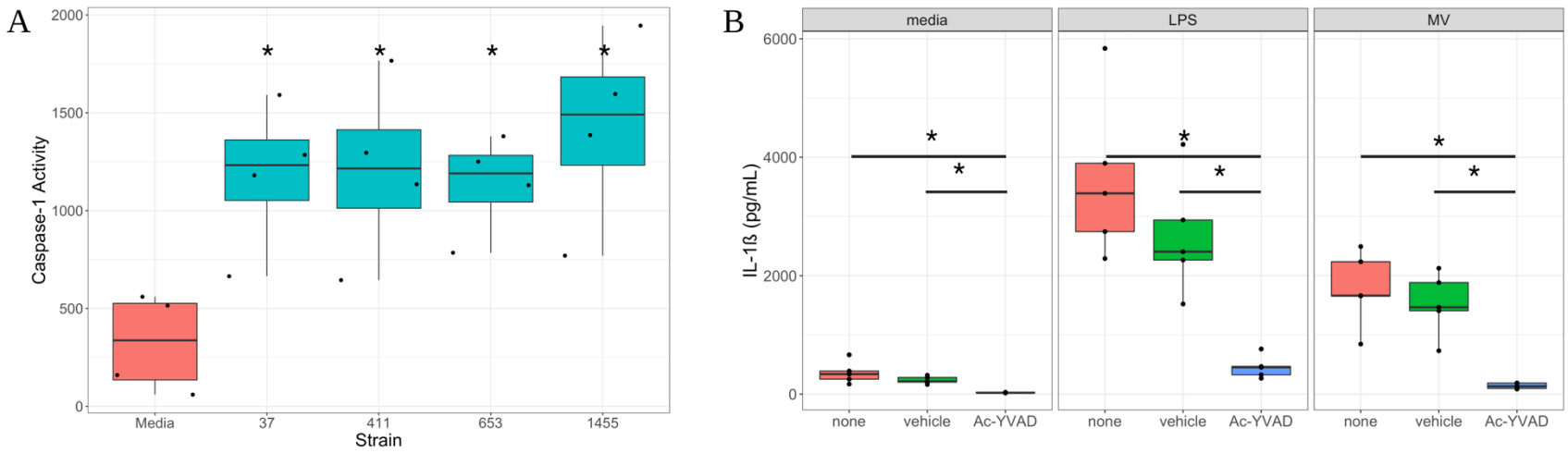

Figure 3.3: Caspase-1 is critical for the host response to GBS MVs.

A.) THP-1 derived macrophages were unstimulated or treated with MVs for 25 hours. Supernatants were then assessed for caspase-1 activity using a caspase-1 GLO assay. Relative light units (RLU) were obtained from a GLO Max Navigator. Data represent the amount of caspase-1 activity (caspase-1 activity = (RLU GLO) - (RLU AC)) from paired samples. B.) THP-1 derived macrophages were pre-treated media, ethanol (vehicle), or Ac-YVAD-CHO for 30 minutes prior to stimulation with LPS, media, or MVs for 25 hours. Supernatants were then assessed for IL-1B concentration using ProcartaPlex bead-based assays. Individual black dots indicate a single biological replicate (n = 4 for each group). Statistical significance is defined as $p<0.05$ as calculated by ANOVA with a Tukey post-hoc and indicated by (*).

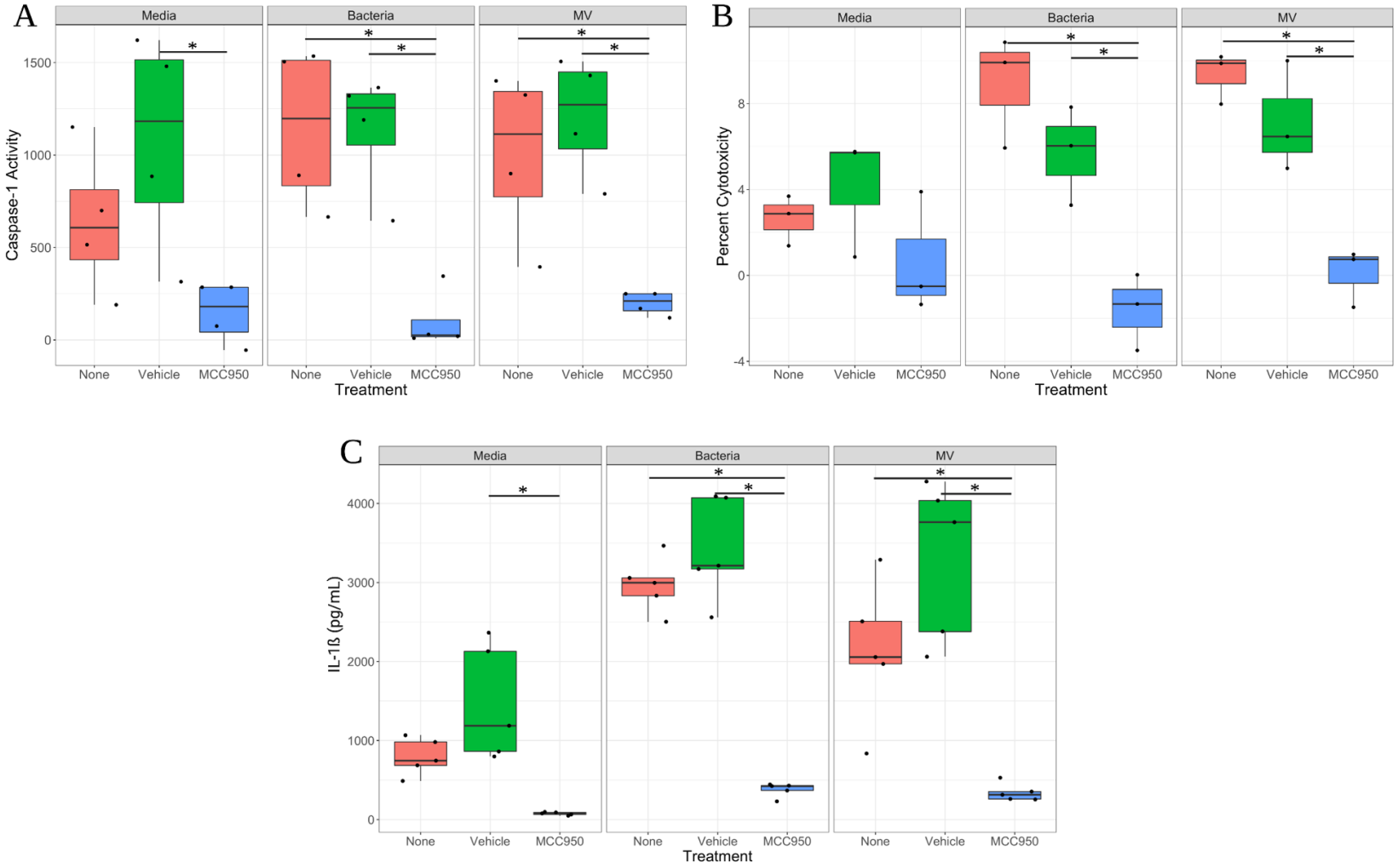

Figure 3.4: Inhibition of NLRP3 Ablates Caspase-1 Activity in response to MVs.

Figure 3.4 (cont'd)

THP-1s were treated with the NLRP3 inhibitor MCC950 or DMSO for 30 minutes prior to treatment with bacteria, MVs, or media for 25 hours. A.) Caspase-1 activity was determined using the Caspase-1 GLO assay. Caspase 1 activity = ((GLO reagent) – (Ac-YVAD-CHO + GLO Reagent)). Individual points represent individual biological replicates (n = 4 each group). B.) Supernatants were assessed for cytotoxicity using the CyQuant LDH Assay. Individual black dots indicate a single biological replicate (n = 3 for each group). C.) IL-1ß contained in supernatants was quantified using ProcartaPlex IL-1ß single plex assays. Individual points represent individual biological replicates (n = 5 each group). Statistics were determined using an ANOVA with a Tukey's HSD post-hoc test. Significance was defined as $p<0.05$ and denoted with an (*).

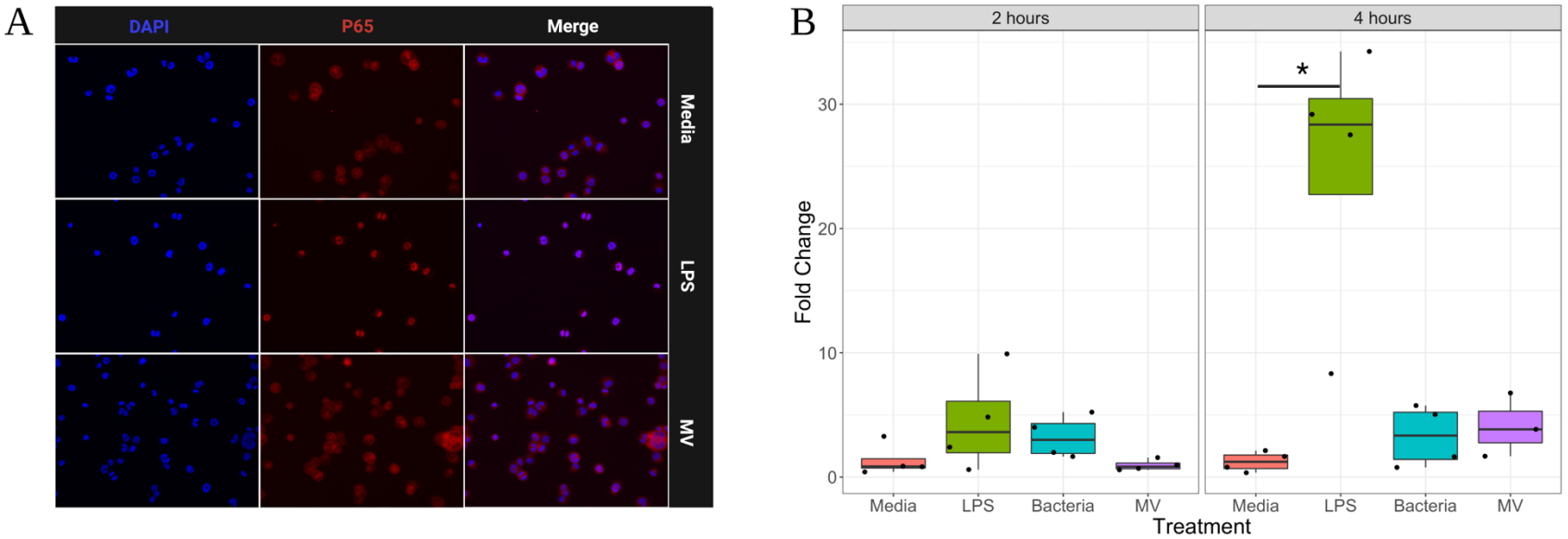

Figure 3.5: MVs do not prime human macrophages.

A.) Differentiated THP-1 derived macrophages were untreated or treated with LPS or MVs for 2 hours prior to fixation and immunofluorescence staining for NF-kB subunit p65 (stained red). Nuclei are stained using DAPI (blue). Shown here are representative images (n = 5) taken at 40x magnification. B.) Expression of pro-IL-1ß mRNA from THP-1s treated with bacteria, MVs, LPS, or media was quantified using Taqman probes at 2 and 4 hours after treatment. Fold change values were calculated relative to respective media controls. Each individual dot represents a single biological replicate (n = 4/group). Statistics are calculated using a t-test or Wilcoxson test when appropriate. Significance was defined as $p<0.05$ and denoted by (*).

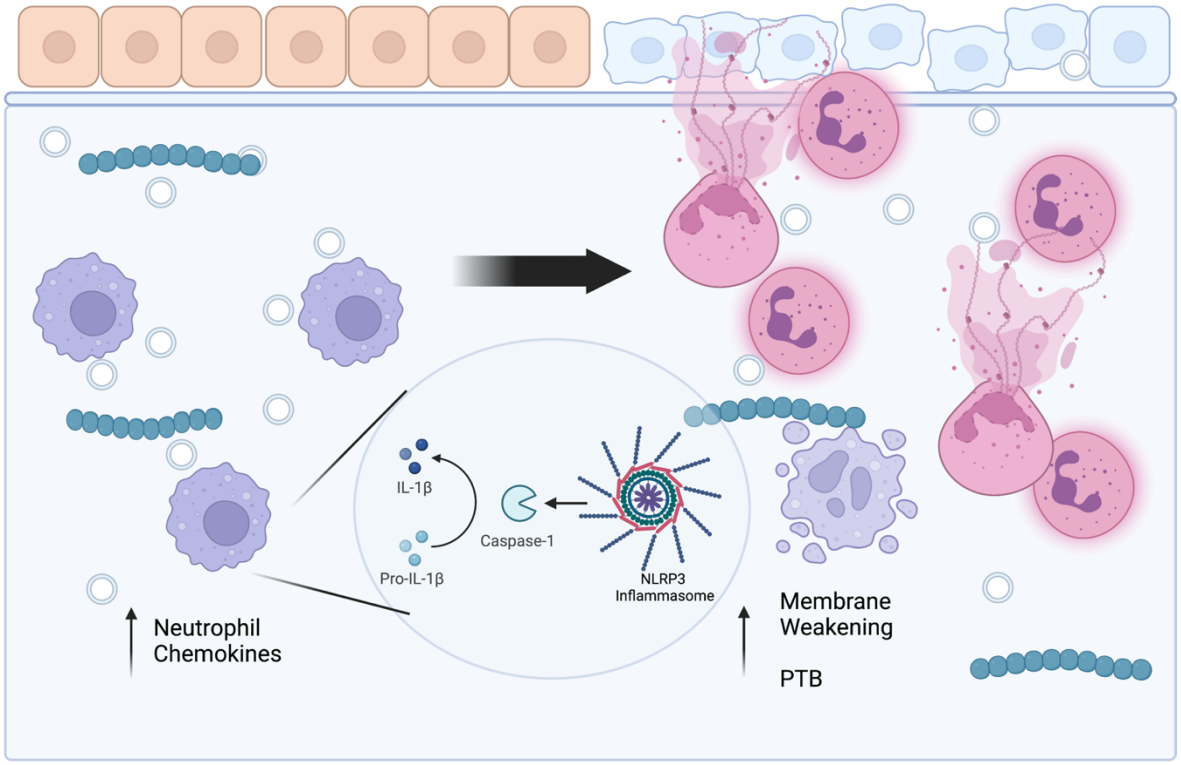

Figure 3.6: Model of GBS MV induced chorioamnionitis.

GBS is a frequent cause of chorioamnionitis. As sentinel cells at the maternal-fetal interface, macrophages play a critical role in shaping how inflammatory responses are initiated. We show here that macrophages respond to MVs by releasing proinflammatory cytokines and chemokines, many of which recruit neutrophils to the site of infection. Additionally, we show that MVs activate the NLRP3 inflammasome, triggering release of the pyrogen IL-1β. Together these processes promote an influx of neutrophils and leukocytes into the site of infection. In cases such as chorioamnionitis, neutrophils undergo processes including NET-osis, which promote tissue weakening and subsequent preterm birth. Taken together these findings demonstrate mechanistically how MVs may promote preterm birth and chorioamnionitis *in vivo*.

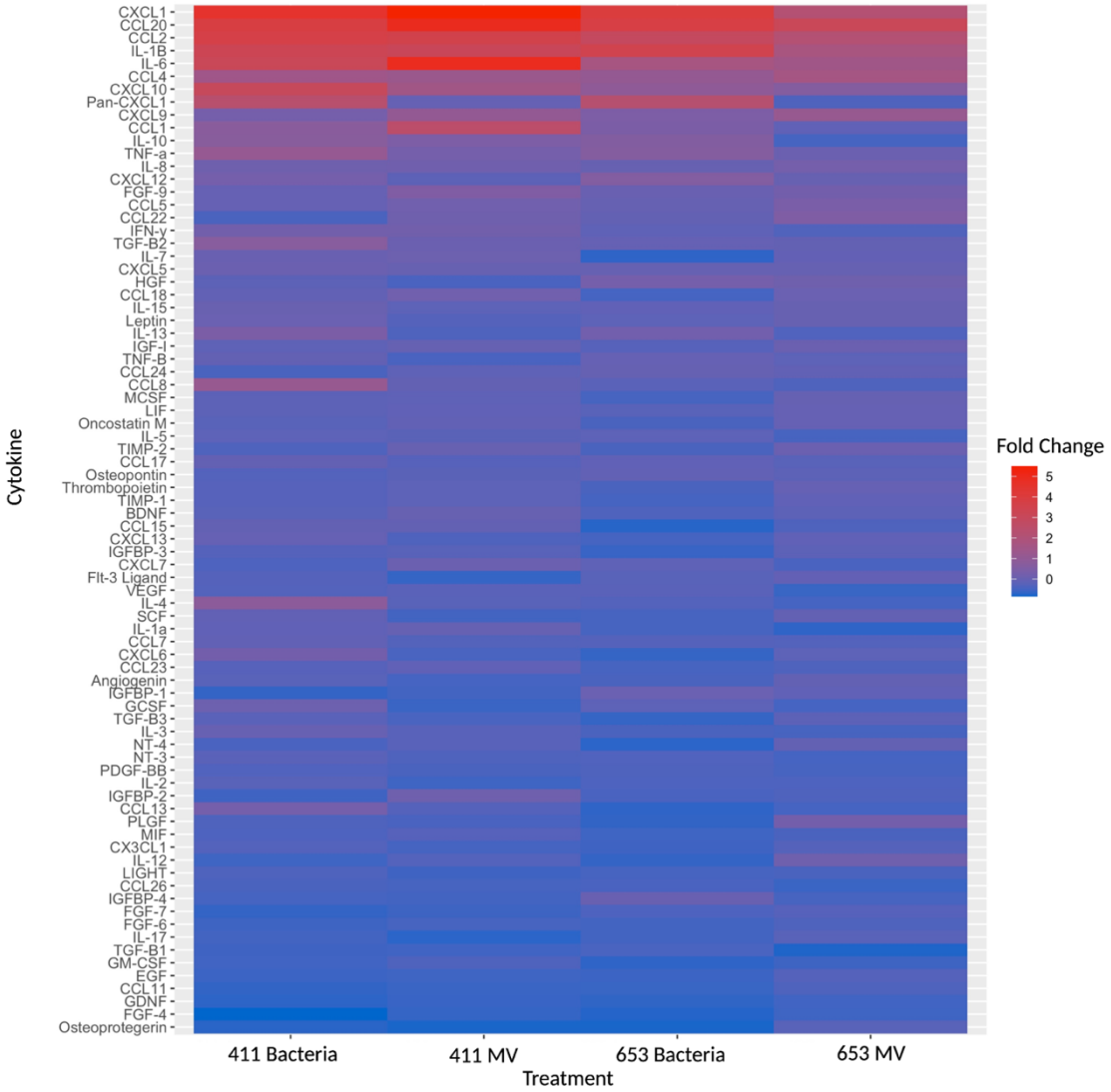

Figure S 3.7: Profiling of cytokine responses elicited towards MVs.

THP-1s were treated with bacteria (multiplicity of infection (MOI) = 10) or MVs (MOI 100) for 25 hours prior to supernatant collection. Cytokine production was analyzed using a human cytokine antibody microarray (Abcam). Shown here is semi-quantitative densitometry analysis (ImageJ) of cytokine production for all 80 cytokines examined. Color denotes fold change relative to untreated controls. All groups were performed in biological duplicate. Boxes indicate mean fold change for each condition.

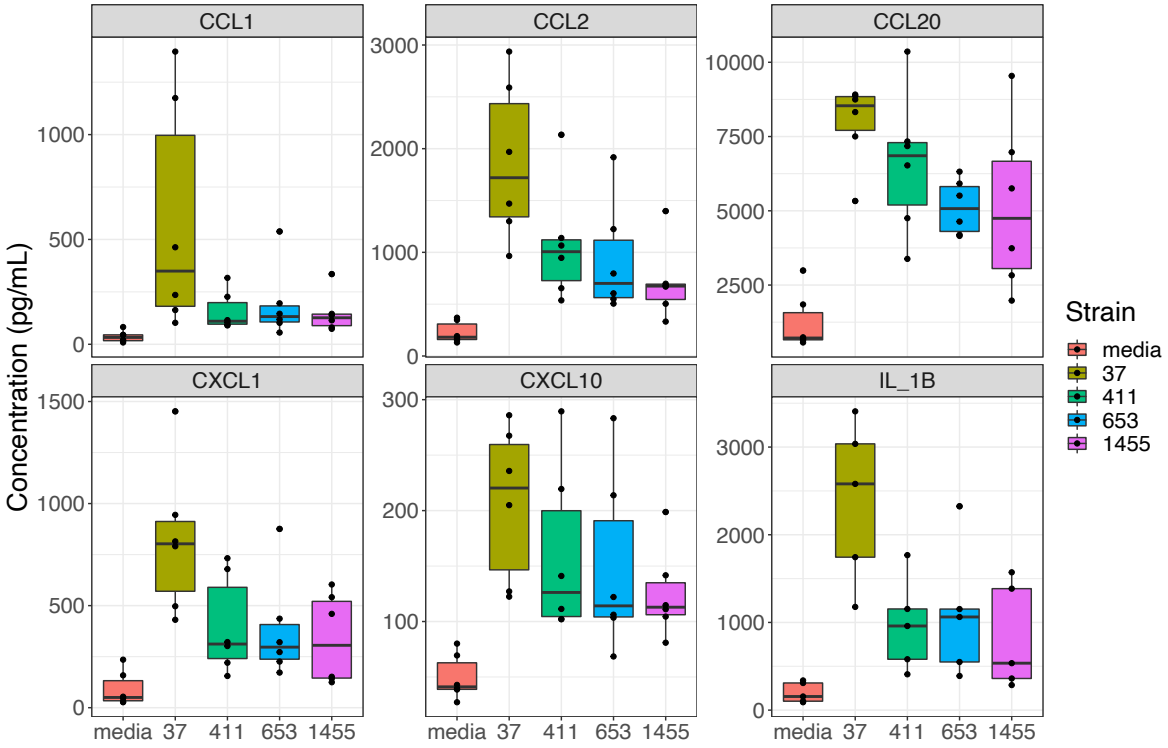

Figure S 3.8: Bacteria elicit proinflammatory immune responses from THP-1 macrophages.

Supernatants from THP-1 derived macrophages, which were untreated or treated with bacteria (MOI 10) for 25 hours were assessed for cytokine production using ProcartaPlex multiplex bead-based assays. Each black dot indicates a single biological replicate (n = 5-6 for each group). Data were analyzed by one-way ANOVA with a Tukey HSD post hoc test, or for non-parametric data, a Kruskal Wallis test with a Dunn Test post hoc test. *, $p < 0.05$ relative to untreated.

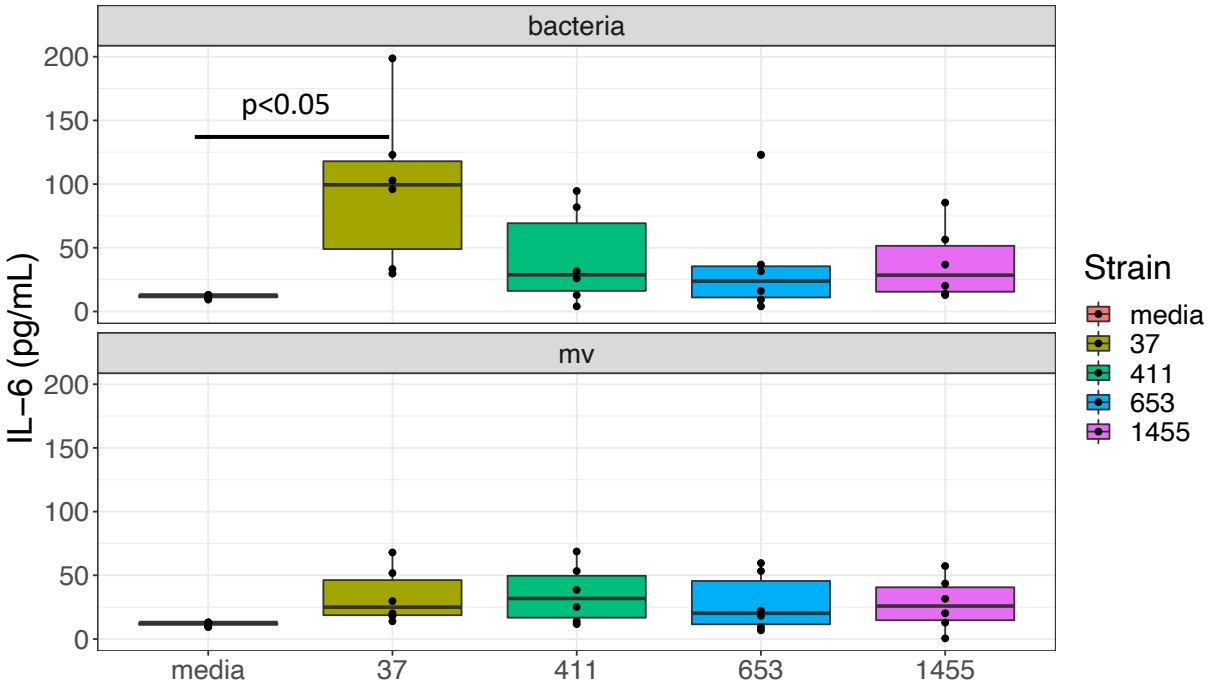

Figure S 3.9: IL-6 is not produced in response to GBS MVs.

Supernatants from unstimulated or MV-treated THP-1 derived macrophages were assessed for IL-6 using ProcartaPlex multiplex bead-based assays. Individual black dots indicate a single biological replicate (n = 5-6 for each group). Statistics were determined by one-way ANOVA with a Tukey HSD post hoc, or for non-parametric data, a Kruskal Wallis test with a Dunn post hoc test. All comparisons to mock were not significant unless noted with a specific p-value.

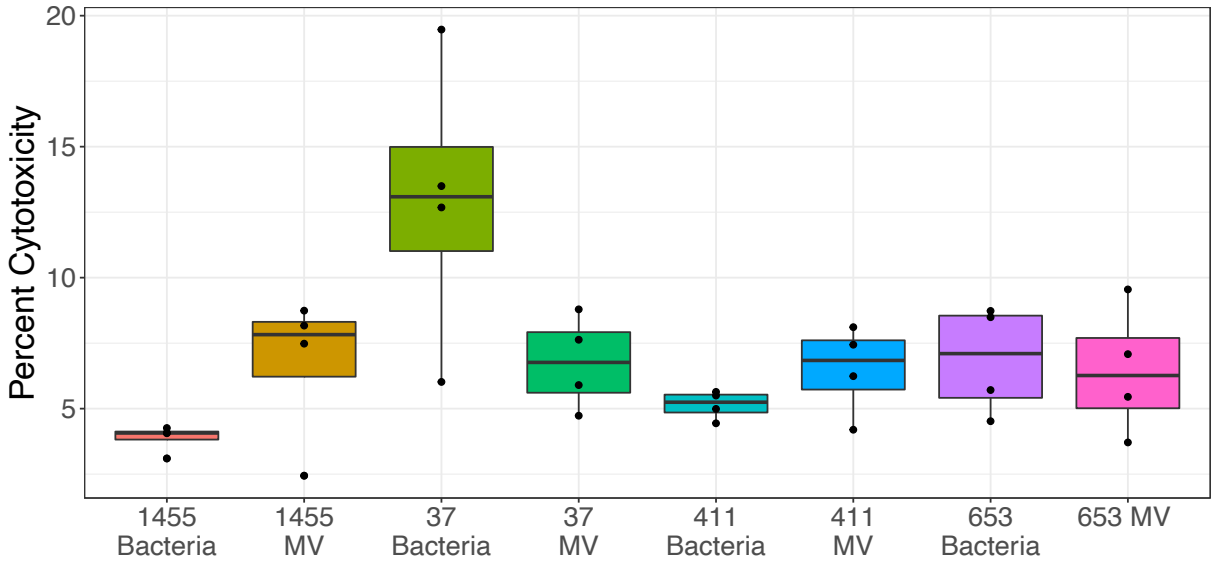

Figure S 3.10: MVs induce a low amount of cell death in THP-1 macrophages.

THP-1 derived macrophages were unstimulated or treated with MVs for 25 hours. Supernatants were assessed for cytotoxicity using the CyQuant LDH Assay. Percent Cytotoxicity is expressed as a percentage relative to untreated cells. Each black dot represents a single biological replicate (n = 4 /group). Data were anlalyzed using either a one-way ANOVA with a Tukey HSD post hoc test (MV treated groups), or a Kruskal Wallis test with a Dunn Test post hoc (Bacteria-treated groups). Significantly different comparison within groups (P-value < 0.05) are denoted with (*). All other comparisons were not significantly different.

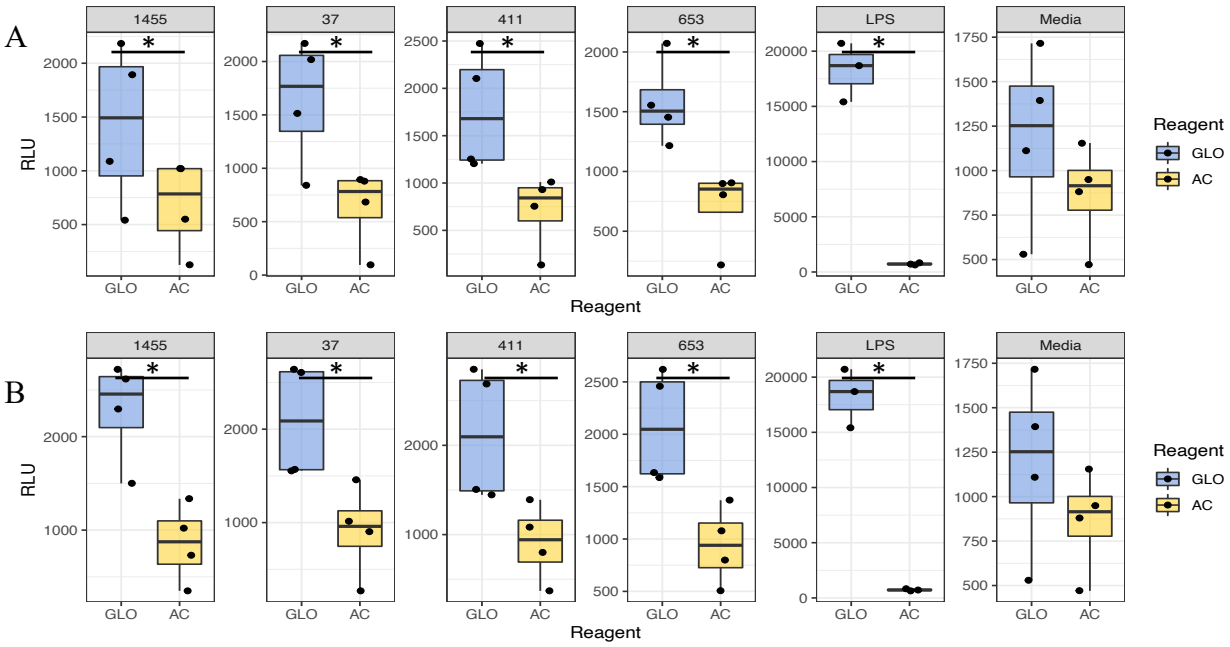

Figure S 3.11: MVs induce Caspase-1 activity.

Supernatants from THP-1 derived macrophages which were unstimulated or treated with bacteria, MVs for 25 hours. Alternatively, cells were stimulated with LPS for 2 hours. Supernatants were then assessed for caspase-1 activity using a caspase-1 GLO assay. A.) Activity from THP-1s treated with bacteria. B.) Activity from THP-1s treated with MVs. Relative light units (RLU) were determined using a GLO Max Navigator. Individual black dots indicate a single biological replicate (n = 3-4 for each group). Statistics were determined using a two-sided, paired t-test. P-value < 0.05 relative to mock treatment is denoted with a (*).

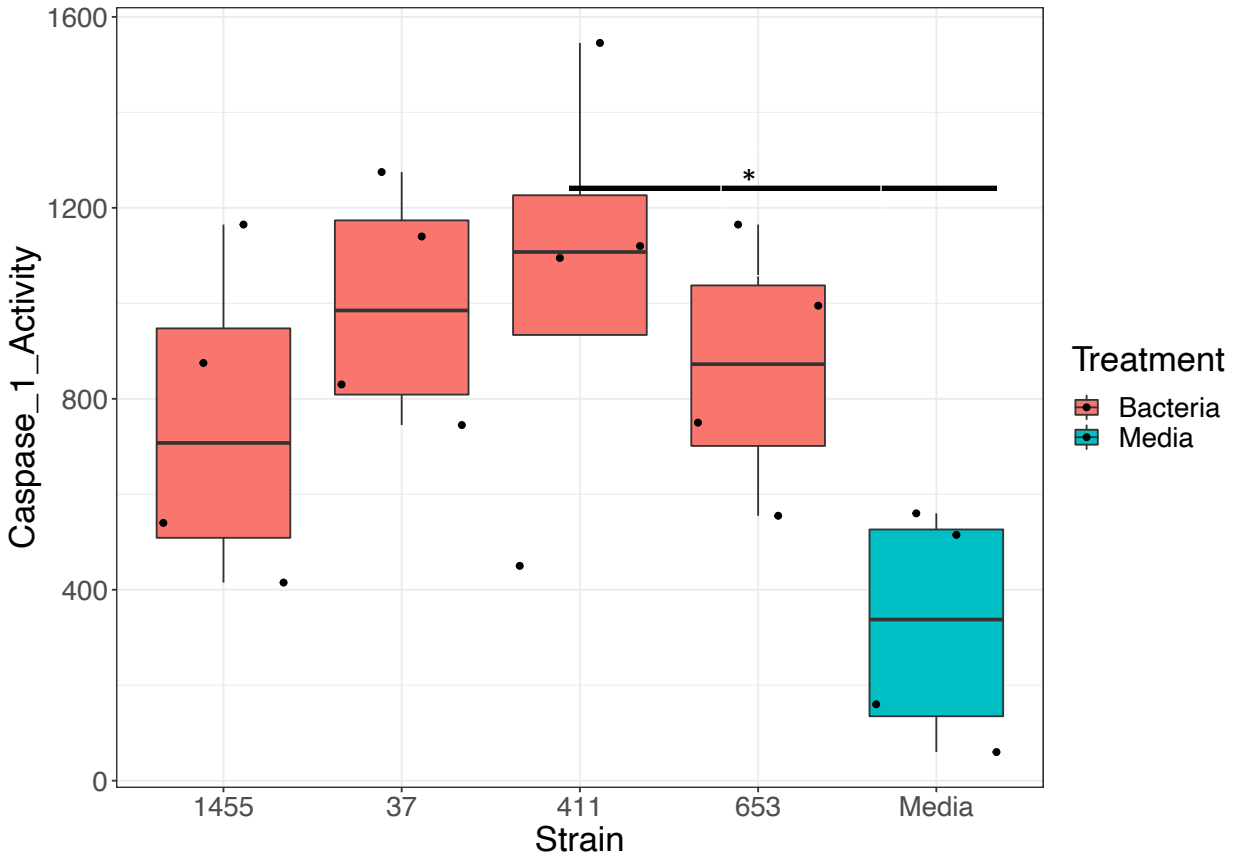

Figure S 3.12: Bacteria induce Caspase-1 activation in THP-1s.

THP-1 derived macrophages were unstimulated or treated with bacteria for 25 hours. Supernatants were then assessed for caspase-1 activity using a caspase-1 GLO assay. Data represent the amount of caspase-1 activity (caspase-1 activity = (RLU GLO) - (RLU AC)) from paired samples. Individual black dots indicate a single biological replicate (n = 4 for each group). Statistical significance is defined as $p<0.05$ as calculated by ANOVA with a Tukey post-hoc and indicated by (*).

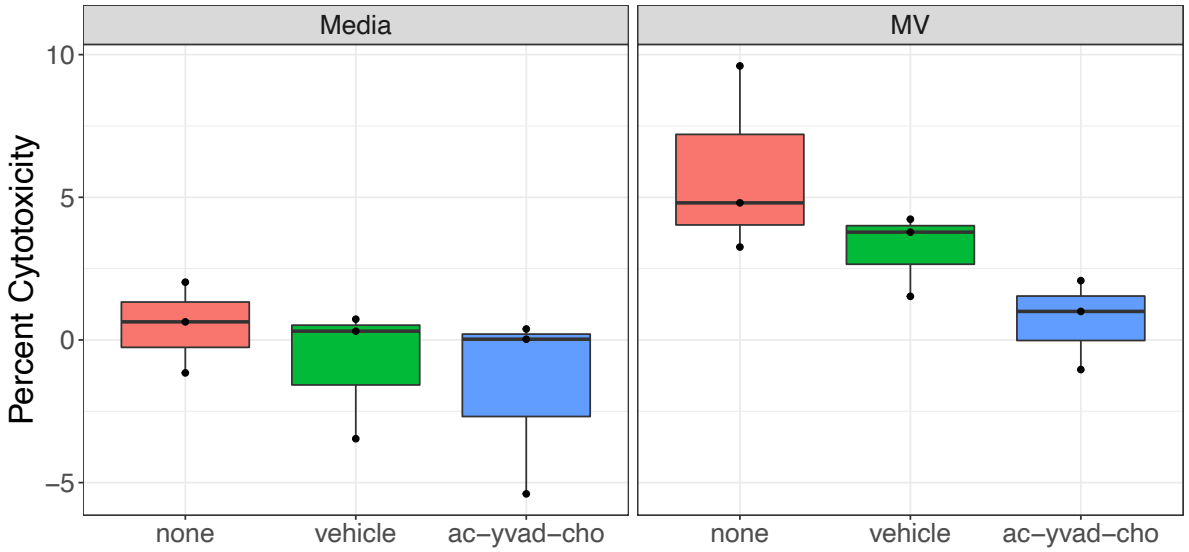

Figure S 3.13: Caspase-1 inhibition does not impact cell death responses.

THP-1s were untreated, treated with ethanol, or Ac-YVAD-CHO for 30 minutes. Supernatants from THP-1 derived macrophages, which were subsequently unstimulated or treated with MVs for 25 hours were assessed for cytotoxicity using the CyQuant LDH Assay. Individual black dots indicate a single biological replicate (n = 3 for each group). Statistics were determined using either an ANOVA with a Tukey HSD post hoc. No significant difference relative to non-pretreated cells were detected for either group.

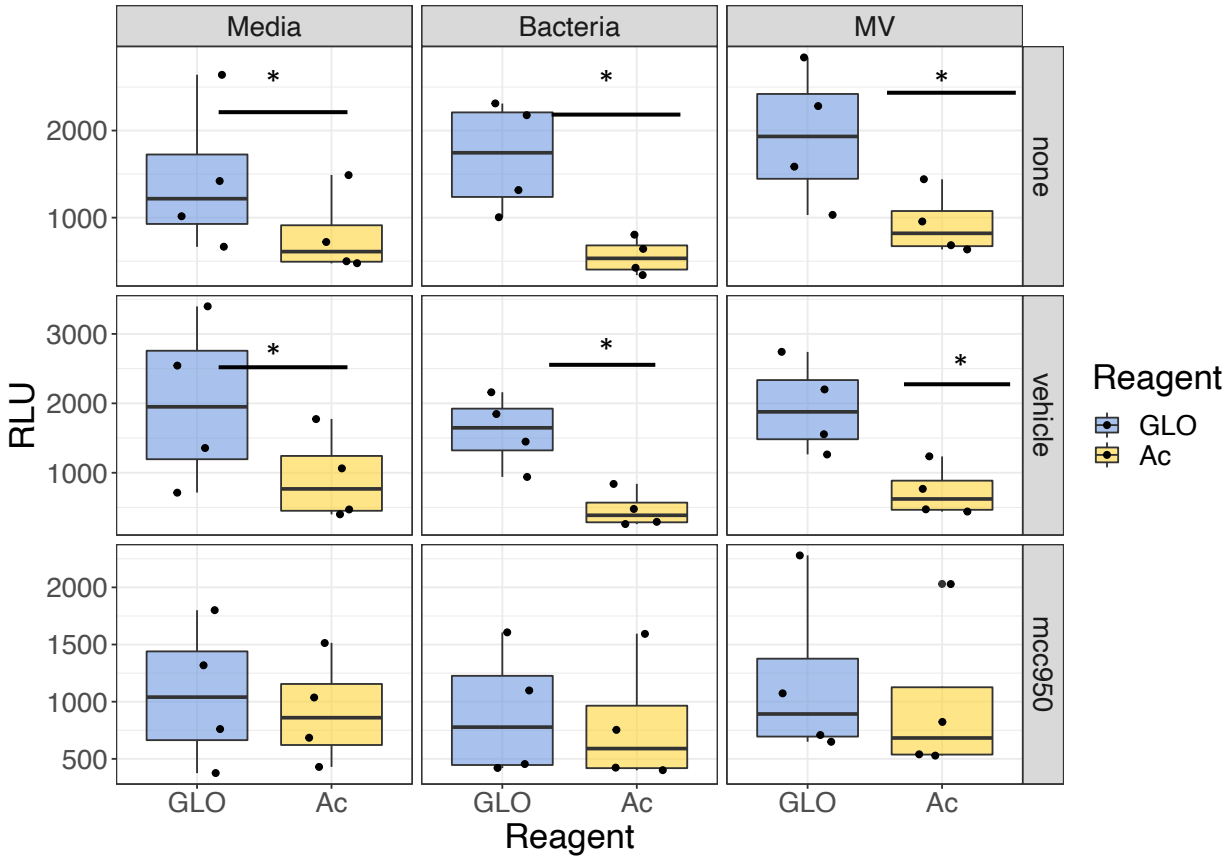

Figure S 3.14: Inhibition of NLRP3 prevents Caspase-1 Activation in Response to MVs. THP-1s were treated with the NLRP3 inhibitor MCC950 prior to treatment with bacteria, MVs, or media. Caspase-1 activity was determined using the Caspase-1 GLO assay. Individual points represent individual biological replicates (n = 4 each group). Statistics were determined using an ANOVA with a Tukey's HSD post-hoc test. Significance was defined as $p>0.05$ and denoted with an (*).

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CHAPTER 4

Group B Streptococcal Membrane Vesicles Do Not Impact Ascending Infection

ABSTRACT

Group B *Streptococcus* is an opportunistic pathogen that asymptomatically colonizes the vaginal tract of 30% of women. Numerous studies have shown that GBS can cause *in utero* infections resulting in chorioamnionitis, preterm birth, or fetal death. Currently, it is believed that GBS causes *in utero* infections by ascending past the cervix and subsequently infecting gestational tissues. While a growing number of studies have indicated that this process can occur *in vivo*, the specific bacterial factors that mediate ascension are not well characterized. Recent studies have shown, however, that GBS MVs can trigger preterm birth, choriodecidual inflammation, and fetal death *in vivo.* Additionally, we have shown that GBS MVs contain virulence factors that are known to promote ascending infection; hence, we sought to determine the role of MVs during ascending infection. Using a mouse model of ascending infection, we show that co-treatment of mice with MVs and bacteria does not appear to promote ascending infection or in utero inflammatory responses. Although previous reports demonstrated that intraamniotic administration of MVs leads to adverse pregnancy outcomes, we show here that vaginal administration of MVs has no effect on pregnancy success or inflammatory responses *in vivo.* Similarly, mice that were vaginally pretreated with MVs prior to infection display no changes in ascending infection. Taken together, these data suggest that GBS MVs do not substantially contribute to ascending infection in this murine model, and hence, they likely play a different role in commensalism and/or the disease process.

INTRODUCTION

Complications arising from preterm birth are a leading cause of death for children under the age of 5 (1). Current estimates indicate that roughly 1 in 10 live births are preterm, with approximately 40 percent of these preterm births being caused by *in utero* infections (2, 3). Many pathogens including Group B *Streptococcus* (GBS) are known to cause *in utero* infections (4, 5).

GBS is an opportunistic pathogen that asymptomatically colonizes the vaginal tract of approximately 30% of women (6). Elderly patients and neonates are at highest risk for invasive GBS infections, which typically present as sepsis or meningitis (5, 7). Recent studies have also indicated that GBS also causes nearly 57,000 fetal infections globally each year (5, 8). In addition, GBS can be found in about 5% of Caesarian-section-delivered placentas of healthy, term births (5, 8), suggesting that because *in utero* GBS infections are often subclinical, they are greatly underreported.

Several animal models have demonstrated that GBS causes *in utero* infections following ascension of the vaginal tract into the gravid uterus (9-11). In both murine and non-human primate models, *in utero* GBS infections are characterized by high bacterial burden and inflammation of gestational tissues that can lead to preterm birth (12, 13). Notably, GBS infections result in a high degree of neutrophil infiltration into infected tissues (13). Neutrophils and macrophages that encounter GBS undergo cell death by NET-osis and MET-osis (neutrophil and macrophage extracellular traps, respectively), which is characterized by extrusion of decondensed chromatin DNA and antimicrobial proteins (14). This form of cell death promotes inflammation and weakens tissues, thereby contributing to preterm birth (13, 15, 16). The

bacterial factors that promote ascending infection by this non-motile pathogen, however, remain relatively uncharacterized.

To date, only two bacterial virulence factors have been shown to play a direct role in GBS ascension from the vaginal tract into the uterus (9). The GBS hyaluronidase, HylB, promotes ascending infection through its immunomodulatory properties: by cleaving hyaluronan fragments into toll-like receptor (TLR)2/4 antagonists, GBS blocks proinflammatory signaling (17), averting an immune response that would otherwise clear the GBS. Indeed, hyaluronidase deletion mutants promoted reduced levels of uterine inflammation and bacterial load compared to controls, suggesting that this factor is critical for ascension (9).

The second factor known to promote ascending infection is the capsular polysaccharide biosynthesis enzyme, CpsE (11). This enzyme promotes the production of capsular polysaccharides in serotype V strains and aids in biofilm formation as well as evasion of phagocytosis. Dams infected intravaginally with a GBS *cpsE* knockout strain displayed reduced bacterial densities and damage of gestational tissues (11). While the mechanism that allows CpsE to facilitate ascending infection remains unclear, the ability to promote biofilm formation and immune evasion likely play critical roles.

GBS also modulates host processes to support its ability to cause ascending infection *in vivo.* By stimulating integrin signaling pathways, GBS promotes vaginal epithelial cell exfoliation, which in turn permits ascension (10). Likewise, when epithelial exfoliation is prevented, GBS is unable to ascend (10). While this mechanism contrasts with that of other pathogens (18), it is believed that by promoting vaginal exfoliation, GBS can invade deeper into the vaginal tissue to cause a more robust ascending infection (10). This suggests that while GBS

possesses specific bacterial factors that can promote ascending infection, it can also alter the host environment to facilitate its pathogenesis.

Although many other GBS virulence factors are known, few have been shown to directly impact ascension. Several studies have shown that GBS produces membrane vesicles (MVs) that contain virulence factors, including HylB (17, 19). When injected directly into the amniotic cavity of pregnant mice, MVs alone caused preterm birth and intrauterine fetal death, suggesting they contribute to pathogenesis *in vivo* (20). Interestingly, while models of neonatal sepsis show that hemolytic MVs can exacerbate disease, no prior studies have examined whether MVs contribute to ascending infection (21).

Here, we hypothesized that MVs play a role during ascending infection. To test this, we used a model of ascending vaginal infection of pregnant mice, co-treating with exogenous MVs and bacteria. Additionally, we examined whether vaginally-administered MVs contribute to host responses *in utero.* We found that GBS MVs do not appear to impact ascending infection or inflammatory responses at the maternal-fetal interface. These data suggest that MVs may play a role in alternative disease states rather than ascension.

MATERIALS AND METHODS

Strains and Media

GBS strains GB0037 (GB37) and GB0411 (GB411) were isolated as previously described, with names abbreviated for clarity (22). Both GB37 and GB411 were recovered from the blood of neonates with early onset disease and were previously characterized by multilocus sequence typing (MLST) and capsular serotyping as described (23, 24). GB37 is a sequence type (ST)-1 strain, whereas GB411 belongs to ST-17. Strains were cultured using Todd-Hewitt Broth (THB) or Todd-Hewitt Agar (THA) (BD Diagnostics, Franklin Lakes, NJ, USA) overnight at 37°C with 5% CO_2.

Membrane Vesicle (MV) Isolation and Purification

Isolation and purification of MVs was performed as we described previously (19). Following overnight growth in THB, GBS cultures were diluted into fresh THB and grown to late logarithmic phase. Cultures were centrifuged at 2000 x g for 20 minutes and supernatants re-centrifuged at 8500 x g for 15 minutes. Supernatants were filtered through a 0.22µm filter, concentrated using Amicon Ultra-15 centrifugal filters (10kDa cutoff) (MilliporeSigma, Burlington, MA, USA), and ultracentrifuged for 2 hours at 150,000 x g at 4°C. Pellets were resuspended in phosphate buffered saline (PBS), purified using qEV Single size exclusion columns (IZON Science, Christchurch, New Zealand), and re-concentrated using Amicon Ultra-4 centrifugal filters (10 kDa cutoff) (MilliporeSigma, Burlington, MA, USA). Aliquots were stored at -80°C until use.

Model of Ascending Infection

Murine experiments were approved in accordance with the Michigan State University Institutional Animal Care and Use Committee (protocols 201800176 and 202200021). Mice were housed in a temperature controlled, 12:12h light/dark cycle room. Timed matings were performed using 6-10 week old C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME, USA); the presence of a copulatory plug was considered gestational day (GD) 0.5. The murine model of ascending infection was adapted from previous publications (11, 13). On GD 13.5 or 15.5 (when indicated), mice were sedated using 3% isoflurane infused with oxygen at a flow rate of 2 L/min, challenged intravaginally with 10^5 colony forming units (CFU) of GBS, 2 x 10^9 MVs, or 10^5 CFU GBS plus 2 x 10^9 MVs. All treatments were suspended in 25uL of PBS containing 10% gelatin and pipetted into the vaginal tract using a P200 micropipette. After challenge, the mouse hindquarters were elevated for 5 minutes to ensure that treatments were absorbed, and mice were subsequently returned to their cages. Infected dams were monitored every 12 hours for signs of distress or preterm birth (e.g., hunched posture, non-responsiveness, vaginal discharge, or the presence of pups in the cage). Mice were sacrificed at 48 or 72 hours post infection, or as soon as when signs of preterm birth or distress were observed.

Tissue Homogenization and Bacterial Enumeration

Mice were euthanized using isoflurane and subsequent cervical dislocation. Fetuses and placentas were removed and weighed prior to CFU determination. To enumerate CFUs, fetuses or placentas were transferred to tubes containing 2.8mm ceramic beads (Omni international, Kennesaw, GA, USA) and 1mL of PBS, and were homogenized using an OMNI Bead Ruptor 12 (Omni international, Kennesaw, GA, USA) at a speed of 5m/s for 30 seconds. Homogenates

were serially diluted in PBS, plated onto THA using glass beads, and incubated overnight at 37°C with 5% CO_2. CFUs were determined by counting plates with 20-200 colonies and normalizing to the weight of each tissue.

Flow Cytometry

After the mice were euthanized, the uterine horns were dissected from the body cavity, and the fetal and placental tissues removed. One uterine horn was used for flow cytometric analysis as previously described (25). Briefly, uterine tissues were minced and incubated in digestion buffer containing RPMI 1640, 0.05% Liberase TH (Roche Diagnostics Corp., Indianapolis, IN, USA), and 100U/mL DNase1 (Sigma-Aldrich, St. Louis, MO) for 1.5-2 hours at 37°C with 5% CO_2. Throughout the digestion incubation, tissues were periodically passed through a 27-gauge needle to facilitate digestion. After incubation, the cells were passed twice through a 70μm nylon cell strainer (Falcon, Tewksbury, MA) to remove large tissue fragments. Tissue digestion was quenched using flow staining buffer (FSB; 2% fetal bovine serum and 0.01% sodium azide in PBS). Cell suspensions were pelleted at 500 x g for 5 mins and the red blood cells were lysed using Ammonium-Chloride-Potassium (ACK) lysis buffer (150mM NH_4, 10mM $KHCO_3$, 0.1mM Na_2EDTA, in distilled water). Cells were resuspended in FSB and aliquoted into U-bottom 96 well plates.

For staining, cells were first incubated with Live/Dead Blue stain (Thermofisher) followed by Fc Block (BioLegend®, San Diego, CA, USA). Cell-surface staining was performed with anti-mouse CD45-BUV395 (clone 30-F11), CD11b-Percp-Cy5.5 (clone M1/70), CD11c-PE (clone N418), Ly6C-BV510 (clone HK1.4), Ly6G-BV711 (clone 1A8), CD64-FITC (clone X54-5/7.1), CD24-APC (clone M1/69), and F4/80-APC-Cy7 (clone BM8), all of which were

purchased from Biolegend®. MHCII-ef450 (clone M5/114.15.2; Invitrogen, Waltham, MA, USA) was also used. Cells were washed twice and fixed in 1% paraformaldehyde (PFA) diluted in FSB. Spectral flow cytometry was performed using a Cytek Aurora spectral cytometer (Cytek BioSciences, Fremont, CA) with eUltracomp compensation beads for single color controls. 100,000-200,000 events were collected for each well and analysis was performed using Kaluza 2.1 software (Beckman Coulter, Brea, CA). Gating strategy was performed as described previously (26).

Data Analysis and Statistics

Data were analyzed, plotted using R (v1.4.1106), and assessed for normality using a Shapiro-Wilk test. For experiments involving two comparisons, data were analyzed by the Mann-Whitney test. Data in experiments involving multiple comparisons were analyzed by ANOVA with a Tukey HSD post-hoc test. Significance was defined as $p < 0.05$.

RESULTS

Bacterial dosage and timing of infection impact pregnancy outcomes

Previous studies have used a variety of doses and gestational ages in ascending GBS infection models (9, 11, 13). Therefore, we performed a pilot experiment to assess the impact of gestational age and inoculum dose on pregnancy. Dams were vaginally inoculated on GD13.5 with 10^3, 10^5, or 10^8 CFU, or on GD15.5 with 10^8 CFU of GBS and euthanized 72 hours after inoculation or when signs of distress were observed, whichever came first. At both gestational ages, intravaginal inoculation caused ascending infections. Strikingly, dams inoculated with the highest dose of GBS on either GD13.5 or GD15.5 developed intrauterine infection but responded to infection in dramatically different ways. Those infected later remained healthy (n=2), whereas those inoculated earlier either succumbed to infection (n=1) or expelled fetuses preterm (n=1) (Figure 4.1).

When inoculated on GD13.5, GBS dose impacted rates of ascending infection, preterm birth, and maternal mortality, as well as timing of onset of disease. Increasing doses of GB37 induced a higher frequency of dams exhibiting preterm birth with detectable GBS in gestational tissues (Figure 4.1). While the lowest dose (10^3 CFUs) resulted in preterm birth in 25% of infected dams, 75% of dams had no detectable CFUs in gestational tissues. In contrast, the highest bacterial dose (10^8 CFUs) caused preterm birth or death in 100% of the animals; in both animals of this group, the bacteria induced severe maternal sepsis and mortality within 36-48 hours of infection. Due to the rapid onset of symptoms and concerns about animal welfare, we were unable to collect tissues for downstream analyses and discontinued this dosage. At a dose of 10^5 CFUs, GBS induced maternal mortality in 50% of dams, and in one of four dams, the onset of preterm birth occurred between 60 and 72 hours after inoculation. Finally, one of the

four mice at this dose exhibited no morbidity, despite a detectable infection in gestational tissues. Given these data, we reasoned that infection with 10^5 CFU would allow us to effectively model the effects of GBS MVs on preterm birth and ascending infection.

Vaginally administered MVs do not impact ascending infection

To determine whether GBS-derived MVs affected ascending infection of GBS *in vivo,* we used the invasive GB411 strain while reasoning that this strain would contribute to lower mortality rates than was observed with GB37. We established four experimental groups: 1) PBS control, 2) MVs (dosage of 2×10^9 MVs), 3) GBS (dosage of 1×10^5 GBS), 4) MVs (dosage of 2×10^9 MVs) + GBS (dosage of 1×10^5 GBS). Dams were inoculated intravaginally on GD13.5 and sacrificed after 48 hours. As an indicator of maternal or fetal wastage, we first assessed maternal, fetal, and placental weight. We found no alteration in maternal weight in the 48 hours following inoculation (Figure 4.2). Similarly, none of the treatments caused changes in placental or fetal weights (Figure 4.3).

To determine whether MV impacted uterine or fetal bacterial load, we measured CFU in fetuses and placentas following infection. While slight decreases in bacterial load were found for the MV + GBS group compared to our GBS group, these differences were not statistically significant (Figure 4.4). Taken together, these data suggest that GBS MVs did not impact GBS ascension in this model.

Vaginally administered MVs do not alter in utero inflammatory responses

Although GBS MVs did not directly contribute to ascending infection, we sought to determine whether they induce an inflammatory response at the maternal fetal interface. To test this, we examined total leukocytes, eosinophils, monocytes, macrophages, and neutrophils by flow cytometry in control and treated mice (Figure 4.5). As expected, GBS infected dams displayed a high degree of leukocyte infiltration, including an abundance of neutrophils, compared to PBS treated controls. Interestingly, GBS dramatically reduced the proportion of eosinophils in the uterus. However, MVs alone failed to induce alterations in immune cell populations. Additionally, MVs did not alter uterine leukocyte proportions when co-inoculated with GBS. These results suggest that MVs do not alter immune cell populations at the maternal fetal interface in this model (Figure 4.5).

Pretreatment with MVs does not impact ascending infection

The co-treatment model suggests that MVs may decrease ascending infection; however, we found no statistical difference between mice treated with bacteria alone and mice treated with bacteria and exogenous MVs. (Figure 4.4). Because the MVs may need more time to exert effects in this model system, we pretreated mice vaginally with MVs (2×10^9) or PBS on GD12.5 followed by GBS challenge on GD13.5. Mice were sacrificed 48 hours later. Pretreatment with MVs did not alter maternal weight (Figure 4.6), fetal weight, or placental weight relative to PBS-pretreated controls (Figure 4.7). Despite observing no significant difference in bacterial load in the fetuses of the PBS and MV pretreated dams, the bacterial load in placentas decreased by 51% in mice with MV pretreatment; however, this difference was also not statistically significant (Figure 4.8).

DISCUSSION

Previous studies have highlighted the detrimental effects of GBS MVs on pregnancy outcomes and neonatal health; however, no study has examined the impact of MVs on ascending infection (20, 21). We report here that an overabundance of MVs at the time of vaginal infection does not impact bacterial ascension or inflammatory responses at the maternal-fetal interface using the models presented here. While our study has not demonstrated any significant alterations in ascension with MV supplementation, we did observe a slight decrease in bacterial ascension when mice were pretreated with MVs prior to infection. These data suggest that in our models of ascending infection, MVs play a minimal role during ascending infection.

Three factors have been shown to promote ascending GBS infections *in vivo.* One of these factors, hyaluronidase, we previously found to be present in GBS MVs at a high level (9). This factor promotes ascending infection by cleaving proinflammatory hyaluronic acid fragments components into TLR2/4 antagonists, ultimately preventing host recognition of infection (9, 17). We found that although MVs contain hyaluronidase, supplementing GBS with additional MVs during infection does not promote ascending infection (19). Notably, we did not observe any alterations in ascending infection when MVs were supplemented prior to infection or during infection, further suggesting that MVs do not play a prominent role in ascending infection in our model system. It remains possible that MV-derived hyaluronidase is inactive. If inactive, this could explain the lack of higher levels of ascending infection. Further studies are therefore necessary to demonstrate that MV-derived virulence factors are biologically active.

Previous studies have indicated that GBS MVs can exacerbate disease in both neonatal models of infection and *in utero* infection models (20, 21). Bolus doses of MVs into the amniotic cavity of pregnant mice results in severe pregnancy complications, with most pregnancies

resulting in fetal death or preterm birth (20). Similarly, injecting MVs together with live GBS resulted in higher levels of neonatal death compared to bacteria alone (21). The data from these experiments combined with those generated in our prior study (Chapter 3) indicate that GBS MVs can induce potent inflammatory responses that can lead to increased morbidity and mortality. Nonetheless, direct injection into the amniotic fluid bypasses numerous defenses normally present during GBS infections, including those found in the extraplacental membranes and the cervix. Our results suggest that these barriers may be sufficient to block MV-induced inflammation at the maternal-fetal interface. While bacterial infection resulted in a pronounced increase in neutrophil and leukocyte infiltration, no significant differences in leukocyte infiltration were observed between mice infected with GBS alone and mice infected with GBS and MVs. This finding suggests that MVs present in the vaginal tract do not contribute to inflammation at the maternal-fetal interface. It remains unclear, however, if vaginally administered MVs can ascend into the gravid uterus, which could prevent any potential effects at the maternal-fetal interface. Therefore, additional studies are warranted to assess MV ascension *in vivo*.

While the findings of our study are important, limitations do exist. Notably, our study is underpowered. This is exacerbated by high variability in infection phenotypes, low sample size, and small effect size. Higher numbers of replicates are therefore warranted to fully rule out any effects of MVs during ascending infection. While it is unclear why infection phenotypes are so variable, several factors may impact these outcomes. Microbiome, host variation, and sex-based differences have all been shown to impact infection phenotypes *in vivo* (27-30). Additionally, our analysis of the inflammatory was not exhaustive. It remains possible that MVs could be affecting other tissues including the vaginal tract, cervix, or the gestational membranes.

Similarly, it remains possible that MVs alter gene expression in non-immune cells, leukocytes, or both. Due to the lack of an observable effect on pregnancy outcome, fetal, or maternal morbidity or mortality in this model, other models may need to be developed to address these questions.

Another critical limitation is that we currently do not have access to a strain of GBS that does not produce MVs. While some have shown that in Group A Streptococcus deletion of the *covRS* homolog *csrRS*, results in reduced MV production, these mutants display highly irregular gene expression (31, 32). This is largely because CovRS is a two-component regulatory system that regulates over 100 genes in GBS (31). Therefore, it is difficult to segregate whether effects of virulence defects are due to a lack of MV production or to altered expression of other virulence genes. Therefore, we relied on supplementing GBS with an overabundance of MVs to observe possible additive effects. While it is unknown if GBS produces MVs during infections, it is likely that dams treated only with GBS have endogenous production of MVs, which could confound our conclusions if endogenously produced MVs contribute to ascension.

Our data indicate that although GBS MVs play important roles in other models of infection, they do not impact ascending infection in our model. We demonstrate that neither co-treatment or pre-treatment with GBS contributes to ascending vaginal infections *in vivo*. Furthermore, we show that MVs alone do not promote immune cell recruitment *in utero,* suggesting that vaginally produced MVs do not affect immune responses at the maternal fetal interface.

FIGURES

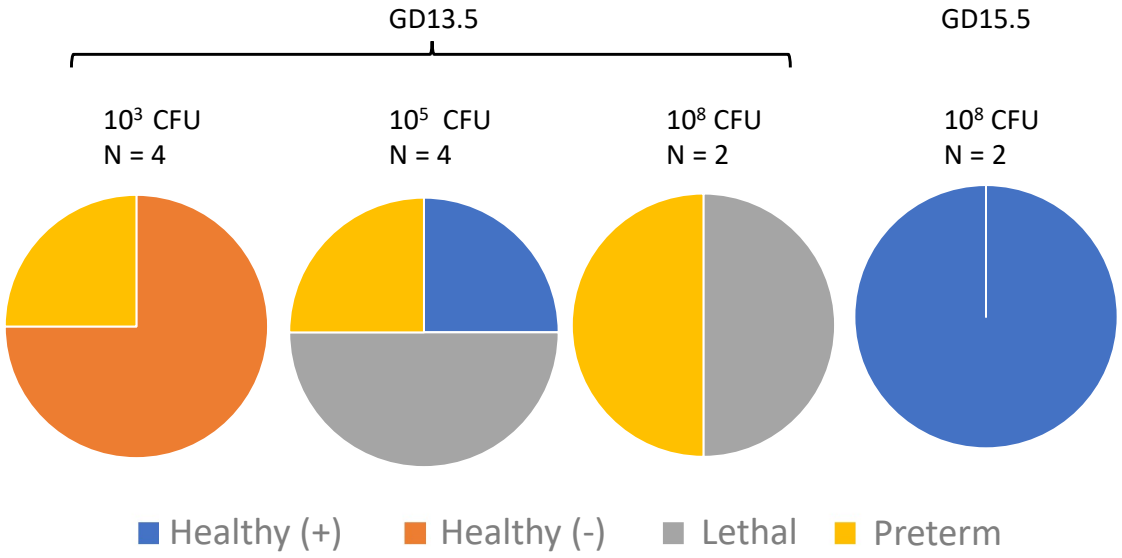

Figure 4.1: Both GBS dose and day of intravaginal infection impact maternal mortality and pregnancy outcome.

Pregnant dams were infected intravaginally on either gestational day (GD) GD13.5 or GD15.5 with 10^3, 10^5, or 10^8 CFU of GBS on GD 13.5, or with 10^8 CFU on GD15.5. Dams were euthanized either 72 hours after infection, or when signs of distress were observed, whichever came first. When possible, gestational tissues were assessed for bacterial load. Healthy (+): Healthy dams with detectable GBS in either maternal or fetal tissue; Healthy (-): Healthy dams with no detectable GBS in maternal or fetal tissues; Lethal: dams that succumbed to infection; Preterm: dams who delivered preterm.

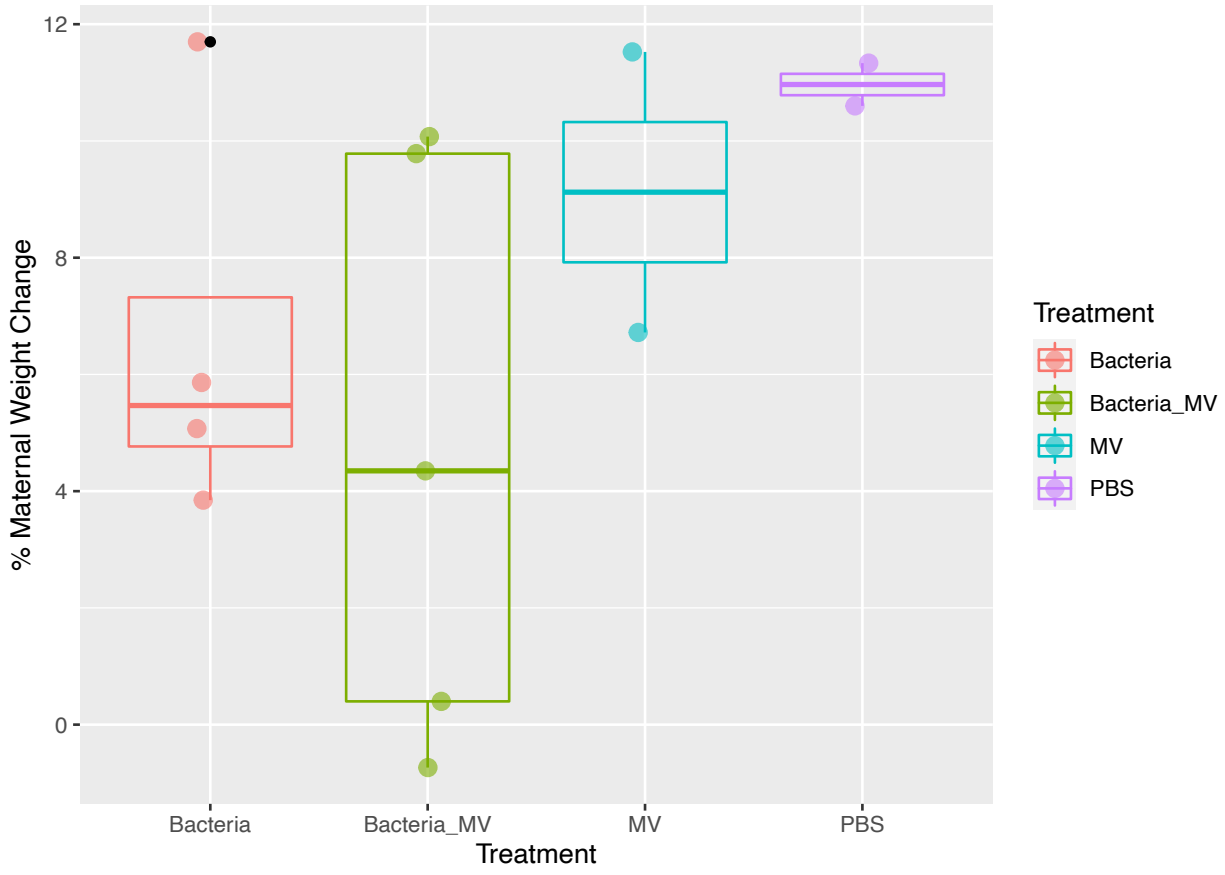

Figure 4.2: Intravaginal inoculation with bacteria or membrane vesicles (MVs) do not alter maternal weight gain.

Dams were infected on GD13.5 with PBS (n = 2), MVs (n = 2), GBS (n = 4), or GBS+MVs (n = 5) and sacrificed on GD15.5. Maternal weights were weighed prior to infection (GD13.5) and at sacrifice (GD15.5). Weight change was calculated relative to weight on GD13.5. Individual points indicate a single biological replicate. Statistics were performed using a one-way ANOVA followed by a Tukey HSD post-hoc test. Significance was set at $p < 0.05$. No significant differences were observed.

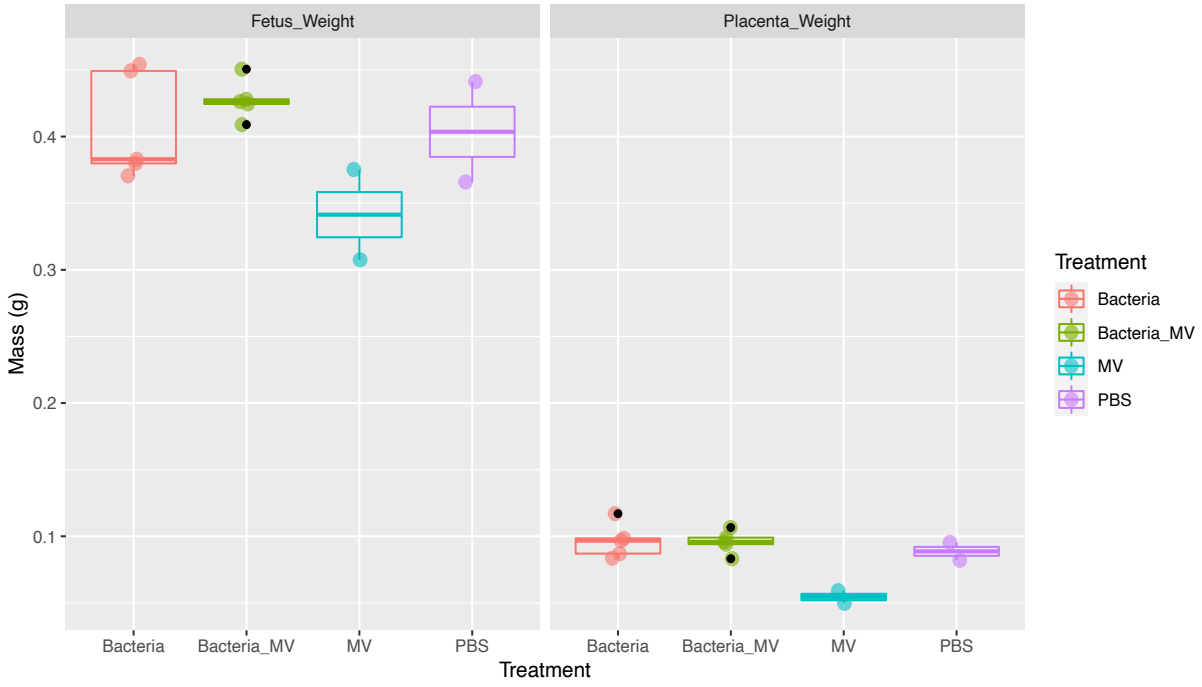

Figure 4.3: Membrane vesicles (MVs) do not alter gestational tissue weight

Dams were infected on GD13.5 with PBS (n = 2), MVs (n = 2), GBS (n = 5), or GBS+MVs (n = 5) and sacrificed on GD15.5. Fetal and placental weights of the fetus closest to the vaginal tract are displayed. Individual points represent a single biological replicate. Statistics were performed using a one-way ANOVA followed by a Tukey HSD post-hoc test. Significance was set at $p < 0.05$. No significant differences were observed.

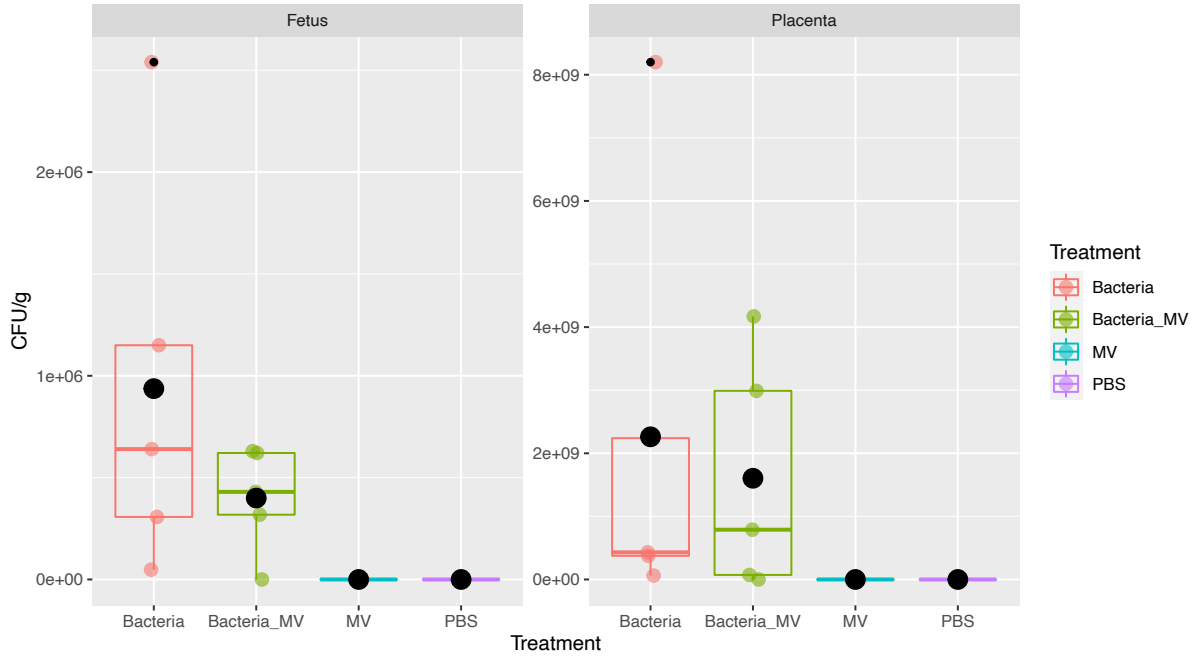

Figure 4.4: Membrane vesicles (MVs) do not alter ascending infection.

Dams were infected on GD13.5 with (n = 2), MVs (n = 2), GBS (n = 5), or GBS+MVs (n = 5) and sacrificed on GD15.5. The first fetus and placenta closest to the vaginal tract were dissected, homogenized, and plated onto THA for bacterial enumeration. CFUs were normalized to total tissue weight. Individual colored points indicate a single biological replicate, while large central black dots indicate mean CFU/g for a treatment group. Statistics were performed using a Mann-Whitney test between GBS treated and GBS + MVs group with significance set at $p < 0.05$. No significant differences were observed.

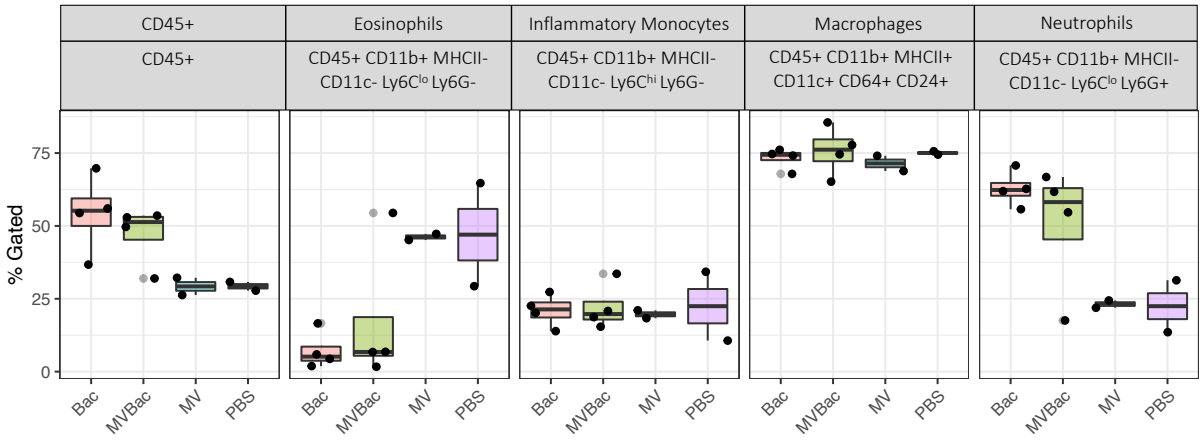

Figure 4.5: Membrane vesicles (MVs) do not alter inflammatory responses at the maternal-fetal interface

Dams were infected on GD13.5 with PBS (n = 2), MVs (n = 2), GBS (n = 4), or GBS+MVs (n = 4) and sacrificed on GD15.5. Uterine horns were dissected, digested, and stained for flow cytometry. Major cell types analyzed are shown by plot headings. Individual black dots represent an individual biological replicate.

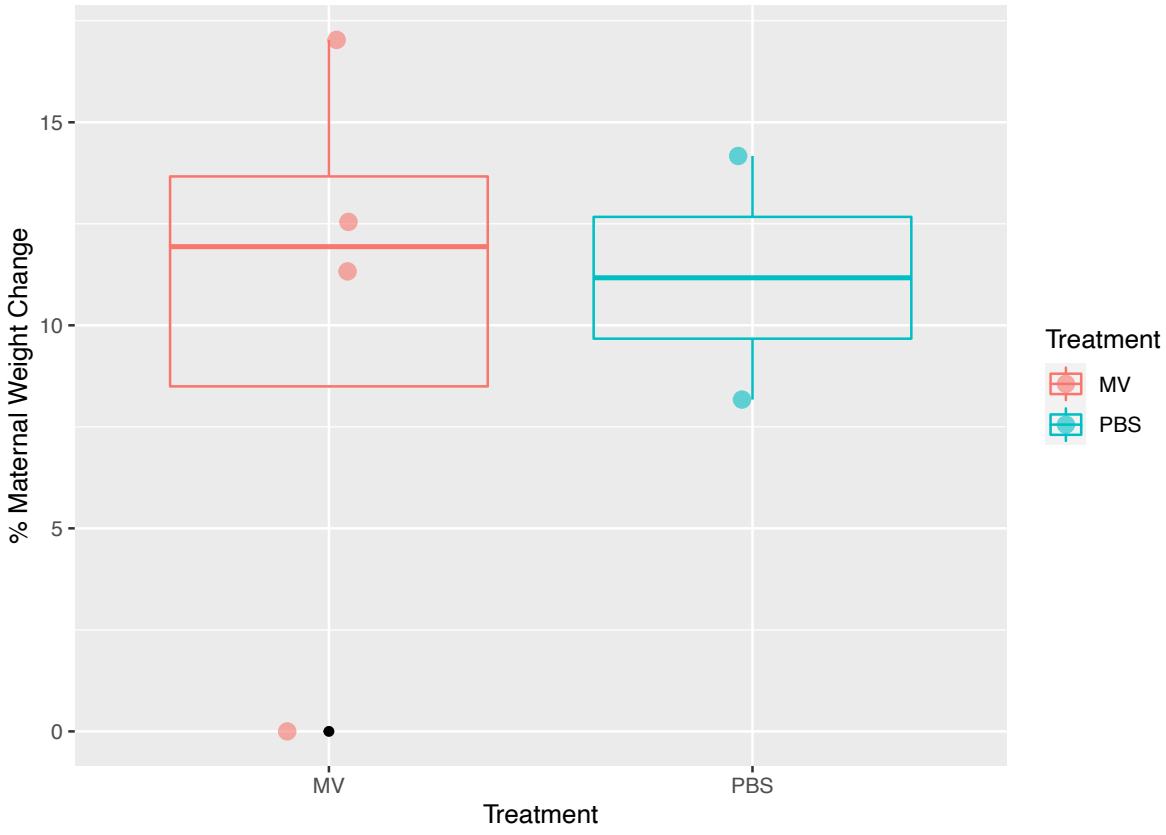

Figure 4.6: Pretreatment with MVs does not alter Maternal Weight

Pregnant dams were pretreated with MVs (n = 4) or PBS (n = 2) on GD12.5 and subsequently infected with GBS on GD13.5. Dams were sacrificed on GD15.5. Maternal weight change was calculated relative to GD12.5 weights. Individual biological replicates are indicated by colored dots.

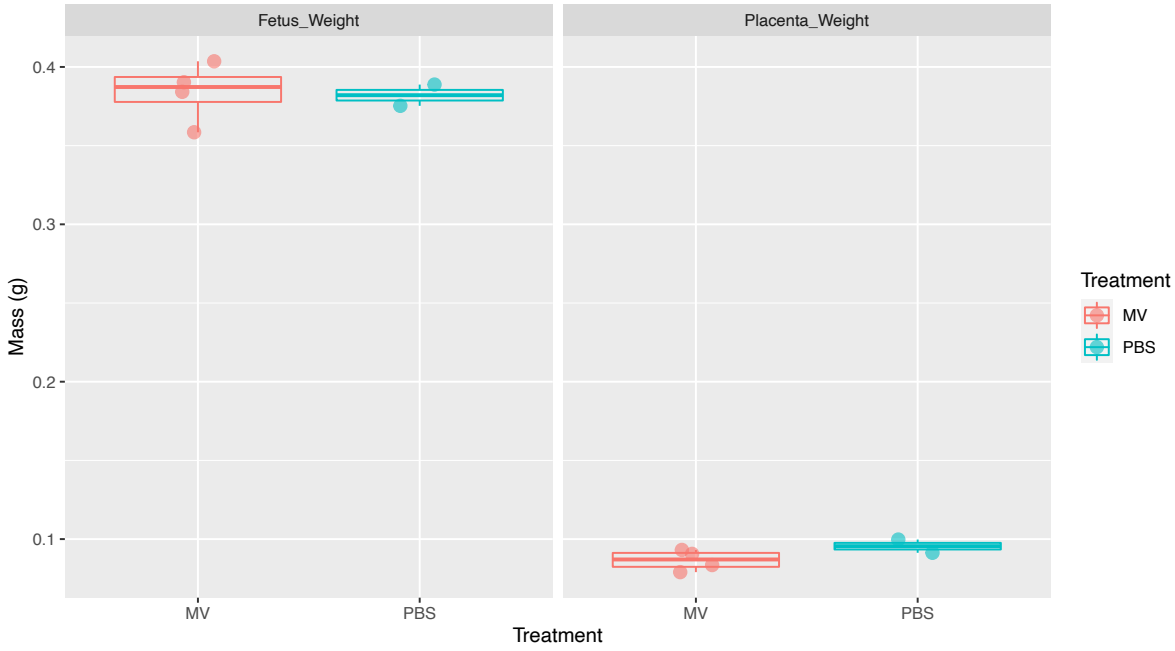

Figure 4.7: Pretreatment with MVs does not alter Fetal or Placental Weight

Pregnant dams were pretreated with MVs (n = 4) or PBS (n = 2) on GD12.5 and subsequently infected with GBS on GD13.5. Dams were sacrificed on GD15.5. Fetal and placental tissues were dissected and weighed. Individual colored points indicate a single biological replicate.

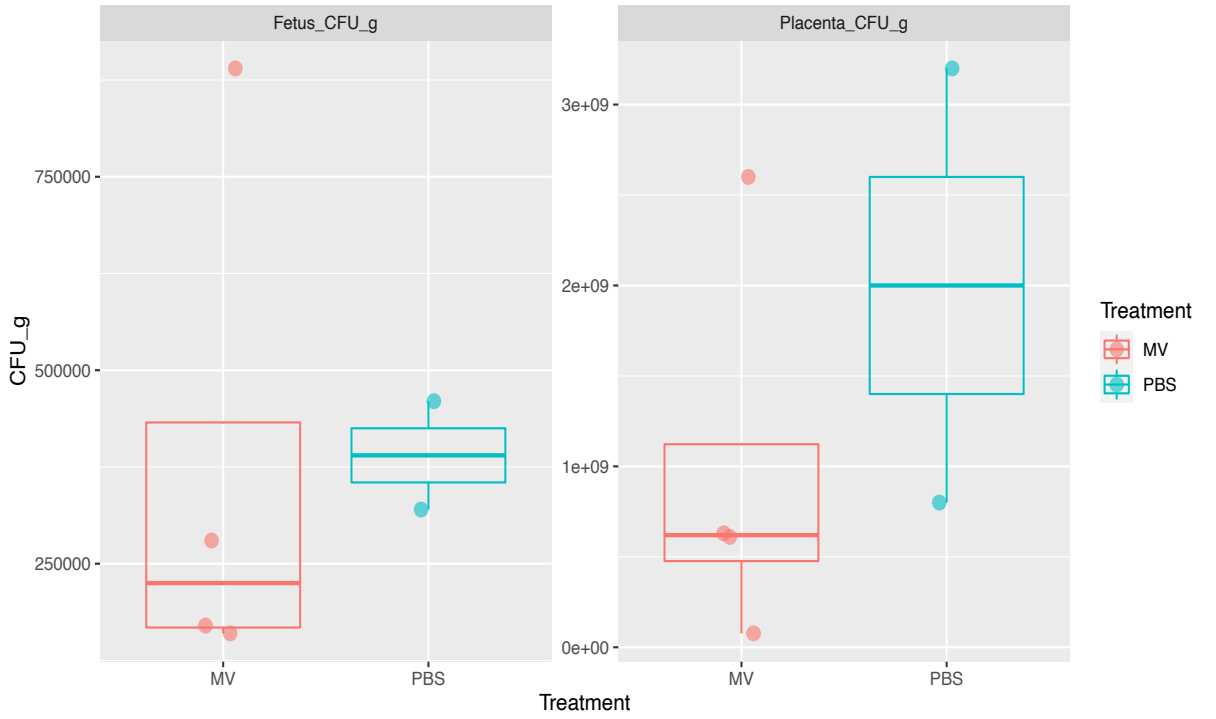

Figure 4.8: Pretreatment with MVs does not alter Bacterial Ascension

Pregnant dams were pretreated with MVs (n = 4) or PBS (n = 2) on GD12.5 and subsequently infected with GBS on GD13.5. Dams were sacrificed on GD15.5. The first fetus and placenta closest to the vaginal tract were dissected, homogenized, and plated onto THA for bacterial enumeration. CFUs were normalized to total tissue weight. Individual colored points indicate a single biological replicate.

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CHAPTER 5
Conclusions and Future Directions

While progress has been made in preventing early onset neonatal disease, GBS associated pregnancy complications remain a leading cause of morbidity and mortality worldwide. Growing evidence suggests that these pregnancy complications occur at a high frequency and range in severity (1). Still, only a fraction of vaginally colonized women develop pregnancy complications. While GBS-specific vaccines are in the later stages of development, no treatment has been shown to prevent GBS associated pregnancy complications. Therefore, there is a pressing need to understand which microbial factors promote adverse pregnancy outcomes so that targeted therapeutics can be developed (2). The primary focus of this dissertation was to understand how GBS derived MVs promote adverse pregnancy outcomes. A prior study showed that GBS MVs can promote preterm birth and intrauterine fetal death (3); however, little was known about MV production and composition across diverse GBS strains, or how these different MVs interact with host cells. Consequently, this dissertation aimed to address these critical knowledge gaps.

In the first study (Chapter 2), we sought to determine how strain diversity impacts MV production and protein composition. Although MVs were produced universally, the level of MV production varied in both a strain- and lineage-dependent manner. Specifically, we demonstrated that MV protein composition is dictated by lineage, or sequence type, with strains in the same lineage displaying more similar protein profiles. An examination of the specific proteins important for lineage-specific divergence uncovered multiple immunomodulatory virulence factors that were variably present across these lineages, with a greater abundance in the lineage associated with enhanced virulence. Together, these findings suggest that MVs have lineage-specific functions that could impact virulence and pathogenicity.

While this study greatly expanded our knowledge of GBS MVs, it has brought more questions forward. In our proteomics analysis, we showed that MVs had varying levels of multiple virulence associated proteins. While a previous study suggested that some of these proteins are functional, additional studies are needed to confirm whether the proteins identified in MVs are functional. Similarly, future studies should be conducted to determine if the varied composition of MVs across lineages confers lineage specific functions. Confirming protein activity could provide insight into the potential roles that MVs are playing during infection and biogenesis. For instance, we identified several proteins that may contribute to MV biogenesis including phage proteins, division associated proteins, and cell wall remodeling enzymes. Follow up studies examining whether these specific pathways contribute to MV biogenesis in GBS are therefore warranted. Since we also demonstrated MV composition variation across isolates, it is likely that other classes of macromolecules are also variably present. RNA and lipids have been identified in GBS MVs previously (3), raising the possibility that these macromolecules could also be differentially packaged across strains of different lineages

The second study (Chapter 3) further expanded our understanding of how MVs interact with the host immune system, by assessing how human macrophages respond to MVs representing different lineages. Regardless of lineage, MVs elicited a potent proinflammatory immune response characterized by an upregulation of proinflammatory cytokines and chemokines. For instance, production of the proinflammatory pyrogen IL-1ß was upregulated and dependent on the NLRP3 inflammasome, suggesting that NLRP3 is a major sensor of GBS MVs. Together these results have defined the host response to GBS MVs and identified NLRP3 as a novel sensor of GBS MVs.

Although this study elucidated important proinflammatory pathways that are activated in response to GBS MVs, many questions remain. While our model assessed a variety of proinflammatory and anti-inflammatory cytokines, our search was not exhaustive. A more unbiased RNA-seq based approach could yield more detailed information regarding the host response to GBS MVs in the future. Furthermore, a more complex cell model containing additional cell types, in monoculture or in co-culture, may yield more information about the host-response to GBS MVs when compared to the THP-1 derived macrophages. Although we identified NLRP3 as a novel sensor of GBS MVs, many of the cytokines that were upregulated in response to MVs are not known to be regulated by NLRP3 or IL-1 signaling pathways, suggesting that other sensors are likely responding to MVs as well. A more comprehensive assessment of pattern recognition receptors that respond to GBS MVs is therefore warranted. Moreover, it is unknown what MV associated pathogen associated molecular patterns or danger associated molecular patterns activate this pathway. Even though hemolysin and RNA, which have been found in GBS MVs and were shown to activate this system (3), no prior studies have examined which of these molecules is responsible for NLRP3 activation.

The goal of the final study (Chapter 4) was to examine the impact of GBS MVs on ascending infection. Using a murine model of ascending infection, we demonstrated that vaginally administered MVs do not promote ascending infection or induce key inflammatory responses at the maternal-fetal interface. Although MVs did not play a substantial role in disease using this model of ascending infection as was shown for other model systems, our data suggest that they could play alternative roles in disease progression.

Although we showed that the addition of exogenous MVs did not impact ascending infection *in vivo*, we lacked sufficient statistical power to make a definitive conclusion. Due to

the small effect size and high variability of this model, the high sample size needed to achieve appropriate power would make this study cost prohibitive. Indeed, it is possible that MVs affect ascending infection, however, our findings suggest that this impact is minimal in this model. Another issue with this model is that live bacteria likely produce MVs *in vivo*, and hence, those bacteria that had ascended into the uterus could have produced the MVs, thereby initiating the immune responses. This hypothesis would support our observation that exogenous MVs did not ascend into the uterus and promote inflammatory responses. Interestingly, use of other, suboptimal models has shown that MVs can impact disease outcome (3, 5), therefore, more sensitive models are needed to better assess the role of MVs during infection. Using a more sensitive model in combination with heat killed bacteria and exogenous MVs will also enhance our understanding of how MVs affect the host-response during infection. While heat killed bacteria elicit a diminished immune response, they are deficient in MV production, making them an adequate model for assessing the relative contribution of MVs in stimulating an immune response. Nonetheless, the lack of other GBS antigens on live cells may further confound these studies.

Although our studies have greatly contributed to our understanding of GBS MVs, these future studies could further help us understand the role of MVs in GBS pathogenesis. That being said, our studies have identified novel MV associated virulence factors that lay the foundation for mechanistic studies. Additionally, we have identified several proteins that were more highly abundant in lineages causing invasive disease, suggesting that these MV associated proteins may serve as potential biomarkers of invasive disease. Our studies further demonstrate that MVs interact with the host immune system in a consistent manner despite having lineage specific functionality, which may be useful for immunotherapeutic targeting. Lastly, although our studies

did not find that MVs contributed to ascending infection, our results will inform future studies examining the role of MVs during active infections. Taken together, our studies have greatly expanded our understanding of how GBS MVs contribute to disease progression and their role in shaping host-pathogen interactions.

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