

Decoding the Innate Immune Response to Group B Streptococcus Infections at the
Maternal-Fetal Interface

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Abstract

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Bacterial infections, including Group B *Streptococcus* (GBS) in pregnancy are a common cause of early preterm birth and severe neonatal morbidity and mortality. The virulence factors expressed by a particular GBS strain are one important factor in determining invasion; host defenses at the maternal-fetal interface are likely equally important in clearing GBS before invasion can occur. The central hypothesis of the dissertation is that differences in immune cell composition and recruitment, expression of immune cell checkpoints, and cell death profile govern GBS infection at the maternal-fetal interface and clinical outcomes. I found that an early GBS infection would induce the expression of immune checkpoint proteins in the placental chorioamniotic membranes and decidua to counteract the cytokine and chemokine response of immune infiltration. Moreover, bacterial invasion into the amniotic fluid was associated with IL-1 signaling and inflammatory host cell death (pyroptosis, ferroptosis) at the maternal-fetal interface. In contrast, a resolved GBS infection was linked to a mild-inflammatory response of cytotoxic granules, likely produced by uterine NK cells and/or CD8⁺ T cells. Surprisingly, there was a prominent inflammatory signature in the uterine muscle and preterm labor rate in the resolved infection group. Future studies should focus on understanding how preterm birth therapeutics might support the function of innate immune cells in the maternal decidua to combat pathogens inducing preterm birth.

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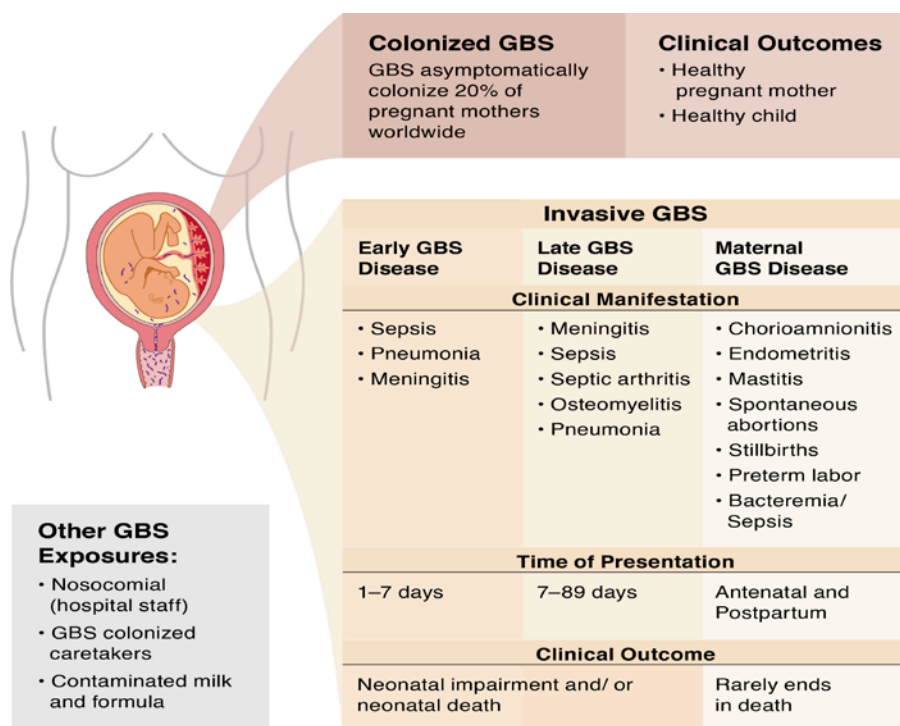
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Chapter I: Introduction

History of Group B Streptococcus (GBS)

Group B Streptococcus (GBS) is a gram-positive coccus that can exist as a commensal microbe of the human gastrointestinal and lower genital tract that can also cause invasive disease. In 1935, GBS was specifically linked to asymptomatic colonization of the human vagina, as compared to other streptococcal species [1]. It was not until 1938 when GBS linked to three fatal cases of postpartum sepsis [2], which established that an asymptomatic colonizing strain could become invasive and cause maternal morbidity and

Figure 1.1. Clinical Outcomes of GBS Colonization and Invasive Disease



This figure illustrates the spectrum of disease that can occur with invasive GBS disease in the mother and neonate, along with the approximate time of presentation. Neonatal invasive disease is characterized as Early-on-set GBS and Late-on-set GBS disease. GBS colonization of mothers is a leading risk factor for development of neonatal disease with other potential GBS exposure.

mortality. In the 1970s, studies began to identify GBS as a major cause of sepsis and meningitis in neonates and infants [3-7] (Figure 1.1). Subsequent studies identified the link between GBS colonization of the vagina and GBS bacteriuria with maternal morbidity (sepsis, postpartum endometritis), preterm birth, and stillbirth [8-13]. These studies clearly defined GBS as an important perinatal pathogen linked to adverse maternal and neonatal outcomes.

Maternal GBS Colonization

Maternal GBS colonization is a major risk factor for GBS invasive disease. The true rate of GBS colonization of the rectovaginal tract is difficult to ascertain but has been estimated in many cross-sectional studies to occur in approximately 20% of pregnancies globally [12, 14]. In a 2017 meta-analysis, global colonization rates of healthy pregnant women were estimated to be highest in the Caribbean (35%) with lowest rates in Southern and Eastern Asia (13% and 9%, respectively) [15]. Within continents, rates of GBS colonization can vary widely with a prevalence of GBS detection in vaginal swabs of 14% in Western Africa and 25% in Southern Africa [15]. Across Europe, Australia and North America, rates of GBS detection range between 15-21%. Higher rates of GBS colonization in pregnant individuals have also been reported in individual studies from South Africa (37%), Tunisia (27%), southern Ethiopia (24%), the Dominican Republic (26.6%), and Western Australia (24%) [16-20]. An updated meta-analysis was performed in 2022, which predicted that rectovaginal colonization of GBS affects nearly 20 million pregnant women globally annually based on estimates of 2020 [21-24]. Overall, many countries have documented rates of GBS vaginal colonization that place at least one-fifth

to one-quarter of pregnancies at risk for GBS invasive disease. The overall burden of maternal GBS colonization is likely larger than currently estimated.

There is a strong link between maternal vaginal colonization and invasive disease. Colonization of the vagina also has the potential to ascend into the intrauterine space to infect the placenta and fetus, resulting in preterm birth and/or stillbirth. Several studies have identified GBS in the amniotic fluid or placental chorioamniotic membranes isolated from mothers in preterm delivery (129). In 2017, maternal GBS colonization was roughly estimated to account for up to 3.5 million GBS related preterm birth [12]. In 2022, the number of annual preterm births due to invasive GBS disease was more precisely estimated at 0.5 million births globally based on country-specific estimates of preterm birth and maternal GBS colonization [21]. Similarly, to maternal colonization, the overall burden of invasive GBS is underestimated.

GBS Invasive Disease

An invasive GBS infection is defined as bacteria isolated from normally sterile sites, such as the blood, cerebral spinal fluid (CSF), amniotic fluid (AF), peritoneal fluid, and the placenta. Studies have shown that the ability of GBS to invade sterile sites is facilitated by the expression of a spectrum of virulence factors that enable bacterial adhesion and invasion of host cells. During fetal life, GBS may ascend from the lower genital tract into the uterus to infect the fetus, resulting in preterm birth, stillbirth, and/or fetal injury. Neonatal invasive disease can also occur after birth if the neonate is exposed to colonized care providers or infected breast milk/formula. GBS invasive disease imparts both adverse short- and long-term outcomes for the child that underscores the need for diverse preventive and treatment measures.

Adverse Neonatal Outcomes

Invasive disease in neonates is broadly categorized into GBS early onset disease (EOD) and late onset disease (LOD). The working definition of EOD has narrowed over the past 10 years to specify whether GBS invasive disease occurred in the first 72 hours of life, which is more common. In EOD, bacterial infection is acquired either prior to delivery through an ascending GBS infection or during delivery from exposure to vaginal fluid. With the evolution of the narrowed definition of EOD, the diagnosis of LOD varies and may now include invasive disease starting after 72 hours -7 days of life.

Infants with EOD generally present with nonspecific symptoms that may include low 5-minute APGAR scores (<5), perinatal encephalopathy, respiratory distress, apnea, tachycardia, hypotension, and persistent pulmonary hypertension of the newborn [25]. The most common clinical finding in EOD patients is GBS bacteremia and/ or sepsis without a focus [26, 27]. Central nervous involvement in cases of EOD is comparably rare and occurs in only 10% of cases (although GBS is isolated from the CSF in ~4% of cases)[27]. Neonates with LOD tend to present with a broader spectrum of clinical findings than those with EOD including lethargy, poor feeding, irritability, grunting and fever. Encephalopathy, bulging fontanelle, nuchal rigidity, and seizures are worrisome signs for central nervous system involvement [4, 7]. Bacteremia is one of the most common findings in LOD and has been reported to occur in 93-94% of cases [27, 28]. Meningitis, another common diagnosis, is more prevalent in infants with LOD as compared to EOD (21-34% of cases) [27, 28]

A recent study estimated that there were 231,000 cases of EOD and 162,000 cases of LOD globally in 2020 [21]. The overall mortality of invasive GBS infections in infants is 3-

6% in high income countries with a greater case fatality in EOD (6.9%) versus LOD (5.4%) in the United States [26, 27, 29]. Mortality is also higher in preterm compared to term neonates diagnosed with EOD (19% vs 2%) and LOD (8% vs 3%) [27]. Early onset sepsis (all infectious causes) is highest amongst infants born between 22-28 gestation in the United States with GBS being the second most frequent pathogen isolated [30]. Collectively, GBS EOD and LOD were responsible for approximately 91,000 child deaths worldwide in 2020 [21].

In countries that introduced prenatal screening with a policy of intrapartum prophylaxis antibiotics (IAP) administration, the incidence of EOD in term infants has decreased significantly (1.8 cases/1000 live births in 1990 to 0.23 cases/1000 live births in 2015 [31]. The U.S. Centers for Disease Controls and Prevention issued a practice guideline in 1996 (updated in 2002, 2010, 2019), which advised administering IAP to pregnant people screening positive for GBS rectovaginal colonization or who had a positive urine culture [32]. Interestingly, a study conducted at 18 centers in the U.S. after initiation of the guidelines revealed that approximately half of invasive GBS cases in infants were born to pregnant people who had negative prenatal screening and no clinical indications for IAP, highlighting a major limitation of this current practice [30]. Additionally, IAP has not decreased rates of GBS LOD. Again, this underscores the need for more preventative and treatment measures.

Adverse Maternal Outcomes

Invasive GBS infection can also cause maternal illness. Maternal invasive GBS diseases are classified as GBS isolated from a sterile site in pregnant and postpartum women with some studies requiring additional clinical signs such as suspicion of sepsis or fever [13]. GBS infections in women can result in chorioamnionitis, postpartum endometritis and pregnancy complications, in particular preterm labor and stillbirth. The incidence of invasive maternal GBS disease was reported to be 0.38 per 1000 pregnancies and 0.23 per 1000 maternities (pregnancies resulting in live or stillbirth) in high-income countries including the United States, France, Ireland, and United Kingdom [13]. However, an updated data analysis estimated the risk of GBS maternal disease to be 0.29 per 1,000 deliveries based on the prior review and additional studies from England [21, 22]. Thus, global estimates of GBS infection range from 33,000 to 40,500 a year for invasive maternal disease from each analysis, respectively [12, 13]. Both studies were likely to underestimate the prevalence of GBS maternal disease as there was minimal data from low and middle-resource countries, and both lacked non-systemic adverse outcomes including chorioamnionitis and postpartum endometritis.

GBS infection of placental and uterine tissues leads to maternal morbidity. Postpartum endometritis is an infection of the decidua that can also affect the myometrium, which can increase the length of hospital stay and antibiotic administration. Maternal colonization by GBS increases the risk for development of postpartum endometritis [33, 34]. Interestingly, meconium staining of the amniotic fluid represents another risk factor for GBS-associated endometritis as GBS growth is enhanced by meconium [35, 36]. Prior to IAP, GBS was one of the most common bacteria isolated from blood cultures in cases of postpartum

endometritis [37]. After the introduction of IAP, rates of postpartum endometritis associated with GBS infection have decreased [38, 39].

Antenatal invasive GBS can infect the fetus *in utero* which results in stillbirths and miscarriages [40, 41]. Stillbirths due to invasive GBS were confirmed with isolation from fetal blood, lung aspirates, and CSF. It was reported in 2015 that GBS causes up to 12.1% stillbirths globally [42]. A 2022 meta-analysis revealed that Sub-Saharan Africa and Central and South Asia are predicted to have the highest burden of stillbirths and a global burden of 46,200 cases per year [21, 24]. In 2014, it was determined that mothers with invasive GBS had higher rates of stillbirth than the national average in the UK [43]. There remains a major gap of knowledge of GBS induced stillbirth cases with less known about GBS infections leading to miscarriages.

GBS Classification of Invasive and Colonizing Strains

GBS taxonomy has evolved over time which include many different methods for isolate categorization and association with colonization and disease-causing strains (Figure 2). Serotyping by capsule, a highly decorated, sialic-acid rich capsular polysaccharide coating, is a common method for characterization [44, 45]. The GBS capsule helps pathogens evade host immune responses through molecular mimicry as eukaryotic cells also often present sialic acid, which is recognized by the immune system as “self” [46-50]. Ten capsular serotypes (Ia, Ib, II- IX) of GBS have been identified to date [51].

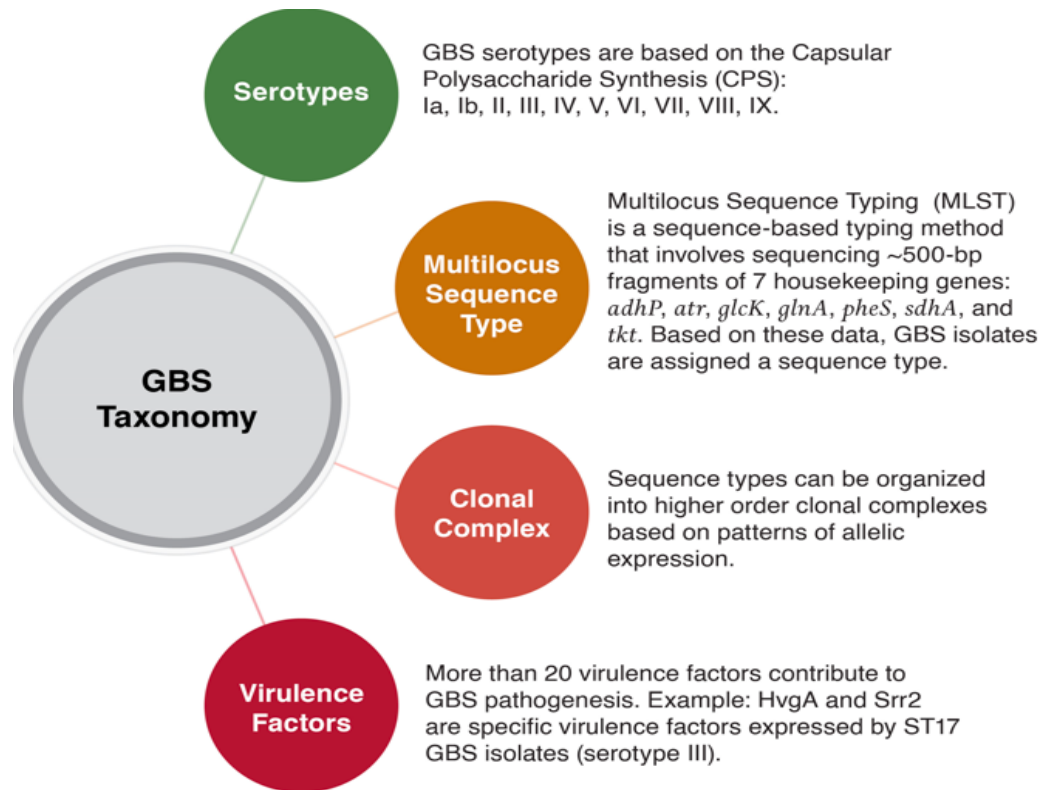
A further innovation in GBS taxonomy was by multi-locus sequence typing (MLST), which reflects a comprehensive strategy for classifying GBS isolates into DNA sequence types (ST) based on the sequences of seven housekeeping genes (*adhP*, *atr*, *glcK*, *glnA*, *pheS*,

sdhA, and *tkt*) [52]. The MLST scheme is further characterized by the grouping of multiple ST into clonal complexes (CCs), which are defined as lineages that share six or seven alleles [53]. Currently, there are more than 2,200 defined GBS sequence types [54]. Some ST and serotypes are associated with each other more frequently than would be expected by chance. However, ST and serotypes are not necessarily concordant; a strain with a particular sequence type might exist with multiple serotypes, and a given serotype might contain strains of different sequence types [52, 53].

The five main serotypes (Ia, Ib, II, III and V) account for 98% of GBS detected in rectovaginal samples; the most prevalent serotype worldwide is serotype III, which is also responsible for a large proportion of invasive disease [12, 55, 56]. Using MSLT, vaginal colonization has been closely associated with the ST1, ST19 and ST23 sequence types as compared to invasive strains [57, 58]. It was also determined that CC1, CC10, CC19, and CC23 - clonal complexes with CCL1 as the most frequently linked clonal complex to colonization as compared to individuals with invasive disease[59].

Much like maternal rectovaginal colonization, there are specific sequence types and clonal complexes associated with invasive neonatal disease. The most common serotype isolated from any invasive GBS EOD or LOD disease (CNS, non-CNS disease) is type III and the second most common is type Ia [28, 60]. Serotype III has a higher frequency in CNS disease compared to other serotypes (~79% of isolates from CNS) [60]. For GBS LOD, serotypes Ia and III are associated with 99.7% of cases [27]. MLST based clonal complex CC17 is increasingly associated with serotype III, LOD, and multidrug resistance (against tetracyclines, aminoglycosides, macrolides, and lincosamides) [61, 62].

Figure 1.2. Classification of Group B *Streptococcus* Strains



This figure illustrates the four main classification of GBS isolates. Researchers and medical providers refer to GBS by categories to elicit those responsible for invasion and disease.

Virulence Factors: Beta-Hemolysin

The presence of GBS virulence factors is also utilized to identify invasive and colonizing strains (Figure 1.2). There are over 20 virulence factors expressed by GBS, which are categorized into various groups and include pore-forming toxins. GBS expresses two known pore-forming toxins: the hemolysin and CAMP factor. GBS is beta-hemolytic (also called “hemolysin”). Hemolysin is a well-known driver of invasive GBS disease.

It was originally assumed that the GBS hemolysin was a secreted protein, as is the case for other Gram-positive hemolysins [63]. The responsible gene was elusive, but

eventually, the *cyI* operon was implicated as important in hemolytic activity [64-66]. Initially, *cyIE*, was putatively linked to the hemolytic phenotype [67]. However, attempts to purify the protein were not fruitful, nor was *cyIE* high in homology to known pore-forming toxins [67]. Later, it was found that *cyIE* itself was not in fact the hemolytic agent but rather an enzyme involved in its biosynthesis: instead, hemolysis was induced by an ornithine rhamnolipid pigment termed granadaene [68, 69].

After identification of the pigment as the β -hemolysin, either the pigment or hyperpigmented strains of GBS were associated with barrier disruption of the placental chorioamniotic membranes and invasion of the placenta, lung, and BBB [68, 70-72]. Invasion of the amniotic cavity by hyperpigmented GBS is also associated with fetal bacteremia and injury [68, 73]. In mice, inoculation of hyperpigmented GBS results in preterm birth and fetal loss [74]. Vertical transmission of pigmented strains has been shown to result in neonatal meningitis and pneumonia [72, 75]. GBS expression of pigment is a major risk factor for perinatal morbidity and mortality.

Finely tuned regulatory mechanisms allow GBS to control expression of its multiple virulence factors, including β -hemolysin based on the host environment. CovR/S (also termed CsrR/S) is the best-characterized two-component system of GBS and a main regulator of β -hemolysin. Generally, GBS CovR is thought to act as a repressor. Deletion of CovR results in a hyperpigmented, hyperhemolytic phenotype resulting from upregulation of the *cyI* operon in the absence of the repressive activity of CovR [68, 76-79]. The Rajagopal Laboratory has created and used the GBS COH1 Δ covR (hyperpigmented; hypervirulence) and GBS COH1 Δ covR Δ cyIE (hypopigmented; less

virulence) in studies investigating the virulence potential of the β -hemolysin in causing invasive disease.

Pathogenesis of Invasive GBS and Spectrum of Disease

Many factors contribute to the transition of GBS from an asymptomatic colonizer in the lower genital tract to a pathogen in the intrauterine space including the requirement for trafficking through the cervix, expression of key virulence factors, and evasion of host immune defenses. Ascending bacteria from the maternal vaginal tract also encounter microbes within the vaginal microbiota that can limit GBS colonization, and a cervical mucus plug with antimicrobial properties. Although the cervical mucus has antimicrobial properties that can eliminate these bacteria, the concentration of antimicrobial peptides alone is insufficient to directly kill GBS without help from other components of cervical innate immunity [80-82]. The major contributors to GBS invasive properties are the virulence and host factors.

Once GBS traffics through the cervix, it reaches the decidua and the placental chorioamniotic membranes in the lower uterine segment. Epithelial and other non-immune cells that GBS encounters in these tissues include maternal decidual stromal cells, chorionic trophoblasts, mesenchymal cells, and amniotic epithelial cells. Although these cell types are considered non-immune, they can respond to microbial threats through pathogen recognition receptors and often participate in the innate immune response to infection. Immune cells are also present in these tissues including macrophages, uterine natural killer cells, and lymphocytes. Neutrophils are also recruited into the decidua and chorioamniotic membranes during infection.

Host Innate Immune Response to GBS

Once GBS reaches the choriodecidual space in the lower uterine segment, it will encounter a population of decidual stromal and immune cells. Resident immune cells include macrophages, dendritic cells, natural killer cells, and T cells. Macrophages are one of the first defenses against bacterial pathogens including GBS. Although GBS is not an obligate intracellular pathogen, it can persist within host cells, such as macrophages [83]. Bacteria that have been phagocytosed but not killed are inherently dangerous to the host, as this can be exploited to escape host immune responses [84]. In cell culture, it has been found that different serotypes and ST of GBS induce different macrophage polarization phenotypes and cytokine profiles [84-86]. Stimulation with GBS has also been shown to cause macrophages transition into a more dendritic cell (DC)-like state [73, 87, 88]. Additionally, internalized GBS that are not killed by the host can themselves induce macrophage killing via both apoptosis and pyroptosis [73, 89-91]. Although infants have deficiencies in complement relative to adults [92], human adult and infant macrophages are both competent to phagocytose and kill GBS; however, for infants this requires prior opsonization [93-95]. Immaturity of infant immunoglobulin repertoires may contribute to poor uptake, and thus control, of GBS. Additionally, infant and adult monocyte-derived macrophages display differential cytokine profiles when challenged with GBS, and it may be that overexpression of Th1 and Th17-associated cytokines as well as IL-10 in infant macrophages may help explain dysregulated immune responses observed in infants [96].

Neutrophils are essential for host control of GBS in several ways including IL-1 β production and generation of neutrophil extracellular traps (NETs) containing antimicrobial peptides [97-99]. GBS can also circumvent neutrophil activity in many ways. The GBS hemolytic pigment, can kill neutrophils directly and inhibit their own killing by NETs [70]. The secreted GBS nuclease A can degrade NETs and hyaluronidase-expressing GBS can evade neutrophil activity by suppressing TLR2/4 signaling [100-103]. Human neonatal neutrophils stimulated with GBS skew towards release of Th1- and Th17-associated cytokines [104]. While this could be helpful for inhibiting viral infection, it may result in excessive inflammatory responses that harm the host. GBS has also been shown to suppress inflammatory signaling in placental neutrophils, which may result in damage due to bacterial dissemination [105].

Mast cells are often associated with allergic responses, but also contribute to other immune functions, one of which is maintaining homeostasis of commensal microorganisms [106]. Mast cells are also now understood to have a key role in the innate immune control of pathogenic bacteria: they can degranulate to release signaling molecules that recruit other immune cells, and they can kill pathogens directly by releasing antimicrobial molecules (reviewed in [107]). Hyperpigmented GBS can induce degranulation of mast cells, promote inflammation, and decrease bacterial titers [108]. Similarly, mast cell chymase has been found to limit GBS dissemination and reduce preterm birth [109]. Chymase can cleave fibronectin, and this observation depended on the presence of the GBS fibronectin-binding protein SfbA, suggesting that fibronectin binding promotes GBS dissemination. Similarly, Factor XIIIa, a clotting factor, has been shown to promote entrapment of GBS in fibrin clots [110]. This occurs because GBS ScpB

binds fibronectin, which is also a component of the clots. Interestingly, female mice express more Factor IIIA than males; thus, in this model, females controlled GBS dissemination better than males [110]. Overall, the role of mast cells in controlling GBS infection has so far been characterized as beneficial to the host, though to varying degrees based on host and bacteria traits.

Nonhuman Primate Model of Pregnancy

We have studied the pathogenesis of invasive GBS disease in the nonhuman primate (NHP) model, which has several advantages in studying the pathogenesis of GBS during pregnancy (Figure 1.3). The NHP pregnancy is the closest model to human pregnancy as they have similar anatomy and physiology, placentation, maternal-fetal interface, and fetal development at birth in comparison to humans. NHP experiments studying the

Figure 1.3: Models to study Group B *Streptococcus* (GBS) Infection

Human



Advantages:

- Species of interest with most applicable findings

Limitations:

- Impractical to gain real time access to amniotic fluid or fetal compartments during GBS infection unless clinically indicated (e.g., amniocentesis)
- Recruitment may be challenging
- Long follow up necessary to capture neonatal outcomes for GBS survivors

Non-Human Primate



Advantages:

- Most comparable to human pregnancy (anatomy and physiology, neonatal and fetal development, host diversity)
- Enables investigation of complex host-pathogen responses that occur in humans, even in the amniotic fluid and fetal compartments using NHP model with indwelling amniotic fluid and fetal catheters
- GBS infection timing, GBS strain and inoculum dose can be tightly controlled

Limitations:

- Model is costly and available only in certain centers

Mice



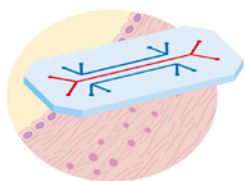
Advantages:

- Cost-effective and efficient model to accelerate research of virulence factors, colonization, invasion, vertical transmission, vaccines, and therapeutics
- Abundant biological and genetic systems available

Limitations:

- Key differences between mouse and human immune system [e.g., human blood is neutrophil rich (50-70%) and mouse blood has fewer neutrophils (15-20%)]
- Key differences between mouse and human pregnancy (e.g., gestational length, mechanism of parturition)

Organ-on-a-Chip



Advantages:

- Mimics cell-cell communication
- Biologically relevant hydrogels and coating mimics the extracellular matrix
- Can replicate autocrine and paracrine effects

Limitations:

- More costly than typical tissue culture experiments
- Limited to investigation of interactions between components present in the device

Varied models are employed to understand GBS colonization, infection, and pathophysiology between mothers and neonates.

impact of many strains of GBS on the pathogenesis of preterm birth and fetal injury have been conducted for more than two decades. Most of these experiments have employed a chronically catheterized NHP model in which amniotic fluid and maternal blood could be accessed multiple times without needing to sedate the animal. In early experiments, GBS was inoculated directly in the amniotic fluid of pregnant rhesus macaques, which demonstrated a temporal progression of the inflammatory cytokine and prostaglandin cascade after GBS infection (273). Some nonhuman primate studies using isogenic strains of GBS expressing specific virulence factors (i.e., β -hemolysin) have demonstrated the marked increase in rates of preterm birth and fetal injury when inoculating hypervirulent strains of GBS (203, 217). Following inoculation of a GBS clinical isolate (COH-1, serotype III) or a hyperpigmented and hypervirulent (COH1GBS Δ covR) GBS strain into the choriodecidual space, there were significantly higher rates of microbial invasion of amniotic cavity (MIAC), fetal bacteremia and preterm labor in pregnant pigtail macaques (274, 277). In the study inoculating the hyperpigmented and hypervirulent (COH1GBS Δ covR) GBS strain, there were higher rates of histological chorioamnionitis of neutrophil infiltration as compared to isogenic strains or saline controls. Interestingly, the GBS hemolytic pigment toxin ability to induce neutrophil cell death and evade killing by neutrophil extracellular traps (NETs) in placental membranes facilitated microbial invasion of the amniotic cavity and fetal injury (203). In another NHP study, choriodecidual inoculation of a hyaluronidase producing GBS inhibited neutrophil function and increased prostaglandin and matrix metalloproteinase activity mediating preterm labor (217). This model highlights how differences in

expression of GBS virulence factors can modify the phenotype of invasive disease in pregnancy.

Dissertation Aims and Central Hypothesis:

Invasive GBS in the uterine space can disseminate to the fetus and/ or induce inflammatory associated outcomes, such as preterm labor. There remains a gap in knowledge on the initial host innate immune response to GBS infection at the maternal-fetal interface and the association with specific clinical outcomes, microbial invasion of the amniotic cavity, and preterm birth. Here, I investigated the early defense mechanisms of inoculated hyper- or hypopigmented GBS strains (COH-1, serotype III, ST 17) at the maternal-fetal interface in our NHP model. **The central hypothesis of the dissertation is that differences in immune cell composition and recruitment, expression of immune cell checkpoints, and cell death profile govern GBS infection at the maternal-fetal interface and clinical outcome.** In chapter 2, I describe the results of spatial immune profiling of the placental chorioamniotic membranes and maternal decidua to capture protein expression in regions infected with GBS. I hypothesized that an early GBS infection induces expression of immune checkpoint proteins in the placental chorioamniotic membranes to counteract the cytokine and chemokine response to prevent destruction of the membranes. In chapter 3, I characterize the spectrum of invasive GBS infections and host innate immune response by quantifying bacterial dissemination and defining infections as progressing, localized to the choriodecidual inoculation site, or resolved. Phenotyping infections in this way is novel and enables the study of innate immunity and cell death pathways induced by a progressing versus resolving GBS infection across the maternal-fetal interface in the placental chorioamniotic

membranes, maternal decidua, and the lower uterine segment of the myometrium (uterine muscle). Finally, in chapter 4, I present the implications of this new understanding of the spectrum of invasive GBS, and host innate immune response for GBS treatment.

Chapter II: Immune Proteins Induced by the Presence of GBS at the Maternal-Fetal interface

Abstract

Background: Preterm birth is a leading cause of neonatal mortality, which is often caused by intrauterine infection and inflammation. We have established a nonhuman primate model of Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) infection-associated preterm birth. Immune checkpoints are modulators of the immune response by activating and suppressing leukocyte function. The objective of this study was to spatially profile changes in immune protein expression at the maternal-fetal interface during a GBS infection with a focus on immune checkpoints.

Methods: Twelve nonhuman primates (pigtail macaques, *Macaca nemestrina*) received a choriodecidual inoculation of either: 1) $1-5 \times 10^8$ colony forming units (CFU) of hyperhemolytic/hypervirulent GBS (GBS Δ covR, N=4); 2) an isogenic/ nonpigmented strain (GBS Δ covR Δ cylE, N=4); or, 3) saline (N=4). A Cesarean section was performed at preterm labor or 3 days after GBS inoculation, or 7 days after saline inoculation. Nanostring GeoMx® Digital Spatial Profiling technology was used to segment protein expression within the amnion, chorion, and maternal decidua at the inoculation site using an immuno-oncology panel targeting 56 immunoproteins enriched in stimulatory and inhibitory immune checkpoint proteins or their protein ligands. Statistical analysis included R studio, Kruskal-Wallis, Pearson, and Spearman tests.

Results: Both inhibitory and stimulatory immune checkpoint proteins were significantly upregulated within the chorioamniotic membranes and decidua (VISTA, LAG3, PD-1, CD40, GITR), as well as their ligands (PD-L1, PD-L2, CD40L; all $p < 0.05$). Immunostaining for VISTA revealed positive (VISTA+) cells, predominantly in the chorion and decidua. There were strong correlations between VISTA and amniotic fluid concentrations of IL-1 β , IL-6, IL-8, and TNF- α (all $p < 0.05$), as well as maternal placental histopathology scores ($p < 0.05$).

Conclusions: Differential regulation of multiple immune checkpoint proteins in the decidua at the site of a GBS infection indicates a major perturbation in immunologic homeostasis that could benefit the host by restricting immune-driven pathologies or the pathogen by limiting immune surveillance. Protein expression of VISTA, an inhibitory immune checkpoint, was upregulated in the chorion and decidua after GBS infection. Investigating the impact of innate immune cell expression of inhibitory immune checkpoints may reveal new insights into placental host-pathogen interactions at the maternal-fetal interface.

Introduction

Group B Streptococci (GBS) are commensal gram-positive bacteria that reside in the gastrointestinal and lower reproductive tracts of approximately 18% of pregnant women.[15] In pregnancy, GBS can ascend into the uterus causing infection of the placenta and fetus resulting in preterm birth, stillbirth, invasive neonatal disease, and neurodevelopmental deficits in the neonate.[10, 11, 111-113] In 2015, it was estimated

that GBS was a leading cause of approximately 3.5 million preterm births and 409,000 cases of adverse maternal, fetal, or infant outcomes.[12] The pathogenesis of invasive GBS infections and how innate immune defenses prevent or fail to control a GBS placental infection are poorly understood but may be leveraged to improve maternal-fetal health.

We have established a nonhuman primate model in which GBS are inoculated into the choriodecidual space of the lower uterine segment where ascending bacteria are first thought to contact the placenta.[103, 114-119] The virulence of the GBS strain is paramount for the outcome of a choriodecidual infection in that strains of higher virulence have a greater likelihood of ascension into the uterus, placental invasion and preterm birth or stillbirth.[103, 114, 120-125] A key GBS virulence factor is the ornithine rhamnolipid pigment (β -hemolysin), which imparts hemolytic activity, which is produced by gene products in the *cyf* operon.[64, 67, 122] In our nonhuman primate model, we have previously demonstrated that overexpression of β -hemolysin is associated with adverse perinatal outcomes, such as bacterial invasion and preterm labor. However, spatial immune events along the path of invasive bacterial infection from the decidua through the chorioamniotic membranes are not well-characterized or understood.

Interrogation of the spatial immune protein expression in the placenta also presents the opportunity to evaluate the role of the amnion, chorion, and decidua in activating and restraining the inflammatory response. The maternal-fetal interface defined by the maternal decidua adjacent to the fetal chorion plays a key role in maintaining fetal tolerance. Immune checkpoint proteins act to inhibit or activate various aspects of T cell function (proliferation, activation) and have been studied extensively in oncology, but

recent evidence indicates that they may play a role in adverse pregnancy outcomes, such as preeclampsia, recurrent pregnancy loss and villitis of unknown etiology.[126-129] Whether their placental expression may be regulated in the course of acute chorioamnionitis to counteract the deleterious effects of the inflammatory cascade is unknown.

Our study objective was to spatially profile immune proteins in the placental chorioamniotic membranes and decidua following an experimental GBS choriodecidual infections in a nonhuman primate model. We hypothesized that an early GBS infection induces expression of immune checkpoint proteins in the chorioamniotic membranes to counteract the cytokine and chemokine response that has the potential for recruiting T cells into the membranes to maintain tolerance of the fetal allograft. Understanding the balance of inflammatory versus tolerance-promoting immune proteins in the amnion, chorion, and decidua would be helpful to gain insight into how the placenta responds to infection.

Methods

Ethics Approval

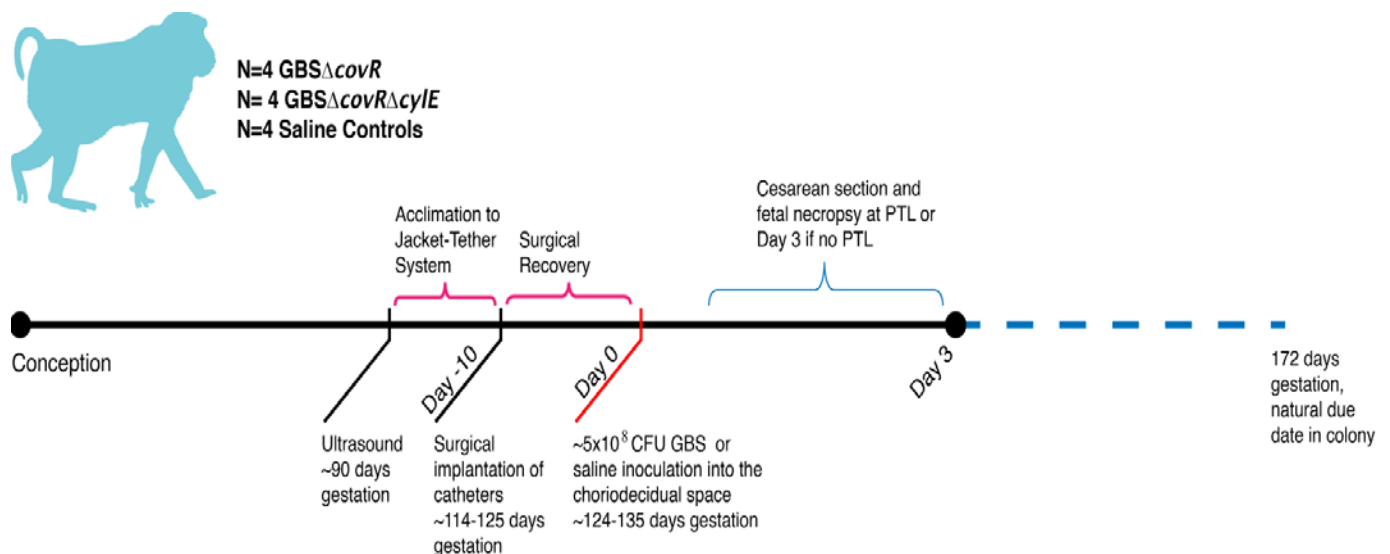
All animal experiments were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Research Council and the Weatherall report, “The use of non-human primates in research.” The University of Washington Institutional Animal Care Use Committee approved the protocol (Permit

Number: 4165-01, last approved 02/09/2021). All surgery was performed under general anesthesia and all efforts were made to minimize suffering.

NHP Model Study Design

In this study, eleven pregnant NHP (*Macaca nemestrina*) had catheters surgically implanted via laparotomy into the maternal femoral vein, amniotic fluid, and choriodecidual interface in the lower uterine segment (between uterine muscle and fetal membranes, external to amniotic fluid). Another pregnant NHP was an uncatheterized control. Animals were randomly selected to receive one of three experimental treatments: i.e., choriodecidual inoculation of approximately $1-5 \times 10^8$ colony forming units (CFU) of either GBS COH1 Δ covR (N=4; hyperpigmented strain) or GBS COH1 Δ covR Δ cyIE (N=4; isogenic, nonpigmented strain). The study design is shown in Figure 2.1 with details regarding inoculum CFU, gestational age at inoculation, and interval from inoculation to delivery, and inoculum Table 2.1. Results obtained from these animals were compared

Figure 2.1 Study Design for Chapter 2



This figure shows the study design. The x-axis reflects both days with respect to inoculation and is not to scale. The approximate days since conception are indicated in text. There were four nonhuman primates (NHP) in each experimental group: saline, GBS Δ covR, and GBS Δ covR Δ cyIE.

to saline controls (N=4; choriodecidual and amniotic fluid saline inoculations and one non-catheterized control) that were performed previously [70, 115].

Table 2.1 Experimental Design for each Animal

Group Name	Animal ID	Inoculation Amount (CFU)	Gestational Age at Necropsy (Days)	Interval Between Inoculation and Necropsy (Days)
Saline	A03140	N/A	139.4	7
Saline	L07201	N/A	139.4	7
Saline	M05062	N/A	155.4	91.5
Saline	M99183	N/A	143.4	7
GBS Δ covR	A10033	1.3 x 10 ⁸	135.6	1
GBS Δ covR	A12263	5.6 x 10 ⁸	130.6	0.25
GBS Δ covR	Z08140	4 x 10 ⁸	146.4	1
GBS Δ covR	A10016	1.3 x 10 ⁸	131.9	3
GBS Δ covR Δ cylE	A09114	5 X 10 ⁸	132.4	3
GBS Δ covR Δ cylE	A10212	5 X 10 ⁸	133.4	3
GBS Δ covR Δ cylE	A10219	5 X 10 ⁸	138.5	3
GBS Δ covR Δ cylE	A10223	5 x 10 ⁸	132.4	3

This table shows the experimental details for each individual animal which includes the inoculation amount, gestational age at necropsy, and interval between inoculation and necropsy.

Sample collection and processing

Preterm labor (PTL) was defined as progressive cervical dilation associated with increased uterine activity (>10,000 mmHg•sec/hr sustained over at least 2 hours). Cesarean section was performed at the following endpoints to allow for tissue collection following: 1) preterm labor, 2) three days after GBS inoculation if no preterm labor was observed, or 3) 7-days after saline inoculation.[70] A 3-day endpoint to assess the effects of GBS on placental tissues and fetal injury was chosen to study the earliest events in the pathway of infection/inflammation associated with preterm birth. The 7-day endpoint for saline controls was chosen at the inception of our research program and provides a close

gestational age match for the current study. After the Cesarean section, fetuses were euthanized by barbiturate overdose followed by exsanguination and fetal necropsy. Clinical outcomes are summarized in Table 2.2.

Amniotic fluid (AF) was sampled frequently before (-24 and -0.25 hours) and after GBS inoculation (+0.75, +6, +12, +24 hours and then every 12 hours until repeat C-section for fetal necropsy). Samples were kept on ice and centrifuged at 4°C for 5 minutes at 48 x g immediately after collection and the supernatant was frozen and stored at -80°C for future analysis. For cytokine and prostaglandin (PG) analysis, samples of AF were collected in EDTA (BD biosciences, San Jose, California, USA) tubes. The peak AF cytokine concentration was identified at the sampling time point where each individual cytokine was highest in each animal post-inoculation. Next, the peak AF cytokine concentration was correlated with immunoprotein expression in each placental region of interest (ROI) targeted in this experiment.

Intraamniotic pressure was continuously recorded using an implanted amniotic fluid pressure catheter (SPR-524, ADInstruments, Colorado Springs, Colorado, USA) and digitized with a Powerlab System (ADInstruments) connected to a desktop computer. Amniotic fluid pressure signals were processed using custom software to eliminate noise due to respiration or position changes. The area under each contraction (mmHg·sec/hr) was summed for each hour allowing calculation of the hourly contraction area, a measure of uterine activity. Finally, we calculated the mean hourly contraction area [(mmHg x sec)/hr]; HCA] over a 24-hour period. We used the peak mean HCA for each animal to test correlations between uterine activity and immunoprotein antigen counts in each placental ROI.

Table 2.2 Uterine Activity, Clinical Outcomes, Placental Histopathology Score and Fetal Sex for Each Experiment

Group Name	Animal ID	Peak Hourly Contraction [(mmHg·sec)/hr]	Average	Maternal Stage	Maternal Grade	Microbial Invasion of the Amniotic Cavity	Preterm Labor	Fetus Sex
Control	L07201	242.17		N/A	N/A	No	No	Male
Control	M99183	325.63		0	0	No	No	Male
Control	A03140	2747.12		N/A	N/A	No	No	Female
Control	M05062	N/A		0	0	No	No	Female
GBS Δ covR	A12263	7298.72		1	2	Yes	Yes	Male
GBS Δ covR	A10016	3522.29		3	2	Yes	Yes	Female
GBS Δ covR	Z08140	1417.12		N/A	N/A	Yes	Yes	Male
GBS Δ covR	A10033	6586.53		2	2	Yes	Yes	Female
GBS Δ covR Δ cyIE	A10212	2372.80		0	0	No	No	Male
GBS Δ covR Δ cyIE	A10223	2818.25		0	0	No	Yes	Female
GBS Δ covR Δ cyIE	A10219	2254.38		0	0	No	No	Male
GBS Δ covR Δ cyIE	A09114	N/A		2	1	No	No	Male

This table shows uterine activity peak hourly contraction area (mean 24-hour), maternal stage and grade, and clinical outcomes including presence of microbial invasion for the amniotic cavity, sex of the fetus, and preterm labor for each animal. Uterine activity was not obtained in two animals due to instrument malfunction after catheterization. Placental Redline score was not obtained in three cases.

GBS strains and bacterial enumeration

The GBS hyperhemolytic/hyperpigmented Δ covR and isogenic nonpigmented Δ covR Δ cyIE strains were derived from wild type (WT) GBS COH-1, an ST-17 clone belonging to capsular serotype III which was obtained from an infected newborn and were previously described. [70] Routine cultures of GBS were grown in tryptic soy broth (TSB) or tryptic soy agar (TSA, Difco Laboratories) at 37°C in 5% CO₂. For inoculations in the NHP model, GBS strains were grown to mid-log phase (O.D.600 = 0.5) and approximately 1-5 X 10⁸ CFU in 1mL PBS was inoculated into the choriodecidual space, as described previously.[130] For bacterial enumeration, AF (200 μ L) from each sampling timepoint

was serially diluted and 10-fold dilutions were plated on TSA, incubated overnight at 37°C, 5% CO₂ and enumerated to determine bacterial invasion.

Confirmation of GBS from infected animals

The hyperpigmented GBS Δ covR strain has an orange color when plated on TSA and nonpigmented GBS strains are white in color [122]. We also note that Δ covR and Δ covR Δ cylE strains of GBS are spectinomycin-resistant because the gene *covR* was replaced with a gene conferring spectinomycin resistance in these strains [77]. To confirm that the GBS strains recovered from infected NHP were the correct strains, a few colonies obtained from each sampled tissue and fluid per experiment were patched on selective medium (i.e., TSA containing spectinomycin), and the level of CAMP factor activity was tested on sheep blood agar plates with the inoculum strain included in parallel.

Cytokine measurement

Cytokine concentrations were determined using Luminex multiplex cytokine kits (Millipore Sigma, Burlington, MA), following manufacturer's instructions. I-TAC, IFN- γ , IL-1 β , IL-6, IL-8, IP-10, MCP-1, MIG and TNF- α were detected. Prostaglandin E₂ (PGE₂) and Prostaglandin F₂-alpha (PGF₂ α) were determined using commercially available human EIA kits (Cayman Chemical, Ann Arbor, Michigan, USA) following manufacturer's instructions. Cytokine data was incomplete for two animals due to insufficient samples.

Amniotic Fluid Processing

Amniotic fluid (AF) was sampled before (−24 and −0.25 h) and after pathogen inoculation (+0.75, +6, +12, +24 h and then every 12 h until Cesarean section for fetal necropsy) to culture for GBS and assay for inflammatory mediators. For cytokine analysis, samples of amniotic fluid were collected in EDTA tubes. Samples were centrifuged for 5 min at 1,200 rpm immediately after collection and the supernatant was frozen and stored at −80°C. Tissues were weighed at necropsy, homogenized in sterile PBS and 10-fold serial dilutions were plated on TSA to count bacterial colonies; plates were incubated overnight at 37°C, 5% CO₂ and enumerated as described [131, 132]. For inflammatory cytokine analysis, organ homogenates were diluted 1:1 in lysis buffer [150 mM NaCl, 15 mM Tris, 1 mM MgCl₂, 1 mM CaCl₂, 1% Triton X-100, supplemented with Complete Mini, EDTA-free protease inhibitor cocktail (Roche, Basel, Switzerland)] and incubated overnight at 4°C. Lysates were centrifuged at 4,000 rpm for 20 min at 4°C, and supernatants were stored at −80°C.

Nanostring GeoMx Digital Spatial Profiling Protein Assay

GeoMx digital spatial profiling (DSP) was performed at NanoString Technologies in Seattle, WA. Formalin-fixed, paraffin-embedded placental sections from animals from each group were incubated with fluorescent probes and eventual multiplex cocktail of primary antibodies with photocleavable oligonucleotides (i.e., a validated DSP human-immune oncology protein panel; NanoString Technologies). Fluorescent antibodies used to highlight features of the histology and draw the ROIs included: 1) anti-pan cytokeratin-Alexa Fluor 488 (Pan-CK, clone AE1/AE3; Novusbio, cat# NBP2-33200AF488); 2) anti-

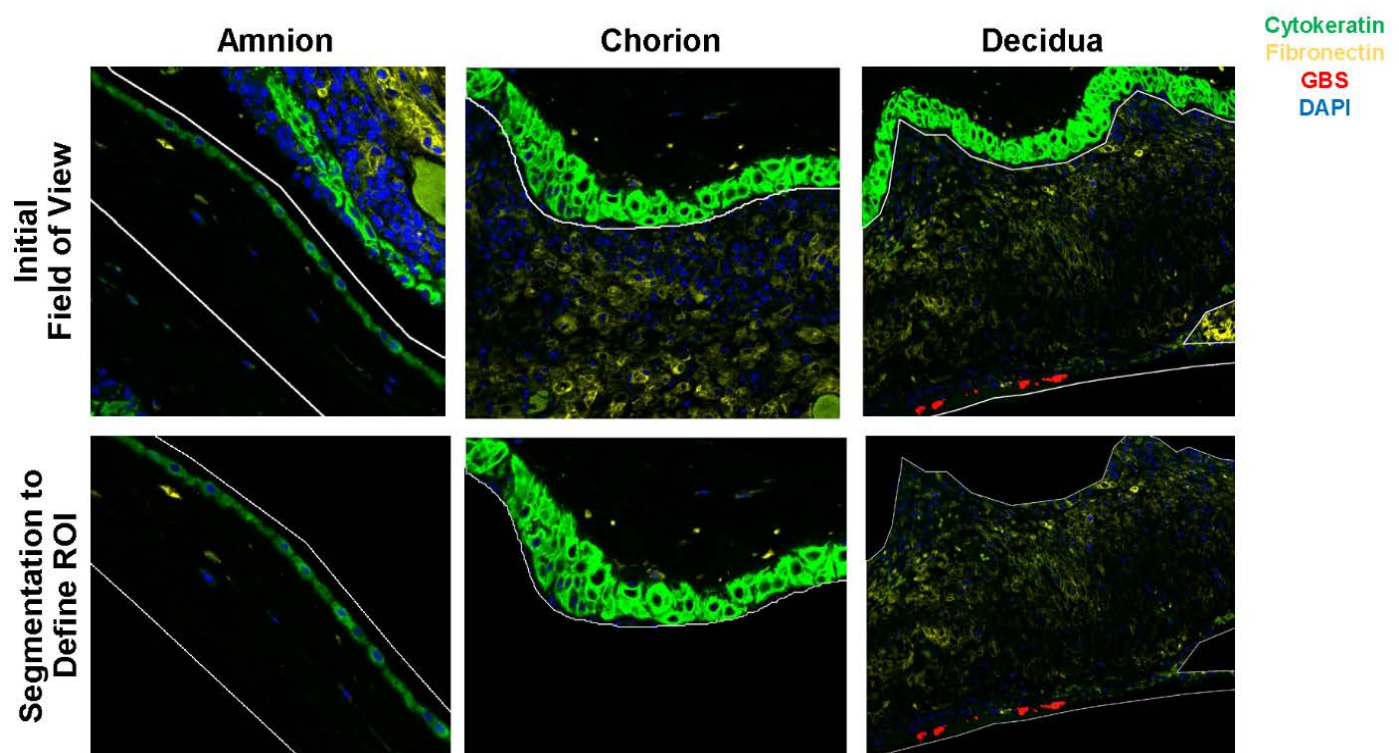
fibroblast activation protein-Alexa-Fluor 594 (FAP, clone SP325; Abcam; cat# ab311827); 3) SYTO 83 for nuclei visualization (ThermoFisher, Waltham, Ma); and 4) anti-GBS-Alexa Fluor 647 (Abcam; cat# ab53584). Sections were magnified 20 times, and ROIs comprising the decidua, chorion, and amnion from each animal were selected based on tissue morphology (Figure 2.2). The 56 immune protein panel of oligoconjugated antibodies was applied to the tissue. Each ROI was then exposed to UV illumination with a double digital mirror device molecule, which cleaved the DNA oligonucleotides into the aqueous layer above the tissue slice. The oligonucleotides in the eluent were collected via microcapillary aspiration and transferred to an individual well of a microtiter plate. Oligonucleotides were then hybridized to Nanostring nCounter optical barcodes to permit *ex situ* digital counting of each analyte. Briefly, hybridization of oligonucleotides to optical barcodes was performed at 65°C in a thermocycler. After hybridization, samples were processed using the nCounter prep station and digital analyzer. Data were normalized to technical controls and area. To generate signal/noise ratios, data were calculated relative to isotype controls.

The 56 immune protein panel was designed to target antigens from common immunology and immuno-oncology proteins. These proteins include VISTA, TIM-3, TGFB-1, SMA, S100B, PTEN, PR, PD-L2, PD-L1, PD-1, PanCk, OX40L, NY-ESO-1, MART1, LAG3, Ki67, IDO1, ICOS, HLA-DR, Her2/ErbB2, GZMB, GITR, $\gamma\delta$ -TCR, FOXP3, Fibronectin, Fap α , ER α , EpCAM, CTL-A4, CD86, CD80, CD8, CD68, CD66b, CD56, CD45RO, CD45, CD44, CD40L, CD40, CD4, CD34, CD3, CD27, CD25, CD20, CD163, CD14, CD127, CD11c, Beta-2-microglobulin, BCL-2, B7-H3, ARG1, and 4-1BB CD137. In addition to these 56 immune proteins, the panel included six control antibodies: mouse IgG1, mouse

IgG2a, rabbit IgG, GAPDH, Histone H3, and ribosomal S6. Antibodies against stimulatory checkpoint proteins and protein ligands include CD27, CD40, CD40L, CD137, OX40L, GITR and ICOS. Inhibitory checkpoint antibodies in the panel targeted B7-H3, CTLA-4, IDO1, LAG-3, PD-1, PD-L1, PD-2, TIM-3, ARG1 and VISTA.

Data was normalized using the Ribosomal S6 protein, which correlated more consistently with the tissue area in each region (amnion, chorion, decidua) than GAPDH or Histone H3.

Figure 2.2 Segmentation of the amnion, chorion and decidua was performed using the Nanostring GeoMx® Digital Spatial Profiling platform



Row **(A)** demonstrates the initial field of view using fluorescent microscopy and the regions of interest drawn to segment the amnion, chorion, and decidua. Row **(B)** demonstrates the segmented tissue regions prior to UV cleavage and capillary collection of the probes from the area of interest.

Immunohistochemistry validation of DSP Protein Assay

Immunohistochemistry staining was performed using two antibodies, VISTA and MPO, on placental chorioamniotic tissues. Tissues were stained with rabbit anti-human VISTA (D1L2G™) XP® 1:200 dilution (cat#64953, Cell Signaling, Danvers, Massachusetts, USA) antibody, rabbit anti-human MPO 1:100 dilution (cat #PA5-16672, ThermoScientific, Waltham, MA) or rabbit IgG (cat # AB-105-C, R&D Systems, Minneapolis, MN) isotype controls. Briefly, slides were prepared from formalin fixed and paraffin embedded specimens of placental tissue. Dewaxing was performed in a xylene series, and antigen retrieval was performed in a citrate buffer with heat for 10 minutes. Following antigen retrieval, the slides were blocked with 10% normal goat serum. Staining was performed using the BOND automated IHC Stainer (Leica, Wetzlar, Germany). Immunohistochemical cell staining was quantified using Visiopharm software (Visiopharm, Hørsholm, Denmark). Regions of interest (ROI) in the decidua were drawn manually, and the percent positive staining was calculated

Statistical analysis. A p-value less than 0.05 was considered significant. Analyses were performed using R studio. Statistical tests included the Kruskal-Wallis and Pearson's correlation. The hypothesis focused on differences between the GBS Δ covR and saline groups, therefore, correction for multiple comparisons was not performed.

Results

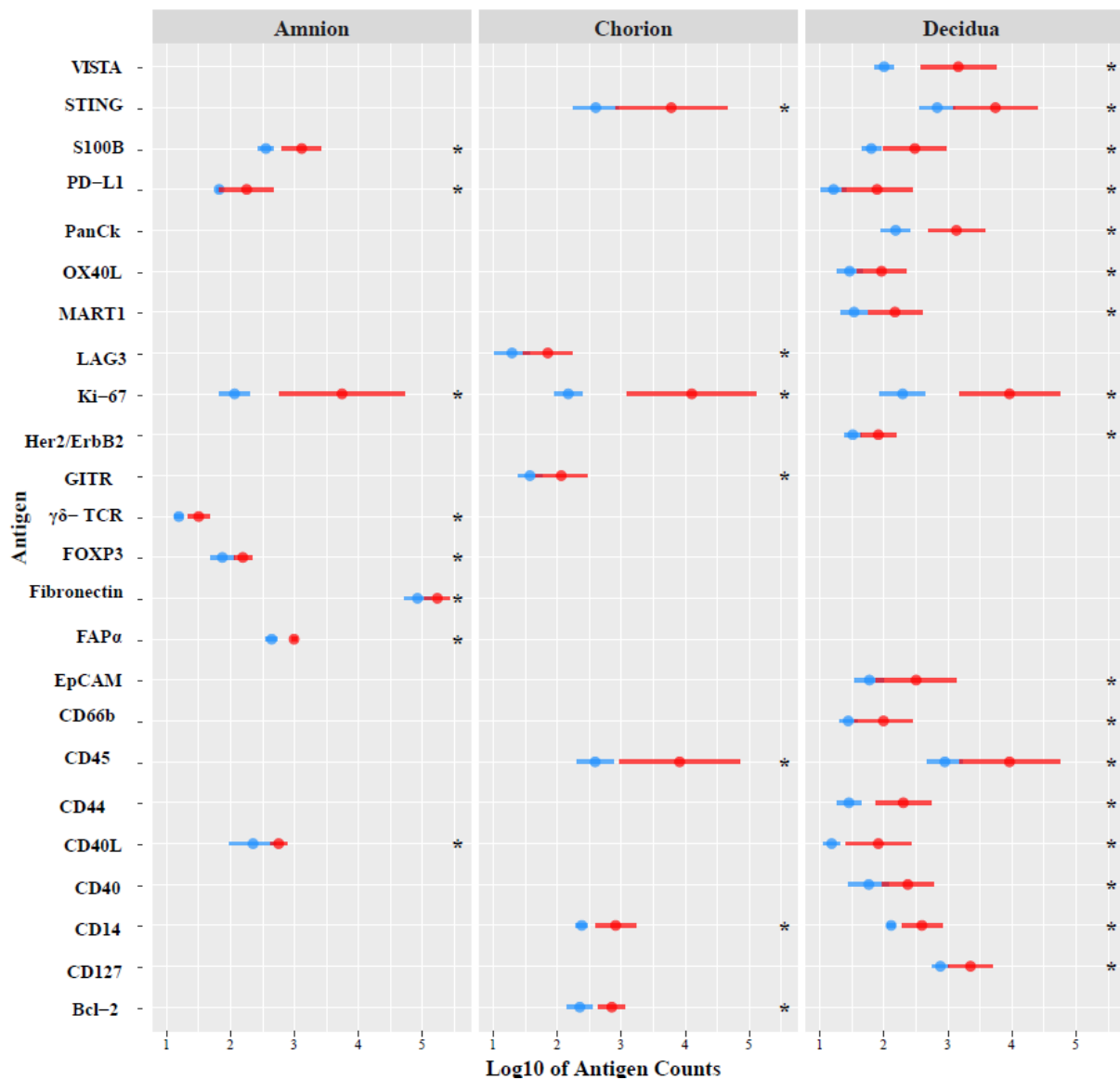
Digital spatial profiling (DSP) in GBS model demonstrates differentially expressed immune checkpoints at the site of infection

Immunoprotein expression was evaluated within the amnion, chorion, and decidua at the site of the GBS infection for the primary comparison between *GBS Δ covR* and saline controls (Figure 2.3). All immunoproteins found to be significantly differentially expressed were upregulated in the *GBS Δ covR* group versus saline controls. The greatest number of upregulated immunoproteins was in the decidua with a lesser number of differentially upregulated proteins in the amnion and chorion (Figure 2.3; Table 2.3). Inhibitory immune checkpoint proteins/ligands were significantly upregulated in all ROIs including VISTA (decidua), LAG-3 (chorion), and PD-L1 (ligand for PD-1, amnion and decidua; all $p < 0.05$). In addition to overexpression of inhibitory immune checkpoint proteins, many stimulatory checkpoint proteins/ligands that activate B and T cells (OX40L, CD40, CD40L, GITR) were significantly upregulated (all $p < 0.05$). Across all ROIs, Ki67 was also significantly differentially upregulated indicating widespread cell cycle activity and/or proliferation ($p < 0.05$, each ROI).

Next, we investigated the role of the GBS β -hemolysin in directing the shift in immune checkpoint expression by comparing immunoprotein expression in *GBS Δ covR* (overexpressing β -hemolysin) versus *GBS Δ covR Δ cyIE* (lacking β -hemolysin) exposed tissues. In this comparison, the profile of immunoprotein expression was very similar to that obtained when comparing *GBS Δ covR* versus saline controls, which underscored the importance of the hemolysin to GBS virulence and induction of host immunologic response (Figure 2.4; Table 2.4). In both comparisons, there was overexpression in

GBS Δ covR of immune checkpoint proteins/ligands PD-L1 (amnion), GITR (chorion), and VISTA (decidua). In contrast, when antigen counts were analyzed for the comparison of

Figure 2.3. Differential Expression of DSP Antigens by Region of Interest for GBS Δ covR versus Saline



The log₁₀ S6 normalized antigen counts for GBS Δ covR (red) and saline controls (blue) are shown on the x-axis by region of the chorioamniotic membranes with the mean (dot) and $\pm$ 1 standard deviation (bar). Although the entire GeoMx panel included 56 antigens, we show only the 24 antigens (y-axis) that were significantly different between the GBS Δ covR and saline controls in either the amnion, chorion or decidua using Kruskal-Wallis ($p < 0.05^*$, $.01^{**}$, $.001^{***}$).

Table 2.3 Differentially Expressed Immunoproteins in the Amnion, Chorion and Decidua at the Site of a GBS Δ covR Inoculation

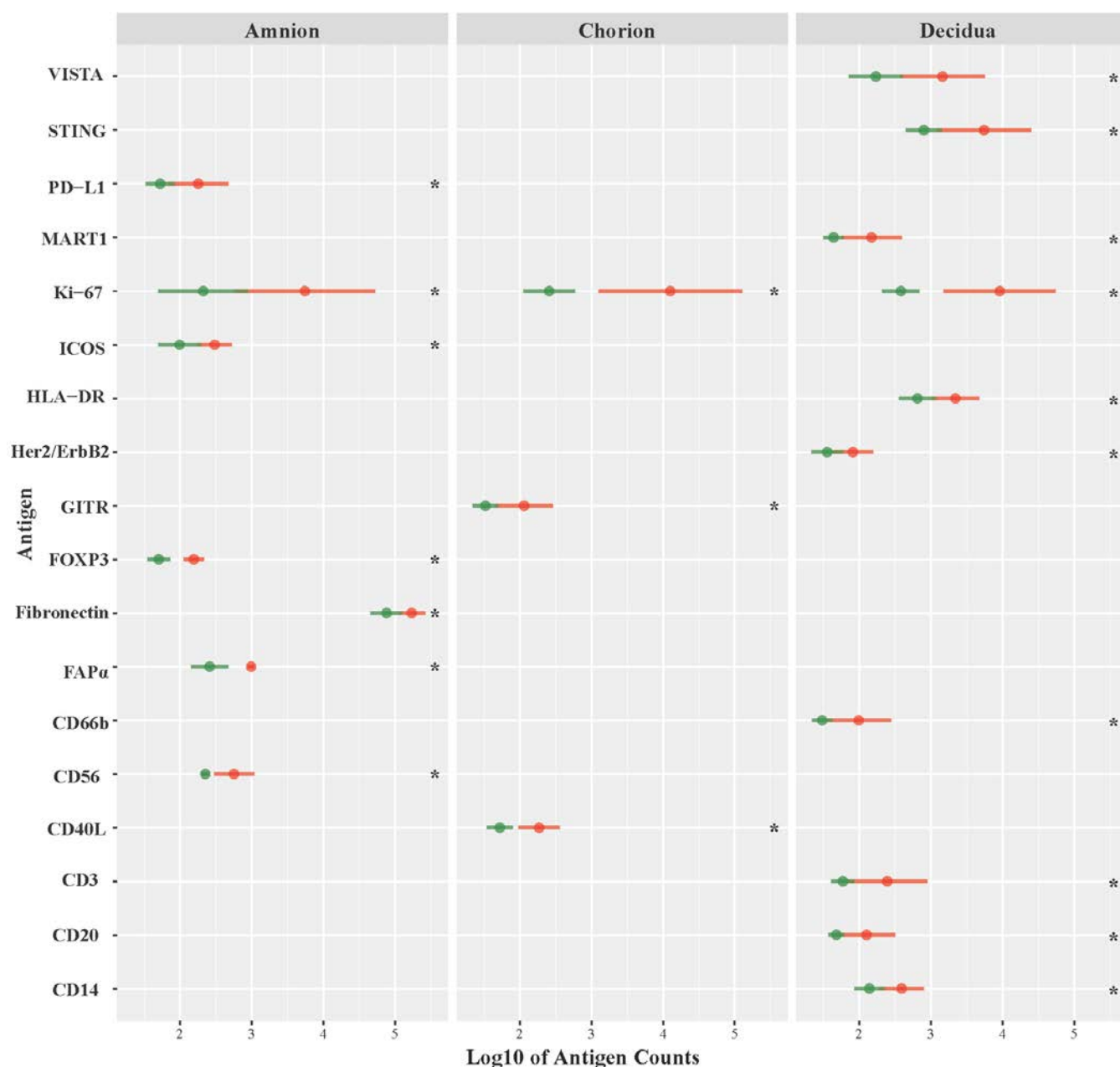
Antigen	Mean Expression for Saline Controls	Mean Expression for GBS Δ covR	Pooled Standard Deviation	Standard Differences between GBS Δ covR and Saline Controls	Kruskal-Wallis Statistic	Kruskal-Wallis p-value
<i>Amnion</i>						
FAP α	2.64	2.99	0.07	5	5.33	0.02
S100B	2.55	3.11	0.23	2.43	5.33	0.02
$\gamma\delta$ -TCR	1.19	1.5	0.13	2.38	4.08	0.04
Ki-67	2.06	3.74	0.71	2.37	5.33	0.02
FOXP3	1.87	2.19	0.17	1.88	4.08	0.04
Fibronectin	4.92	5.23	0.2	1.55	4.08	0.04
CD40L	2.35	2.75	0.27	1.48	4.08	0.04
PD-L1	1.82	2.25	0.3	1.43	5.33	0.02
<i>Chorion</i>						
Ki-67	2.17	4.1	0.72	2.68	5.33	0.02
Bcl-2	2.35	2.85	0.2	2.5	5.33	0.02
CD14	2.38	2.91	0.23	2.3	5.33	0.02
CD45	2.59	3.91	0.7	1.89	4.08	0.04
STING	2.6	3.78	0.66	1.79	4.08	0.04
LAG3	1.29	1.85	0.33	1.7	5.33	0.02
GITR	1.57	2.06	0.31	1.58	4.08	0.04
<i>Decidua</i>						
Ki-67	2.29	3.96	0.6	2.78	5.33	0.021
PanCk	2.18	3.13	0.35	2.71	5.33	0.021
VISTA	2.00	3.16	0.43	2.7	5.33	0.021
CD44	1.45	2.3	0.33	2.58	5.33	0.021
CD14	2.11	2.59	0.22	2.18	5.33	0.021
CD40L	1.18	1.91	0.37	1.97	5.33	0.021
MART1	1.53	2.17	0.33	1.94	5.33	0.021
S100B	1.80	2.48	0.36	1.89	5.33	0.021
Her2/ErbB2	1.51	1.91	0.22	1.82	4.08	0.043
CD127	0.88	3.35	0.26	1.81	4.08	0.043
STING	2.83	3.74	0.51	1.78	40.8	0.043
CD45	2.95	3.96	0.58	1.74	4.08	0.043
CD40	1.76	2.37	0.36	1.69	4.08	0.043
CD66b	1.44	1.99	0.33	1.67	4.08	0.043
OX40L	1.46	1.96	0.3	1.67	5.33	0.021
PD-L1	1.21	1.89	0.41	1.66	4.08	0.043
EpCAM	1.77	2.5	0.47	1.55	4.08	0.043

This table lists the significantly differentially expressed immunoproteins within the amnion, chorion and decidua for the GBS Δ covR versus saline control group. Within each region of interest, the antigens were listed in descending order by the standard difference between GBS Δ covR and saline controls.

antigen was significantly differentially expressed in the *GBSΔcovRΔcyIE* group ($p<0.05$).

Next, we investigated the role of the GBS β -hemolysin in directing the shift in immune checkpoint expression by comparing immunoprotein expression in *GBSΔcovR* (overexpressing β -hemolysin) versus *GBSΔcovRΔcyIE* (lacking β -hemolysin) exposed tissues. In this comparison, the profile of immunoprotein expression was very similar to that obtained when comparing *GBSΔcovR* versus saline controls, which underscored the importance of the hemolysin to GBS virulence and induction of host immunologic response (Fig. 4; Table 2.4). In both comparisons, there was overexpression in *GBSΔcovR* of immune checkpoint proteins/ligands PD-L1 (amnion), GITR (chorion), and VISTA (decidua). In contrast, when antigen counts were analyzed for the comparison of *GBSΔcovRΔcyIE* versus saline controls, only the gamma-delta T cell receptor ($\gamma\delta$ -TCR) antigen was significantly differentially expressed in the *GBSΔcovRΔcyIE* group ($p<0.05$).

Figure 2.4. Differential Expression of DSP Antigens by Region of Interest GBS Δ covR Versus GBS Δ covR Δ cy/E



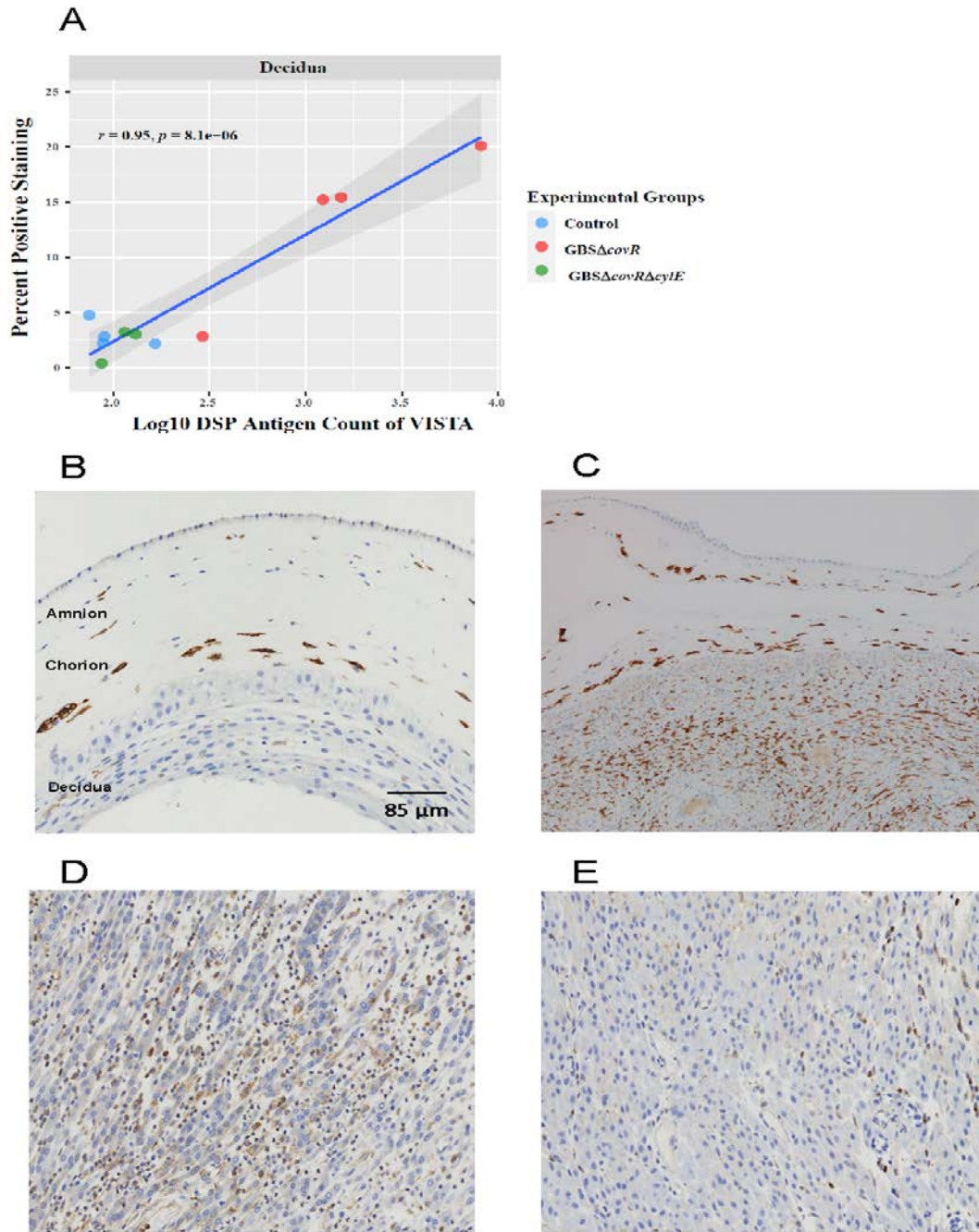
Differential Expression of DSP Antigens by Region of Interest GBS Δ covR Versus GBS Δ covR Δ cy/E. The log10 S6 normalized antigen counts for GBS Δ covR (red) and GBS Δ covR Δ cy/E (green) are shown on the x-axis by region of the chorioamniotic membranes with the mean (dot) and $\pm$ 1 standard deviation (bar). Although the entire GeoMx panel included 56 antigens, we show only the 18 antigens (y-axis) that were significantly different between the GBS Δ covR and saline controls in either the amnion, chorion or decidua using Kruskal-Wallis (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

Table 2.4. Differentially Expressed Immunoproteins in Amnion, Chorion, and Decidua for Each Experimental Group Contrast.

Significant DSP Antigen	GBS Δ covR vs. Control			GBS Δ covR vs GBS Δ covR Δ cyIE		
	Amnion	Chorion	Decidua	Amnion	Chorion	Decidua
Bcl-2		X				
CD3						X
CD14		X	X			X
CD20						X
CD40			X			
CD40L	X		X		X	
CD44			X			
CD45		X	X			
CD56				X		
CD66b			X			X
CD127			X			
EpCAM			X			
FAP α	X			X		
Fibronectin	X			X		
FOXP3	X			X		
$\gamma\delta$ -TCR	X					
GITR		X			X	
Her2/ErbB2			X			X
HLA-DR						X
ICOS				X		
Ki-67	X	X	X	X	X	X
LAG3		X				
MART1			X			X
OX40L			X			
PanCk			X			
PD-L1	X		X	X		
STING		X	X			X
S100B	X		X			
VISTA			X			X

This table shows the differentially expressed immunoproteins for each experimental group contrast. An X indicates a significant ($p < 0.05$) comparison within a specific region of interest (amnion, chorion, decidua).

Figure 2.5. VISTA Correlation and Expression by DSP and Immunohistochemistry



Panel **(A)** shows the relationship between $\log_{10}$ DSP antigen counts and percent positive VISTA immunostaining (DAB, brown stain) out of the total area stained (DAB + haematoxylin blue stain) for GBS Δ covR (red) and GBS Δ covR Δ cylE (green), and controls (blue) in the decidua. The Pearson coefficient is shown (r). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. VISTA **(B)** and CD163 **(C)** immunostaining of the amnion, chorion and decidua are shown to demonstrate morphologic overlap between the staining profiles in a saline control. Immunohistochemistry targeting VISTA in the decidua is shown for GBS Δ covR **(D)** and GBS Δ covR Δ cylE **(E)** groups to show the cellular influx of VISTA+ cells in the GBS Δ covR group.

Finally, we validated the DSP profile using immunohistochemistry. The percentage of immunohistochemical staining in decidua and the DSP antigen counts in decidua were highly and significantly correlated for VISTA ($r=0.95$; $p=8.1e-6$; Fig. 5A]. Cell staining for VISTA was widely expressed in chorionic macrophages in the saline control animals, which was verified by CD163 staining (Figure 2.5B-C). However, VISTA immunostaining was significantly higher in the decidua in the GBS $\Delta covR$ group compared to the isogenic GBS strain and saline (Figure 2.5D-E).

Peak AF cytokine concentration correlated with immune checkpoint expression

To determine whether spatial profiles of expressed immunoproteins in the placental chorioamniotic membranes and maternal decidua are related with AF inflammatory proteins, we correlated AF cytokine concentrations with immunoprotein antigen counts in the amnion, chorion, and decidua. First, we evaluated the AF cytokine distribution in the samples for which we had digital spatial profiling data[114]. As expected, there was a significantly increased concentration of multiple inflammatory cytokines (IL-6, I-TAC, IL-8, IL-1 β , MCP-1, TNF- α) in the GBS $\Delta covR$ group versus saline controls (all $p<0.05$).

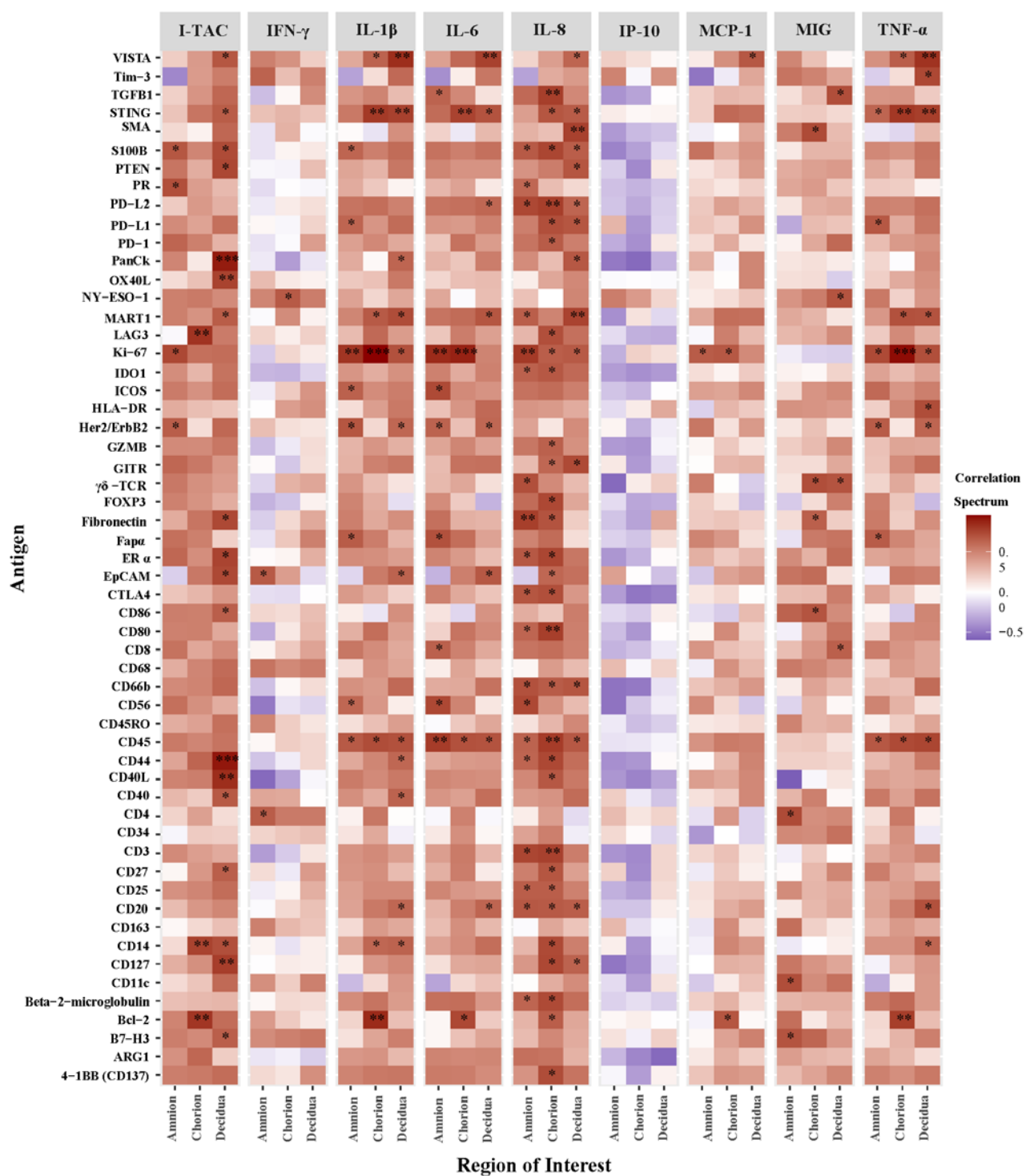
Next, we correlated AF cytokines with immune protein antigen counts expressed at the site of GBS inoculation within the amnion, chorion, and decidua (Figure 2.6). Among the ROI, chorion and decidua had the greatest number of correlations with peak AF cytokine concentrations. Of the immune checkpoint proteins in our DSP panel, VISTA had the greatest number of significant correlations with peak AF cytokine concentrations (I-TAC, MCP-1 IL-1 β , IL-6, IL-8, and TNF- α) within either the chorion or decidua. Of the AF

cytokines evaluated, IL-8 had the greatest number of significant correlations with overall DSP antigen counts and immune checkpoint proteins captured by the DSP (i.e., inhibitory: VISTA, PD-1/PDL-1/PDL-2, LAG3, CTLA4; stimulatory: GITR, CD27, CD40L CD137; $p < 0.05$). There were strikingly different patterns of correlation between T cell chemokines and stimulatory/inhibitory immune checkpoint proteins. For example, peak AF concentration of I-TAC was highly and significantly correlated with multiple immune checkpoint antigens (inhibitory immune checkpoints: VISTA, B7-H3; stimulatory immune checkpoints: OX40L, LAG3, CD27, CD40/CD40L; all $p < 0.05$). In contrast, there were few correlations between MIG and immune checkpoint antigens and none for IP-10. Overall, these findings reveal the selective and coordinated regulation of immune checkpoint proteins in placental chorioamniotic membranes with cytokine elevations in the amniotic fluid.

Uterine activity was moderately correlated with expression of few immunoproteins but not of immune checkpoints.

Our NHP model was designed to measure intraamniotic fluid pressure through implanted catheters to obtain quantitative and continuous measures of uterine contraction activity.[114, 115, 133, 134] We asked whether uterine activity, measured by the peak hourly contraction area in 24 hours, correlated with immune checkpoint or other immunoprotein antigen counts in the amnion, chorion, and decidua. Of the 168 antigens analyzed across all three ROIs in the GBS Δ covR versus saline contrast, only the counts for three antigens had moderate correlations with peak hourly contraction area in the decidua: fibronectin ($\rho = 0.66$, $p = 0.04$), CD127 ($\rho = 0.68$, $p = 0.035$) and phosphatase tensin

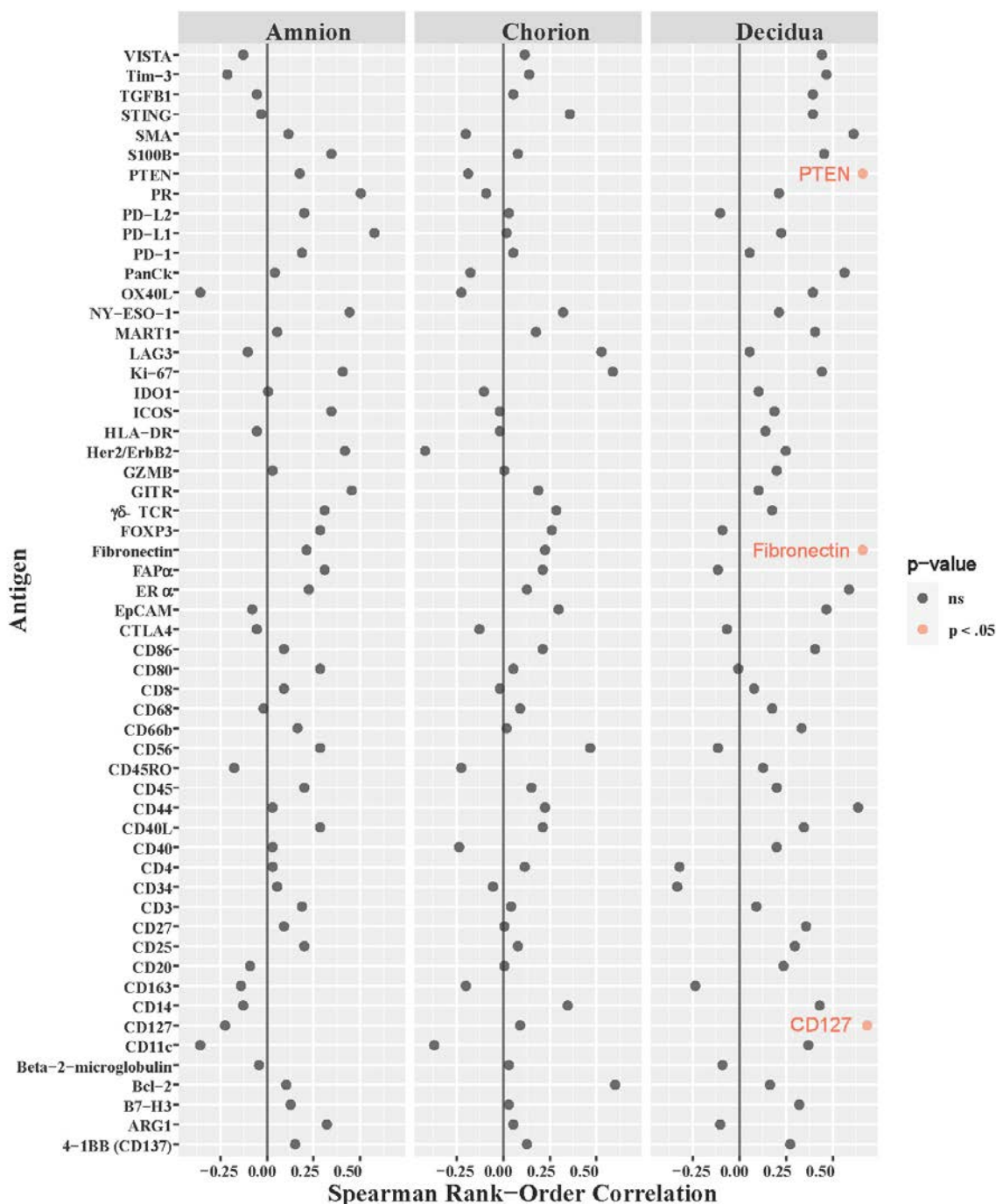
Figure 2.6. Heatmap of Correlations Between DSP Antigen Counts and Amniotic Fluid Cytokine Concentrations by Region of Interest



Log10 cytokine concentrations were correlated to log10 S6 normalized counts of immunoproteins (y-axis) for GBS Δ covR, GBS Δ covR Δ cyIE and controls. All 56 immunoproteins have been included, to evaluate the correlation in either the amnion, chorion or decidua using Spearman rank-order correlation (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

checkpoint protein expression and uterine activity.

Figure 2.7. Correlations Between DSP Antigen Counts and Peak 24-hour contraction area



The peak average hourly contraction area per day was correlated to log10 S6 normalized counts of immunoproteins (y-axis) for GBS Δ covR, GBS Δ covR Δ cyIE and controls. All 56 immunoproteins have been included, to evaluate the correlation in either the amnion, chorion or decidua using Spearman Rank order correlation coefficient (*, p < 0.05; **, p < 0.01; ***, p < 0.001).

Table 2.5. Antigens Significantly Correlated with Red Maternal Stage Score.

Antigen	Amnion		Chorion		Decidua	
	ρ	p-value	ρ	p-value	ρ	p-value
Bcl-2	-	-	0.7	0.02	-	-
CD20	-	-	-	-	0.7	0.02
CD45	0.7	0.03	0.8	0.009	0.9	0.003
Ki-67	0.9	7×10^{-4}	0.9	7×10^{-4}	-	-
NY-ESO-1	0.7	0.03	-	-	-	-
PD-L2	-	-	-	-	0.7	0.02
STING	-	-	0.8	0.02	0.7	0.02
Tim-3	-	-	-	-	0.7	0.02
VISTA	-	-	-	-	0.7	0.02

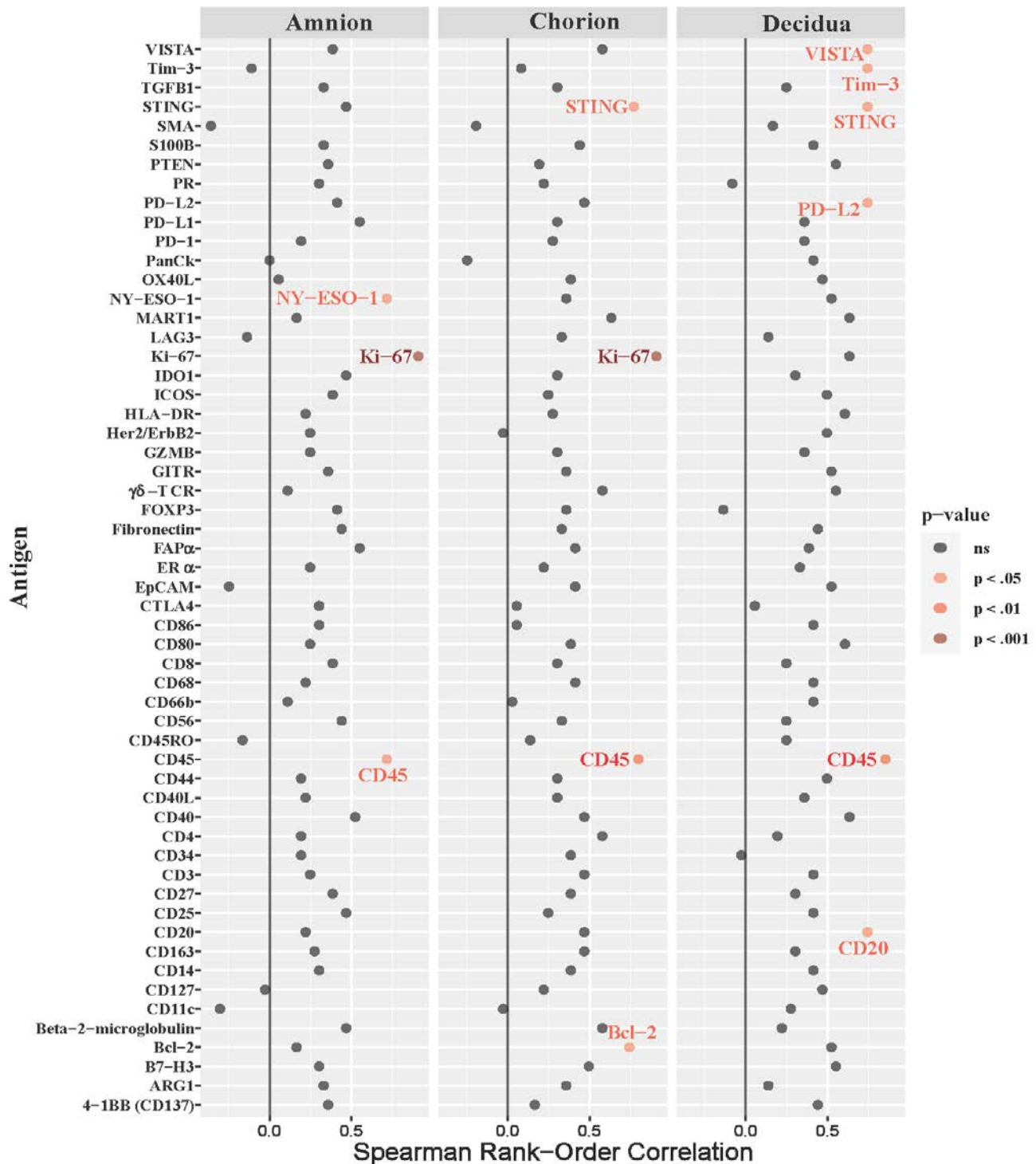
The table shows the ρ (Spearman correlation coefficient) and p-value within each region of interest for each antigen that had a significant correlation.

Placental Redline histopathologic scoring correlated with only inhibitory immune checkpoint expression

Next, we asked whether immune checkpoint expression correlated with histopathological evidence of placental inflammation, as quantified by the Redline criteria[135]. Redline scoring was performed by a pediatric and placental pathologist with focus on scores assigned to maternal stage and maternal grade. The maternal stage score reflects the

duration of the inflammatory response based on the presence of neutrophils spread in segmented tissues, while the maternal grade score reflects the maternal and fetal inflammatory response based on the presence of abscesses in different tissue areas. When analyzing both maternal stage and grade, only inhibitory immune checkpoints (VISTA and Tim-3) were significantly correlated with either the stage or grade score in the chorion and decidua (maternal stage, Figure 2.8, Table 2.5; maternal grade; all $p < 0.05$). The maternal stage Redline score also correlated with PD-L2 expression in the decidua. No stimulatory immune checkpoints correlated with maternal stage or grade scores.

Figure 2.8. Correlations Between DSP Antigen Counts and Placental Redline Maternal Stage Score



Placental Redline Maternal Stage scores were correlated to log10 S6 normalized counts of immunoproteins (y-axis) for GBS Δ covR, GBS Δ covR Δ cyIE and controls. All 56 immunoproteins have been included, to evaluate the correlation in either the amnion, chorion or decidua using Spearman Rank order correlation coefficient (*, p<0.05; **, p<0.01; ***, p<0.001).

Discussion

Summary of major findings

Immune checkpoints are critical for maintaining immunologic homeostasis and can be co-opted within tumors and during infection to facilitate immune evasion. In our nonhuman primate model, we could spatially profile the immune checkpoint expression at the maternal-fetal interface exposed to a GBS experimental infection. Our data indicates that an early and spatial immunologic response occurs in the placental chorioamniotic membranes and decidua, which upregulates expression of multiple inhibitory immune checkpoint proteins (VISTA, PD-L1, LAG3) and a few activating checkpoint proteins (OX40L, CD40/CD40L, GITR) during a GBS Δ covR inoculation. The lack of differentially expressed immune checkpoints in the placental membranes and decidua between saline and GBS Δ covR Δ cyIE groups reflects the importance of β -hemolysin in driving immune checkpoint expression. The broad spectrum of stimulatory and inhibitory immune checkpoint proteins was highly, positively correlated with several AF inflammatory cytokines (e.g., IL-8) and the Redline maternal stage/grade scores indicating an association between these classes of immune proteins and the inflammatory response within the AF and at the maternal-fetal interface.

The inhibitory immune checkpoint VISTA had the highest differential expression in the decidua, which was due mainly to VISTA expression on infiltrating neutrophils. In contrast, there were relatively few significantly expressed immunoproteins in the amnion and chorion, underscoring the importance of decidual immune cells in the placental/maternal

host response to infection. Overall, our data reveals the complexity of pro- and anti-inflammatory responses at the site of a GBS infection with an intriguing role for VISTA and other immune checkpoint proteins to modulate the maternal immune response during acute chorioamnionitis.

Role of immune checkpoints in normal and adverse pregnancy outcomes

The immunologic balance required to maintain tolerance to semi-allogeneic fetal cells is complex. Immune checkpoint proteins have previously been detected within gestational tissues and peripheral blood in normal and abnormal pregnancies. In the term placenta, immune checkpoints are expressed by a diverse repertoire of cells at the maternal-fetal interface including cytotrophoblast cells, stromal decidual cells, and decidual lymphocytes with a changing profile of expression across gestational age[136]. Investigation of immune checkpoints revealed elevated levels of soluble PD-L1 (sPD-L1) and Galectin-9 (Gal-9) in peripheral blood of pregnant women and placental chorionic villous tissues, suggesting that inhibitory immune checkpoint expression may be important for normal pregnancy[137]. Further studies suggested a low sPD-L1 may be a biomarker for spontaneous abortion [138]. Dysregulation of immune checkpoints has been associated with adverse pregnancy outcomes. Expression of immune checkpoints is also increased in the pro-inflammatory state of preeclampsia (PD-1/PD-L1 system, TIM-3, CD40/CD40L) [139, 140]. During early stages of pregnancy, LAG-3 CTLA4 T⁺ regulatory cells have been found in the periphery and decidua and have been postulated to inhibit proliferation of tolerance against the fetus[141]. Dysregulation of imm

une checkpoints has also been associated with recurring pregnancy loss. Increased expression of immune checkpoints LAG-3 and CTLA4, and decreased expression of CD276 have been demonstrated to negatively affect implantation and contributes to overactivation of the immune system in the endometrium, preceding tissue of the decidua [141]. Data from this study indicate that immune checkpoint upregulation occurs during an early GBS infection within the placental chorioamniotic membranes and decidua, which may favor either the host or pathogen.

Role for inhibitory immune checkpoint proteins in GBS infection within chorioamniotic membranes

Multiple activating and inhibitory immune checkpoint proteins were upregulated in the placental chorioamniotic and decidua tissues after GBS inoculation (i.e., VISTA, PD-L1, LAG3, OX40L, GITR). Regulation of VISTA expression was a key finding in our study, as it demonstrated the highest difference between experimental groups and broadest correlation with AF cytokine concentration and maternal placental redline stage scoring of the immune proteins in the DSP panel. VISTA is an inhibitory immune checkpoint expressed primarily on myeloid derived immune cells, including macrophages, monocytes, neutrophils, and both lymphoid and myeloid subsets of dendritic cells and at lower levels on naïve CD4⁺ and CD8⁺ T cells [142]. Studies have shown that deficiency of VISTA leads to dysregulation of macrophage chemotaxis due to downregulation of chemokine receptors (CCR2, CCR5, MCP-1 receptor)[143]. Chemotaxis of myeloid cells are crucial for host response during bacterial infection, which is partially regulated through expression of VISTA. After GBS infection with the hypervirulent strain (GBS Δ covR),

VISTA immunostaining was significantly increased in the decidua with morphology suggestive of neutrophil and macrophage expression. The relationship between neutrophil influx and immune checkpoint expression in the decidua may be critical for defining the chemotactic response.

Immune checkpoint proteins control T cell activation and when expressed by neutrophils can impair the function of cytotoxic CD8⁺ T cells [144]. In humans, CD4⁺ and CD8⁺ T cells within the maternal decidua represent approximately 30-40% and 45-75% of leukocytes in humans[145]. Studies have demonstrated that both CD4⁺ and CD8⁺ T cells from human third trimester decidua have a highly differentiated effector memory phenotype and express higher levels of immune checkpoints (PD-1, TIM-3, CTLA-4 and LAG-3) in comparison to peripheral blood[144, 146]. Our findings of the regulation of immune protein expression after GBS infection within the chorion and decidua may be important in understanding how decidual CD4⁺ and CD8⁺ T cells balance the role of preventing an allogeneic response against the fetus while protecting against infection[144, 147, 148].

The association of immune checkpoint expression with pro-inflammatory cytokine concentrations is comparable to literature in oncology describing a tumor microenvironment. A hallmark feature of our GBS NHP model is neutrophil influx into the decidua and chorioamniotic membranes with release of neutrophil extracellular traps after inoculation of GBS Δ covR [114]. In tumor microenvironments, neutrophils infiltrate different cancers and express inhibitory immune checkpoints, including ARG1 and PD-1/PDL1, which restrict immune surveillance of malignant cells [149-152]. Chemotactic cytokines recruiting neutrophils (e.g., IL-8) into tumors have been investigated as

potential prognostic markers for the effectiveness of cancer drugs targeting inhibitory immune checkpoints.[153, 154] In addition, neutrophil release of NETs within a tumor is also linked to secretion of immune checkpoint proteins, a finding associated with a poorer prognosis in several cancers including pancreatic ductal adenocarcinoma [70, 149, 152, 154, 155]. Our data supports further studies to investigate the key role of immune checkpoint proteins in the immune response of an evolving infection in the chorioamniotic membranes and decidua. Whether immune checkpoints may promote pathogen survival or activate host responses is unknown.

Role for stimulatory immune checkpoint proteins in GBS infection within chorioamniotic membranes

Many stimulatory immune checkpoint proteins/ligands were significantly increased in the amnion, chorion and decidua, but primarily the decidua, in the GBS Δ *covR* versus saline group [GITR, OX40L, CD40, CD40L, tumor necrosis factor receptor superfamily (TNFRSF) receptors]. TNFRSF receptors are well characterized as proinflammatory markers that can bind TNFs, trigger cell death and primarily expressed on myeloid and/or lymphocytes[156, 157]. The corresponding ligands are found on a multitude of cells, including OX40L on antigen presenting cells and CD40L on activated T and B cells, platelets, monocytic cells, natural killer cells and basophils. Surprisingly, in our study TNFRSFR receptors and ligands did not correlate with AF TNF- α concentrations. In contrast to inhibitory immune checkpoints (e.g., VISTA), not one stimulatory immune checkpoint correlated significantly in antigen counts with either AF cytokine concentrations, uterine activity or placental histopathology scores. This may suggest that

the inhibitory immune checkpoint VISTA has a more consistent and greater change in expression, which could facilitate immune evasion by bacteria.

Clinical and Research Implications

This study has provided insight into the role of immune checkpoint proteins in the activation of adaptive immune cells in normal pregnancy and GBS chorioamnionitis. Late preterm birth is often characterized by infiltration of CD8⁺ T cells into the chorioamniotic membranes, a condition called chronic chorioamnionitis [158, 159]. Investigating the regional expression of immunoproteins is critical to understanding the innate and cellular response and developing therapeutics that can manipulate the natural immune response to prevent adverse pregnancy outcomes. It is possible that inhibitory immune checkpoints upregulated during bacterial-host pathogen interactions in the decidua may prevent T cell infiltration over a few days but could be overwhelmed if the bacteria persist. Further research is necessary to determine how stimulatory and inhibitory immune checkpoint proteins may exclude or allow maternal T cells to infiltrate the chorioamniotic membranes.

Strengths and Limitations

Immune checkpoint proteins are potential therapeutic targets for improving pregnancy outcomes following an infectious encounter. These proteins have been demonstrated to be upregulated in other acute and chronic infections, such as malaria, listeria, HIV, and hepatitis B [160]. However, there are no prior studies to our knowledge that reveal changes in immune checkpoint expression at the maternal-fetal interface during an infection. Therefore, our study is the first to correlate regional expression of immune

checkpoint proteins after GBS infection within the chorion and decidua with inflammation, uterine activity and histopathologic changes in the placental chorioamniotic membranes. As best practices for data normalization are not yet established for this new technology, the extensive testing of different normalization methods and validation with data from other platforms is a study strength. Finally, interrogating correlations between the chorion, decidua, and AF cytokines revealed crosstalk between these compartments.

There are also several limitations to this study, including the number of animals and immunoproteins analyzed. This panel focused mainly on adaptive immune checkpoints and lacked several immune checkpoint receptors or ligands that would allow us to evaluate an entire immune checkpoint axis (e.g., OX40-OX40L; GITR-GITRL). Other proteins of interest not in the panel include signal-regulatory protein- α (SIRP α), an inhibitory receptor expressed on myeloid phagocytes, and LILRB2, found on both innate (monocytes, macrophages, basophils, and DCs) and adaptive (CD4⁺ T cells) cells [161]. These limitations can be addressed using the latest version of Nanostring GeoMx® and targeting the whole transcriptome. Additionally, this data does not allow us to elicit the functions of these immune proteins in the context of a GBS Δ *covR* infection but instead highlights the differential regulation of multiple immune checkpoint proteins. Finally, this experiment has a modest sample size in each experimental group and should ideally be validated in a human cohort; given the small N and exploratory nature of the investigation, multiple hypothesis correction was not performed. However, a limited study of two immune checkpoints in the peripheral blood of pregnant women and chorionic villous tissues suggested higher expression of PDL-1 in syncytiotrophoblast cells that validates in part the dynamic nature of immune checkpoint expression in the placenta[137]. More

studies on the role of immune checkpoints in the placenta following infection are warranted.

Conclusions

Pregnancy requires an intricate balance of tolerance to fetal allograft while being able to respond to infection. Our study findings reveal the complexity of the pro- and anti-inflammatory response at the site of a GBS infection in the placental chorioamniotic membranes and decidua, which underscores the importance of the decidua and chorion in the host response. Expression of VISTA, an inhibitory immune checkpoint, within the decidua may represent an important inhibitory immune checkpoint at the site of an infectious stimulus that alters pathogen response by other immune cells.

Chapter III: Spectrum of GBS Invasive Infections in the Uterine Cavity

Abstract

Inflammation and infection are the causes of the majority of early preterm births. Host innate immune responses at the maternal-fetal interface that contribute to sterile intra-amniotic inflammation (SIAI), bacterial clearance, or invasive infections at the maternal-fetal interface remain poorly understood. We have used Group B Streptococcus (GBS) as a model for pregnancy associated bacterial infections because it is a well-known cause of preterm birth, stillbirth, and invasive neonatal disease. In a chronically catheterized nonhuman primate (NHP) model, we investigated host innate immune responses correlating with invasive and resolving GBS infections in the choriodecidual space adjacent to the uterine muscle (myometrium) and the placental chorioamniotic membranes. The GBS infection was classified as either progressing (invasive into the amniotic fluid), localized (bacteria present at the choriodecidual inoculation site), or resolved (minimal to no bacteria detected) based on the extent of bacterial dissemination. Temporal and spatial RNA and protein profiles in the chorioamniotic membranes/decidua and myometrium were investigated by histopathology, multiplex protein assays, total RNA-Seq, Nanostring nCounter, and digital spatial profiling. Preterm labor occurred in 2/5 (40%) of progressing infections and 4/5 (80%) of resolved infections. Progressing GBS infections were characterized by neutrophilic infiltration, elevation of pro-inflammatory cytokines, and activation of inflammatory cell death pathways (pyroptosis, ferroptosis) within the chorioamniotic membranes and decidua. In contrast, the resolved GBS infection group was characterized by minimal inflammation and upregulation of cytotoxic

granule gene expression [i.e., granzyme A (GZMA)]. Digital spatial profiling identified the decidua as the region of inflammatory pathway activation in the progressing group and increased cytotoxic granule gene expression in the resolving infection group. Interestingly, the myometrium in the resolved GBS infection group exhibited evidence of greater inflammation and lymphocyte activation. A greater inflammatory response in the myometrium of GBS-resolved infections is observed, and the higher rate of preterm labor in this group suggests that bacterial killing at the maternal-fetal interface may induce uterine inflammation that ultimately leads to preterm labor. Further investigation is required to determine if the killing of bacteria in the decidua by uNK and CD8⁺ T cells, with propagation of the inflammatory response into the myometrium, may explain cases of preterm labor associated with SIAI, which were previously not thought to be linked to intrauterine infections.

Introduction

Most early preterm births are associated with either sterile intra-amniotic inflammation (SIAI) or microbial invasion of the amniotic cavity (MIAC), which share a common pathway characterized by elevated inflammatory cytokines in the amniotic fluid, like interleukin-1 β (IL-1 β). Group B Streptococcus (GBS, *Streptococcus agalactiae*) is a gram-positive bacterium that is a significant cause of infection and inflammation, leading to preterm birth, stillbirth, and neonatal sepsis. GBS is a common colonizer of the vaginal tract; however, in pregnancy, GBS can ascend into the lower uterus and traffic into the decidua and across the placental chorioamniotic membranes to invade the amniotic fluid [21]. An

estimated 500,000 preterm births and 46,000 stillbirths annually have been linked to GBS infections worldwide [21, 125, 162-165].

The relatively inaccessible maternal-fetal interface has made it challenging to understand the host's innate immune responses that direct GBS bacterial clearance or aid in bacterial propagation and invasive disease. A framework for the progression or resolution of inflammation and infection have evolved over the years to incorporate several factors: 1) the type of cells responding to the infection (i.e., neutrophils), 2) the class of chemical modulators produced, 3) degree of activation of innate immune sensors (i.e., toll-like and nod-like receptors, TLR and NLR, respectively), 4) mechanism of host cell death, and 5) how dying stromal and immune cells are removed (efferocytosis) [166-168]. An invasive infection at the maternal-fetal interface is more likely to be driven by a hyperinflammatory response with induction of pyroptosis or ferroptosis, and lysis of the host cell with release of pathogen-associated and danger-associated molecular patterns (PAMPs and DAMPs, respectively) [169]. Which mechanisms are involved in determining the outcome of host-pathogen responses at the maternal-fetal interface is unknown.

Our group has previously published studies in a pregnant nonhuman primate (NHP) model that a GBS choriodecidual inoculation can either become invasive into the amniotic cavity or resolve over time [70, 115]. Therefore, we have used a chronically catheterized nonhuman primate (NHP) model to examine host innate immune responses associated with experimentally invasive and resolving GBS infections in the choriodecidual space, which lies adjacent to the uterine muscle (myometrium) and the placental chorioamniotic membranes. To elicit the temporal and spatial host response to bacterial dissemination and resolution within the uterus and the surrounding lower uterine segment of

myometrium, we inoculated GBS in our chronically catheterized nonhuman primate (NHP) pregnant model. Here, we characterized the range of GBS infections inoculated into the choriodecidual space as being either progressive (invasive, extending into the amniotic fluid), localized (bacteria persisting primarily at the inoculation site), or resolved (with minimal to no bacteria detected at the choriodecidual inoculation site and amniotic fluid). The objective of this study was to determine the host innate immune factors and transcriptional profile that characterized the persistence or resolution of a GBS infection at the maternal-fetal interface in our NHP model. We hypothesized that a highly inflammatory immune profile, associated with IL-1 signaling and inflammatory host cell death (pyroptosis, ferroptosis, necroptosis), was linked to the progression of infections, whereas a mild or non-inflammatory signature was associated with the resolution of infections.

Methods

Research Animals and Ethics Approval

In this study, animal experiments were conducted in full compliance with the guidelines outlined in the Guide for the Care and Use of Laboratory Animals by the National Research Council, and “The Use of Non-Human Primates in Research” in the Weatherall report. The study protocol was approved by the University of Washington Institutional Animal Care and Use Committee, under Permit Number 4165-01, with the most recent approval on May 7, 2025. Surgeries were performed under general anesthesia, and every precaution was taken to minimize animal distress. All experimental animals utilized were pregnant (Table 3.1).

Table 3.1: Animal Demographics, Sex, Pathogen, Time of Inoculation

Animal ID	Group	Strain	Dam Gestational Age at Inoculation (days)	Interval Between Inoculation and Delivery (days)	Fetus	
					Sex	Weight (g)
CTL4	uninfected	N/A	127.3	1.10	male	242.0
CTL5	uninfected	N/A	133.4	1.00	female	264.0
CTL6	uninfected	N/A	135.3	1.04	female	299.5
RES1	resolved	GBS Δ covR Δ cyIE	132.4	1.04	male	267.0
RES2	resolved	GBS Δ covR Δ cyIE	129.4	3.04	female	299.0
RES3	resolved	GBS Δ covR	132.4	3.25	male	366.0
RES4	resolved	GBS Δ covR	128.4	1.17	male	236.0
RES5	resolved	GBS Δ covR	129.3	1.05	male	200.0
LOC1	localized	GBS Δ covR	130.4	1.06	female	310.0
LOC2	localized	GBS Δ covR	130.3	1.05	male	310.0
LOC3	localized	GBS Δ covR	135.4	1.00	male	351.5
LOC4	localized	GBS Δ covR	132.4	1.00	male	344.8
LOC5	localized	GBS Δ covR Δ cyIE	135.4	3.09	male	340.0
LOC6	localized	GBS Δ covR Δ cyIE	129.4	3.04	male	N/S
PRO1	progressing	GBS Δ covR	130.4	0.23	male	299.0
PRO2	progressing	GBS Δ covR	134.4	1.21	female	263.0
PRO3	progressing	GBS Δ covR	127.4	1.00	female	255.7
PRO4	progressing	GBS Δ covR Δ cyIE	131.4	3.04	female	299.0
PRO5	progressing	GBS Δ covR	129.4	2.03	female	197.0

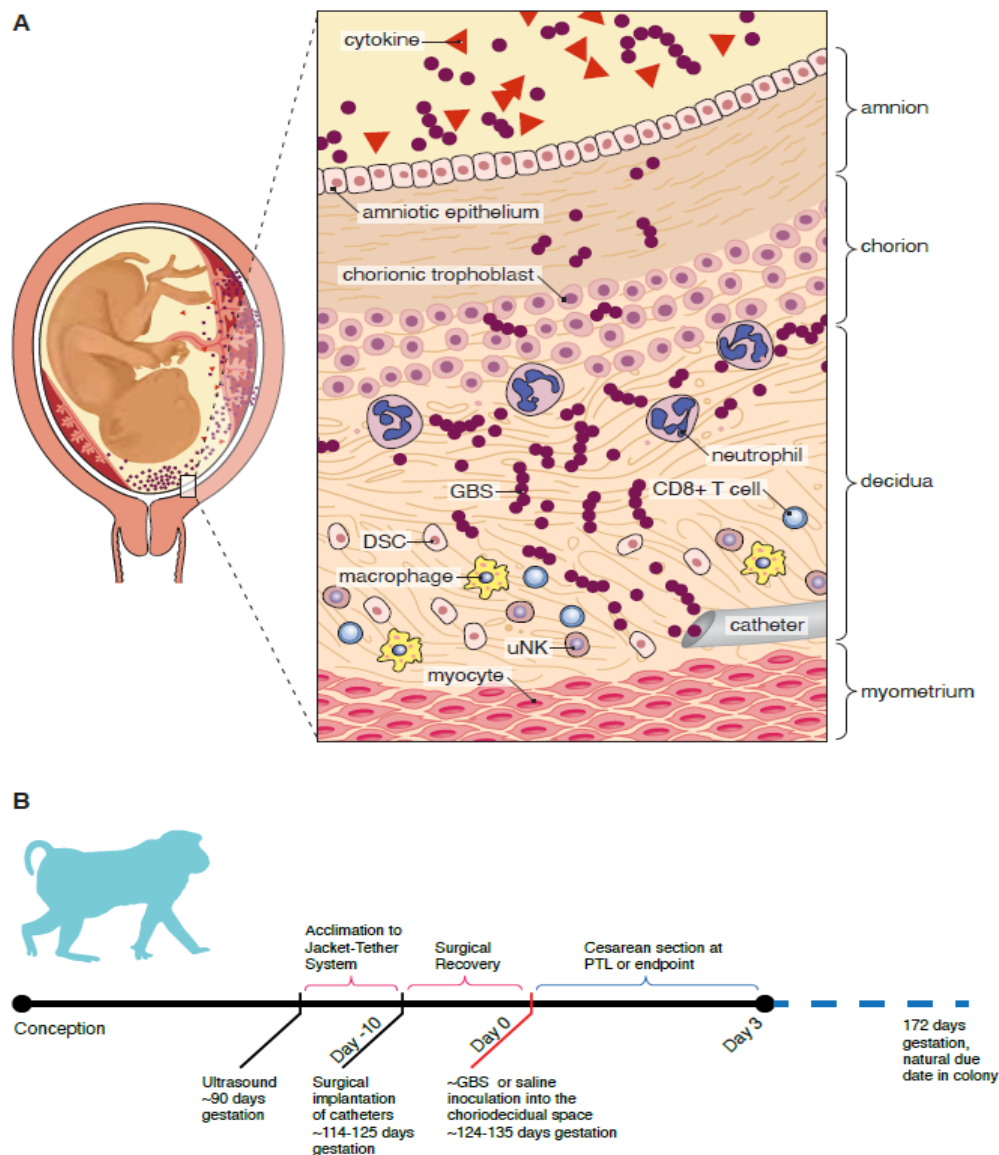
Abbreviations: NS, not sampled; N/A, not applicable

This table presents the demographics of the animals used in this study, including the gestational age of the mother, study duration, sex of the fetus, and weight for each animal.

Pregnant Nonhuman Primate Model of PTL

The chronically catheterized pregnant nonhuman primate (NHP) model of preterm labor (PTL) has been used in our lab for over two decades. Pregnant NHPs underwent surgical implantation of catheters via laparotomy into the maternal femoral vein, amniotic fluid, and choriodecidual interface in the lower uterine segment between 16- and 20-weeks' gestation (Figure 3.1). The NHPs received either a choriodecidual inoculation of the GBS strain COH1 Δ *covR* of $1-5 \times 10^8$ colony-forming units (CFU; n=11) or GBS strain COH1 Δ *covR* Δ *cyIE* of $1-5 \times 10^8$ CFU (n=5). Experimental controls were also catheterized and received saline inoculations (n=3).

Figure 3.1: The Nonhuman Primate Model of Group B *Streptococcus* (GBS) Infection



This figure illustrates the choriodecidual inoculation site of GBS and the surrounding placental and myometrial tissues, including the amnion, chorion, decidua, and lower uterine segment of the myometrium (**A**). A typical experimental study design schematic is shown in (**B**) with time on the x-axis in relation to the inoculation day, which is referred to as Day 0. A Cesarean section was performed at the time of preterm labor or on Day +1 or Day +3 of the study to follow the natural course of GBS infection.

Preterm labor and Cesarean section

In this study, cervical dilation and effacement or cervical dilation with increased uterine activity ($>10,000$ mmHg•sec/hr sustained over at least 2 hours) were used to define preterm labor. Cesarean sections were performed at either 1) preterm labor, 2) designated endpoint of 1- or 3-days post inoculation, or 3) 1 days after saline inoculation. Fetuses were euthanized by barbiturate overdose prior to exsanguination and fetal necropsy. Tissue collection took place at the endpoint, followed by complete gross and histopathologic examination. A 1- or 3-day endpoint was designed to investigate the earliest events and effects of GBS infection on placental tissues, fetal injury and infection/inflammation mediated preterm birth pathways. Recently, one-day control NHP experiments with saline choriodecidual inoculation were performed to more closely match the study design of the GBS 1-day inoculations designed to investigate early events in the pathogenesis of GBS invasive disease at the maternal-fetal interface. There was no inflammation and minimal signs of preterm labor in the one-day controls.

Intraamniotic pressure was continuously recorded using an implanted amniotic fluid pressure catheter (SPR-524, ADInstruments, Colorado Springs, Colorado, USA) and digitized with a Powerlab System (ADInstruments). The Amniotic fluid pressure signals were analyzed using custom software to eliminate noise due to respiration or position changes. The area under each contraction (mmHg•sec/hr) was summed for each hour, allowing calculation of the hourly contraction area, a measure of uterine activity. Finally, we calculated the mean hourly contraction area [(mmHg x sec)/hr; HCA] over a 24-hour period. Cervical dilation and length were recorded at baseline and delivery. Preterm labor was defined as cervical change with/ or uterine contractions.

Bacteria Growth and Confirmation of Infection

The GBS isogenic strains COH1 Δ *covR* and COH1 Δ *covR* Δ *cylE* were derived from wild type GBS COH-1, an ST-17 clone belonging to capsular serotype III, recovered from a neonate with meningitis. Single colonies of minimally passaged GBS from blood agar plates were grown in tryptic soy broth or tryptic soy agar 37°C in 5% CO₂ overnight. The GBS strains were grown to mid-log phase (O.D.₆₀₀=0.5) and prepared to a concentration of approximately $1-5 \times 10^8$ CFU in 1mL PBS before inoculation into the choriodecidual space.

Aliquots of the prepared inoculum were plated on tryptic soy agar to confirm strains and assess the precise inoculation amount. On tryptic soy agar, COH1 Δ *covR* appears orange and COH1 Δ *covR* Δ *cylE* appears white. Both strains were spectinomycin-resistant, and thus were subculture on selective medium (i.e., TSA containing spectinomycin). In addition to inoculum, amniotic fluid, fetal blood, homogenized placental (chorioamniotic membranes and decidua) and fetal tissues (lung, brain, fetal blood) were plated on TSA plates and enumerated. The level of CAMP factor activity was measured on sheep blood agar plates with the inoculum strain included in parallel.

Sample Collection and Processing

Amniotic fluid (AF) was collected throughout the experimental study. AF was sampled at baseline (-24 and -0.25 hr) and post GBS inoculation (+0.75, +6, +12, +24 h and then every 12 h until Cesarean section for fetal necropsy). Viable GBS bacteria were determined by AF serial dilutions plated on TSA, incubated overnight at 37°C, 5% CO₂ and enumerated. Additional aliquots were flash frozen in -80 °C. For detection of low GBS amounts in AF, qPCR (DNA quantification) was performed later on endpoint AF samples.

For quantification of cytokine and chemokine concentration, AF samples were collected in EDTA. Immediately after sample collections, samples were centrifuged for 5 min at 48 X g. The supernatants were frozen and stored at -80 °C. Similarly, to AF, viable GBS in fetal blood were determined by serial dilutions plated on TSA, incubated overnight at 37°C, 5% CO₂ and enumerated.

In all experiments, fetal and placental tissues (chorioamniotic membranes and decidua at the inoculation site and lower uterine segment of myometrium) were either flash frozen and stored in -80°C, preserved in RNA later, then frozen at -80°C, or placed in formalin-fixed paraffin-embedded molds. Flash-frozen samples were used for the quantification of cytokine and chemokine concentrations. Samples stored in RNA later were used for gene expression analysis, Total RNA sequencing, and nCounter, as well as the detection of bacterial genomic DNA. Formalin-Fixed embedded tissues were utilized for Nanostring GeoMx Digital Spatial Profiling Whole Transcriptome Analysis.

Cytokine Quantitation

Immune mediators at the tissue level were revealed using multiplex and simplex immunoassays validated for primate species. Cytokine and chemokine concentrations (IL-1 β , IL-6, IL-8, and TNF- α) were determined by Luminex multiplex technology using commercially available non-human primate cytokine kits (Millipore, Billerica, MA). IFN β was determined using commercially available human ELISA kits (PBL Assay Science, cat #46100-1).

GBS DNA qPCR in Amniotic Fluid, Myometrium and Chorioamniotic Membranes/ Decidua

GBS qPCR was used to detect both trace and total amounts of bacterial genomic DNA in AF and tissue samples. Primers and probes were designed using Integrated DNA Technologies (San Diego, CA) based on validated experiments on the surface immunogenic protein (SIP) found in GBS [170]. SIP is conserved in all GBS strains, including COH1 Δ covR and COH1 Δ covR Δ cytE. The TaqMan Fast Advanced Master Mix (Fisher Scientific, cat# 444964) was used for amplification. qPCR was performed on the QuantStudio 3 machine (Thermofisher Scientific, cat. #A28132) according to the manufacturer's instructions. Samples were run in triplicate. Positive results were defined as any sample with an average Ct value of 38 cycles or less.

Name	Sequence
GBS SIP Forward	GTTCCAGCAGCTAAAGAGGAAG
GBS SIP Reverse	CCGGTGCTACTTTAGCTACTGG
GBS SIP Probe	5'-FAM-CACCAGCTTCTGTTGCCGCTGAAACACCAGC-BHQ1-3'

Genomic DNA from amniotic fluid and placenta tissues was extracted by the QIAamp DNA mini kit (Qiagen, cat# 51306). For amniotic fluid, equal volumes of samples and AL buffer were mixed. 1:10 Proteinase K was added to the volume of AL buffer (e.g., 20 μ L Proteinase K to 200 μ L AL buffer, 40 μ L Proteinase K to 400 μ L AL buffer). Samples were incubated at 56 C for 10 minutes. DNA was eluted in 50 μ L Qiagen elution buffer.

DNA was extracted from myometrium homogenates prepared in TRIZOL. Tissue samples were mixed with 500 μ L of Back Extraction Buffer (BEB; 4M Guanidine Thiocyanate,

50mM Sodium Citrate NaCl and 1 M Tris base, prepared in ddH₂O) and centrifuged at 12,000 xg for 30 minutes. The aqueous upper phase was resuspended with 400 ul of ice-cold isopropanol to precipitate DNA. Samples underwent two wash steps which included centrifugation (12,000 xg for 15minutes at 4C) and resuspension with 500% ethanol. The final DNA pellet was dissolved in 30 uL of Qiagen elution buffer. For all amniotic fluid and myometrium DNA concentration was quantified using a Nanodrop spectrometer.

Definition of GBS Groups in Pigtail Macaque

GBS-infected NHPs were categorized into three groups based on bacterial enumeration (viable GBS) and GBS DNA (total GBS) counts in AF. Animals with a progressing GBS infection had viable counts [minimal concentration of 5×10^5 (colony-forming units per mL; CFU/mL)] and at minimal 1×10^6 genomic DNA equivalence per mL (GE/mL; total GBS DNA amount) of GBS in the AF. A localized GBS infection was defined by positive culture results at the inoculation site in the choriodecidual space or the chorioamniotic membranes but no viable counts in the AF; a trace amount of GBS DNA in the AF by qPCR (1×10^1 to 1×10^3 GE/mL) was determined. This was deemed to represent an infection that was intermediate between progressing and resolved. In contrast, a resolved GBS infection group was defined by no viable counts of GBS in the choriodecidual space, chorioamniotic membranes, or AF by bacterial plating or qPCR.

RNA isolation

High-quality RNA was required to investigate transcriptome changes using the total RNA-sequencing and nCounter platforms. Tissue samples stored in RNA Later were combined

with TRIzol (1 mL) and homogenized by the Precellys Evolution machine (Bertin Technologies, cat#K002198-PEVO0-A.0). RNA was extracted from tissue samples using RNeasy mini-Kit (Qiagen, cat#74104). The concentration of RNA was determined by the Qubit 4.0 fluorometer (ThermoFisher, cat#Q33238) and integrity of RNA (RIN) was measured by Agilent 4200 TapeStation (Agilent, cat#G2991BA).

Total RNA seq and Analysis

Total RNA seq was performed to capture a broad and unbiased view of transcriptional changes in the placenta and myometrium. The Roche KAPA RNA HyperPrep kit with RiboErase HMR (cat#8098131702) was used for library preparation with RNA input of 100 ng and RIN >3. RiboErase HMR was used to reduce rRNA in total RNA samples. Libraries were prepared following the standard manufacturing protocol. Library quality was evaluated using Qubit and TapeStation. Constructed libraries were sequenced on NovaSeq 6000 Illumina platform, producing 2x100nt stranded paired end reads.

Raw total RNA sequencing fastq files were aligned to the *Macaca nemestrina* reference genome from Ensembl (Mnem_1.0, INSDC Assembly GCA_000956065.1) using STAR 2.7.8a in Partek, a bioinformatics software suite (Partek, Inc.). All samples had a quality score greater than 35, so no bases were trimmed, and 100% of sequencing bases overlapped with read lengths of the reference genome. Genes were filtered out of the count matrix if the sum of reads across all the samples was less than 10.

Downstream analysis of total RNA-sequencing count data was completed with the R statistical computing environment (R version 4.4.0). Gene counts were filtered by a row

mean of 10 or greater, then normalized using edgeR package to implement Trimmed Mean of M-values (TMM) normalization for technical biases. The voom function log-transforms the data and models the mean-variance relationship which stabilizes the variance across different levels of gene expression. Differential Gene Expression (DGE) analysis was carried out between GBS infection groups and saline controls. Comparisons were based on a linear model fit for each gene using limma. P-values were obtained using limma's moderated t-test with empirical Bayes variance shrinkage. Criteria for all DGE analyses were an absolute fold change of 1.5 and a p-value < 0.05.

Nanostring nCounter and Analysis

nCounter was employed to validate and investigate immune changes at the maternal-fetal interface. A custom-code set of 30 cell death genes and Nanostring nCounter NHP Immunology V2 was created for analysis of total RNA from chorioamniotic membrane samples. Total RNA extracted from the myometrium samples were analyzed with the original Nanostring nCounter NHP Immunology V2 gene expression code set (Nanostring, cat#11500276). RNA hybridization reactions were completed based on the manufacturer's protocol. Hybridized probe and RNA complexes were immobilized on nCounter Cartridges using the nCounter Prep Station and quantified with nCounter Digital Analyzer (Nanostring, cat#MAN-C0035-07).

Downstream analysis of raw nCounter data was completed in nSolver software (4.0). Background thresholding correction was applied based on negative controls, where probe counts below one were set to a value of 1. Normalization was completed using positive controls for technical variation and housekeeping genes for biological variation. The

normalized count matrix was log2-transformed in the R Statistical Computing Environment in the voom function. Comparisons were based on a linear model fit for each gene using the limma package. P-values were obtained using limma's moderated t-test with empirical Bayes variance shrinkage. Criteria for all DGE analyses were an absolute fold change of 1.5 and a p-value < 0.05.

Nanostring GeoMx Digital Spatial Profiling Whole Transcriptome Analysis

Transcriptional profiles of the fetal and maternal placenta were more clearly delineated at a spatial resolution. Formalin-fixed, paraffin-embedded chorioamniotic membranes and decidua tissue were sectioned into microarray slides. The samples underwent deparaffinization and overnight *in situ* hybridization with biological UV-cleavable oligonucleotide probes based on the manufacturing protocols. The microarray slides were incubated with morphological fluorescent antibodies used to highlight key histological features and draw the regions of interest (ROIs), amnion, chorion, and decidua. The morphological antibodies were 1) anti-pan cytokeratin-Alexa Fluor 488 (Pan-CK, clone AE1/AE3; Novusbio, cat# NBP2-33200AF488); 2) anti-fibroblast activation protein-Alexa-Fluor 594 (FAP, clone SP325; Abcam; cat#ab311827); 3) SYTO 83 for nuclei visualization (ThermoFisher, Waltham, Ma); and 4) anti-GBS-Alexa Fluor 647 (Abcam; cat# ab53584). After selection of ROIs on GeoMX DSP instrument (Nanostring, cat#MAN-10152-01), UV light was illumined and photocleaved DNA oligos were collected via aspiration.

Library preparation was completed based on manufacture's protocol (Nanostring, # MAN-10150-01). Constructed libraries were sequenced on NovaSeq 6000 Illumina platform, producing 2x100nt stranded paired end reads. Raw FASTQ files were transformed to

DCC files by DRAGEN via BaseSpace Sequence Hub. Downstream analysis of data was completed with the R statistical computing environment (R version 4.4.0). DCC, sample annotation and PKC files were compiled to develop spatial objects for analysis with NanoStringNCTools and GeoMxTools packages. standR package was implemented for quality control, normalization and batch correction with compatible subsequent linear modeling analysis (eg. edgeR and limma-voom). Preliminary gene-level quality control occurred to visualize and filter out genes that were found in less than 10% of ROIs with a minimal count of 1.5. ROIs were not filtered based on area or nuclear count, as the library size to cell count distribution follows a relatively smooth pattern. ROIs with a library size less than 20,000 were filtered. Gene counts were further filtered by a row mean of 10 or greater, then normalized using edgeR package to implement Trimmed Mean of M-values (TMM) normalization for technical biases. The voom function log-transforms the data and models the mean-variance relationship which stabilizes the variance across different levels of gene expression. Differential Gene Expression (DGE) analysis was carried out between all GBS infection groups and saline controls at every ROI. Comparisons were based on a linear model fit for each gene using limma. P-values were obtained using limma's moderated t-test with empirical Bayes variance shrinkage. Criteria for all DGE analyses were an absolute fold change of 1.5 and a p-value < 0.05.

CIBERSORT Estimation of Single-Cell Frequencies in Placental Chorioamniotic Membranes

To estimate the relative frequencies of various cell types within bulk placental tissue samples, we applied CIBERSORT, a computational deconvolution algorithm that uses

gene expression signatures to infer cell composition. A reference signature matrix was constructed from a single-cell RNA-sequencing (scRNA-seq) atlas of the pigtail macaque placenta that we have developed, which included transcriptional profiles of distinct placental cell populations such as trophoblast subtypes, endothelial cells, Hofbauer cells, and stromal populations. This matrix was curated to optimize cell type resolution while minimizing cross-cell-type gene expression overlaps.

Bulk RNA-seq datasets from placental chorioamniotic membranes containing decidua were normalized and inputted into CIBERSORT along with the reference signature matrix. The algorithm was run in a relative mode with 1000 permutations to estimate the proportion of each cell type in the bulk samples. The output provided a quantitative approximation of cell type composition across samples, enabling comparative analyses of cellular heterogeneity under different physiological or pathological conditions.

Results

Phenotyping the spectrum of GBS infections

We allowed the natural progression of GBS infections in our chronically catheterized nonhuman primate (NHP) model to better characterize the spectrum of invasive GBS infections at the maternal-fetal interface (Table 3.2). The endpoint of the studies was concluded either to prevent the pregnancy ending in stillbirth or preterm birth, or at the predesignated experimental end point. NHPs were classified based on bacterial counts (colony-forming units per mL; CFU/mL) and/or genomic DNA (genomic equivalents per mL; GE/mL) in the amniotic fluid (AF) and the inoculation site collected at the end of the study (Table 2). A progressing GBS infection is defined by the presence of viable bacterial counts (CFU/mL) in the AF at the end of the study. This indicates dissemination of GBS from the inoculation site and active replication/dissemination within the uterine cavity. In contrast, a resolved GBS infection has no viable counts in the AF at the end of the study. Furthermore, resolved infections had minimal to no GBS bacteria at the inoculation site, supporting the clearance of GBS at the maternal-fetal interface. An intermediate infection was labeled as localized, with 0 (CFU/mL) viable counts and trace amounts of GBS genomic DNA in the AF (GE/mL). Most of these animals also had a positive bacterial swab or tissue culture test result at the inoculation site. In comparison to the resolved and progressing GBS infections, the localized infections at the endpoint were captured at an intermediate point with the potential to transform into a progressing or conversely resolved infection if given more time.

Microbial invasion of the amniotic cavity (MIAC) was the primary outcome of the study and the defining feature of a progressing GBS infection. However, other adverse pregnancy outcomes, including preterm labor and fetal infection, were observed. All the fetuses of mothers identified as having a progressing GBS infection had bacteria in their tissues, blood, lungs, and/or brains, further indicating the spread from the inoculation site to the fetus (5/5; Table 3.2). Interestingly, preterm labor was identified in 40% (2/5) of the NHPs in the progressing group as compared to 80% (4/5) of the NHPs in the resolved GBS infections. Both the resolution and progression of GBS infections in the uterine cavity are associated with unfavorable obstetric outcomes.

Representative histological sections of the chorioamniotic membranes, decidua, and the lower uterine segment of the myometrium were prepared for the progressing, resolved, and localized GBS infections, as well as the saline controls (Figure 3.2). Widespread neutrophil infiltration was seen in the layers of the chorioamniotic membranes and decidua in the progressing GBS infection group, in contrast to minimal neutrophil infiltration observed in the resolved GBS infection group. Unlike within the chorioamniotic membranes and decidua, there was high lymphocytic infiltration in the myometrium of the resolved GBS infection adjacent to the inoculation site; interestingly, this wasn't observed in the progressing GBS infections. Two localized GBS infections, "High" and "Low", were shown to represent the variation amongst this group. The "Low" localized GBS recapitulates a pattern similar to the resolved, while the "High" resembles the progressing group. The immune cell infiltration patterns of the GBS infection groups in these tissues suggested a reflective chemokine expression profile that can account for these differences.

To determine the cytokine profile associated with progressing and resolved GBS infections, a 14-plex panel of cytokines/chemokines was quantified at 24 hours in the chorioamniotic membranes and decidua, and the lower uterine segment of the myometrium at the endpoint (Figure 3.2). AF was serially sampled over time, with the onset of significant changes beginning 24 hours post-inoculation of GBS. IL-1 β and tumor necrosis factor (TNF) were statistically different between the resolved and progressing GBS infection groups in the AF at 24 hour. Again, IL-1 β with interleukin-six (IL-6) was statistically different in the chorioamniotic membrane and decidua amongst the GBS groups. However, there were no inferential differences in the myometrium between the resolved and progressing GBS infection groups. IL-1 β appears to be the major cytokine driving the progressing GBS infection group.

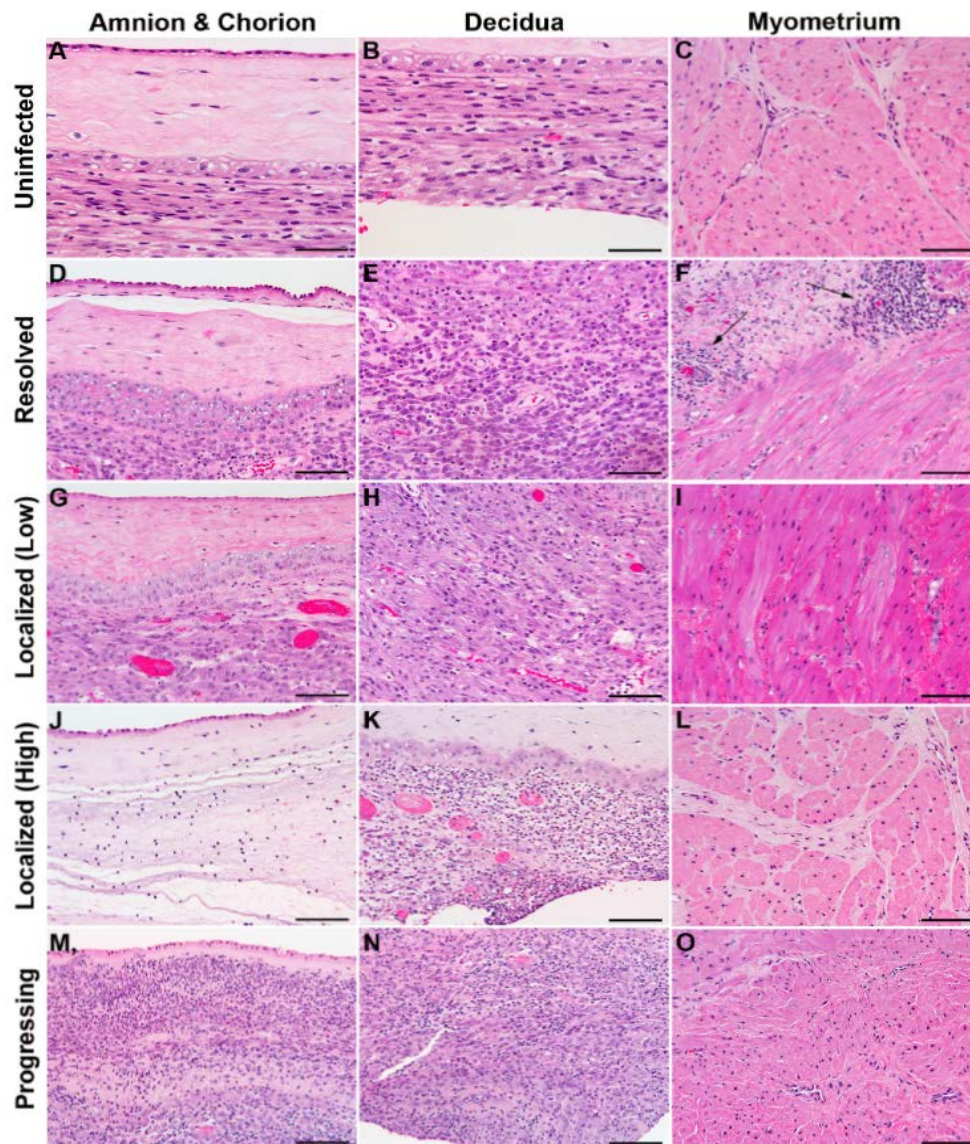
Table 3.2 Preterm Labor, qPCR, and Culture Results by Group

Animal ID	Group	PTL	Amniotic Fluid		Membrane Inoculation Site		Fetus		
			qPCR (GE/ml)	Peak culture (CFU/mL)	Tissue (CFU/mL)	Swab	Blood (CFU/mL)	Lung (CFU/mg)	Meninges (CFU/mg)
CTL4	uninfected	no	0	0	0	0	0	0	0
CTL5	uninfected	no	0	0	0	0	0	0	0
CTL6	uninfected	no	0	0	0	0	0	0	0
RES5	resolved	no	0	0	0	0	0	0	0
RES4	resolved	yes	0	0	0	0	0	0	0
RES1	resolved	yes	0	0	N/S	N/S	0	0	0
RES2	resolved	yes	0	0	0	0	0	0	0
RES3	resolved	yes	0	0	0	0	0	0	0
LOC1	localized	yes	1.5E+02	0	1.60E+04	+++	0	0	0
LOC2	localized	yes	3.3E+02	0	0	+++	0	0	0
LOC3	localized	yes	6.1E+02	0	1.00E+02	+++	0	0	0
LOC4	localized	no	7.0E+02	0	0	+++	0	0	0
LOC5	localized	no	7.9E+02	0	N/S	N/S	0	0	0
LOC6	localized	no	1.1E+03	0	0	0	0	0	0
PRO1	progressing	yes	1.5E+06	2.9E+07	0	+++	N/S	2.1E+06	7.3E+03
PRO2	progressing	yes	1.8E+06	9.4E+07	N/S	+++	6.5E+03	6.0E+05	0.0E+00
PRO3	progressing	no	2.5E+06	7.9E+05	2.10E+04	0	0	2.6E+04	1.8E+04
PRO5	progressing	no	4.1E+07	1.0E+07	4.00E+02	+++	0	3.6E+04	1.3E+06
PRO4	progressing	no	6.5E+07	9.2E+06	N/S	+++	4.2E+01	1.1E+06	8.4E+05

Abbreviations: CFU, colony-forming unit; qPCR, quantitative polymerase chain reaction; PTL, preterm labor; GE, genome equivalence

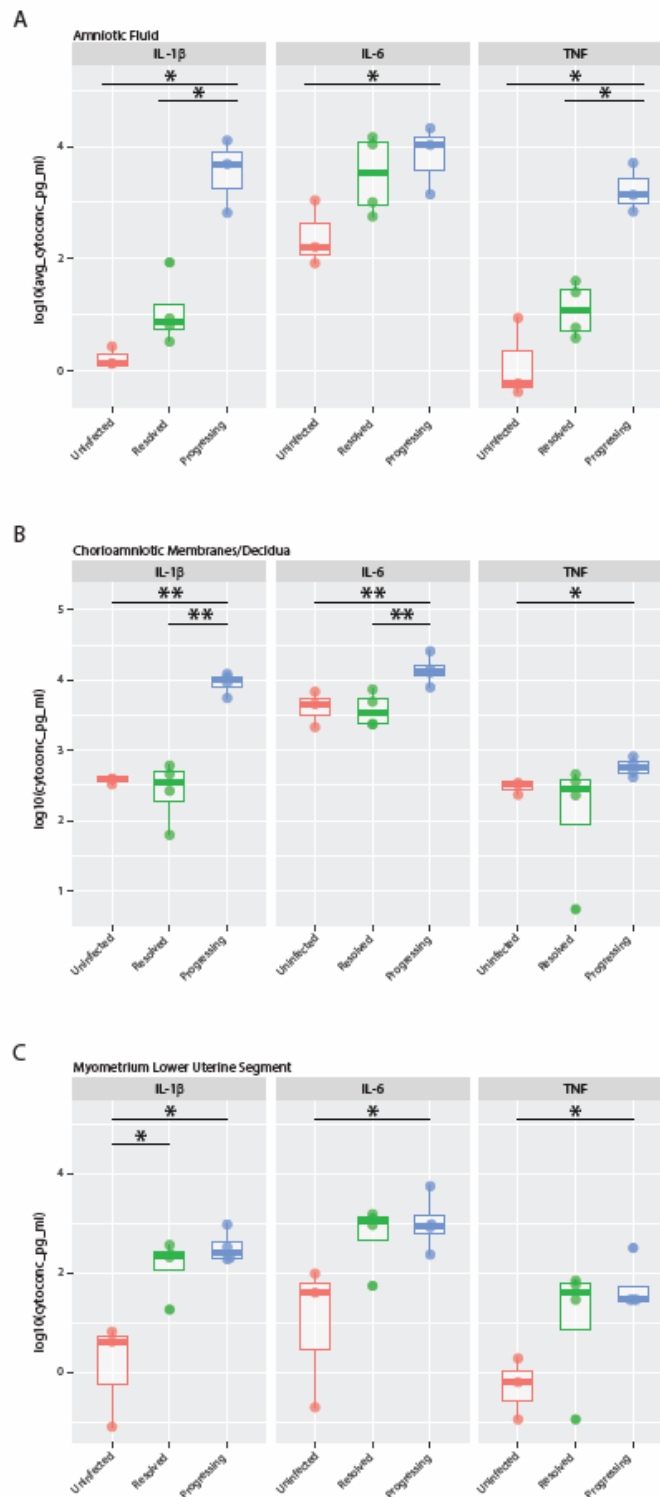
This table reveals the adverse clinical outcomes addressed in this study: GBS dissemination and preterm labor. Qualitative and quantitative amounts of GBS in AF, at the membrane inoculation site, and fetuses are reported.

Figure 3.2 Histopathology of the chorioamniotic membranes, decidua, and myometrium in the GBS infection groups



Representative histological findings in the chorioamniotic membranes/ decidua and myometrium from uninfected controls (**A-C**), and GBS infections determined to have either resolved (**D-F**), remained localized at the choriodecidual inoculation site (**G-L**), or progressed (**M-O**) to invade the amniotic cavity and fetus. Images in the far-left column (**A, D, G, J, M**) show the amnion and chorion, (**B, E, H, K, N**) the decidua, and (**C, F, I, L, O**) the myometrium. No significant inflammation was observed in the chorioamnion or decidua of an uninfected animal (**A, B**), a resolved infection (**D, E**), or some localized GBS infections (**G, H**) designated as “Low” to indicate minimal to no neutrophilic infiltration. In contrast, neutrophilic inflammation was present in the amnion, chorion, and decidua from a different pregnancy with localized infection designated as “High” infiltration (**J, K**) and an animal with a progressing infection (**M, N**); the intensity of chorioamniotic inflammation was most severe in the progressing infection. The myometrium shows no significant inflammation except in the resolved GBS infection group (**F**), in which dense aggregates of lymphocytes (**arrows**) are present in the connective tissue between and overlying smooth muscle cells—scale bars: 100 μ m.

Figure 3.3 Inflammatory cytokines in the amniotic fluid, chorioamniotic membranes/decidua, and myometrium



Select pro-inflammatory cytokine and chemokine concentrations are shown in the amniotic fluid over time **(A)**, chorioamniotic membrane/decidual tissue at the inoculation site **(B)**, and the lower uterine segment of myometrium **(C)**. Data was analyzed using the non-parametric Kruskal-Wallis test. ** $p < 0.01$, **** $p < 0.0001$.

Progressing GBS infection is characterized by inflammation in the chorioamniotic membranes and decidua

Transcriptomic analysis was performed to investigate the gene expression at the initial location of GBS in the chorioamniotic membranes and decidua (Figure 3.4). Principal component analysis reveals the inherent clusters of the GBS infection groups, progressing and resolved, based on their gene expression profiles (Figure 3.4A). Seven hundred twelve genes were differentially expressed at the inoculation site in the progressing infection group versus resolved, with the bulk of genes (423) upregulated in the progressing GBS group ($\log_2FC > 1.5$, $p < 0.05$; Figure 3.4B). Progressing GBS infections were characterized by expression of highly inflammatory genes including *CSF3*, *MMP8*, *CCL20*, *CXCL8*, *CLEC4D*, *PROK2*, *IL1B*, *OSM*, *CD177* and *IL18RAP* ($\log_2FC > 2$, $p < 0.05$). These genes induce the production of cytokine and chemokine, receptors for pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) and reflect neutrophil activation.

Next, we used a hierarchical clustering technique to determine key features of the progressing versus resolved group, which clustered genes based on data points (medoids) where groups were most similar (Figure 3.4C). Over-representation analysis on clusters 1 and 2 using WebGestaltR (KEGG pathways; Nonredundant GO Biological Process, GO Molecular Function, and GO) demonstrated enrichment in granulocyte activation, leukocyte migration and positive regulation of cytokine production in the progressing GBS infection (adjusted p val with false discovery rate (FDR) correction < 0.05 ; Figure 3.4 C, D, E) [171]. Genes characteristic of pyroptosis (*IL1B*, *IL1A*, *NLRP3*, *IRF1*, *CASP4*, and *IL18*) and ferroptosis (*IL1B*, *IL1A*, *SLC39A14*, *PTGS2*, *ACSL1*, *SLC39A8*, *SLC11A2*, *ALOX5AP*, *GCH1*, *TXNRD1*, *TFRC*, *ACSL4* and *STEAP3*) cell

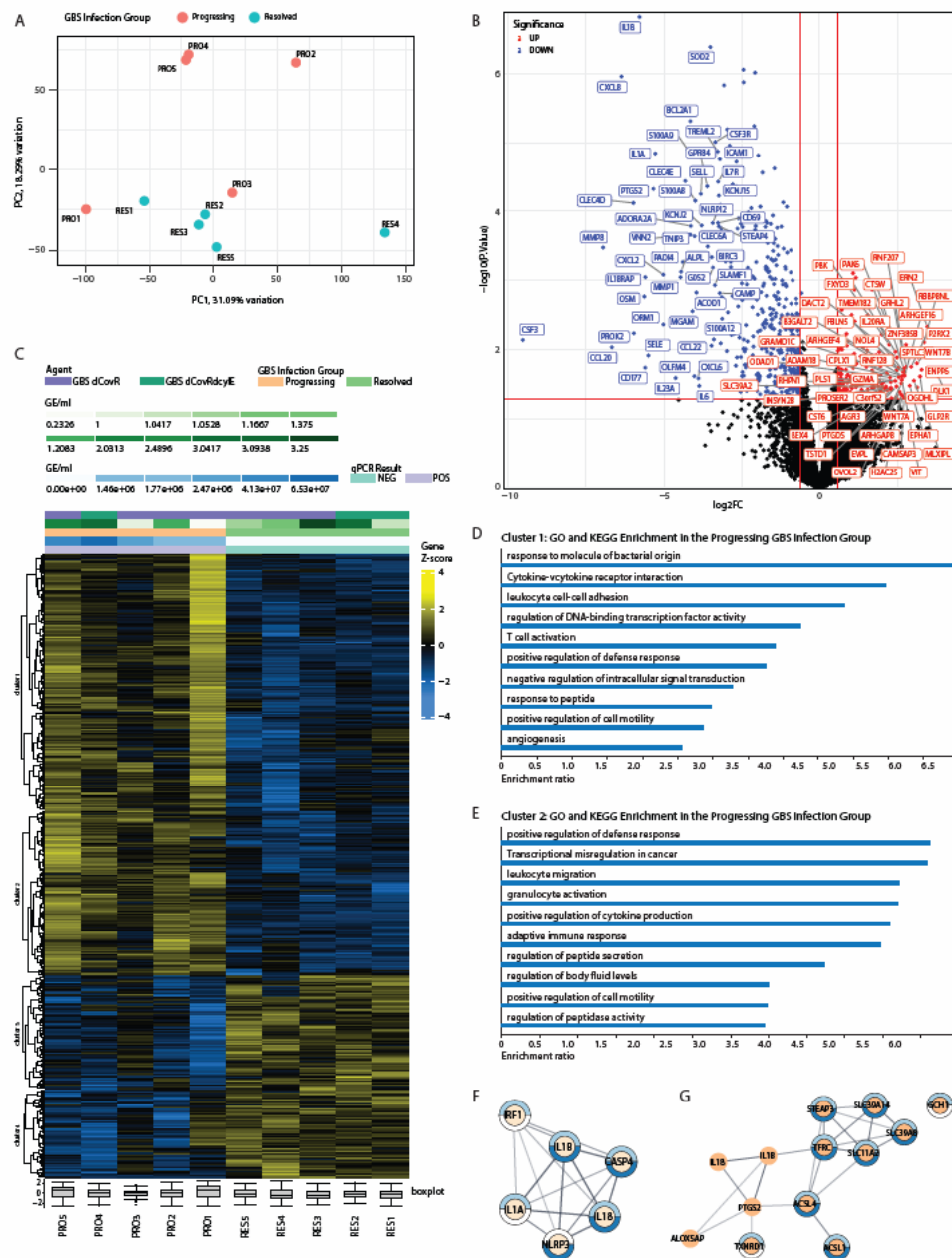
death pathways were identified and enriched in the progressing GBS infections (Figure 3.4F and 3.4G). These results were validated using digital gene counting (Nanostring nCounter technology) on the same samples (Table 3.3). Progressing GBS infections are defined by a pro-inflammatory innate immune response.

Cytotoxic granule-associated genes were upregulated in resolved GBS infections within the chorioamniotic membranes and decidua

In contrast, resolved GBS infections were not associated with an inflammatory gene signature in the chorioamniotic membranes and decidua. The top upregulated genes were *DLK1*, *ERN2*, *RBBP8NL*, *ENPP6*, *GLP2R*, *SPTLC3*, *OGDHL*, *WNT7B*, *MLXIPL* and *P2RX2* ($\log_2FC > 2$, $pval < 0.05$; Figure 3.4B). These genes are generally associated with cell cycle regulation and differentiation (*DLK1*, *RBBP8NL*, *WNT7B*), stress response (*ERN2*), and metabolism (*ENPP6*, *GLP2R*, *SPTLC3*, *OGDHL*, *MLXIPL*, *P2RX2*). While PAM cluster and overrepresentation (ORA) analysis with general GO terms and KEGG pathway did not originally reveal significant pathways, we performed functional overrepresentation network enrichment analysis with the expert-reviewed GO terms UniProt-GOA curated database (GO: BiologicalProcess, GO: ImmuneSystemProcess, GO: MolecularFunction) on the PAM clusters of the resolved GBS infection groups using ClueGo on the Cytoscope platform (Figure 3.5 A-B; $\log_2FC > 1.5$ and $p < 0.05$) [172-174]. Next, network enrichment analyses were conducted on the most differentially expressed genes with the STRING application (Figure 3.5 C; adjusted p-value with FDR correction < 0.05) [175]. Cytolytic mechanisms, including natural killer and T cell-mediated cytotoxicity, were identified using ClueGo and STRING pathways. The specific cytotoxic

granule-associated genes were *CTSW*, *GZMA*, *KIR2DL4*, *KLRB1*, *PRF1*, *NKG7*, and *PIK3R6* ($\log_2FC > 1.5$ and $p < 0.05$). These same genes are found to be upregulated in the resolved GBS infection group using nCounter technology (Table 3.3). Other enriched pathways were related to lipid metabolism, regulation of the cell matrix, and epidermal cell differentiation, commonly associated with antioxidant mechanisms and placenta development. At the inoculation site, GBS resolved infections induced responses that relatively reverted the membranes and decidua to homeostasis by the induction of membrane integrity and cytotoxic granule mechanisms.

Figure 3.4 Transcriptional profiling of the chorioamniotic membranes and decidua from GBS infection groups



A Principal Component Analysis shows resolved (blue), localized (red), and progressing (green) GBS infections (**A**). Volcano plot of differentially expressed genes (DEG) between resolved (right side) versus progressing (left side; $\log_2FC > 0.58$; $p < 0.05$, panel (**B**)). P-values were obtained using limma's moderated t-test with empirical Bayes variance shrinkage. Heatmap representation of hierarchical clustering of DEG between resolved and progressing GBS infections is shown in panel (**C**); columns indicate different biological replicates. Over-representation enrichment analysis was performed on clusters. The enriched Gene Ontology (GO) and KEGG pathways are shown in for clusters 3 and 4 (**D**, **E**; adjusted p-value with false discovery rate (FDR) correction < 0.05). Pyroptosis (**F**) and ferroptosis (**G**) related genes were selected from DEG and analyzed using the STRING database to reveal protein-protein interaction pathways upregulated in the progressing vs. resolved GBS infection groups. Functional enrichment analysis confirmed these results (light and dark blue circles indicate genes in pathways; FDR-adjusted $p < 1 \times 10^{-8}$).

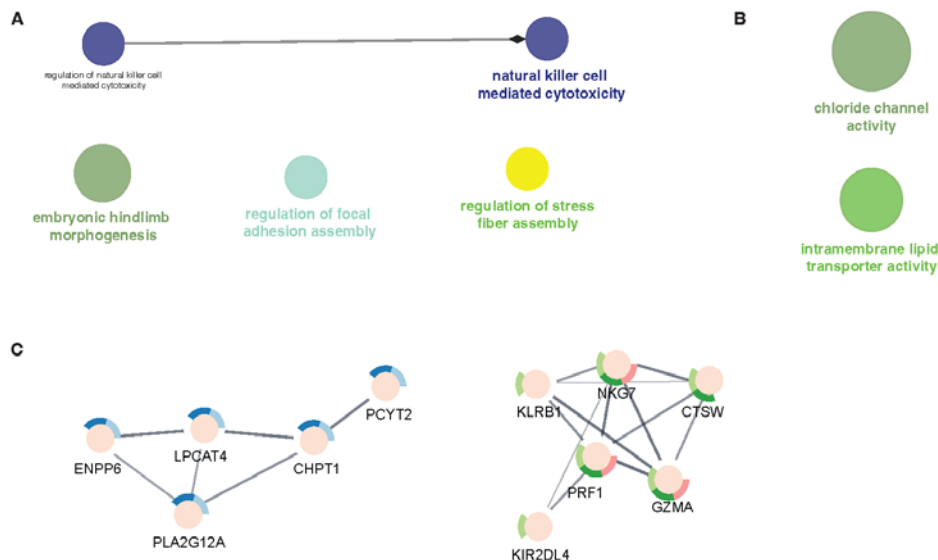
Table 3.3 nCounter of Differently Expressed Genes at the Chorioamniotic Membranes and Decidua Between Resolved versus Progressing GBS Infection

Probe	LogFC	P value
GZMA	5.31	0.006
GZMB	3.26	0.024
PRF1	3.24	0.013
GNLY	3.15	0.035
CCL24	2.55	0.010
PTGER3	2.42	0.044
NT5E	2.25	0.048
KLRB1	2.18	0.040
TWIST2	2.04	0.048
HDAC4	1.87	0.004
BAD	1.67	0.027
AIFM2	1.53	0.009
SMAD5	1.5	0.040
KEAP1	1.49	0.001
HRAS	1.48	0.020
C1QBP	1.38	0.021
LTBR	1.37	0.006
CISD1	1.36	0.008
SKI	1.36	0.030
BAX	1.33	0.025
GTF3C1	1.33	0.047
DDX50	1.26	0.039
ATF2	1.24	0.018
ERCC3	1.24	0.025
USP9Y	1.2	0.024
ANP32B	1.2	0.040

Probe	LogFC	P value
IL8	-57.65	0.002
CXCL3	-26.7	0.003
IL1A	-21.37	0.001
DEFB4A	-20.53	0.011
S100A9	-20.48	0.002
CCL20	-18.86	0.009
IL1B	-18.59	0.001
S100A8	-16.81	0.005
SELL	-11.84	0.001
CCL3	-11.18	0.005
CXCL2	-10.95	0.016
PTGS2	-9.35	0.002
IL7R	-8.25	0.001
GBP1	-8.24	0.030
TNFAIP3	-7.83	0.013
CLEC4E	-7.36	0.002
TNFAIP6	-6.39	0.013
TRAF1	-6.01	0.021
ICAM1	-5.97	0.002
CD274	-5.93	0.009
CLEC4A	-5.75	0.000
LCP2	-5.48	0.005
PLAUR	-5.25	0.013
SLAMF1	-5.23	0.011
IL1RN	-4.95	0.025
PLAU	-4.88	0.016

This table reveals the top genes in the resolved and progressing GBS infection groups in the bulk tissue near the inoculation site, the chorioamniotic membranes, and decidua.

Figure 3.5 Networks of Resolved GBS Infection versus Progressing GBS infection



ClueGo network visualizations of functionally enriched biological and immunological processes of cluster 3 (**A**), and cluster 4 (**B**). Select STRING Protein–protein interaction (PPI) pathways highlight the interactions of the DEG upregulated in the resolved GBS infection group. Functional enrichment analysis on the network identified enrichment in cytolytic granule and granzyme-mediated programmed cell-death signaling a (pink [CL:16323] and green[CL:16226]; FDR-adjusted $p < 0.02$) and lipid metabolism (light[KW-0443] and dark[CL:102777] blue; FDR-adjusted $p < 0.02$) (**C**).

Inflammatory and cytotoxic genes were determined within the amnion, chorion, and decidua

The chorioamniotic membranes and decidua have unique cellular components and roles. Functionally, the amnion is in direct contact and the last barrier to the amniotic fluid; chorion maintains immune tolerance between the maternal decidua and the fetal-derived amnion-chorion layers, and the decidua comprises the predominant maternal immune and stromal cells. We investigated the spatial gene expression differences between the resolved and progressing GBS infection groups in our regions of interest (ROIs): amnion,

chorion, and decidua (Figure 3.6A). In all layers, the progressing GBS infections are characterized by exacerbating inflammatory responses, with varying responses present in the ROIs of the resolved group (Figure 3.6; Tables 3.4, 3.5, 3.6).

Innate immune inflammatory markers were upregulated in the progressing NHP group within the amnion, chorion and decidua. A broad spectrum of chemokines were induced throughout the membranes and decidua with *CXCL8* consistently expressed in each ROI (amnion: *CCL3*, *CCL2*, *CXCL3*, *CCL20*; chorion: *CXCL2*, *CXCL16*, *CCL20*; decidua: *CCL2*, *CCL8*, Table 3.4, 3.5, 3.6). Other significant cytokine markers included *IL6* in chorion and *IL1B* in the chorion and decidua. Anti-inflammatory genes, *SOD2*, *TNFAIP3*, and *NFKBIA*, were also consistently elevated in all layers (Table 3.4, 3.5, 3.6). Over-representation enrichment analysis confirmed the enrichment of inflammatory pathways in each ROI, such as activation of the adaptive immune response, response to IL-1, TNF, and chemokine and cytokine-cytokine receptor interaction (Figure 3.6 B, C, D). The interferon signaling pathway is also upregulated in the chorion and decidua (*IFNGR1*, *IFNGR2*, *IRF1*, *STAT1*). The robust inflammatory response at the membranes is associated with bacterial persistence and proliferation.

Inflammatory cell death markers, ferroptosis, and pyroptosis were also identified at a regional level in the progressing GBS group. Canonical genes in the ferroptosis pathway were elevated in all ROIs: amnion (*SLC39A8*, *FTH1*), chorion (*SLC39A8*, *ALOX5AP*, *ACSL1*, and *FTH1*), and decidua (*ACSL1*, *IL1B*, *SLC39A8*, and *ALOX5AP*; Tables 3.4, 3.5, 3.6). Pyroptosis pathway genes were induced in the chorion (*IL1B*, *IRF1* and *CASP4*) and decidua (*IRF1*, *IL1B*, and *CASP4*; Table 3.5, 3.5, 3.6). Inflammatory cell death genes

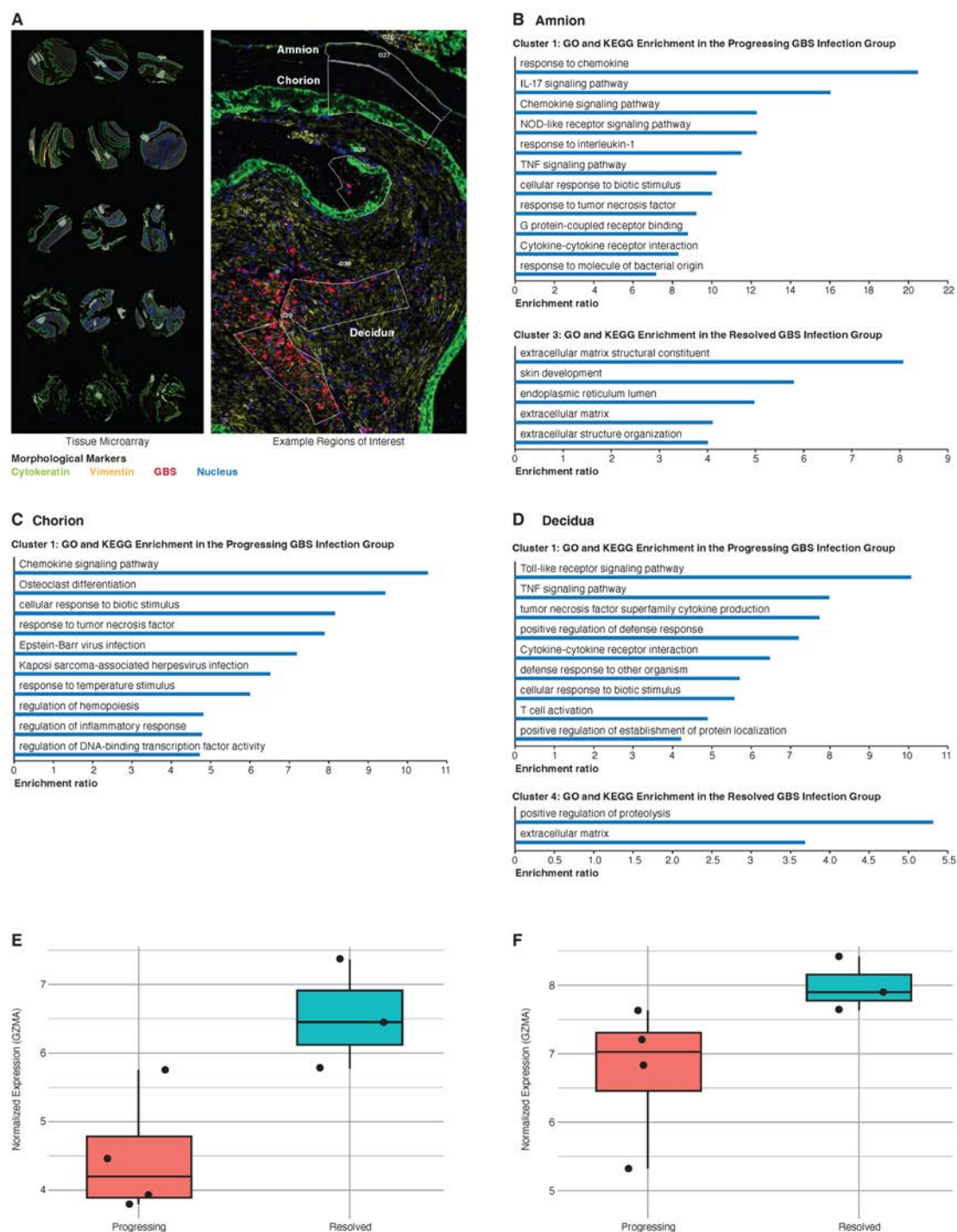
were localized primarily in the chorion and decidua during the progression of GBS infection.

Conversely diverse biological processes were induced in the resolved infection group spatially, and thus, very few functional enrichment pathways were seen. The top 10 genes in the decidua were associated with placenta development (*IGFBP1*, *CITED*), tissue coagulation (*F3*), myeloid progenitor inhibitory factor 1 (*CCL23*), neutralization of TGF- β superfamily (*FST*), removal of misfolded proteins (*CLU*), cytotoxicity (*GZMA*), and extracellular matrix (*COL8A1*, *ACTA2*, *EFEMP1*). Genes responsible for placental growth through insulin-like growth factors were upregulated (*APOE*, *IGFBP1*, *IGFBP7*, *LGALS1*, *PDIA6*, *TIMP1*), indicating an attempt to restore homeostasis. In addition to *GZMA*, another known cytotoxicity gene, *GZMB*, is upregulated in the decidua of the resolved GBS infection group (Figure 3.6D). Overall, there were limited immune genes upregulated in the decidua of the resolved GBS infection group, yet granzyme-dependent cytotoxicity was increased.

Extracellular matrix changes relatively dominate the chorion and amnion of the resolved GBS infection. The top 10 genes upregulated in the chorion of the resolved GBS infection were associated with extracellular matrix production and interaction (*COL1A1*, *COL1A2*, *EMILIN1*, *EFEMP2*, *LOXL2*, *FXRD5*, *CAV1*, *TGM2*), suppression of ferroptosis (*LPCAT4*), and magnesium transportation (*TMEM94*). Functional over-representation enrichment analysis revealed extracellular matrix changes also in the amnion of the resolved GBS infection (Figure 3.6 B, C). These wound healing and extracellular remodeling genes include *IGFBP5*, *SPARC*, *DCN*, *COL1A2*, *COL1A1*, *COL3A1*, *COL5A2*, *COL5A1*, and *COL6A3*). M2 Macrophage wound healing marker, CD163, was upregulated in the

amnion resolved group. Furthermore, we observe that ferroptosis inhibitor genes were enhanced in the resolved GBS infection, specifically *GXP4* and *APOD* in the decidua, and *HSPB1* and *LPCAT4* in the chorion (Table 3.4, 3.5, 3.6). Again, genes upregulated in the resolved group were indicative of anti-inflammatory, pro-resolving, and attempt to remodel the placenta following the infection to maintain homeostasis.

Figure 3.6 Digital Spatial RNA Profiling in the Amnion, Chorion, and Decidua



RNA expression across the entire transcriptome was captured using the NanoString GeoMx Digital Spatial Profiling platform. **(A)** Cluster-over-representation of differentially expressed genes (FDR-adjusted $p < 0.05$) was conducted between resolved and progressing GBS infections within the amnion **(B)**, chorion **(C)**, and decidua **(D)**. Boxplot of *GZMA* and *GZMB* gene expression between resolved and progressing GBS infection within the decidua **(E)**.

Table 3.4 Differentially Expressed Genes in the Amnion between the Resolved and Progressing GBS Infection group

Gene	log2FC	P Val	Gene	log2FC	P Val
COL1A2	3.6482	0.0005	CXCL8	-3.8478	0.0041
COL1A1	3.6176	0.0005	SOD2	-3.5972	0.0000
COL3A1	2.8372	0.0055	TNFAIP3	-2.7799	0.0004
IGFBP5	2.1736	0.0011	HILPDA	-2.7018	0.0093
COL5A2	1.8961	0.0003	CCL3	-2.6329	0.0016
DCN	1.8887	0.0051	PNP	-2.3867	0.0003
COL5A1	1.8509	0.0035	SAT1	-2.3237	0.0052
CD163	1.7814	0.0147	CD44	-2.2662	0.0002
SERPINH1	1.6846	0.0008	CCL20	-2.2612	0.0063
NR4A1	1.6804	0.0086	C15orf48	-2.2230	0.0011
S100A6	1.6628	0.0343	G0S2	-2.1476	0.0435
SPARC	1.6569	0.0460	TCIM	-1.9566	0.0043
CEBPZOS	1.6420	0.0185	FTH1	-1.9381	0.0147
CREB3L1	1.5946	0.0318	SERPINB6	-1.9294	0.0125
DKK1	1.5017	0.0052	NDRG1	-1.9229	0.0310
ZNF764	1.4276	0.0095	NFKBIA	-1.8797	0.0040
CRIP1	1.4100	0.0142	SLC39A8	-1.8279	0.0016
DDRKG1	1.3965	0.0109	PNRC1	-1.8170	0.0010
GLUL	1.3866	0.0323	CRABP2	-1.7875	0.0279
REXO2	1.3831	0.0074	CXCL3	-1.7097	0.0126
CITED2	1.3783	0.0160	RIPK2	-1.7052	0.0073
PTK7	1.3750	0.0140	KRT6A	-1.6057	0.0200
TOMM7	1.3596	0.0063	WFDC2	-1.5750	0.0251
CAV1	1.3221	0.0111	LAMB3	-1.4036	0.0266
TXNDC5	1.3131	0.0025	CCL2	-1.4008	0.0461
RAB2A	1.3110	0.0065	CCNG2	-1.3863	0.0064
COL6A3	1.2850	0.0379	EIF1	-1.3124	0.0112
KRT24	1.2832	0.0309	DDIT4	-1.2865	0.0477
S100A4	1.2767	0.0143	SIK1	-1.2842	0.0310
GPRC5A	1.2487	0.0468	WTAP	-1.2814	0.0032
TUBA1C	1.2467	0.0269	TM4SF1	-1.2719	0.0316
HERPUD1	1.2358	0.0104	SLC25A3	-1.2621	0.0082
SNRPD2	1.2279	0.0432	LAMA3	-1.2576	0.0256
RHOA	1.2226	0.0421	EIF1B	-1.1351	0.0299
DUX5	1.2107	0.0154	PABPC4	-1.0946	0.0182

This table shows the top 25 genes in the resolved and progressing GBS infection groups at the amnion.

Table 3.4 Differentially Expressed Genes in the Chorion between the Resolved and Progressing GBS Infection group

Gene	log2FC	P Val	Gene	log2FC	P Val
COL1A1	2.358	0.005	CXCL8	-4.368	0.001
LPCAT4	2.226	0.000	SOD2	-4.258	0.000
TMEM94	1.793	0.005	G0S2	-3.874	0.001
CAV1	1.781	0.001	S100A9	-3.520	0.002
PTK7	1.747	0.001	CCL3	-2.984	0.001
EMILIN1	1.697	0.001	BCL2A1	-2.757	0.009
EFEMP2	1.683	0.001	TNFAIP3	-2.723	0.000
LOXL2	1.669	0.001	CEBPB	-2.700	0.000
FXYD5	1.663	0.002	ALOX5AP	-2.571	0.015
COL1A2	1.633	0.047	NFKBIA	-2.565	0.000
TGM2	1.623	0.045	FTH1	-2.539	0.005
IGFBP6	1.602	0.026	ZFP36	-2.486	0.000
SMPDL3A	1.546	0.009	HLA-B	-2.480	0.001
STAB1	1.516	0.013	SRGN	-2.427	0.000
SBSN	1.515	0.002	CD44	-2.278	0.000
CEMIP	1.503	0.004	SLC39A8	-2.205	0.000
CUZD1	1.472	0.029	CSF3R	-2.062	0.017
CAMK2N1	1.469	0.001	C15orf48	-2.016	0.004
DLG5	1.451	0.006	HLA-G	-2.015	0.002
PKP2	1.438	0.002	TAP1	-1.962	0.001
SORT1	1.437	0.033	MT2A	-1.877	0.038
VEGFB	1.422	0.003	GMFG	-1.846	0.002
UXS1	1.402	0.023	PNP	-1.801	0.002
INHA	1.387	0.035	CXCL2	-1.766	0.004
LY6G6C	1.377	0.016	IER3	-1.765	0.004
TMED3	1.367	0.012	SLC7A2	-1.738	0.032
RGL1	1.352	0.008	ETS2	-1.735	0.001
LHFPL6	1.347	0.005	RNF19B	-1.734	0.003
RDH13	1.340	0.014	BTG2	-1.689	0.009
SPCS3	1.338	0.004	PTX3	-1.679	0.048
GIPR	1.332	0.029	SEMA4A	-1.643	0.008
KRTDAP	1.306	0.017	RNF149	-1.642	0.004
KRT24	1.303	0.033	CD14	-1.633	0.012
HSPB1	1.298	0.012	MARCKS	-1.608	0.001
TM4SF4	1.284	0.004	GBP1	-1.600	0.022

This table shows the top 25 genes in the resolved and progressing GBS infection groups at the chorion.

Table 3.6 Differentially Expressed Genes in the Decidua between the Resolved and Progressing GBS Infection group

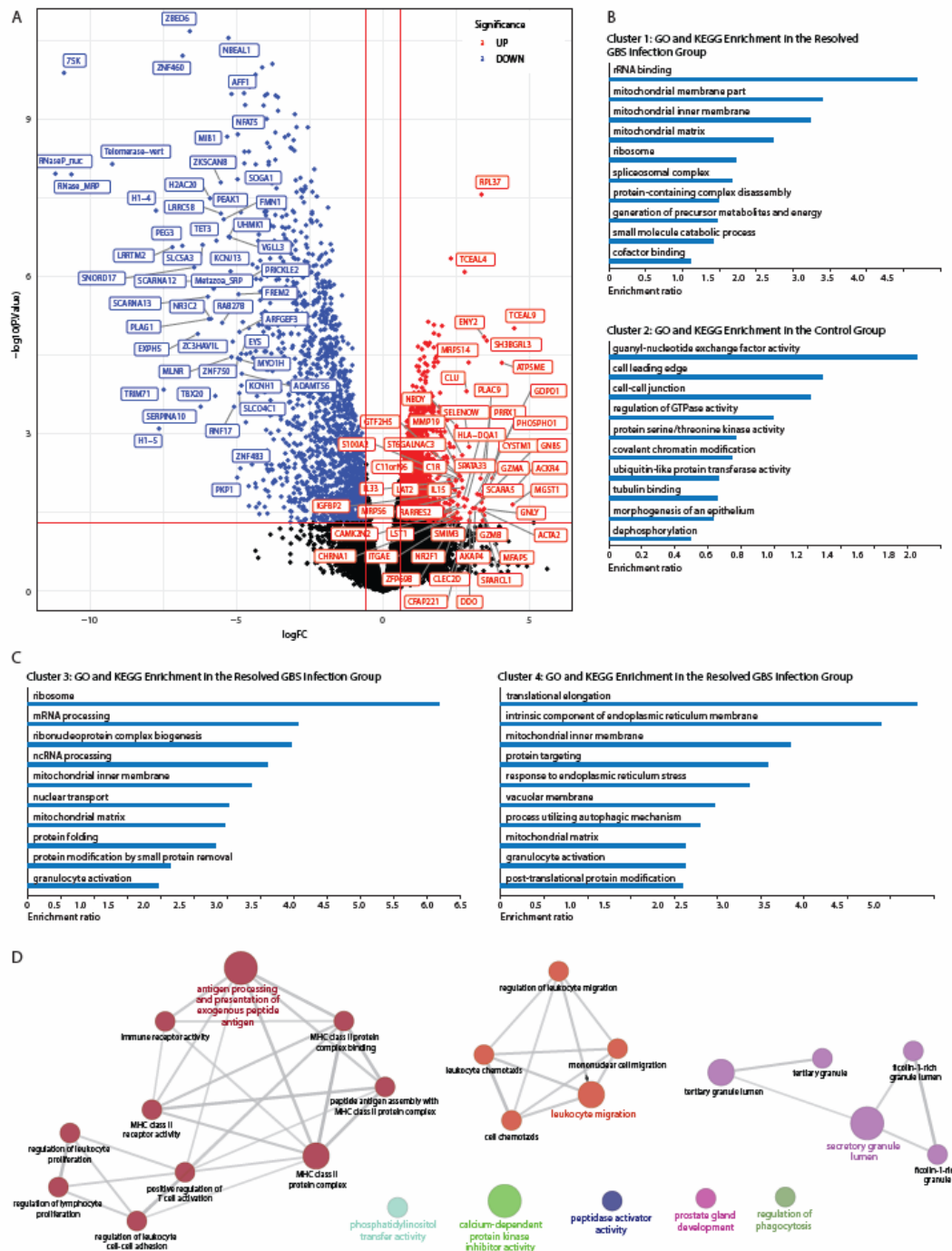
Gene	log2FC	P Val	Gene	log2FC	P Val
IGFBP1	2.768	0.050	IL23A	-3.828	0.000
F3	2.518	0.013	CXCL8	-3.675	0.002
CITED2	2.482	0.001	S100A8	-3.571	0.001
CCL23	2.447	0.039	SOD2	-3.346	0.000
COL8A1	2.376	0.020	ICAM1	-3.227	0.000
CLU	2.236	0.016	PLEK	-3.176	0.000
FST	2.233	0.000	GBP1	-2.958	0.000
ACTA2	2.226	0.002	CSF3R	-2.846	0.004
GZMA	2.170	0.006	G0S2	-2.844	0.015
EFEMP1	2.144	0.002	OSM	-2.801	0.001
MEST	2.075	0.009	HCAR2	-2.637	0.012
FADS2	2.015	0.001	S100A9	-2.630	0.015
IFI27	2.001	0.029	NFKBIA	-2.609	0.000
TIMP1	1.845	0.034	ALCAM	-2.586	0.000
CCDC80	1.741	0.034	MMP8	-2.520	0.003
RNF144A	1.732	0.014	BCL2A1	-2.485	0.015
LGALS1	1.720	0.017	NAPSA	-2.446	0.010
HTRA3	1.664	0.013	TLR2	-2.431	0.000
FSCN1	1.611	0.009	IL1RAP	-2.422	0.000
REXO2	1.572	0.008	NCF1	-2.346	0.004
PDIA6	1.557	0.005	BCL3	-2.324	0.000
PPIC	1.544	0.022	TNFAIP3	-2.260	0.004
GJA1	1.534	0.011	DRAM1	-2.209	0.000
APOE	1.513	0.034	RNF19B	-2.186	0.001
NID1	1.492	0.034	ALOX5AP	-2.115	0.048
ITIH5	1.398	0.037	SEMA4A	-2.055	0.003
DDOST	1.369	0.029	FCGR2C	-2.052	0.004
EMILIN1	1.341	0.018	KDM6B	-2.023	0.000
PCOLCE2	1.324	0.024	CSF2RB	-1.997	0.002
OAT	1.284	0.026	IRF1	-1.994	0.000
ADAMTS5	1.274	0.043	RHOH	-1.925	0.007
NDUFA13	1.256	0.024	CFLAR	-1.891	0.003
GZMB	1.247	0.045	NFKB2	-1.853	0.001
MTCH1	1.225	0.029	ACSL1	-1.847	0.010
IGFBP7	1.166	0.049	IL23A	-3.828	0.000

This table shows the top 25 genes in the resolved and progressing GBS infection groups at the decidua.

Cytotoxic gene expression is upregulated in the resolved GBS infection versus saline controls

In order to determine if the resolved GBS infection group reverts to normal conditions, we investigated the resolved GBS infections compared to saline controls (Figure 3.7). Three thousand seven hundred twenty-nine genes were differentially expressed between resolved GBS infection and saline groups, with most contributing from the resolved GBS infection group ($\log_2FC > 0.58$, $pval < 0.05$). The top 10 genes were *ACKR4* (cytokine scavenger), *TCEAL9* (transcription factor), *MGST1* (redox-sensitive repressor of ferroptosis), *ATP5ME* (mitochondrial respiratory chain), *CYSTM1* (cysteine-rich transmembrane module), *SH3BGRL3* (cytoskeleton), *PLAC9* (placental development), *GNLY* (cytotoxicity), *MFAP5* (components of the ECM), and *ENY2* (transcription factor). Hierarchical PAM clustering and overrepresentation analysis revealed that in the resolved GBS infections, pathways associated with protein formation, autophagic mechanisms, and granulocyte activation (KEGG pathways; Non-redundant GO Biological Process, GO Molecular Function, and GO; Figure 3.7 B-D) were identified. To better characterize the immune response, overrepresentation network enrichment analysis using the UniProt-GOA curated database was performed on the top genes upregulated in the resolved group. Resolved GBS infections were enriched in regulation of phagocytosis, leukocyte cell migration, antigen processing and presentation, and secretory granule lumen (ClueGo; GO: BiologicalProcess, GO: ImmuneSystemProcess, GO: MolecularFunction). Again, cytotoxic genes, including *GNLY*, *GZMA*, *GZMB*, *ITGAE*, and *FCER1G*, were upregulated in the resolved group (Figure 3.7B). Cytolytic processes were depicted at the chorioamniotic membrane and decidua of the resolved GBS infection.

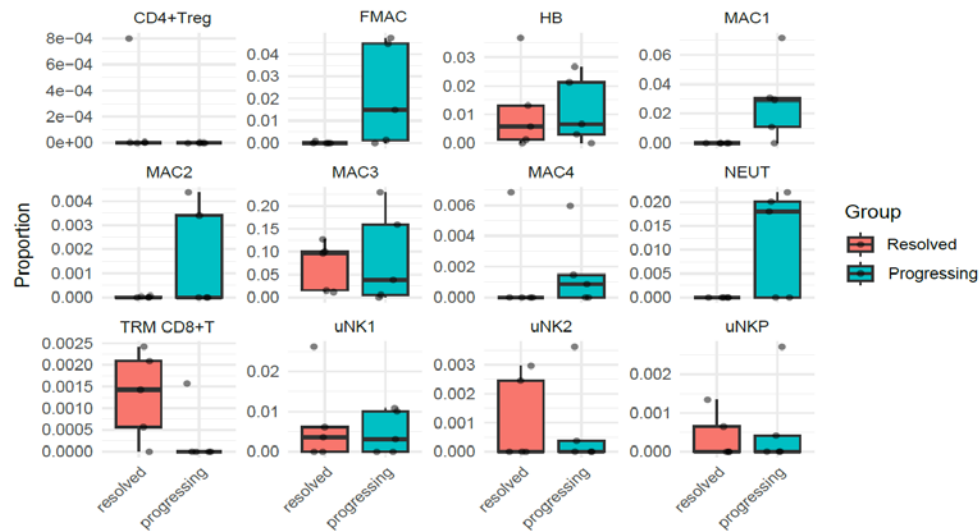
A Volcano plot of DEG is shown contrasting RNA-Seq profiles from the resolved GBS infections (right panel) with uninfected controls (left panel; $\log_2FC > 0.58$; $p < 0.05$). **(A)** Cluster over-representation enrichment analysis of DEG within the resolved vs. uninfected group (FDR-adjusted $p < 0.05$; **B-C**). A ClueGo network visualizations of functionally enriched biological and immunological processes in the DEG of the resolved vs. uninfected group ($\log_2FC > 1$; $p < 0.05$; **D**). Node size reflects enrichment significance (Bonferroni adjusted $p < 0.05$).



Neutrophils and M1 macrophages are enriched at the chorioamniotic membranes of the progressing GBS infection groups

Deconvolution of cell proportions at the chorioamniotic membranes and decidua was determined with CIBERSORT technology and our latest single-cell RNA-Seq placental *Macaca nemestrina* atlas (Figure 3.8) [176]. A Welch's t-test was performed on immune cells between the GBS infection groups to determine statistical significance. Neutrophils and M1 macrophages were significantly increased in the progressing GBS infection group, which were ultimately ineffective for bacterial clearance (pval <0.05). We hypothesize that the cells responsible for the transcriptomic signature of cytotoxic proteins (i.e., *GZMA*) were either a uNK and/or a tissue resident memory CD8⁺ T cell (TRM CD8⁺ T cell) in the placenta. There were no statistical differences in the proportion of uNK cell types (uNK1, uNK2, uNKP) and tissue-resident CD8⁺ T cells between the progressing and resolved GBS infections by deconvolution analysis of the bulk RNA-Seq signatures using a single-cell RNA-Seq atlas of the pigtail macaque placenta as a reference. CIBERSORT analysis revealed statistically augmented immune populations, neutrophils and M1 macrophages, in the progressing GBS infection group, but none in the resolved group.

Figure 3.8 Bulk tissue deconvolution of cell populations in the chorioamniotic membranes and decidua



The proportion of single cells is shown for discrete populations within the chorioamniotic membranes for resolved and progressing infections based on a single-cell RNA-Seq atlas of the pigtail macaque placenta in late gestation. Abbreviations: TRM, Tissue Resident Macrophage; FMAC, Fetal Macrophage; Hoffbauer, HB; Macrophage, MAC; NEUT, neutrophils; uNK, uterine Natural Killer

Inflammation in the myometrium of a resolved GBS infection

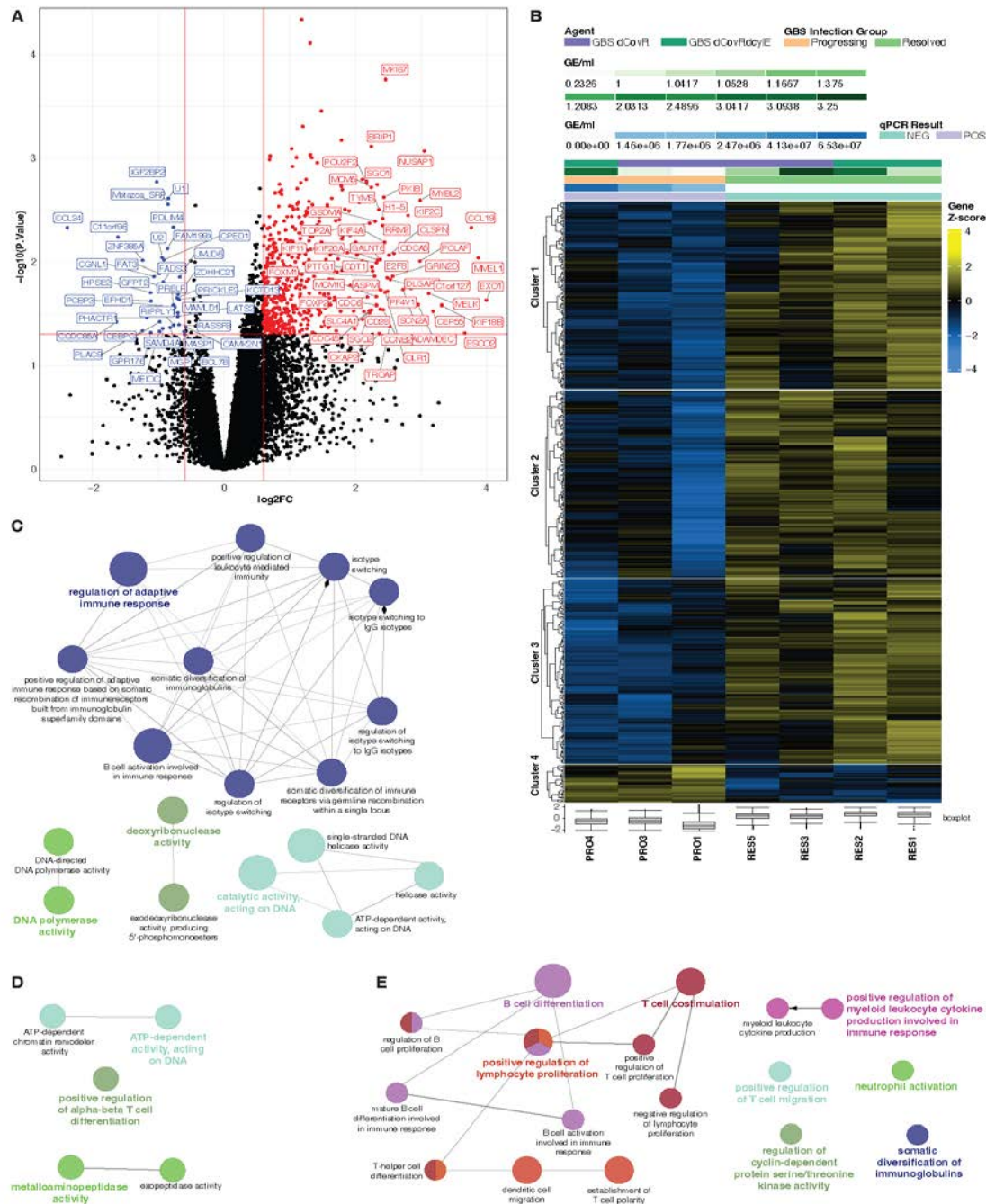
To capture changes in the gene expression of neighboring tissues close to the site of inoculation, we examined the lower uterine segment of the myometrium (Figure 3.9). Surprisingly, the DEGs between the resolved and progressing GBS infections revealed a DNA repair and inflammatory profile in the resolved group. The top ten genes were related to DNA repair (*EXO1*), cell division (*ESCO2*, *KIF18B*, *CEP55*, *MELK*, *NUSAP1*, *MYBL2*, *PCLAF*), neuropeptide degradation (*MMEL1*), and chemokine (*CCL19*) expression. To elucidate the specific immune responses of the DEGs, functional network enrichment on the PAM clusters with the UniProt-GOA curated database was performed (GO: ImmuneSystem and GO: MolecularFunction). In addition to the cell cycle mechanism, adaptive immune responses such as B cell activation, diversification of immunoglobulins,

and isotype switching were enriched in clusters 1 and 3 of DEGS upregulated in the resolved GBS infection group. DEGs induced in the resolved GBS infection groups identified T cell differentiation, migration, and co-stimulation in clusters 2 and 3. Other immune responses induced were neutrophil activation, myeloid leukocyte cytokine production, and metalloaminopeptidase activity. There was no functional enrichment of genes induced in the progressing GBS infection group using PAM hierarchical clustering and ORA of general GO terms, UniProt-GOA curated database, and STRING pathways. Findings indicated an innate and adaptive activated immune response in the resolved GBS infection group as compared to the relatively quiescent myometrium in the progressing group.

Again, we compared the resolved group to saline controls. The top genes were related to antimicrobial mechanisms (*PLA2G2A*, *PI3*, *LCN2*), chemoattractant (*CCL19*, *CCR2*, *PF4V1*), initiation of anti-inflammatory responses (*TLR10*), and calcium signaling and muscle contraction (*PCP4*, *ACTA1*). In the resolved GBS infection (Figure 3.10; $\log_2FC < 1.5$, $pval < 0.05$), functional enrichment pathways of the upregulated genes were related to DNA repair, humoral immune response, phagocytosis, T cell activation, and granulocyte activation. Overrepresentation network analysis using the UniProt-GOA curated database revealed similar patterns of pathways upregulated in dendritic cell chemotaxis, pattern recognition pathways, regulation of leukocyte and B cell function, and leukocyte activation (Fadj, $p < 0.05$). GBS DNA in the myometrium was measured to determine if the innate and adaptive immune response is more prevalent in the resolved GBS group due to a higher trace amount in this tissue as compared to the progressing group. There was no statistically significant difference in the GBS amount between the

GBS infection groups in the myometrium. This suggests the inflammatory signature of the resolved group in the myometrium is not due to the presence of GBS in this tissue (Figure 3.11).

Figure 3.9 Transcriptional profiling of the lower uterine segment myometrium from GBS infection groups.



A Principal Component Analysis shows resolved (blue), localized (red), and progressing (green) GBS infections (**A**). A volcano plot of differentially expressed genes (DEG) between resolved (right panel) vs. progressing GBS infection (left panel; $\log_2FC > 0.58$; $p < 0.05$; **B**). Gene counts are TMM normalized, and p-values were obtained using limma's moderated t-test with empirical Bayes variance shrinkage. A heatmap demonstrates the hierarchical clustering of DEG between resolved and progressing GBS infections (**C-E**). A ClueGo network visualization of each cluster are functionally enriched immunological processes in DEG upregulated in Resolved GBS infections versus Progressing ($\log_2FC > 0.58$; $p < 0.05$). Node size reflects the term enrichment significance (Bonferroni-adjusted $p < 0.05$).

A

B

Cluster 2: GO and KEGG Enrichment in the Resolved GBS Infection Group

- mismatch repair
- postreplication repair
- humoral immune response
- DNA replication
- cell cycle G1/S phase transition
- regulation of response to DNA damage stimulus
- nucleic acid phosphodiester bond hydrolysis
- chromosomal region
- cellular response to external stimulus
- mitotic cell cycle phase transition

C

Cluster 3: GO and KEGG Enrichment in the Resolved GBS Infection Group

- phagocytosis
- Tuberculosis
- NOD-like receptor signaling pathway
- immune responses regulating signaling pathway
- T cell activation
- granulocyte activation
- leukocyte migration
- DNA replication
- cell leading edge
- side of membrane

D

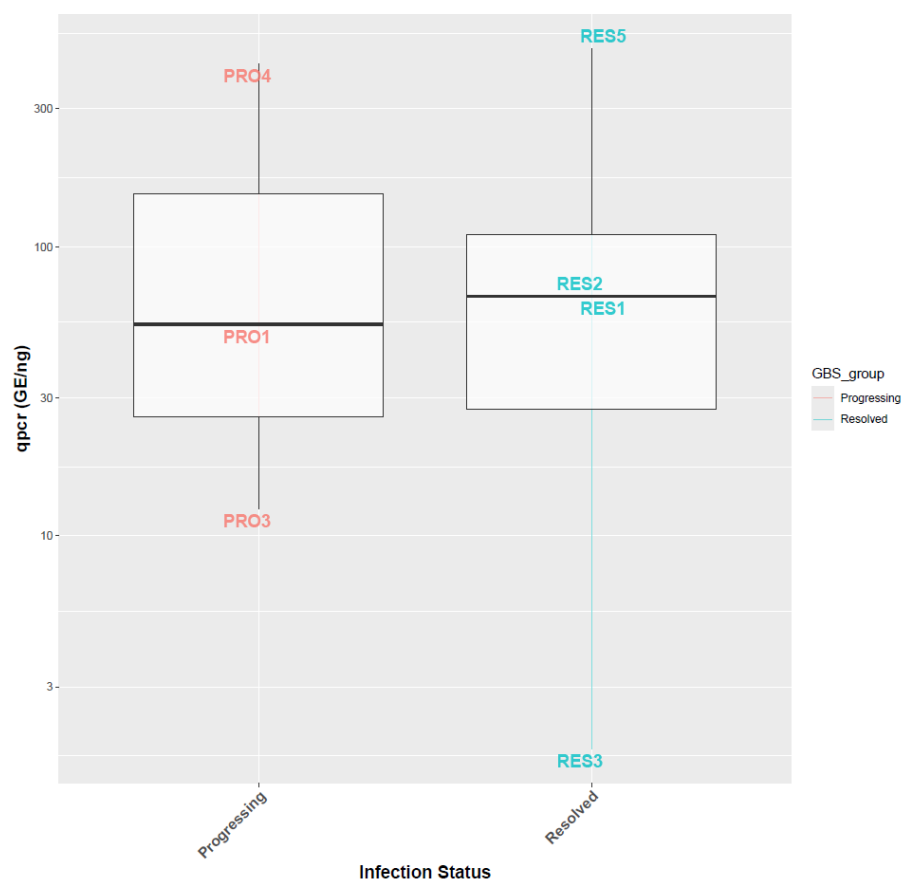
Cluster 4: GO and KEGG Enrichment in the Resolved GBS Infection Group

- humoral immune response
- adaptive immune response
- positive regulation of cytokine production
- leukocyte differentiation
- defense response to other organism
- positive regulation of defense response
- antigen binding
- granulocyte activation
- endopeptidase activity
- divalent inorganic cation homeostasis

E

(A) Volcano plot of DEG between Resolved and controls ($\log_2FC > 0.58$; p value < 0.05). **(B-D)** Cluster over-representation enrichment analysis of DEG within the resolved GBS infection groups versus controls (FDR-adjusted $p < 0.05$). **(E)** A ClueGo network visualization of functionally enriched immunological processes in DEG upregulated in Resolved versus controls ($\log_2FC > 1$; $p < 0.05$). Node size reflects the term enrichment significance (Bonferroni-adjusted $p < 0.05$).

Figure 3.11 GBS load in the Lower Uterine Segment of Myometrium



A box plot of the amount of genomic DNA (GE/ng) in the myometrium of the resolved and progressing GBS infection groups.

Discussion

Our results indicate that GBS infections at the maternal-fetal interface can progress and disseminate into the amniotic fluid or resolve with varying profiles of gene and protein expression. IL-1 β was the hallmark cytokine observed in AF at 24 hours and chorioamniotic membranes and decidua of the progressing GBS infections compared to resolved infections. Total RNA-Seq expression profiles in the progressing GBS group revealed a broad pro-inflammatory response in the chorioamniotic membranes, reflecting acute chorioamnionitis. We focused on the gene expression profile exhibited by the resolved GBS infections, as this provided novel insights into how the host's immune and stromal cells at the maternal-fetal interface can successfully defend against GBS bacteria. Interestingly, cytotoxic granule genes (*GZMA*, *GZMB*) were upregulated in the GBS resolved group, which signaled a potential role for cytotoxic uterine NK cells (uNK), NKT cells, or CD8⁺ T cells in bacterial clearance. Spatial resolution detected the cytotoxic signature of the resolved group in the decidua and the proinflammatory signature of the progressing GBS infection within all layers of the chorioamniotic membranes and decidua. Remarkably, the myometrium of the resolved GBS infection was enriched with a pro-inflammatory signature of adaptive immune activation that correlates with a high preterm labor rate. Therefore, this study supports the notion that sterile intraamniotic inflammation can occur following a resolved bacterial infection and contribute to preterm labor by affecting immune leukocyte cell differentiation, migration, and activation in the lower uterine segment of the myometrium.

Neutrophils and macrophages play a pivotal role in bacterial clearance. However, in this study, the progressing GBS infection group was enriched with neutrophils and

macrophages, as confirmed by histological sections and gene expression, at all layers of the chorioamniotic membranes and decidua, and overall, in estimated cell frequencies by CIBERSORT, but was not associated with the eradication of GBS. The effectiveness of phagocytosis and bacterial sequestration by these cells is associated with the virulent potential of a GBS strain [86, 99, 103, 177]. For example, the overexpression of β -hemolysin, hyaluronidase, β -protein, and serotype 3 GBS isolates is commonly associated with greater resistance to clearance by these immune cells, as opposed to mutant strains that lack these virulence factors and serotype V isolates. The strains used in this study were previously investigated, and it was determined that neutrophil recruitment is not successful in resolving the infection due to a β -hemolysin-producing GBS strain (serotype III, ST-17). Although the GBS pigment can induce the formation of neutrophil extracellular traps these were shown to be ineffective in trapping bacteria, leading to invasive infections in 60% of GBS Δ *covR* animals (3/5). With the use of improved molecular techniques and a larger study sample size, our current study found that dams infected with the hypervirulent GBS Δ *covR* strain was evenly distributed among the progressing (4/11, 36%), localized (4/11, 36%), and resolved groups (3/11, 27%). This suggests that inoculation with even a hypervirulent GBS Δ *covR* strain does not always result in a progressing infection, and that host mechanisms for resolving infections at the maternal-fetal interface may take divergent paths. Ultimately, the presence of neutrophils and macrophages were not always linked to bacterial clearance at the maternal-fetal interface suggesting that other cells and pathways may also be important.

The programmed death of these immune and certain stromal cells is thought to play a role in the outcome of infection. While cell death, including inflammatory processes, is a

normal and expected placental development and maintenance, dysregulation during infection can contribute to the ongoing inflammation and preterm labor [178]. In this study, we found that the pyroptosis and ferroptosis pathways were significant markers in the progressing group compared to the resolved GBS infection group. In contrast, the resolved GBS infection group showed overall induction of ferroptosis suppressor genes, specifically in the chorion and decidua. Noninflammatory cell death markers of apoptosis were found in both groups and thus were not a defining feature of either the progressing or resolved groups.

Pyroptosis and ferroptosis are highly inflammatory forms of programmed cell death characterized by inflammasome activation and iron-dependent lipid peroxidation, respectively. The GBS β -hemolysin toxin has been shown to cause pyroptosis in macrophages, and the mechanism exacerbates fetal injury and death during pregnancy [73]. Recently, GBS has been found to induce ferroptosis in macrophages as well [179]. In the same study, ferroptosis was found to be beneficial in the host response to controlling a GBS infection, resulting in increased mouse survival. We attributed the different roles of ferroptosis in infection outcome in this study to the state of pregnancy. A similar trend has been reported in non-pregnant mouse models, where pyroptosis is beneficial in containing infection [180]. However, both pyroptosis and ferroptosis have been linked to the development of preeclampsia and dysregulation of trophoblast cell activity during pregnancy [178, 181, 182]. This study indicates that both pyroptosis and ferroptosis pathways are also associated with adverse pregnancy outcomes.

The characteristics of a resolved GBS infection at the chorioamniotic membrane and decidua in this study align with similar findings in other controlled intracellular bacterial

infections at the maternal-fetal interface. Cytolytic-associated genes, specifically granzyme A (*GZMA*), and cytotoxicity pathways were enriched in the resolved GBS group. The proteins associated with these upregulated genes are markers of activated cytotoxic cells (killer cell immunoglobulin-like receptor 2DL4: *KIR2DL4*, killer cell lectin-like receptor B1: *KLRB1*,) and components of the cytotoxic granules that are produced (cathepsin w: *CTSW*; perforin-1: *PRF1*; natural killer cell granule protein 7: *NKG7*, and phosphoinositide-3-kinase regulatory subunit 6 *PIK3R6*)[183, 184]. Cytotoxic granules comprise effector proteins, including granulysin (*GNLY*), perforins, and granzymes that can induce pores, antimicrobial, and cytolytic activity. Granzymes are serine peptidases that can induce programmed cell death in microbes by inducing reactive oxygen species, thereby limiting their antioxidant defenses and disrupting the biosynthetic and metabolic pathways required for survival [185]. *GZMA* induces the death of microbes by inducing caspase-dependent programmed cell death. These effector granules have been found to kill several types of bacteria, including *Escherichia coli* (E.Coli), *Mycobacterium tuberculosis*, and *Salmonella typhimurium* [186-190]. *In vitro* studies of maternal decidual and peripheral NK cells demonstrated the selective transfer of *GNLY* via nanotubes to kill intracellular *Listeria monocytogenes* in extravillous trophoblasts, macrophages and dendritic cells. The importance of the *GNLY* gene was confirmed by the transgenic introduction into mice that resulted in a significant reduction of the *L. monocytogenes* load in the placenta as compared to wild-type mice that lack this gene. Furthermore, lipopolysaccharide (LPS) was also found to induce cytotoxic and activated placental CD8⁺ T cells in the placenta, which transitioned to an exhausted CD8⁺ T cell phenotype at 24 hours [191]. In this study, we found that both *GZMA* and *GZMB* genes were

upregulated in the decidua of resolved GBS infections, suggesting the roles of uNK and cytotoxic CD8⁺ T cells in bacterial clearance of intracellular GBS at the maternal-fetal interface.

Estimated cell compositions of uNK or CD8⁺ T cells were not statistically different between the resolved and progressing infections. This suggests that these cell types were not responsible for bacterial killing in the resolved GBS group as we hypothesized. While not statistically significant, there is a relatively increased expression of tissue-resident CD8⁺ T cells in the resolved GBS compared to the progressing group. Due to the specificity of *GZMA* and *GZMB* to these cytotoxic cell types, we believe these cells are still the origin of this signature. At the maternal-fetal interface, these cytotoxic proteins are primarily localized in uNK, cytotoxic T cells, and $\gamma\delta$ T cells [192-195]. We proposed that while the composition of these cells may remain unchanged, their ability to respond during infection may be challenged amongst GBS infection groups. Recent studies have shown that GNLY⁺ decidual NK cell concentration and activation in both humans with acute chorioamnionitis and NHPs with LPS or *E. coli* infection are not altered compared to their healthy controls [195]. The uNK and CD8⁺ T cytotoxicity functions can also be suppressed by immune checkpoints on infiltrating innate immune cells, as well as the production and signaling of TGF- β by macrophages, and the regulation of regulatory T cells [196, 197]. These suppression mechanisms may be responsible for the limited cytotoxic signature found in the progressing GBS group and the lack thereof, allowing activation in the resolved group.

More studies are needed to investigate the effectiveness of cytotoxic bacteria killing in the uterine cavity in relation to these various factors. However, this estimation would

require validation of immune cell proportions, ideally before an infection occurs with correlation after an infection occurs. This isn't possible in a pregnancy model, which is another factor that makes understanding the outcomes of these complex host-pathogen infections so difficult.

In addition to bacterial dissemination in the uterine cavity, preterm labor is a major clinical concern and observation within this study. Many of the key drivers of preterm and term labor include prostaglandins, hormones (such as estrogen, oxytocin), cytokines, and matrix metalloproteinases. Dysregulated inflammation in response to microbial infections or inflammatory cytokines in the sterile intraamniotic and uterine cavity is linked to preterm labor [198, 199]. In this study, we found preterm labor occurred in both GBS infection groups, but at a higher prevalence of 80% in the resolved group (4/5) compared to 40% in the progressing group (2/5).

Preterm labor that occurred in the progressing group is closely associated with chorioamnionitis, an established cause of preterm labor in the literature. Intraamniotic GBS infections have been shown to cause inflammation in the fetal membranes and maternal placenta, which leads to weakening [118, 200]. The substantial tissue injury and inflammation of the membranes that occur during chorioamnionitis in response to IL-1 signaling can explain this phenomenon in our progressing animals. Inhibition of IL-1 β signaling is a potential therapeutic option for GBS disease [201]. Recent studies have demonstrated that prophylaxis IL-1 signaling inhibition to heat-killed GBS leads to improved pregnancy outcomes, decreased preterm labor, reduced IL-1 β expression in the fetal membranes and myometrium, and an increase in viable pups [202]. Further investigation into the inhibition of IL-1 signaling and its impact on preterm labor, as well

as the active replication and dissemination of GBS, is warranted, especially for progressing invasive infections.

Notably, this is the first study to identify an association between bacterial resolution at the membranes that results in sterile intraamniotic infection, myometrial inflammation, and a high incidence of preterm labor. We identified a transcriptomic signature in the myometrium of the resolved GBS group, indicative of B and T cell activation and differentiation, neutrophil activation, and lymphocyte proliferation. The histological finding of lymphocytic infiltration in the myometrium of the resolved GBS group validated this inflammatory gene profile. Surprisingly, there was no statistical difference in cytokine and chemokine expression of the panel we tested between the progressing and resolved GBS infections at the time of Cesarean section. However, we cannot exclude that an early difference in cytokine/chemokine signature occurred. Furthermore, matrix metalloproteinase activity is also enriched in the myometrium of NHPs with resolved GBS infections, another labor-inducing pathway. This study supports the hypothesis in the field that infections that resolve and are deemed sterile are associated and may be a major contributor to spontaneous preterm birth [203].

Literature indicates that myometrial inflammation is a consequence of preterm labor rather than a cause [204]. Inflammatory processes in the myometrium has been linked to immune cell infiltration and tissue-resident cells in the decidua [205]. Several of our proposed cell types responsible for bacterial clearance at the decidua in the resolved GBS infection group, iNKT, and effector T cells, activation, have been linked to preterm labor [206, 207]. These cells in the decidua are probably inducing preterm labor, myometrial inflammation, and leukocyte infiltration. *In vivo* murine models demonstrated

that T cell activation at the maternal-fetal interface and in the myometrium not only caused preterm labor but also induced B-cell activation and macrophages at these tissues[208]. Similar responses could occur with the resolution of GBS infection and the activation of macrophages, T and B cells in myometrium, suggesting that this signature is a consequence of the resolution of bacteria and likely T cell activation in the adjacent tissue. The link between myometrial inflammation and the resolution of bacterial infections at the maternal-fetal interface merits greater focus and investigation.

Moreover, inhibition of myometrial inflammation has been successful in preventing preterm labor and its associated pathways. Broad-spectrum inhibitor (BSCI), FX125L, which inhibits signaling through the cell-surface type-2 somatostatin receptor (SSTR2) and chemokine function, has been found to prevent contractions, crosstalk between macrophages and myocytes, and cytokine secretion in an *in vitro* LPS-stimulated myocyte model [209]. Our lab demonstrated that the prophylactic use of the same BSCI can prevent preterm labor in our NHPs inoculated with GBS; however, myometrial tissues were not investigated [117]. It is possible that BSCI was successful in preventing preterm labor through the suppression of inflammation in the myometrium.

Understanding the spectrum of invasive GBS disease is crucial for the development of therapeutics that prevent the spread of bacteria and reduce the risk of preterm labor. The current interventions for GBS disease are antibiotics, with no available treatment for preterm labor. Progressing GBS infections were characterized by inflammation, including pyroptosis and ferroptosis, which further amplified the secretion of IL-1 β at the chorioamniotic membranes and decidua. The resolved GBS infections had a strong inflammatory response in the myometrium. Thus, therapeutics that inhibit both the IL-1

signaling closely associated with GBS dissemination at the chorioamniotic membrane and decidua, as well as the inflammation and infiltration of innate and immune cells in the myometrium of resolved GBS infections, need to be studied.

The advantages of the NHP model for studying host factors include its similarity to pregnancy and the structure of the maternal-fetal interface, as well as its outbred, genetically diverse nature. We examined host-pathogen interactions by assessing multiple uterine and fetal tissues, serial fluid sampling, and investigating clinical outcomes of concern. Furthermore, advanced technology, such as spatial whole-genome transcriptional profiling, was incorporated to provide high granularity. This study has characterized the spectrum of infection outcomes of invasive GBS, including progression and resolution, at the maternal-fetal interface, a new approach to understanding the range of disease. Furthermore, we have revealed a close association between GBS dissemination and IL-1 β production in the membranes. For the first time, we determined the resolution of GBS results in inflammation in the myometrium and a higher prevalence of preterm labor, associated with cervical effacement and shortening. Therapeutics need to address both mechanisms that can occur in the chorioamniotic membrane, decidua, and myometrium in response to GBS.

Several limitations and considerations should be acknowledged and considered in this study, including the sample size and the timing of tissue collection. In this study, we had a limited number of animals, particularly those that received a *GBS Δ covR Δ cyIE* inoculation. NHP studies are conservative for justifiable ethical and financial reasons and therefore result in small sample sizes. While there were limited *GBS Δ covR Δ cyIE* used in this study, animals that received this isolate were again evenly distributed between

localized (2/5), resolved (2/5) and progressing (1/5). The addition of more animals inoculated with *GBSΔcovRΔcyIE* would increase the overall sample size, providing a more robust analysis of the results, irrespective of the strain. Furthermore, tissue samples were taken at the end point; thus, our results reflect the immune response at a single point in time. Therefore, the phenotype for resolved GBS infections was captured after the killing of GBS. These results may be missing some of the earliest events associated with infection resolution, however, the transcriptomic signature is suggestive that cytotoxic cells are playing an important role in this process. Lastly, we utilized two specific GBS strains, *GBSΔcovR* and *GBSΔcovRΔcyIE*, that were designed to understand the role of the β -hemolytic pigment in perinatal infections; other virulence factors expressed by GBS were not examined in our study. The mechanisms of infection resolution for other pathogens may differ.

Conclusions

Adverse clinical outcomes occur in both a progressing and resolving GBS infection at the maternal-fetal interface. In the chorioamniotic membranes, protein and transcriptional profiling indicated that progressing GBS infections were associated with an inflammatory IL-1 β response and pyroptosis and ferroptosis cell death pathways. In contrast, resolved GBS infections were associated with a less inflammatory and cytotoxic granular (granzymes) transcription, likely related to bacterial killing, likely by uNK and/or CD8+ T cells. Conversely, a greater inflammatory response and higher preterm labor rate in resolved versus progressing infections in the myometrium suggests that bacterial killing in the choriodecidual space may lead to preterm labor and induce an inflammatory

response in the myometrium. The interaction of activated uNK and CD8⁺ T cells at the junction of the decidua and myometrium requires further investigation to understand how bacterial killing occurs at the maternal-fetal interface and might stimulate preterm labor. Additional studies are also needed to test preterm birth therapeutics that target the underlying mechanisms driving each infection outcome.

Chapter IV: Discussion and Conclusion

Invasive GBS during pregnancy is a leading cause of fetal injury, preterm birth, and stillbirth. While GBS is a common commensal of the vaginal and gastrointestinal tract, it can ascend and disseminate in the uterine cavity to the chorioamniotic membranes, maternal decidua, myometrium, amniotic fluid (AF), and the fetus. However, there is a gap in knowledge of host-pathogen interactions of GBS and innate immune response, as it is challenging to investigate the inaccessible maternal-fetal interface during pregnancy. To gain insight, we utilize the well-established chronically catheterized non-human primate (NHP) model to investigate the innate immune response that contributes to preterm labor and microbial invasion of the amniotic cavity in response to a GBS inoculation. Choriodecidual inoculation was chosen to capture the host's innate immunity at the initial location where microbes undergo an ascending infection. In this dissertation, I investigated both the hypervirulent β -hemolytic GBS-producing strains, *GBS Δ covR* and an isogenic strain that lacks this virulence factor, *GBS Δ covR Δ cyIE*. The goal was to determine the early molecular and cellular immune pathways that govern adverse clinical outcomes.

In chapter 3, I explored the first main objective of the dissertation, which is to spatially resolve immune protein expression, with an emphasis on immune checkpoints, at the amnion, chorion, and decidua. Inhibitory and activating immune checkpoint proteins/ligands were markedly increased in all layers, especially VISTA, an inhibitory protein, between *GBS Δ covR* and saline, and *GBS Δ covR* versus *GBS Δ covR Δ cyIE* in the decidua. Moreover, the modulator checkpoint proteins were more consistently associated

with peak cytokine concentrations in the AF and the placental redline maternal stage, score for chorioamnionitis. VISTA emerged as an essential immune inhibitor due to its correlation with both factors and the highest expression overall.

When VISTA is expressed on neutrophils, it impairs the function of cytotoxic CD8⁺ T cell. In this study, we verified VISTA expression on the infiltrating neutrophils as opposed to macrophages and monocytes. Our findings suggest that the regulation of VISTA after GBS infection within the chorion and decidua may indicate a potential role in how decidual CD4⁺ and CD8⁺ T cells balance the need to prevent an allogeneic response against the fetus while protecting against infection. Previously, we have shown a statistical increase in CD8⁺T cell infiltration at the chorioamniotic membrane and decidua in animals that received a GBS Δ *covR* inoculation, corresponding to the same animals with high neutrophil infiltration. The data from this study and previous published work suggest that the intense neutrophil infiltration into GBS could counteract the effect of CD8 T⁺ cells. The role of CD8⁺T cells at the maternal-fetal interface in acute infection is essential to consider.

In chapter 4, I focused on the host immune roles that contribute to the spectrum of invasive disease at the maternal-fetal interface, specifically the resolution or progression of GBS into the amniotic cavity. Analysis was conducted for the chorioamniotic membranes and the surrounding uterine muscle (myometrium), an understudied tissue within the field. An infection that spreads to the amniotic fluid and fetus can be defined by an inflammatory environment driven by IL-1 signaling, cell death pathways, pyroptosis, and ferroptosis in the fetal membrane and maternal decidua. In contrast, a resolved GBS infection was associated with a mild immune response that entailed cytotoxic effector

proteins (granzymes) known to contribute to bacterial killing, likely by uNK and/or CD8+ T cells. Surprisingly, a greater inflammatory response and a higher preterm labor rate occur in resolved versus progressing infections in the myometrium. This suggests that the bacterial killing by cytotoxic cells in the choriodecidual space may also induce preterm labor and an inflammatory response in the neighboring tissue, the myometrium.

Both chapters 3 and 4 highlight the potentially important roles of cytotoxic cells, CD8+ T, $\gamma\delta$ -T, natural killer and natural killer T (NKT), at the maternal-fetal interface during GBS infection. However, the exact mechanism and types of cytotoxic NK and T cells in response to GBS during pregnancy in the uterine cavity are unknown. Targeted *in vitro* and *in vivo studies* are needed to validate the specific cells and pathways that influence GBS clearance. Furthermore, studies that elucidate the cross-talk between bacterial clearance and cytotoxic cell activity in the chorioamniotic membranes and myometrium would help understand the role of these cells in pregnancy and acute infection.

Importantly, I hope that the results of this dissertation work will guide the development of potential interventions to treat the spectrum of GBS disease and other intrauterine infections during pregnancy. Results in Chapter 3 suggest that future studies using immune checkpoint inhibitors (ICIs), such as JNJ-61610588, which block VISTA and remove the suppression of cytotoxic immune cells at the maternal-fetal interface, may be beneficial. However, there is hesitation in using ICI in pregnancy; preclinical studies in mice and other animal models are required to evaluate reproductive toxicity [210].

Other more promising treatments that could be studied include a combination of specific immunomodulators. The role of IL-1 signaling has been greatly attributed to GBS spread at the maternal-fetal interface in this and previous studies. Our lab is currently using

RYTVELA, an allosteric antagonist of the IL-1 receptor, to prevent preterm labor induced by GBS in our NHP model. I hypothesize that using a combination of RYTVELA and a broad-spectrum chemokine inhibitor (BSCI) to prevent the spread of bacteria and reduce the risk of preterm labor could be effective. In this study, a comparison group would need to include the use of two immunomodulators with antibiotics, as inhibition of inflammatory pathways may inhibit the necessary cytotoxic pathways and not eradicate the bacteria in its entirety. My thesis adds to the literature that balancing the control of a bacterial infection and inflammatory response at the maternal-fetal interface requires a multi-faced approach.

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