Roles of Group B Streptococcal membrane proteins in virulence and protection

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A dissertation

submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

University of Washington

2024

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Program Authorized to Offer Degree:

Pathobiology

University of Washington

Abstract

Roles of Group B Streptococcal membrane proteins in virulence and protection

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Group B Streptococcus (GBS) asymptomatically colonizes the rectovaginal tract in up to 20% of women worldwide. During pregnancy, GBS is a leading cause of invasive infections associated with adverse pregnancy outcomes including preterm births, neonatal infections, and stillbirths. In addition to its role as a pregnancy-associated pathogen, GBS infection rates in non-pregnant adults are also rising. While the field has made significant progress in defining GBS virulence factors and their roles in infection, many remain undefined. GBS membrane-associated and secreted proteins are more likely to interact with the host, making these proteins especially important during infection. Importantly, the localization of these proteins also makes them attractive targets for therapeutic design, which is relevant because current GBS treatment regimens are limited to antibiotics and no prophylactic vaccine is approved for use. Here, I describe the importance of two GBS membrane proteins: the serine protease HtrA and the alpha-like surface proteins.

Many bacterial pathogens encode homologs of high temperature response A (HtrA) serine proteases, which regulate virulence and stress responses. While the function of GBS HtrA was previously unknown, here I show that HtrA-deficient GBS displayed attenuated virulence during

systemic infection and resulted in fewer adverse pregnancy outcomes in mice. Proteomic analysis of isogenic HtrA-proficient and -deficient strains identified 110 proteins that may be HtrA regulated, including the novel Streptococcal surface immunogenic protein (Sip). Interestingly, HtrA's virulence effect is partially linked to Sip, since deletion of both proteins led to a similar rate of adverse pregnancy outcomes compared to either single deletion. Further, I observed that Sip can be directly cleaved by HtrA. These findings support a system wherein HtrA, partially through the effects of Sip, can regulate GBS disease pathogenesis.

Alpha-like proteins (Alps) are a family of adhesins found exclusively on the surface of Streptococci. In GBS, there are six chimeric Alps variants that are allelically expressed., with alpha C (αC) and Rib being the most abundant. Here, I assessed the immunogenicity and efficacy of the clinical GBS-NN vaccine produced by fusion of the N-termini of surface exposed αC and Rib proteins. GBS-NN immunized mice exhibited heightened vaccine-specific antibody titers in serum compared to adjuvant controls. GBS-NN-specific antibodies also bound to native conformations of αC and Rib on GBS clinical isolates. Mice immunized with GBS-NN experienced reduced disease severity during both systemic and pregnancy-associated challenge. In addition to improving fetal survival in immunized dams, maternally-derived GBS-NN-specific antibodies were detected in full-term pups and contributed to improved pup survival following intranasal GBS challenge.

Together, the insights gained from this thesis highlight the importance of membrane associated proteins in GBS pathogenesis and provide examples of the broad range of host-pathogen interactions they can confer. They may also inform the development of novel anti-GBS strategies such as a small molecule inhibitor targeting HtrA and lend support for further testing of maternal vaccination strategies utilizing GBS-NN, which is currently under human clinical trials. Together with antibiotics, these novel strategies could improve GBS disease outcomes worldwide.

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

TABLE OF CONTENTS

LIST OF FIGURES

CHAPTER 1. Introduction

CHAPTER 2. The serine protease HtrA cleaves surface immunogenic protein (Sip) and regulates Group B *Streptococcus* virulence

LIST OF FIGURES

CHAPTER 3. A recombinant alpha-like protein vaccine (GBS-NN) provides protection in murine models of Group B *Streptococcus* infection

LIST OF TABLES

ACKNOWLEDGEMENTS

There are so many people who helped to make this work possible, and I will attempt to thank many of them here. First and foremost, a huge thank you to my advisor – Lakshmi Rajagopal – for giving me the opportunity to join her lab a quarter early and for fostering a truly team-focused and positive environment. Doing science here has not only trained me as a scientist, but also lengthened my publication record and taught me a lot about organization, communication, and collaboration. Lakshmi is a power-house who is highly respected in the Streptococcal biology and broader bacterial pathogenesis fields, and I am lucky to have a strong female mentor who I can look up to for the rest of my scientific career.

To all members of the Rajagopal lab past and present, thank you for your support and collaboration throughout my PhD. A special and heartfelt thank you to all who saw me through the transition to my main thesis project at the start of the COVID-19 pandemic. This was a difficult time, and I am especially for your support during that period. I am especially grateful to Anna, who was stretched in similar ways as a graduate student during this time. I was lucky to have another student in the lab, and that we were able to support each other during the peaks and valleys of both research and graduate school. Thank you to Michelle, our resident expert in *in vivo* models and immunology for helping me stretch my brain to make space for immunology. I simply would not have gotten through graduate school without our scientific conversations or venting sessions, so thank you for always giving your time and providing me space to be seen and heard. To Kavita, who by some twist of fate had studied another HtrA, thank you for the support you gave as my thesis took off, and for always validating my thoughts and ideas. We shared many laughs and caffeine breaks throughout my PhD, and while our coffee breaks are now on the phone or on FaceTime, it's been special to receive your support from afar. Phoenicia was a true force who always managed to jump from project to project, helping with whatever she could. I am so grateful for all of your assistance in the lab, and for your friendship always. Thanks for always putting things into perspective for me, and for making time for coffee, lunch, and happy hours. To Austyn,

who has been a consistent source of support both scientifically and personally, thank you for always being there with a hug or a sassy remark when I needed it. You remind me to celebrate all good things both big and small and aren't afraid to speak up for yourself or for others. Thank you for welcoming Juniper into your home when we're on vacation, and for giving me so many good memories both in and outside of the lab.

To Ravin, who joined our lab with perfect timing and stepped in as a secondary mentor to me. I am so grateful for your help not only with molecular biology, but also for reminding me to dream big but be realistic. Thank you for sharing your advice, which is always insightful and well-intentioned, and for being honest in all things. Your guidance has helped to turn some interesting data into a story, and I am grateful for the time and effort you've invested in me and my project. Thank you for challenging me to focus on the important details without getting lost in everything else, and for helping me to become a better and more direct scientific writer. To Claire K. and Joy, while you joined the lab towards the tail-end of my thesis, know that I have greatly appreciated your support and positivity during this phase. Claire, thank you for jumping into the world of protein biology and for helping me polish up the final experiments so I could prioritize writing. Thanks for also keeping the science moving so the project can continue when I leave. I enjoy our conversations, especially when they revolve around food and result in teasing from Ravin. Joy, thank you for being a constant source of good vibes during my PhD. While we didn't see each other frequently, it always made my day to catch up at seminar or symposium. More recently, thank you for being an exceptional desk buddy and giving me thoughtful advice as my PhD comes to a close. Your opinions and advice have been so valuable and appreciated.

To Shayla and Grace, two incredible undergrads who sought the opportunity to help with my project, I have had the best time teaching, mentoring, and working with you. Teaching someone to work in a lab is difficult, and it is often harder to teach someone how to think scientifically and do research. You both worked so hard to learn as much and as quickly as you could, volunteering full days of your time simply because you believed in yourselves, and you

believed in me. Thank you for investing in yourselves as I invested in you. Your work ethic and tenacity paid off in spades, and the volume of work that I have been able to accomplish during my PhD is a direct reflection of you. Shayla, while medical school will be difficult, I know you have what it takes to not only make it through, but to do it well. You will be an excellent doctor because you are detail-oriented, focused, and compassionate. Grace, your work ethic and dependability will make you a major asset for your next lab, but when you're stressed about learning the details of a new field, don't forget that you became an expert on my thesis in less than two months. You are already ready for graduate school, and when the time comes to apply remember that any program will be lucky to have you.

Next, a huge thank you to my committee: Lakshmi Rajagopal, Josh Woodward, Christoph Grundner, and Tom Hawn. The consistent support from all of you made this whole PhD journey so much less stressful than it could have been. Knowing that I would receive nothing but respectful and cooperative mentorship from you all allowed me to enter every committee meeting excited to share my new data and looking forward to hearing your thoughts and suggestions. Thank you for providing critical insight throughout my PhD, for being understanding during my project transition, and for constantly challenging me in a productive and meaningful way. All four of you have made my thesis better.

I have many people to thank in the Pathobiology program. First and foremost, thank you to Lee Ann Campbell and Jennifer Lund, for their fearless leadership and consistent investment in our program and its students. Thank you also to Ernie Lefler for keeping the program operational and for always sending extra reminders to keep us all on track. Thank you to all of the Pathobiology faculty who have seen my work progress throughout my time here – I especially appreciate those who have taken an interest in my project by asking questions and providing me with feedback at symposium or retreat. To all the Pathobiology students, your support, ideas, and friendship throughout this experience have meant the world. I am lucky to be a part of a community of students who are top-caliber scientists but also top-tier people. To all Pathobiology faculty and

students who are involved in the push for improved DEI within our small microcosm of academia, and those who consistently challenge our program to improve each year, thank you. I would also like to thank Andrew Frando and Sean Windle for providing consistent molecular biology technical support and taking part in critical discussions about my work. Finally, a huge thank you to my cohort: Mark Hernandez, Ceejay Boyce, Nika Hajari, Lizette Carrasco, and Amy Lu. Going through this process alongside all of you reminded me that I was never alone and gave me a constant source of support. I appreciate you all so much and am so excited to see what we all accomplish.

At Seattle Children's, thank you to Connie Hughes for her organization and administrative assistance throughout my PhD. Thank you to the Office of Animal Care team for their continued support for not only research animals, but also the researchers who use them. Thanks especially to Cindy Chang and Karuna Patil who are founts of technical knowledge and are always willing to provide training and support. Thank you to Phil Gafken and the Fred Hutchinson Shared Proteomics Core staff for providing their assistance with targeted mass spectrometry and shotgun proteomics methods, and to Joel Nott and the Iowa State University Protein Facility for their help with N-terminal sequencing workflow and analysis.

To my previous advisor – Anna Tischler – thank you for investing in me as an undergraduate and for providing me with the opportunity to do research full time. You gave me my first introductions into hypothesis-driven research, molecular biology, collaboration, scientific writing, time management, record keeping, and so much more. I am where I am today because you fostered my interest in research and instilled in me the drive to continue to persist (no pun intended) when science doesn't go how you expect. I appreciate your mentorship and still see its effects every day. To Christian Mohr, thank you for authenticating my love of microbes and for providing me with the opportunity to be your teaching assistant. Without that experience, I may not have realized my passion for teaching and mentorship, and for that I am eternally grateful.

Finally, to my family: Tom, Michelle, and Rebecca, thanks first for supporting my decision to go to graduate school. That alone is a huge endeavor, and then add on top of that moving across two time zones to Seattle. While it's been all too easy to get lost in the science, thank you for supporting me from afar and for coming out to visit as much as you could. Having your support has made the stress bearable, and I appreciate everything you've done to make this possible. To the Becks: Christine, Phil, and Caity, thank you for being the best in-laws from day one and for your support throughout this journey. Thank you for accepting my decision to move your son across the country, and for always welcoming us back home sporadic as the timing may have been. We're looking forward to being back with you all and are excited to begin that journey. To Jacob: thank you for your willingness to accept this crazy dream and for the constant support you've given me from day one. From doing our laundry, to taking Juniper on her daily walks, and reminding me to schedule appointments and call home, you've helped to make sure that I am functioning so that I could worry about lab. Thank you for also reminding me to see my friends and for ensuring that I made into nature every now and then. While graduate school is not very good for mental health, thanks for prioritizing me when I didn't have the bandwidth to prioritize myself. And to Juniper and Willow: thanks for the snuggles and unconditional love. Getting a dog in graduate school was definitely the correct choice.

CHAPTER 1. Introduction

This chapter was adapted from the following articles with permission under the following license agreement (Creative Commons BY 4.0 DEED):

Brokaw, A., Furuta, A., Dacanay, M., Rajagopal, L., & Adams Waldorf, K. M. (2021). Bacterial and Host Determinants of Group B Streptococcal Vaginal Colonization and Ascending Infection in Pregnancy. *Frontiers in Cellular and Infection Microbiology*, *11*, 720789. DOI: 10.3389/fcimb.2021.720789

Furuta, A., **Brokaw, A**., Manuel, G., Dacanay, M., Marcell, L., Seepersaud, R., Rajagopal, L., & Adams Waldorf, K. (2022). Bacterial and Host Determinants of Group B Streptococcal Infection of the Neonate and Infant. *Frontiers in Microbiology*, *13*, 820365. DOI: 10.3389/fmicb.2022.820365

1.1 *Group B Streptococcus* etiology and disease outcomes

Group B Streptococcus (GBS) (also called *Streptococcus agalactiae*) is a gram-positive, β-hemolytic, chain-forming bacterium that was first characterized by Rebecca Lancefield in the 1930s. Lancefield noted variable hemolytic activity and carbohydrate content in the streptococci that she isolated from humans, animals, and dairy products, which eventually led to the classification of Streptococcal pathogens. Due to its consistent isolation from cow milk, GBS became known as an etiological cause of bovine mastitis before it had any known association with human disease (3). However in the mid-1930s, following discoveries that related streptococci cause tonsilitis and scarlet fever, GBS was identified as a cause of afebrile puerperium or low-grade fever following delivery (4). By the mid-1960s GBS was emerging as a major cause of infant invasive infections (5, 6), leading to a sharp increase in research on this pathogen.

In the time since Lancefield's discovery, great progress has been made in describing GBS as a human pathobiont. GBS is a frequent asymptomatic colonizer of the human rectovaginal tract. During pregnancy GBS can become highly invasive and pathogenic to both mother and

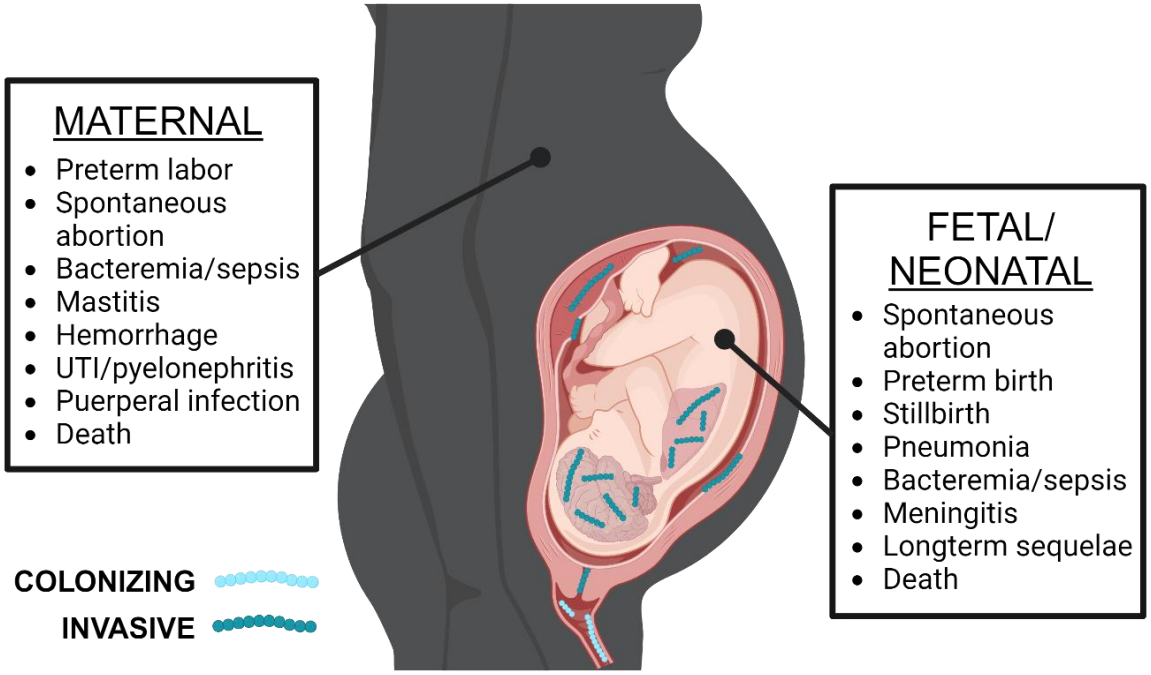

FIGURE 1.1. Maternal and fetal/neonatal disease outcomes associated with GBS perinatal infections. This figure was re-designed and used with permission (1) (CC BY 4.0 DEED).

baby, leading to wide-ranging adverse outcomes (**Figure 1.1**, previous page). In the mother, invasive GBS infections can lead to maternal sepsis and postpartum endometritis (7).

However, invasive *in utero* GBS infections can be especially catastrophic to the fetus, resulting in adverse outcomes such as preterm birth (PTB), stillbirth, and fetal injury or death. Even during the first three months of life, the neonate remains highly susceptible to GBS, resulting in highly invasive infections that are associated with sepsis, pneumonia, and meningitis. In severe cases, exposure to invasive GBS or maternal inflammation may lead to long-lasting sequelae affecting the child's central nervous system and lung function (8-10).

1.2 GBS epidemiology and current prophylactic strategies

GBS colonizes the rectovaginal tract in approximately 20-25% of women worldwide, with rates varying widely in different parts of the world (11). In a meta-analysis pooling estimates of GBS colonization from 85 countries, the prevalence varied between 7-14% in Central America and Asia to 35% in the Caribbean. Europe, North America, and Australia had similar prevalence rates of 15-20%. Interestingly, estimates in Africa varied widely with West and South Africa at 14 and 25%, respectively (12). Fewer studies have assessed GBS carriage rates in healthy young adults, although rates in this population in the United States are also around 20% (13). Beyond limitations on geography and sampling bias, colonization state is generally determined by rectovaginal swab, which also excludes the ability to identify GBS in other niches such as the gastrointestinal tract (14). Due to its asymptomatic nature, it is also difficult to determine whether the prevalence of GBS colonization is changing over time; however, there is evidence that rates of invasive GBS disease in nonpregnant adults are increasing. Between 2008 to 2018 within the Active Bacterial Core surveillance network, the incidence of invasive GBS infection in nonpregnant individuals increased from 8.1 to 10.8 cases per 100,000 adults (15). These studies emphasize that GBS colonization is a global issue and highlight asymptomatic carriage as a risk factor for developing GBS disease.

Although GBS vaginal colonization is generally asymptomatic, it is of particular concern during pregnancy due to the significant risk for adverse fetal and neonatal outcomes. Maternal GBS infection is a leading cause of infection-induced PTB and stillbirth (11). Further, in 2015 more than 20 million neonates were exposed to maternal GBS out of 140 million live births worldwide and half of neonates born to pregnant GBS-colonized women were estimated to become colonized themselves (11). In a conservative analysis of international data from a single year, at least 90,000 infant deaths (< 3 months of age), 10,000 cases of disability in children, 57,000 fetal infections or stillbirths, and 3.5 million cases of PTB were attributed to GBS. A disproportionate burden of these occurred in Africa, which accounts for 54% of annual GBS cases and 64% of all fetal and infant deaths due to GBS (11). In addition to its fetal and neonatal disease burden, GBS can cause serious maternal disease. Recently, it was estimated that there are at least 33,000 annual cases of maternal invasive GBS disease worldwide (11). While the impact of GBS on maternal and infant health systems is clearly large, the elevated burden in the developing world produces major challenges.

Importantly, the prevalence of maternal, fetal, and neonatal disease is likely underestimated. In low- to middle-income countries with the highest rates of invasive GBS disease, the microbiological cause of sepsis, PTB, stillbirth, or death may not be recognized if cultures are not obtained or if formalized pre- or antenatal doctor's visits are inaccessible. In addition, policies for GBS screening are highly variable both geographically and across the economical scale. Screening policies generally fall into two categories: 1) microbiological or 2) risk factor-based screening. Microbiological screening, sometimes known as universal screening, includes programs that tests all mothers for GBS at a specific gestational age. In contrast, factor-based screening is more cautious in that mothers are tested only if they present with symptoms of GBS invasive disease (such as preterm labor) or if they have a history of adverse pregnancy outcomes. While expensive to implement, universal screening may be highly effective in

populations with access to specialized medical professionals; however, risk-based screening is frequently successful in populations with lower rates of colonization (16).

Mothers who test positive for GBS may be administered intravenous antibiotics during labor, an intervention called intrapartum antibiotic prophylaxis (IAP). Unfortunately, IAP policies are also highly variable due to costs, the need for specialized nursing staff, and rising concerns of antibiotic resistance (17). Discrepancies in both screening and antibiotic use can have major effects on GBS case counts and birth outcomes. When symptoms are absent until after delivery, cases may not be attributed to GBS until late in the clinical course when there may be no time to intervene. Conversely, subclinical cases – which may never be attributed to GBS infection – can still lead to lifelong complications if they remain undetected and untreated. Rapid and low-cost testing strategies that can be implemented in low-resource settings would likely allow for more accurate estimates of the GBS burden of disease and might provide opportunities to reduce this burden through current antibiotic strategies.

1.3 GBS serotypes and sequence-types

Many virulence factors influence GBS' ability to colonize or cause severe disease. A key virulence factor is the sialylated capsular polysaccharide (CPS), which provided the basis through which Rebecca Lancefield distinguished GBS (exhibiting group B antigen) from other streptococci (3, 4). To date, there are ten capsular serotypes (Ia, Ib, II-IX), although six account for 98% of GBS colonization worldwide (Ia, Ib, II-V) (12). These same serotypes cover over 99% of overall cases of disease, including EOD and LOD (18). Not unlike colonization rates and disease burden, GBS serotype frequencies vary geographically. For example, 25% of colonizing strains are serotype III worldwide, but this serotype accounts for only 10% of cases in Asian and African countries. Instead serotypes V-IX are more prevalent colonizers in these regions (18). While serotype Ia and III are linked to approximately 30% of GBS maternal invasive cases (7), fetal and neonatal disease is dominated by serotype III which was responsible for 62% of cases. Despite

this major skewing towards serotype III, serotypes Ia, Ib, and V also cause frequent fetal and neonatal disease (9).

GBS can be further clustered through genetic multi-locus sequence typing, which is performed by sequencing seven housekeeping genes including alcohol dehydrogenase (*adhP*, *gbs0054*), phenylalanyl tRNA synthetase (*pheS*), an amino acid transporter (*atr*, *gbs0538*), glutamine synthetase (*glnA*), serine dehydratase (*sdhA*, *gbs2105*), glucose kinase (*glcK*, *gbs0518*), and transketolase (*tkt*, *gbs0268*). The combination of sequences from these seven genomic loci generates a sequence type (ST) (19). Among a global and ecologically diverse sample of strains, four major sequence types have been identified: ST-1, ST-17, ST-19, and ST-23. ST-1 and ST-19 are largely associated with asymptomatic carriage, while ST-17 serotype III strains are responsible for a large proportion of neonatal invasive infections and associated with elevated rates of antibiotic resistance (19-22). However, the rise of antibiotic resistant GBS is not a problem exclusive to ST-17 strains (23), since IAP is the only FDA-approved preventative measure against vertical transmission of GBS. Further, GBS infections in infants and adults are routinely treated with antibiotics. The development of alternative GBS treatment strategies could allow for replacement of antibiotic therapies or enhancement of antimicrobial activity at lower doses, which may alleviate the threat of antibiotic resistance and improve GBS infection outcomes with minimal disruption to the human microbiome.

Beyond the challenges of antibiotic resistance, hypervirulent GBS – including ST-17 strains – have recently been isolated from tilapia (24, 25), and there are rising concerns that tilapia-adapted GBS might serve as a zoonotic route for human transmission (26). In addition to representing a possible reservoir for GBS spillover, tilapia-adapted GBS are terrorizing global fishing industries across South America and Asia. In 2011 alone, streptococcosis caused by GBS led to losses of at least $40 million in China, where an outbreak can cause up to 80% mortality in fish. Luckily, the economic impact of GBS streptococcosis has led to a major push for tilapia

vaccines (27). Since many virulence factors are similar between hosts, this may be the helpful nudge required to prioritize investment in GBS vaccines.

1.4 GBS virulence factors and their regulation

The complex pathogenesis of GBS infections arises due to the large arsenal of virulence factors encoded by this pathogen. Due to its pan-genome, the presence of these virulence factors often varies widely across GBS strains and serotypes. Additionally, many factors undergo drastic changes in expression depending on the host niche encountered. While some virulence factors are implicated in a single disease outcome, such as the role of HvgA in neonatal meningitis caused by hypervirulent ST-17 strains (20) other factors are very broadly associated with GBS pathogenesis and are thus linked to many disease manifestations. One well-defined example of this is the GBS hemolysin, granadaene (also called β-hemolysin/cytolysin), which is implicated in dissemination and damage across many tissues due to its direct cytotoxicity to many types of host cells (28-35). Despite a relatively good understanding of how some GBS factors influence disease outcomes, the impact of many virulence factors on pathogenesis remains undefined. Furthermore, studies aimed to dissect how regulatory pathways allow this pathobiont to transition from an asymptomatic colonizer to a highly invasive pathogen are greatly needed.

During its pathogenic transition, GBS must adapt to new host niches in order to successfully colonize, persist, and disseminate (36). Thus, GBS encodes a plethora of two-component systems (TCS), each comprised of a sensor histidine kinase and DNA-binding response regulator (denoted by S and R, respectively) that grant the ability to respond to diverse environmental stimuli (37). Perhaps the best-studied of these TCS is CovR/S (also known as CsrR/S), which responds to low pH likely encountered in the healthy human vaginal or gastrointestinal tracts (38). CovR/S is considered the master regulator of GBS virulence, and more than a hundred genes undergo transcriptional changes in response to CovR. Interestingly, GBS lacking CovR or CovS are hyper-adherent due to increased expression of cell surface

adhesins (38-40). By regulating this process, CovR/S may ultimately allow GBS to colonize and persist in a host. However, this TCS also regulates expression of the *cyl* operon (41) which encodes factors required for the biosynthesis of granadaene (32, 33, 42). This hemolytic lipid toxin is cytotoxic towards an exhaustive list of host cells including many immune cells, and granadaene-mediated killing of these cells is pro-inflammatory. Thus, this factor is very important during many GBS disease manifestations including pneumonia, sepsis, meningitis, PTB, and invasive infection of adults (29-33, 35, 43-48). While over-production of this pro-inflammatory toxin by "hyperhemolytic" strains is generally associated with reduced GBS persistence (34, 49), strains harboring mutations associated with *covR/S* function are often linked to highly invasive infections (50-52).

Our understanding of these and other TCSs and their role in regulating virulence highlight the importance of adaptation to the host in GBS' ability to transition from colonizing to pathogenic bacterium. Studies aimed to further define virulence factor function and regulation may be beneficial for the design of alternative therapies for GBS treatment or for the design of vaccines that could be used prophylactically against GBS. Chapter 2 of this thesis will summarize the function of the serine protease HtrA and will describe its importance as a factor that post-translationally modifies the abundance of numerous GBS virulence proteins.

1.5 Host-pathogen interactions along the course of GBS infection

1.5.1 Host pathogen interactions during GBS colonization

In addition to regulating its own virulence factors, GBS must also overcome the host immune response to achieve persistent colonization. Vaginal tract colonization induces the recruitment of many immune cells which facilitate bacterial clearance through diverse mechanisms. The production of IL-1β, IL-6, IL-8, IL-23, IL-17, IFN-γ, and TNF-α are associated with reduced GBS colonization (49, 53-55). These proinflammatory mediators are primarily produced by neutrophils recruited through IL-8, CXCL1, and CXCL2 (49, 53, 54), by macrophages

(54), and by vaginal epithelial cells (**Figure 1.2, vaginal tract, panel A**) (53). In addition, resident mast cells contribute to GBS clearance through granadaene-induced release of pre-formed inflammatory mediators, including histamine and β-hexominidase (**Figure 1.2, vaginal tract, panel B**) (34). In mice, vaginal clearance is associated with Th1, Th2, and Th17 responses (49, 54), while in humans IFN-γ production seems to be associated with clearance of serotype III GBS (56). Importantly, multiple studies have identified strain-level differences in immune responses that may partially explain differences in strain and serotype colonization of the vagina (49, 55, 56).

If GBS can withstand these immunological insults and persistently colonize in the vaginal tract, it may have the opportunity to ascend during pregnancy. GBS also encodes TCS that confer protection against innate defenses encountered in the host, which ultimately function to prolong GBS survival (37). The TCS DltR/S is involved in cell wall lipoteichoic acid maintenance, and loss of this system leads to altered cell surface charge that heightens GBS susceptibility to cationic antimicrobial peptides (CAMPs) such as colistin found in the reproductive tract (57, 58). LiaR/S is also important for cell wall integrity and regulates genes involved in cell wall synthesis, cell membrane modifications, and pilus. Thus, mutations in this system also result in increased sensitivity to CAMPs (59). Another well-studied TCS is CiaR/H, and *ciaR* deletion has been linked to hypersensitivity to CAMPs and other factors important for killing of intracellular pathogens (60). GBS may evade these processes by inhibiting endocytic trafficking of host cells through the effects of two factors (peptidase SAN_0039 and hypothetical protein SAN_2180) which are regulated by CiaR/S (61). While GBS uses many pathways to prevent its recognition and killing by host cells, the conditions required to activate each TCS is unknown, and the redundancy of these mechanisms remains unclear.

1.5.2 Host-pathogen interactions during GBS ascending infection

It is puzzling that non-motile GBS is able to ascend from the colonized vagina to the uterus. One mechanism that typically protects a host against ascending infection is vaginal epithelial exfoliation. This process is facilitated by epithelial-mesenchymal transition (EMT), a coordinated loss of epithelial cell tight junctions that leads to the detachment of apical cells which are shed from the epithelium. GBS resident in the vaginal tract binds to α1β1 integrins, which activates a signaling cascade that displaces β-catenin upon the breakdown of adherens junctions. Once displaced, β-catenin translocates to the nucleus and activates transcription of genes associated with EMT (62). *In vivo*, expression of these transcripts results in vaginal exfoliation that is typically associated with eradication of vaginal pathogens (63). However, GBS burden in the murine vaginal tract remained unaltered during EMT-induced exfoliation, and instead this phenomenon was associated with increased ascension to the uterus (62). This strategy seems to be unique to GBS, and details that coordinate this mechanism – such as the GBS factors that engage α1β1 integrin or damage epithelial cell tight junctions to promote EMT – remain undefined.

Most pathogens that cause intra-amniotic infections are vaginal tract commensals, highlighting the importance of ascending infection as a mechanism of invasive disease during pregnancy (64). During ascension, GBS interacts with cervical epithelial cells using its diverse repertoire of adhesins and invasins (65-72); however, many of these interactions have only been assessed *in vitro*. During GBS infection of the murine vaginal tract, IL-17 produced by neutrophils or CD4+ helper T cells may mediate GBS clearance in the mouse cervix (**Figure 1.2, cervix**) (49). Thus, by invading cervical cells GBS can avoid detection by extracellular host immune factors which allows this pathogen to persist and further disseminate.

The cervix undergoes major physiological changes during pregnancy that impact ascending infection. For example, hyaluronan (HA, also called hyaluronic acid) levels increase in the cervix throughout pregnancy and reach their height during parturition. This glycosaminoglycan plays a structural role in the extracellular matrix and can be digested by host hyaluronidases into

smaller fragments that interact with a diverse array of host receptors to regulate a variety of cellular processes (73-75). Host-cleaved low molecular weight HA fragments serve as damage-associated molecular patterns (DAMPs) that bind to toll like receptor (TLR) 2 and 4 to mediate inflammation during tissue injury (76-78). HA is also crucial for the maintenance of epithelial polarization and barrier function, and loss of HA is associated with ascending *Escherichia coli* infection with subsequent PTB (79). GBS exploits this axis by secreting its own hyaluronidase (HylB), which further cleaves pro-inflammatory HA DAMPs into disaccharides that bind to TLR2 and 4 but block recognition of other GBS ligands. This ultimately dampens the host immune response to permit ascension by GBS (80). The protection afforded by HylB is absent during GBS infection in TLR2-, TLR4-, or IL-10-deficient mice, suggesting that these factors are critical to mediate GBS immune suppression through HylB (**Figure 1.2, uterus**). Uterine macrophages were identified as important producers of IL-10 during GBS ascending infection, and *in vitro* treatment of mouse bone marrow-derived macrophages with GBS and IL-10 resulted in impaired production of MIP-1α, MIP-2α, TNF-α, and MCP-1 (81). GBS clinical strains isolated from cases of PTB or invasive neonatal infection may exhibit high HylB activity, which is associated with ascending infection, reduced uterine inflammation, and increased rates of PTB in murine infections (82). While IL-10 appears to be important during GBS ascension, its impact during infections caused by GBS strains with lower HylB activity remains unknown.

These studies describe some GBS and host factors that mediate the arms race occurring as GBS transitions from vaginal colonizer to invader; however, many molecular details remain undefined. Further, insights gleaned from mouse models may not be directly translatable to human disease. There are major anatomical and physiological differences between mouse and human reproductive anatomy. Mice have a small cervix with a relatively small proximity between the vaginal opening and the bifurcated uterus (83), and thus ascending infections in mice progress quite quickly. Despite these limitations, mice models are a tractable tool for studying GBS ascending infection, but findings may not be directly translatable to humans.

1.5.3 Host responses during GBS placental infection

Following ascension across the cervix and into the uterus, GBS encounters a network of immune cells and tissues known as the maternal-fetal interface (84). The placenta and associated maternal decidua are immune cell-rich multi-layered barriers that attach to the uterine wall. This barrier separates maternal and fetal tissues to maintain fetal tolerance. Additionally, the placental disc contains the chorionic villous tree immersed in maternal blood, which is an essential site of gas and nutrient exchange during pregnancy (85). Hematogenous pathogens may invade the maternal-fetal interface through the chorionic villi, ultimately gaining access to fetal circulation. Alternatively, ascending vaginal colonizers may encounter the maternal decidua and chorioamniotic membranes, or the placental disc. In either case, invaders must also cross the chorioamniotic membranes in order to access the amniotic sac and fetus within. Together, these maternal-fetal interface tissues harbor a unique physiological environment with a distinct immune milieu capable of facilitating pathogen clearance or invasive disease.

A variety of immune cells are present in the maternal-fetal interface, and many aspects of their function and protective mechanisms have been studied in the context of GBS infection and chorioamnionitis. Due to its barrier function and complexity, it is difficult to study immune responses within this compartment during pregnancy, and thus many studies utilize *in vitro* approaches. Decidualized immortalized endometrial stromal cells may mimic the decidua, but these cells are also immortalized. GBS induces multiple cell signaling mediators in this decidualized cell line, including MAPK linked to NFkB activation and c-JNK linked to the p38 pathway (86). Studies also suggest that paracrine signaling molecules derived from the choriodecidua, such as prostaglandin E_2, can alter the effects of neighboring immune cells within the same compartment (87). In a more complex model utilizing *ex vivo* choriodecidual tissue punches, IL-1α and IL-1β exhibited similar paracrine signaling and affected the production of defensins in the amnion (88). Despite the use of models that do not fully account for the anatomical complexity of the placenta, these studies highlight the importance of cell signaling in

the regulation of effective placental immune responses against GBS. While these studies were still able to observe the effects of cellular crosstalk, they are unlikely to model conditions that are relevant *in vivo*. Thus, follow up studies should look for similar phenotypes in more relevant primary cells. *Ex vivo* analyses of placental immunity should aim to model the complex maternal-fetal interface by incorporating multiple cell types and signals that would be encountered *in vivo*. To this end, an engineered "fetal membrane on a chip" approach contains multiple biologically relevant cell types and can be easily adapted to incorporate other stimuli such as GBS, cytokines, hormones, prostaglandins, nutrients, or inhibitors (89).

While *in vitro* technologies are improving, the best way to study immune responses is in a host. Thus, animal models of pregnancy-associated GBS infection and GBS chorioamnionitis are essential to inform placental immunity against GBS. In a murine model of GBS vaginal inoculation during pregnancy, neutrophil infiltrates in the choriodecidua and placenta produced neutrophil extracellular traps (NETs) that immobilized GBS and repressed GBS growth via lactoferrin-mediated iron sequestration (**Figure 1.2, placenta, panel A**) (90). In another study, vaginal GBS infection of non-pregnant mice triggered NET production in a granadaene-dependent manner (54). Despite the activation of NETs by this factor, hyperhemolytic GBS can evade NETs through direct neutrophil killing (**Figure 1.2, placenta, panel B**) (31). Production of extracellular traps is unique to neutrophils, and primary placental macrophages release macrophage extracellular traps (METs) when exposed to GBS *ex vivo*. METs in the fetal chorioamniotic membranes contained a variety of matrix metalloproteases and were produced in a ROS-dependent manner (**Figure 1.2, placenta, panel A**) (91), suggesting that – in addition to GBS factors – host factors also influence extracellular trap formation. Despite their importance in the anti-GBS immune response, the production of these extracellular traps may also adversely affect pregnancy. The production of matrix metalloproteases and neutrophil elastase can stimulate uterine contractions and is thus associated with chorioamnionitis and PTB (92-94). While the above studies utilized murine models of perinatal GBS infection, another recent study underscores the importance of

immune checkpoint receptors and ligands in the chorioamniotic membranes and decidua of GBS infected pigtail macaques. For the inhibitory checkpoint receptor VISTA, there were high correlations between histological VISTA staining in these tissues and peak amniotic fluid levels of pro-inflammatory cytokines (95), suggesting that immune control in the maternal-fetal interface may affect GBS invasion and disease progression. While these studies emphasize the importance of immune cell crosstalk in the maternal-fetal interface, we do not understand how this crosstalk regulates immune cell function or how functional changes within immune cell subsets may exacerbate or impair GBS disease progression in the maternal-fetal interface. Perinatal animal models and studies utilizing samples from GBS exposed versus unexposed pregnancies can address these complex questions.

1.5.4 GBS and host responses during GBS invasion of the amniotic cavity

After crossing the maternal decidua and chorioamniotic membranes, GBS must invade the amniotic fluid and adapt to this new niche in order to subsequently infect the fetus. Multiple studies have assessed GBS changes in response to amniotic fluid. Changes in the levels of transcripts associated with metabolism of amino acids and carbohydrates – especially fructose – likely represent GBS adapting to new nutritional requirements within this niche; however, some virulence genes also underwent altered transcription (96, 97). During *in vitro* exposure to amniotic fluid, adhesins were downregulated whereas capsule, hemolysin, and IL-8 proteinase were upregulated (96). Recently, a transposon screen identified a novel GBS transcription factor, MrvR, that was important for survival in amniotic fluid. Deletion of *mrvR* reduced GBS survival in amniotic fluid and impaired biofilm formation. Further, pregnant mice vaginally infected by MrvR-deficient GBS exhibited normal vaginal persistence and ascending infection but did not experience PTB (98). While MrvR may promote GBS survival during amniotic fluid invasion in mice, the *in vivo* consequence of other transcriptional changes induced by exposure to amniotic fluid remain unclear.

In the amniotic cavity, immune cells recognize GBS and produce inflammatory mediators that contribute to the development of adverse pregnancy outcomes. In a nonhuman primate model, inoculation of GBS into the amniotic fluid caused levels of IL-1β, TNF-α, IL-6, and IL-8 to rise (99). When these cytokines are individually administered into the amniotic fluid of a rhesus macaque, only primates who received IL-1β or TNF-α developed preterm labor (100); thus, certain inflammatory cytokines that can be induced by GBS are sufficient to stimulate PTB. During GBS choriodecidual infection of pigtail macaques, expression of neutrophil recruiting cytokines and chemokines was also elevated in the amniotic fluid, which was associated with high levels of neutrophil infiltrates in the amniotic membranes of the placenta (31). Further, amniotic fluid prostaglandin production also increased in response to GBS invasion of the chorioamniotic membranes and amniotic cavity (31, 101), which could contribute to preterm labor due to its role in cervical softening. While they do not cause PTB on their own, increases in amniotic fluid IL-6 and IL-8 concentrations are useful predictors of fetal lung injury in a macaque model of GBS chorioamnionitis (102). Together these data highlight the importance of cytokine, chemokine, and prostaglandin production during GBS infection, and describe their associations with the development of PTB or other adverse fetal outcomes. Therapeutics that target or modulate the host immune response could lengthen pregnancy duration and prevent PTB. A broad-spectrum chemokine inhibitor showed efficacy against the development of PTB and neutrophil-driven chorioamnionitis; however, administration in combination with antibiotics will likely be required since immunomodulators alone have not resulted in successful clearance of GBS using preclinical models (103).

1.5.5 Host responses during fetal and neonatal infection

While *in utero* fetal exposure to invasive GBS occurs following ascending or maternal invasive infection, a neonate may also acquire invasive disease from their mother (or another host) who may only be transiently colonized by or exposed to GBS (104). In part, neonatal

susceptibility to GBS can be attributed to the more suppressive nature of their antibacterial immune responses. For instance, while neonatal mice rapidly produce high levels of IL-10 in response to GBS, neonatal mice receiving IL-10 receptor blocking antibody exhibited heightened neutrophil recruitment and reduced bacterial burden (105), suggesting that IL-10 may be counterproductive in the neonatal GBS immune response. In another study, IL-10 production was activated in mice in response to GBS recognition by TLR2, but abrogation of TLR2 or IL-10 signaling improved neutrophil influx to infected tissues and facilitated subsequent clearance of GBS (106). Interestingly, human infants receiving colostrum that is high in IL-10 were twice as likely to become GBS colonized (107), so this phenomenon may also be relevant during human neonatal GBS infection. Together, these data indicate that regulation of neutrophils through an axis involving IL-10 is important for neonatal susceptibility to GBS. Future studies assessing this axis further may allow for the identification of biomarkers capable of identifying infants at high risk of GBS invasive disease or may enable the development of host-directed therapies that may block IL-10 mechanisms to improve infection outcomes in infants.

Beyond IL-10, neonates also have intrinsic immune deficiencies that prevent the productive clearance of GBS and contribute to the development of severe disease outcomes. Compared to adults, neonates have defective Th1 immune responses against GBS. This occurs in part due to impaired production of IL-12 and IL-18, which limits the downstream production of IFN-γ that is typically associated with GBS clearance (108). Stimulation of neonatal cells with exogenous IL-12 and IL-18 enhances their production of IFN-γ (109), and exogenous administration of IFN-γ prolonged survival of GBS infected neonatal mice and partially cleared GBS sepsis (110). Neonates also have reduced pools of Th17 cells and thus, cord blood cells produce less IL-17 in response to GBS than cells purified from adult blood (111). Interestingly, administration of exogenous IL-17 led to an increase in IFN-γ production in neonates infected by GBS (112). These data describe two deficiencies in infants that may result in increased likelihood

of GBS infection. Interestingly, both are linked to IFN-γ, suggesting that host-directed therapies capable of activating these responses may help to clear GBS infection in the neonate.

In addition to immune perturbations that limit the development of productive immune responses required for GBS clearance, neonates may also induce exaggerated pro-inflammatory responses that instead promote adverse infection outcomes through tissue damage. Preterm infants produce higher levels of IL-6 in response to bacterial infection compared to adults, which is consistent with the high concentrations of this cytokine during cases of GBS neonatal sepsis (113). Additionally, GBS-stimulated neutrophils induce pro-inflammatory Th1 and Th17 responses in neonatal regulatory T cells through cell-cell contact and the production of soluble mediators. This pro-inflammatory bias within a population that is canonically immunosuppressive suggests that, in a neonate, GBS can curtail protective immunosuppressive responses to instead favor inflammatory responses that exacerbate GBS disease (114). Clearly, neonatal immune responses to GBS are multifactorial, and thus additional insights are needed to understand how neonatal immune deficiencies may simultaneously drive inflammation and dampen protective host defenses during invasive disease. Any immune therapies targeted at neonatal populations should address this important dichotomy.

1.5.6 Dissemination of invasive GBS in the fetus or neonate

Following GBS exposure via inhalation of GBS-infected vaginal or amniotic fluid, the neonatal lung is highly susceptible to invasive disease. GBS must adhere to the lung epithelium in order to invade the cell; however, this first requires the evasion of lung surfactants coating this barrier. While premature and low birth weight neonates are deficient for dipalmitoylphosphatidylcholine (DPPC), a major component of lung surfactant, this factor appears to inhibit the hemolytic and pro-inflammatory effects of GBS granadaene (**Figure 1.3, pneumonia/sepsis panel A**) (43), which may explain why GBS pneumonia is rare in adults but very common in the first few weeks of life (115). In neonatal rabbits, intratracheal treatment with

exogenous surfactant suppressed GBS lung proliferation, reduced pro-inflammatory responses, and contained infection (116), suggesting that this pathway could be targeted therapeutically to restrain GBS at this early infection site.

After evading the first line of pulmonary defense, GBS must adhere to the lung epithelium. To accomplish this, GBS employs a multitude of adhesins including Spb1 pilus backbone protein, BibA, and fibrinogen binding proteins to bind components of the host extracellular matrix (**Figure 1.3, pneumonia/sepsis, panel B**) (68, 117-119). In addition to binding, some of these adhesins also confer additional functions including cellular invasion and immune evasion (120). Currently, it is unclear how redundant function of these and other adhesins and invasins impacts development of disease *in vivo*, and the relative contribution of these proteins in various host sites warrants further study.

Once GBS has established a niche in the lung mucosa, it can penetrate lung endothelial barriers and gain access to the bloodstream. The loss of endothelial barrier integrity is largely mediated by granadaene, which is cytolytic to human alveolar epithelial cells and pulmonary arterial and lung microvascular endothelial cells (43, 45). Granadaene also stimulates the release of the neutrophil chemoattractant IL-8, initiating subsequent pro-inflammatory responses that further compromise barrier function (**Figure 1.3, pneumonia/sepsis, panel C**) (46). Events leading to loss of lung barrier function have also been corroborated in a murine vertical transmission model that recapitulates human maternal-fetal transmission occurring *in utero*. Pregnancies from mice vaginally colonized by hyperhemolytic GBS result in increased pup mortality, elevated bacterial burdens in disseminated sites, and heightened lung inflammation compared to an isogenic strain that does not produce granadaene (121). These studies highlight the importance of GBS granadaene as a key virulence factor that enables invasion of the fetal or neonatal blood vessels. After gaining access to the bloodstream surviving GBS can easily disseminate to other tissues in this hypersusceptible host.

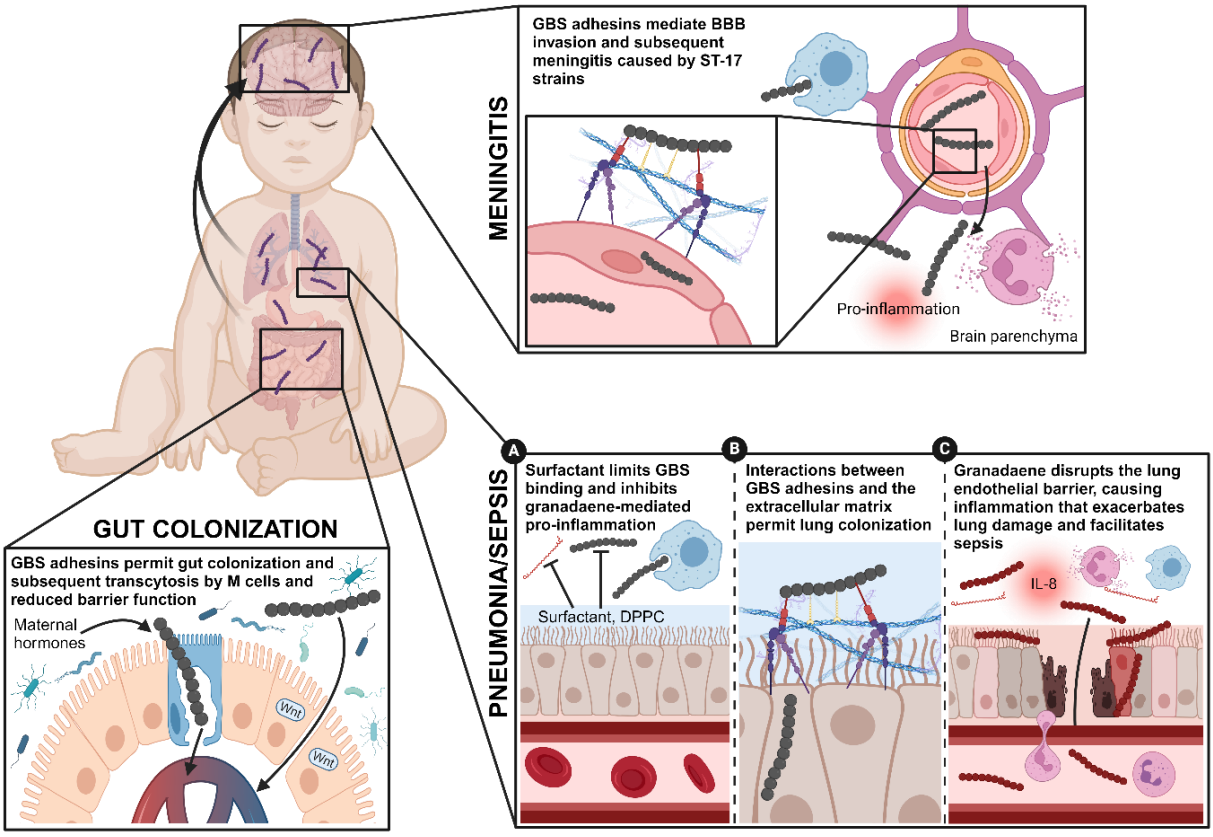

FIGURE 1.3. Host-pathogen interactions in the GBS infected neonate. This figure was partially re-designed and used with permission (1) (CC BY 4.0 DEED).

As an alternative to lung colonization, a neonate may swallow GBS-infected vaginal fluid or breastmilk and become gastrointestinally colonized (122-124). The developing neonatal microbiome is important for regulating gut epithelial function, and a recent study observed that neonatal mice were more susceptible to oral GBS challenge at postnatal day 1 (P1) versus day 5 (P5). This was partially due to the immaturity of the P1 microbiome, since reconstitution of P1 pups with P5 microbiota rescued P1 pup susceptibility. Importantly, Wnt signaling in the neonatal mouse gut results in mis-localization of epithelial junction proteins and ultimately causes reduced barrier function against GBS (**Figure 1.3, gut colonization**); however, the regulation of this process appears to be independent of the developing microbiome. Microbiome composition aside, GBS colonized pups that experienced intestinal barrier disruption were predisposed to subsequent dissemination to other sites (125). While it remains unclear how taxa-level interactions between the gut microbiome and GBS may impact epithelial barrier function in

neonates, further studies interrogating the gut microbiomes of GBS colonized adults versus neonates may also shed light on how differences in microbiome content may impact host susceptibility to invasive GBS infection. Information gleaned in these areas may inform the development of pre- or probiotic therapies for GBS prevention.

To effectively colonize the neonatal gut, GBS that outcompetes the host microbiome utilizes adhesins to attach to the intestinal epithelium. Hypervirulent GBS adhesin (HvgA), which is unique among hyperinvasive ST-17 isolates, is especially important during intestinal colonization. Heterologous expression of HvgA in non-adhesive GBS strains confers binding to intestinal epithelial cells, and oral challenge of mice with HvgA-expressing GBS causes rapid invasion into cecal tissue, suggesting that HvgA is also involved in GBS translocation across the intestinal barrier (20). Another ST-17 surface protein, Srr2, can similarly bind the intestinal wall. Interaction between Srr2 and intestinal M cells enhance GBS transcytosis across the intestinal epithelium, leading to subsequent bloodstream infection. GBS transcytosis across the intestinal barrier is dependent on M cell differentiation, and this process is altered in the neonatal gut due to the high level of maternal hormones that persist early in life (**Figure 1.3, gut colonization**). Thus, the levels of estrogen and progesterone potentiate Srr2-mediated GBS traversal across the intestinal barrier (126). While maternal hormones may be important, male infants are at increased risk for GBS invasive disease and frequently experience more severe disease outcomes (127), suggesting that neonatal gonadotropic hormones are also important factors that influence susceptibility. However, if neonatal gonadotropic hormones affect M cell differentiation, it is unknown how that may impact GBS transcytosis and dissemination.

Following successful gut colonization, GBS can invade the bloodstream even in the absence of transcytosis of the gut epithelium. Srr2 binding to fibrinogen and plasminogen can result in the production of GBS aggregates which may be engulfed by macrophages (128). Inside the macrophage, Srr2 improves GBS intracellular survival and helps to mediate persistence in

the blood (129). Granadaene is another factor that likely facilitates traversal across the gut epithelium and entry into the bloodstream, although this has not been assessed directly.

If successful in the blood, GBS may ultimately gain access to the blood-brain barrier (BBB). Interestingly, the ST-17 factor HvgA that mediates colonization also facilitates adhesion and invasion of the choroid plexus and microvascular endothelial cells of the BBB. Oral challenge of neonatal mice with HvgA-expressing GBS led to invasion of the brain and meningitis (20). In adult mice, a more direct hematogenous route of intravenous GBS infection may also lead to meningitis. While HvgA-deficient GBS replicated well in the blood, they were impaired for BBB invasion (20), suggesting that this factor could be targeted to prevent the development of GBS meningitis in infants experiencing early-stage sepsis. Similar to HvgA, Srr2 also plays a critical dual role in intestinal colonization and BBB invasion; however, Srr2 binds to α5β1 and αvβ3 integrins to mediate BBB adhesion and internalization. These integrins are overexpressed in the postnatal brain, which may partially explain why neonates are hypersusceptible to GBS meningitis. Blocking this interaction prevented GBS translocation across the BBB, demonstrating that Srr2 may also be targeted to prevent GBS meningitis (130). Finally, the BspC adhesin has been shown to brain endothelial cells through the cytoskeletal protein vimentin, and mice infected with BspC-deficient GBS exhibited reduced bacterial burden in the brain, low inflammation, and improved survival (131). While these studies provide evidence supporting the importance of these adhesins for GBS invasion of the gut and brain, as described previously their individual contributions in GBS clinical isolates are not well described (**Figure 1.3, gut colonization**).

On the host side, multidimensional quantitative proteomics of infected whole brains revealed that GBS alters proteomic signatures involved in the upregulation of interferon signaling and leukocyte recruitment. In the brain microvasculature, proteins involved in barrier function, such as maintaining integrity of the BBB after brain hemorrhage, were downregulated (132). This loss of BBB integrity was also mediated by activation of TLR2 and subsequent Snail1 signaling, which ultimately leads to reduced expression of tight junction components (133). Interestingly,

many host signaling proteins that mediate loss of tight junctions in the BBB endothelium are also involved in the loss of vaginal and placental epithelial function via EMT (62, 134). Thus, it may be possible to use a similar strategy to target these factors for prevention of GBS-induced barrier loss in multiple sites. Altogether these studies summarize several unique mechanisms that GBS uses to exploit host defenses in the BBB. However, it is possible that studies of invasive disease in adult mice may not directly inform neonatal disease outcomes. Thus, we emphasize the need for improved GBS invasive disease models that more accurately represent neonatal anatomy and physiology.

1.6 GBS vaccines in human clinical trials

Due to the major global burden of GBS invasive infections, the development of a prophylactic vaccine has been a major goal for researchers ; however, finding investors has proven challenging (135). While many pre-clinical vaccine formulations based on a wide range of GBS virulence factors exist, only two main formulations are currently undergoing human clinical trials. Pfizer is pursuing a hexavalent CPS conjugate vaccine (GBS6) derived from the capsules of serotypes Ia, Ib, II, III, IV, and V (136-139). Clinical trials for safety, tolerability, and immunogenicity have been completed in healthy adults (ClinicalTrials.gov ID: NCT04258995, NCT03170609, NCT03170609), and an addition study that included pregnant women and their infants was just completed (NCT03765073). Due to concerns of suboptimal CPS vaccine immunogenicity and difficulties of upscaling CPS vaccine production, MinervaX is pursuing an alternate protein-based strategy. MinervaX's GBS-NN/NN2 is derived from four N-terminal domains from the chimeric surface Alpha-like protein (Alp) family, of which the vaccine includes five of six GBS variants (2, 140-143). Clinical trials for safety, immunogenicity, and boosting have been completed in healthy adults (NCT02459262, NCT05005247) and a similar trial in elderly participants will be completed soon (NCT05782179). MinervaX has also assessed safety, tolerability, and immunogenicity in pregnant women with and without HIV, as well as their infants

(NCT05154578, NCT04596878). Chapter 3 of this thesis will describe the efficacy of GBS-NN vaccination against GBS using various murine challenge models, and thus GBS vaccines will be discussed more extensively in that section.

1.7 Summary

GBS inflicts an annual public health burden on pregnant women and neonates, which is disproportionately concentrated in low-income countries. Country and region-specific discrepancies between screening guidelines and the use of IAP confounds the accurate detection of invasive GBS disease cases, limiting our ability to control the associated adverse outcomes through timely intervention. The solution to reducing invasive GBS disease in pregnant women and neonates is not simple, but the development of a GBS vaccine in addition to other alternative therapies would be a major step forward in preventing disease.

This section highlights recent discoveries elucidating important interactions between GBS and the host. I have described diverse mechanisms that GBS employs to successfully disseminate to vulnerable host niches during pregnancy and have similarly recounted the varied ways through which the host can impede this pathogen's success. In recent years, major strides have been made to improve our understanding of GBS vaginal colonization, ascending infection, and congenital infection, advancing the strategic development of several candidate vaccines with demonstrated efficacy in animal models or human clinical trials. While it is too early to forecast whether these vaccines will someday receive FDA-approval, their success symbolizes a major step towards reducing the global burden of GBS invasive disease.

In this thesis, I will describe two studies performed to further our understanding of GBS disease pathogenesis and how we may be able to disrupt virulence-associated pathways to ultimately prevent disease. Chapter 2 will describe my work identifying the serine protease HtrA as an important virulence factor that post-translationally regulates global abundance of numerous GBS virulence proteins, including the Streptococcal surface immunogenic protein (Sip) which is

highly immunogenic during infection. Chapter 3 will summarize my work to assess GBS-NN vaccine efficacy using mouse models of GBS infection. Collectively, this thesis emphasizes the importance of membrane proteins for GBS pathogenesis and lays the groundwork for the development of antibacterial strategies that could be used to prevent or treat GBS invasive disease.

CHAPTER 2. The serine protease HtrA cleaves surface immunogenic protein (Sip) and regulates Group B *Streptococcus* virulence

This chapter is re-used from the following article:

Brokaw, A., Wallen, G., Orvis, A., Seepersaud, R., Nguyen, S., Sharma, K., Kwon, H.J., Coleman, M., Quach, P., Twentyman, J., Zielinski, R., Jones, L.A., Lin, C., Gafken, P.R., Rajagopal, L. (2024). The serine protease HtrA cleaves surface immunogenic protein (Sip) and regulates Group B *Streptococcus* virulence. *In preparation.*

2.1 Abstract

Group B Streptococcus (GBS) rectovaginally colonizes up to 20% of women worldwide and is a leading cause of invasive infections during pregnancy, contributing to a significant proportion of preterm births, neonatal infections, and stillbirths each year. Despite its reputation as a perinatal pathogen, GBS infection rates in non-pregnant adults are increasing. While much progress has been made to understand GBS virulence factor transcriptional regulation by two-component systems and the mechanisms that promote disease, other virulence regulators remain understudied. Many bacterial pathogens encode homologs of the high temperature response A (HtrA) family of serine proteases which regulate virulence and stress responses post-translationally, but the function of HtrA in GBS was previously unknown. Here we show that GBS lacking HtrA exhibited attenuated virulence during systemic infection and cause fewer adverse pregnancy outcomes in murine models. Proteomic analysis comparing isogenic HtrA-proficient and -deficient strains revealed 110 proteins that may be regulated by HtrA, including the Streptococcal surface immunogenic protein (Sip). Similar to our observations for HtrA-deficient GBS, a strain deficient for Sip also caused fewer adverse pregnancy outcomes during murine infection. We demonstrated that HtrA can directly cleave Sip, which diminishes Sip-mediated induction of host responses through TLR4 and NFkB. Additionally, the absence of both HtrA and Sip did not further attenuate GBS virulence, suggesting both proteins interact within the same pathway to mediate GBS virulence and pathogenesis. Our findings indicate that HtrA, partially through the action of Sip, regulates GBS virulence.

2.2 Introduction

Approximately 20% of women worldwide are rectovaginally colonized by Group B Streptococcus (GBS, *Streptococcus agalactiae*). Despite being an asymptomatic colonizer, GBS can be a major health threat during pregnancy. It is estimated that approximately 15% of neonates born each year may be exposed to maternal GBS (11), and *in utero* GBS infections are estimated to cause up to 1 million cases of preterm birth (PTB) and at least 50,000 stillbirths annually (10, 11). Invasive infection rates are also high, with GBS causing up to 400,000 cases of pneumonia, sepsis, and meningitis in neonates and infants (10, 144). In addition to this major burden on perinatal health systems, GBS invasive disease rates in non-pregnant adults are also rising (15).

Treatment of GBS infections is currently restricted to antibiotics, such as intrapartum antibiotic prophylaxis which is administered during delivery to reduce the likelihood of vertical transmission (16, 145, 146). While recent research has focused on the development of GBS vaccines (1, 135), it is clear that alternative strategies for GBS prophylaxis and treatment are also needed – especially amid rising concerns of drug resistance (104).

Anti-infection strategies typically target either essential cellular processes or virulence factors. GBS encodes a large arsenal of virulence factors whose impact on disease varies by site of infection or by GBS strain and/or serotype (1, 36, 104, 147). Moreover, GBS encodes multiple two-component systems that respond to variable stimuli and activate transcriptional changes to adapt to new environments (37). Despite some understanding of these pathways in GBS, transcriptional control of virulence occurs through multiple overlapping mechanisms that are difficult to disentangle. The role of the major virulence regulator CovR/S in GBS pathogenesis is very well described (30, 32-35, 38, 40, 42, 48, 50, 53, 148-153), but major knowledge gaps remain surrounding the roles of regulators acting downstream of transcription. Dissecting the roles of such regulators may provide insights for the development of alternative therapeutic strategies that could be used to reduce disease severity or prevent infection.

The high temperature response A (HtrA) family of serine proteases is broadly conserved among bacterial pathogens. These proteins contain a trypsin-like protease domain with a histidine-aspartic acid-serine (H-D-S) catalytic triad essential for enzyme activity, along with one to two PDZ domains (Post-synaptic density protein-95, Drosophila disc large tumor suppressor, Zonula occludens-1), that are involved in substrate binding and self-oligomerization (154-156). HtrA was first described in *Escherichia coli*, which encodes three homologs (DegP, DegQ, and DegS) that degrade misfolded proteins, regulate bacterial responses to heat and membrane stress, and modify interactions with the host (157, 158). More recently, similar roles in regulation of bacterial stress responses and virulence have been illustrated for other bacterial HtrA. Consequently, *htrA* deletion frequently produces strains with partial to full virulence attenuation and heightened susceptibility to stress conditions including oxidative, acid, osmotic, and high temperatures (159-178). Despite the established role of HtrA in bacterial virulence and survival under stress, the precise mechanism by which HtrA contributes to these processes remains largely understudied.

Overall, how HtrA influences these processes appears to depend in large part upon the subcellular localization of the enzyme. With the exception of *E. coli* DegS, Gram-negative bacteria secrete their HtrA homologs, while Gram-positive HtrAs are attached to the cell membrane (154, 155). HtrA secreted from Gram-negative enteropathogens including *E. coli*, *Salmonella enterica*, *Shigella flexneri*, and *Helicobacter pylori* cleave host E-cadherin in the gut lumen to weaken epithelial adherens junctions and promote bacterial translocation into deeper tissues. Here, HtrA can further cleave fibronectin to enhance dissemination (169, 170, 179, 180). In contrast, membrane-anchored HtrA homologs from Gram-negative *S. flexneri* or Gram-positive *Bacillus anthracis, Bacillus subtilis*, *Streptococcus pneumoniae*, *Streptococcus mutans*, and *Streptococcus pyogenes* are frequently linked to the quality control of exported virulence factors such as adhesins, peptide pheromones, or even other proteases (160, 178-193). Thus, the extracellular localization of HtrA confers these proteases with distinct functions at the bacterial

cell surface or in the extracellular environment during infection. Despite extensive studies on the effects of other HtrA homologs on bacterial physiology and pathogenesis, the role of HtrA in GBS virulence and the accompanying host response has not been examined.

In this study, we show that *htrA* deletion attenuates GBS virulence in mice compared to isogenic wild-type, and that the *htrA* deletion strain causes fewer adverse outcomes during pregnancy-associated infection. Proteomic analysis uncovered 110 significant changes in protein abundance due to changes in HtrA expression, the majority of which affected exported proteins. These included factors previously linked to GBS virulence in addition to novel proteins whose functions in GBS pathogenesis remain unknown. One such example is Streptococcal surface immunogenic protein (Sip). Although Sip immunogenicity has been previously described (194-199), here we provide evidence that Sip also affects the development of adverse outcomes during perinatal GBS infection, in part through a mechanism that may involve processing by HtrA. Protease assays revealed that Sip can be specifically cleaved by HtrA, suggesting a relationship between the functions of these proteins. Together, these results support a mechanism whereby HtrA functions as a regulatory protease that controls GBS virulence in part through the processing of Sip, which subsequently alters the host immune response during infection

2.3 Results

2.3.1 HtrA is localized to the GBS membrane

HtrA is a putative serine protease encoded by *GBSCOH1_2002* in strain COH1 GBS (GenBank HG939456.1, CDN67468.1). The domain organization of GBS HtrA is similar to other Gram-positive HtrA homologs. The protein contains an N-terminal signal peptide followed by a putative transmembrane domain, a chymotrypsin-like protease domain [containing a conserved histidine-aspartic acid-serine (HDS) catalytic triad], and a PDZ domain at the C-terminus (**Figure 2.1A**). Based on Hidden Markov modeling, GBS HtrA is predicted to be localized at the membrane with the protein facing extracellularly. In addition, structural modeling of GBS HtrA in Swiss-Model

identified the H126, D156, S237 catalytic triad to be spatially clustered together (**Figure 2.1B**) (200). To confirm HtrA localization in GBS, WT COH1 GBS were grown to mid-logarithmic phase, fractionated to collect cytosol, membrane, and secreted proteins, and probed with GBS HtrA antiserum. Western blot analysis identified a major reactive band of 42 kDa within the membrane fraction of COH1 WT GBS that was absent in the *htrA* deletion strain (COH1Δ*htrA*). In a complemented strain expressing HtrA episomally in the knockout background (COH1Δ*htrA*/pDC123*htrA*), the protein is predominantly observed in the cytosolic and membrane fractions (**Figure 2.1C**). Collectively, these findings support a model whereby GBS HtrA is functionally localized at the bacterial membrane.

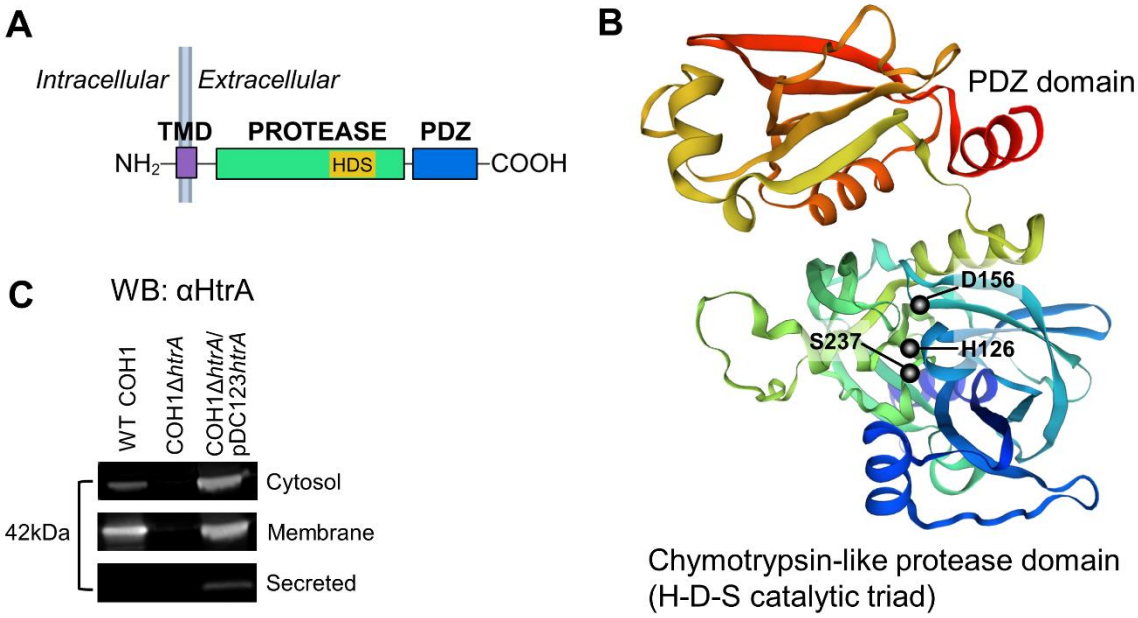

Figure 2.1. HtrA is localized to the GBS membrane. A) GBS HtrA contains a putative transmembrane domain (TMD), protease domain with histidine-aspartic acid-serine (HDS) catalytic triad, and PDZ domain (postsynaptic density protein of 95 kDa; Drosophila disc large tumor suppressor; zonula occludens-1 protein). Protein orientation was predicted by transmembrane hidden Markov modeling (DeepTMHMM). **B)** Annotated ribbon diagram of GBS HtrA (CDN67468.1, pos. 50-360) was generated using the Swiss-PdbViewer build model function (CC BY-SA 4.0 license). The circles added indicate the catalytic triad. **C)** Western blot of cytosolic, membrane, or secreted fractions of proteins isolated from WT COH1, COH1Δ*htrA*, or COH1Δ*htrA*/pDC123*htrA* (over-expressor) GBS. HtrA levels were detected using rabbit serum raised against the recombinant GBS HtrA^{S237A} catalytically-inactive protein. Western blots were performed in biological triplicate, with the panel showing one representative sample.

2.3.2 *HtrA is important for GBS systemic infection and affects sensitivity to neutrophils*

Previous studies show that deletion of *htrA* in other bacterial pathogens can attenuate virulence. This prompted us to ask if expression of *htrA* is required for full GBS virulence. After confirming the COH1Δ*htrA* deletion mutant exhibited no growth defects in broth culture when compared to the isogenic WT strain (**Supplemental Figure A-1a and b**), we tested our *htrA* deletion mutant in a murine systemic infection model to assess its virulence. To this end, mice

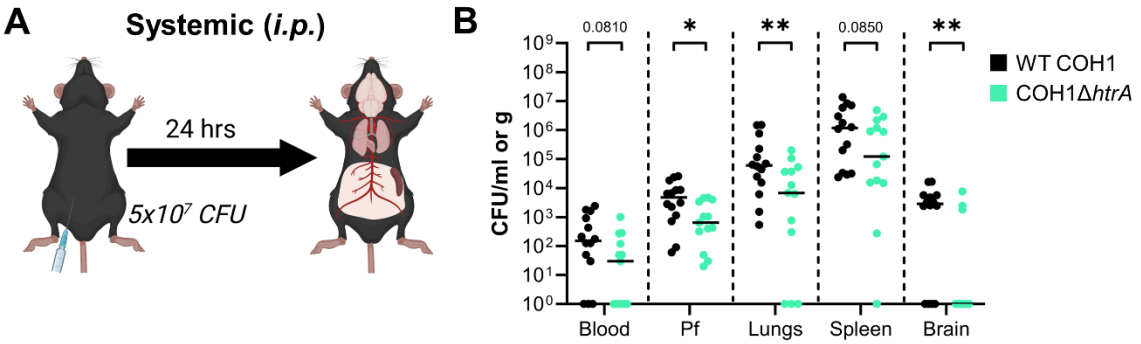

Fig 2.2. HtrA is important for GBS systemic infection and affects sensitivity to neutrophils. A) Schematic of GBS systemic infection murine model. C57BL6/J mice were systemically infected with ~5x10^7 CFU of WT COH1 (n=14) or COH1Δ*htrA* (n=13) intraperitoneally (*i.p.*). CFU were enumerated from blood, peritoneal fluid (Pf), and tissue homogenates of lungs, spleen, and brain 24 hours post-infection. B) CFU were normalized to tissue weight for homogenates and volume for fluids. Significant differences were determined using Mann-Whitney test: ∗ P < 0.05, ∗∗ P < 0.01. Data represent mice from three independent experiments. C) WT COH1 or COH1Δ*htrA* GBS was exposed to neutrophils isolated from fresh human blood (n=9) at MOI 1 for 1 hour. Neutrophils were lysed using 0.1% (v/v) Triton-X and samples were serially diluted and plated to assess GBS viability compared to a control lacking neutrophils. Means ± SEM were assessed for significance using an unpaired t-test : ∗ P < 0.05.

were infected intraperitoneally (*i.p.*) with ~5x10^7 CFU of COH1 WT or COH1Δ*htrA* GBS and were sacrificed 24 hours later to enumerate bacterial dissemination in systemic organs including the blood, peritoneal fluid, lungs, spleen, and brain as described previously (**Figure 2.2A**) (201). We observed that mice infected with COH1Δ*htrA* exhibited reduced GBS burden in the peritoneal fluid, lungs, and brain compared to WT GBS (**Figure 2.2B**). Blood and spleen CFU also trended down but were not significant (p-value < 0.1). Collectively, these findings suggest that *htrA* expression is important during GBS systemic infection.

Extensive literature supports neutrophils as important early responders during GBS infection (31, 46, 49, 53, 54, 90, 202, 203), and HtrA-deficient strains of *Bacillus anthracis* and *Pseudomonas aeruginosa* exhibit reduced survival in the presence of immune cells including macrophages and neutrophils, respectively (163, 164, 204). Thus, we assessed sensitivity of our GBS *htrA* deletion mutant to primary human neutrophils. While the isogenic WT strain survived neutrophil exposure, COH1Δ*htrA* was markedly more sensitive to neutrophil-mediated killing (**Figure 2.2C**). These data indicate that expression of HtrA is important for GBS resistance to neutrophils. Furthermore, due to the importance of neutrophils *in vivo*, heightened susceptibility of COH1Δ*htrA* to these cells may partially explain the attenuation seen during systemic infection.

2.3.3 HtrA mediates adverse outcomes during GBS pregnancy-associated infection

Given that GBS is an important perinatal pathogen, we assessed how *htrA* expression affects pregnancy-associated ascending infection. To this end, pregnant mice were intravaginally (*i.vag.*) infected with ~1x10^8 CFU of COH1 WT or COH1Δ*htrA* GBS at embryonic day 14 (E14) and sacrificed at preterm labor onset or at 72 hours post-infection. Maternal tissues including lower genital tract (LGT) and uterus, and fetal tissues (proximal and distal pups and their respective placentas from each uterine horn) were collected to evaluate GBS dissemination as described previously (**Figure 2.3A**) (81). We also noted the occurrence of adverse outcomes including preterm birth (PTB, defined as vaginal bleeding or pups in cage), stillbirth (defined as dead pups in cage), and intrauterine fetal death (IUFD).

While there were no significant differences in LGT colonization or ascension to the uterus when comparing these GBS strains, elevated GBS burden was seen in the fetal tissues of COH1Δ*htrA* infected mice compared to isogenic WT (**Figure 2.3B**). We observed only one case of PTB among the COH1Δ*htrA* infected dams versus three cases for isogenic WT. Two of these WT-infected dams gave birth to stillborn pups overnight, which were not collected or included in CFU analysis due to confounding factors. Despite the striking increase in COH1Δ*htrA* CFU in fetal

tissues, this strain was significantly attenuated for its ability to cause adverse pregnancy outcomes (**Figure 2.3C**). Thus, HtrA also contributes to GBS virulence during pregnancy.

Previous work from our lab and others indicates that a pro-inflammatory immune response in the maternal-fetal interface is strongly associated with the development of adverse outcomes, especially in the case of PTB (31, 82, 99-101, 103, 205). Therefore, we hypothesized that the increase in adverse outcomes in mice infected with COH1 WT may be due to elevated pro-inflammatory responses compared to mice infected with COH1Δ*htrA*. To this end, we used Luminex to measure chemotactic, pro-inflammatory, and anti-inflammatory factors in infected mouse uterine and placental lysates. We noted significant elevations in the chemokines Gro-α/KC, MIP-1α, MIP1-β, and MIP-2α in placental tissues from dams infected with COH1Δ*htrA* compared to WT. Interestingly, Gro-α/KC levels increased 1000-fold, which may affect the levels of placental neutrophils. The pro-inflammatory cytokine IL-1β was also significantly more concentrated during COH1Δ*htrA* GBS infection compared to WT, and IL-6 and TNF-α exhibited similar trends. Although the elevated chemokine and cytokine responses in COH1Δ*htrA* infected placentas did not support our initial hypothesis, we also detected a concomitant and significant increase in anti-inflammatory IL-10 (**Figure 2.3D**). The induction of this highly divergent immune profile in response to COH1Δ*htrA* GBS infection is interesting, especially because this strain caused very low rates of adverse pregnancy outcomes.

Figure 2.3. HtrA mediates adverse outcomes during GBS pregnancy-associated infection. A) Murine model of pregnancy-associated ascending GBS infection. Pregnant C57BL6/J mice at gestational age E14 were infected with ~1x10^8 CFU WT COH1 or COH1Δ*htrA* GBS (n=13). CFU were enumerated from maternal tissues [lower genital tract (LGT) and uterus] and fetal tissues (proximal and distal pups and placentas) at 72 hours post-infection or at the onset of preterm birth (PTB, blood or pups in the cage prior to the onset of natural labor at ~E20). Pup viability was noted at necropsy. **B)** CFU were normalized to tissue weight and medians are shown. PTB and pup deaths are indicated by open symbols. Significant differences were determined using Mann-Whitney test: ns $P > 0.05$, ∗∗∗∗ $P < 0.0001$. **C)** Frequencies of adverse outcomes (including PTB, stillbirth, and intrauterine fetal death) for each GBS strain. Graphs indicate percentages of adverse pup outcomes out of total pups ± SEM. Contingency tables were used to assess significance via Fisher's exact test: ∗∗∗ $P < 0.001$**. D)** Placental lysates from the above mice were assessed via Luminex to quantify the indicated immuno-analytes. Data was normalized to tissue weight and graphed as means ± SEM. The dashed line indicates mean analyte levels in PBS-treated control mice. Significant differences were determined using two-tailed unpaired t-tests: ∗ $P < 0.05$. Trending statistics ($P < 0.1$) are also indicated.

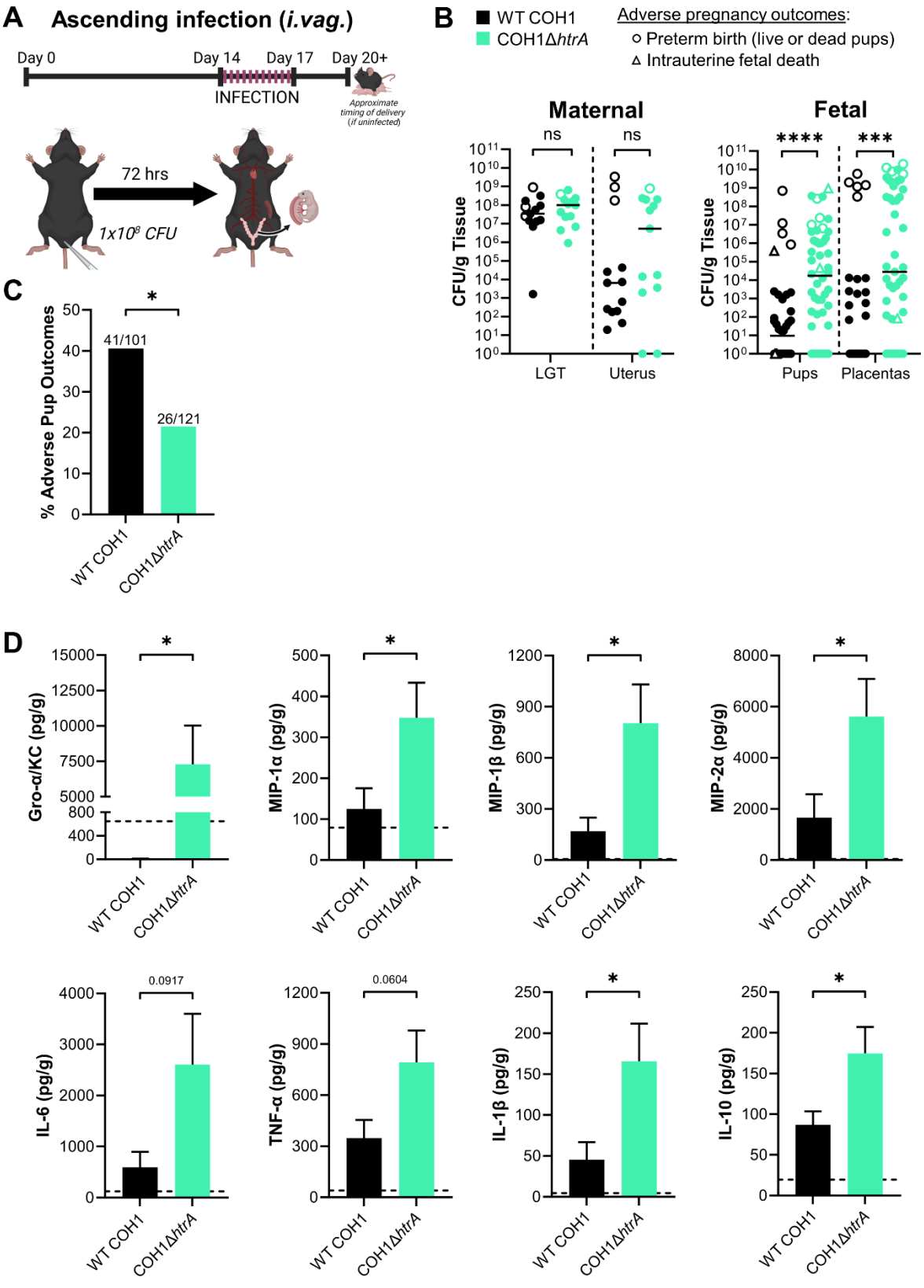

Figure 2.3. HtrA mediates adverse outcomes during GBS pregnancy-associated infection. See full figure legend on next page.

2.3.4 HtrA affects global GBS protein abundance

Having established that HtrA is required for GBS virulence, we speculated that one function of HtrA could be to influence the abundance and distribution of proteins within the GBS proteome. In fact, other homologs of this serine protease have been reported to process a variety of endogenous bacterial proteins (181, 183-185, 187-189, 191, 206, 207). To determine the effects of HtrA on the GBS proteome, we employed quantitative mass spectrometry-based proteomics using tandem mass tag (TMT)-labeling. To maximize identification of GBS proteins dependent upon HtrA, we utilized secreted, membrane, and cytosolic proteins fractionated from WT COH1 (expressing normal levels of HtrA), *htrA* deletion mutant (COH1Δ*htrA*, devoid of HtrA), and *htrA* over-expressing strain (COH1Δ*htrA*/pDC123*htrA*, that overproduces HtrA *in trans*). Fold-change and *P*-value cutoffs were used to identify proteins with significantly different abundance (≥1.5 and ≤0.05, respectively) (**Figure 2.4A**). We expected that we would identify more significant changes in protein abundance when comparing strains with greater relative difference in *htrA* expression (i.e., COH1Δ*htrA* vs. COH1Δ*htrA*/pDC123*htrA* > COH1 WT vs. COH1Δ*htrA*/pDC123*htrA* > COH1Δ*htrA* vs. COH1 WT). Consistent with this idea, the magnitudes of fold-change for Spb1 pilus backbone protein (GBSCOH1_1279) and surface immunogenic protein (Sip, GBSCOH1_0031), for example, increased in this manner (**Figure 2.4B, top row**), suggesting that these proteins may experience a dose-dependent effect based on HtrA levels.

In total, approximately 1,000 GBS proteins were detected by mass spectrometry, accounting for approximately half of the COH1 proteome (~2,003 orfs in the COH1 genome) (**Supplemental Figure A-2**). Of these, 110 (11%) exhibited significant changes in abundance due to a loss or over-expression of HtrA (**Figure 2.4B, dark purple**). We also identified numerous proteins that were significant for only FC or *p*-value, shown in lilac (**Figure 2.4B, lilac**). As expected, HtrA was one of the most significant changes in FC, confirming its effective knock-out and over-expression. Other notable changes in abundance included alcohol aldehyde dehydrogenase (AdhE, GBSCOH1_0053, increased), a hypothetical GBS protein with a

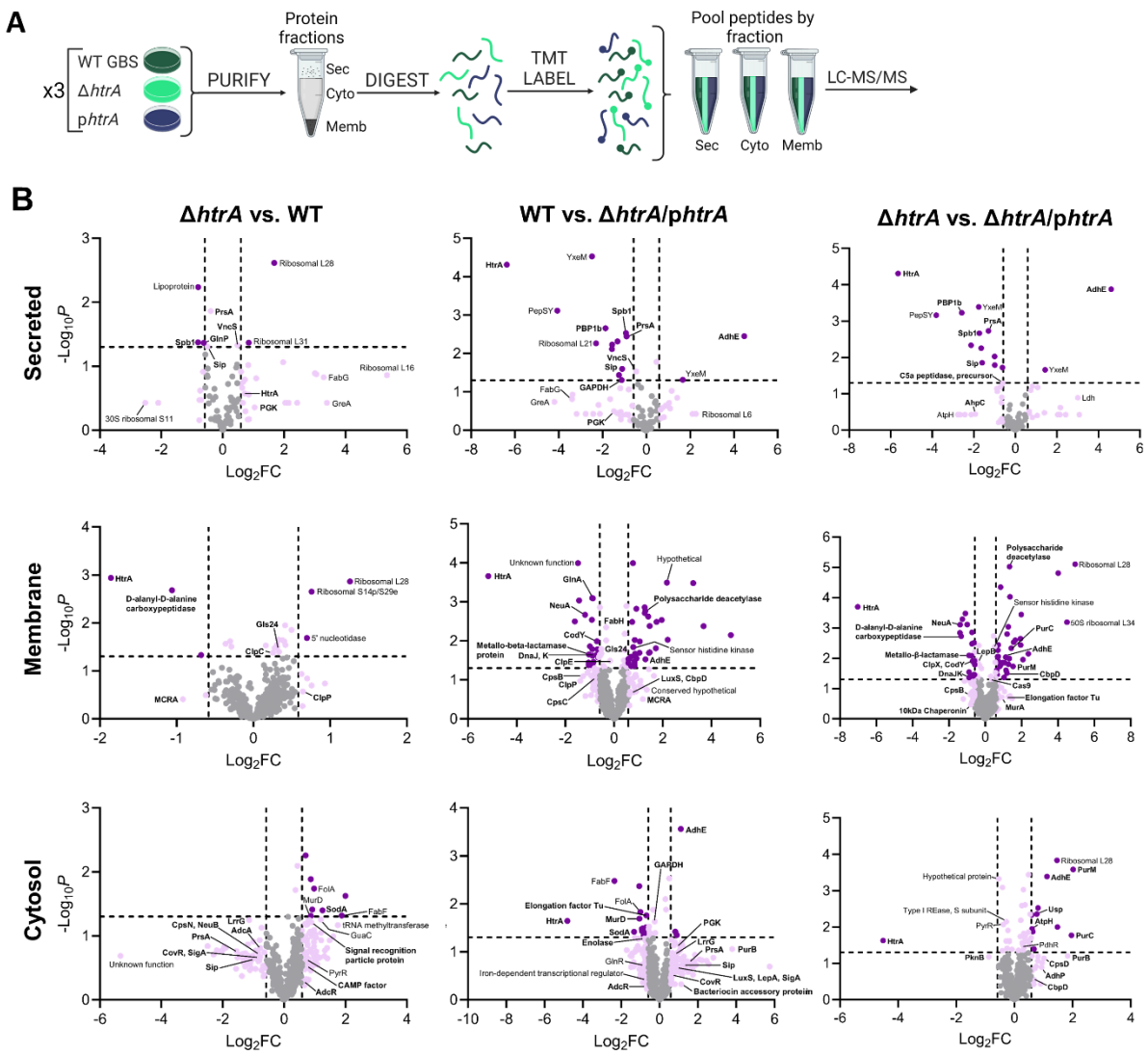

Fig 2.4. HtrA affects global GBS protein abundance. A) Schematic of proteomic study comparing cytosolic, membrane, and secreted fractions of protein isolated from three biological replicates of WT COH1, COH1Δ*htrA*, and COH1Δ*htrA* pDC123/*htrA* GBS grown in TSB. Tandem mass tag (TMT) labels were used to multiplex samples by protein fraction prior to liquid chromatography-mass spectrometry (LC-MS/MS) quantification. B) Matrix of volcano plots for the indicated GBS strain comparisons (left: COH1Δ*htrA* vs. WT, center: WT vs. COH1Δ*htrA*/pDC123*htrA*, and right: COH1Δ*htrA* vs. COH1Δ*htrA*/pDC123*htrA*) assessed for each indicated protein fraction (top: secreted, bottom: membrane). Characters above the strain comparison indicates relative HtrA levels. Significant changes in abundance [fold-change (FC) ≥ 1.5 & *p*-value < 0.05) are shown in dark purple and changes meeting only one criterion (FC ≥ 1.5 or *P*-value < 0.05) are in lilac. Bolded proteins have been linked to bacterial virulence and/or stress responses according to PubMed database searches.

conserved protease-inhibitory PepSY domain (GBSCOH1_0641, decreased), and numerous ribosomal proteins (both directions). Approximately 80% of the observed significant changes occurred in the GBS membrane or secreted fractions, supporting a role for GBS HtrA in the processing of proteins in these compartments. Among these proteins, 17/21 (81%) secreted

proteins were reduced in abundance while 44/73 (60%) membrane proteins were increased in abundance. The opposing directions of these changes in abundance suggests that the impact of HtrA in these compartments may be distinct.

The list of significant changes included many proteins that are minimally annotated in the COH1 genome assembly (GBCO_P1), restricting our ability to fully assess their role in functional or ontology databases. Despite this limitation, KEGG analysis suggests that HtrA may regulate a variety of GBS processes. The top five functional categories were virulence (n=32) (identified through PubMed database searches), ribosome (n=16), ABC transport (n=9), glycolysis/gluconeogenesis (n=5), and purine or pyrimidine metabolism (n=7) (**Figure 2.5A**). The virulence category included many proteins whose roles in GBS pathogenesis are well-established, plus additional proteins whose impact on GBS virulence remains undefined despite their characterization for other bacterial pathogens (**Table 2.1**). While 12 of these proteins displayed increased abundance in strains expressing less HtrA, 17 exhibited decreases (**Figure 2.5B**). The altered abundance of these proteins may explain the attenuation of COH1Δ*htrA* in our mouse models. Finally, we noted that Sip abundance mirrored changes to HtrA levels and that this effect was dose-dependent with respect to *htrA* expression, indicating that interaction with HtrA may be important for proper processing or export of Sip.

Table 2.1. A selection of virulence-associated proteins that exhibit altered abundance due to HtrA.

Gene Locus	Gene product/BLAST result	Proposed pathogenesis role	Organism (ref)
GBSCOH1_2002	Serine protease, HtrA	Virulence-associated protease	See introduction
GBSCOH1_0161	Penicillin-binding protein 1b, putative, PBP1b	Cell wall remodeling Antimicrobial peptide resistance	Various (208)
GBSCOH1_1614	Glyceraldehyde-3-phosphate dehydrogenase, GAPDH	Immunomodulatory protein/Adhesin*	GBS (209-212)
GBSCOH1_0031	Streptococcal surface immunogenic protein, Sip	Immunogenic protective antigen	GBS (195, 196, 213)
GBSCOH1_1279	Pilus island-2b backbone protein, Spb1	Colonization factor Adhesion/invasion	GBS (214, 215)

		Resistance to phagocytosis	
GBSCOH1_0734	Rotamase family protein, PrsA	Chaperone/folding of virulence factors	GAS (216) *Listeria* (165)
GBSCOH1_0053	Aldehyde-alcohol dehydrogenase, AdhE	Ethanol tolerance Adhesin*	*S. pneumo* (217) *S. suis* (218)
GBSCOH1_0094	D-alanyl-D-alanine carboxypeptidase family protein	Peptidoglycan turnover Stress tolerance	*S. pneumo* (219) *Francisella* (220)
GBSCOH1_1064	CMP-N-acetylneuraminic acid synthetase, NeuA	Capsule biosynthesis Complement evasion	GBS (221, 222)
GBSCOH1_0099	Chaperone protein, DnaJ	Immunomodulatory protein*	*S. pneumo* (223)
GBSCOH1_1675	ATP-dependent Clp protease, ATP-binding subunit, ClpP	Modulator of virulence gene expression	*S. pneumo* (224, 225)
GBSCOH1_0098	Chaperone protein, DnaK	Stress tolerance Adhesin*	*S. mutans* (226) *Neisseria* (227)
GBSCOH1_1609	Glutamine synthetase, type I, GlnA	Colonization factor Adhesin*	*S. suis* (228)
GBSCOH1_1526	Transcriptional regulator, CodY	Regulates virulence gene expression	GBS (229)
GBSCOH1_0049	Holliday junction DNA helicase, RuvB	Alters virulence gene expression Promotes intracellular survival	*S. enterica* (230)
GBSCOH1_0364	Copper-translocating P-type ATPase, CopA	Increased tolerance to Cu intoxication	GBS (231)
GBSCOH1_0373	Sensor histidine kinase, BgrS or BaeS	Reduced β antigen expression Cell envelope stress	GBS (232) *E. coli* (233)
GBSCOH1_0747	Phosphoenolpyruvate-protein phosphotransferase, PtsA	Adhesin* Virulence gene regulation	*S. pneumo*, (234, 235)
GBSCOH1_0964	Formate--tetrahydrofolate ligase, Fhs	Unknown – necessary for colonization & dissemination	*S. suis* (236)
GBSCOH1_0023	Phosphoribosylaminoimidaole-succinocarboxamide synthase, PurC	Unknown – disruption in purine biosynthesis attenuates survival	*S. pneumo* (237) *E. coli* (238)
GBSCOH1_1211	Choline binding protein D, CbpD	Competence & fratricide	*S. pneumo* (239)
GBSCOH1_2003	Partitioning protein, ParB family	SOS response Gene regulation	*Pseudomonas* (240)
GBSCOH1_0963	Cardiolipin synthetase, Cls	Promotes lipid remodeling Antibiotic resistance	*S. mutans* (241) *S. aureus* (242)

GBSCOH1_1343	SsrA-binding protein, SmpB	Unknown – disruption attenuates intracellular survival	*S. pneumo* (243) *Listeria* (244)
GBSCOH1_1580	Universal stress protein family, Usp	Adhesin* Stress tolerance	*S. aureus*, (245, 246)
GBSCOH1_1612	Phosphoglycerate kinase, PGK	Adhesin/neutrophil activation*	GBS (247, 248) GAS (249)
GBSCOH1_1290	Nucleotidyl transferase, putative, IspD2	Adhesin*	*S. aureus* (245)
GBSCOH1_1607	Metallo-beta-lactamase superfamily protein, Rnj	Regulates virulence gene expression	*E. faecalis* (250)
GBSCOH1_0329	3-oxoacyl-(acyl-carrier-protein) synthase II, FabF	Fatty acid synthesis	*S. aureus* (251)
GBSCOH1_0715	Superoxide dismutase, SodA	Oxidative stress Intracellular survival	GBS (252) *S. suis* (253)
GBSCOH1_0688	Translation elongation factor, Tu	Adhesin*	*S. aureus* (254)

All proteins above are significant by both FC and *p*-value (Student's t-test, $P<0.05$) cutoffs.
Proteins were categorized as pathogenesis-associated through PubMed database searches.
*Cytosolic protein with published moonlighting functions at the bacterial surface.

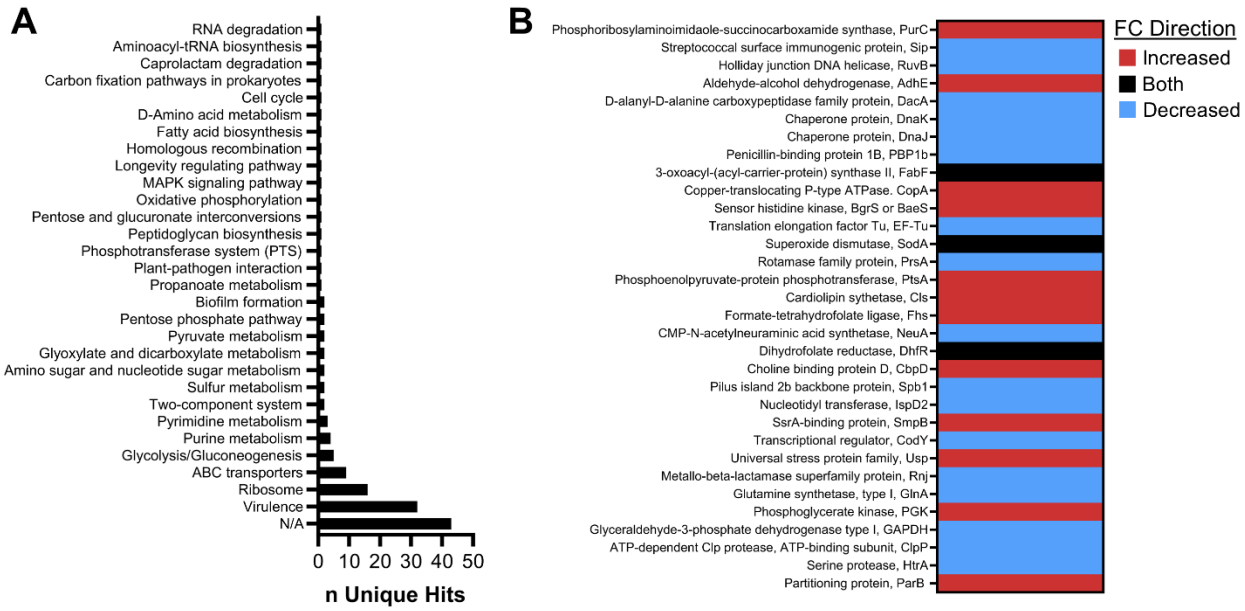

Fig 2.5. KEGG functional summary of direct and indirect HtrA-regulated processes. A) All proteins undergoing significant changes in abundance were categorized based on Kyoto Encyclopedia for Genes and Genomes (KEGG) functional annotations. Frequencies for each functional category are indicated. Proteins were sorted in the virulence category based on results from PubMed database searches. B) Direction of fold-change (FC) for a subset of virulence proteins. All strain comparisons were performed with the strain displaying less *htrA* expression as the numerator.

2.3.5 Sip is a substrate of HtrA

Based on our proteomics study, we selected Sip for further assessment as a potential substrate of HtrA protease. To this end, we generated and purified recombinant HtrA-H6 and its predicted catalytic mutant HtrA^{S237A}-H6 and used these to assess HtrA protease activity. By western blot using an HtrA-specific antibody, purified catalytically-inactive HtrA^{S237A}-H6 was observed as a single band around 42 kDa, whereas HtrA-H6 displayed a full-length 42 kDa protein along with smaller anti-HtrA reactive fragments indicative of HtrA autocleavage (**Supplemental Figure A-3a**), a feature reported for other HtrA homologs (227, 255).

We first examined HtrA protease activity using the model protease substrate β-casein. After co-incubation of β-casein with HtrA-H6 and subsequent separation by SDS-PAGE, processed β-casein fragments of ~15 to <10 kDa were visible. These fragments were not observed following co-incubation with HtrA^{S237A}-H6 (**Supplemental Figure A-4b**, lane 4 versus 5). Densitometric analysis confirmed that full-length β-casein was significantly depleted following exposure to HtrA-H6 but its abundance remained unchanged upon incubation with HtrA^{S237A}-H6. (**Supplemental Figure A-4c**). These findings demonstrate that GBS HtrA functions as a protease, and that the serine residue within the catalytic triad is essential for its protease activity.

We next examined whether HtrA can directly cleave GBS proteins as a means of controlling GBS protein abundance. Based on our proteomics survey, we noted that Sip abundance mirrored changes to HtrA levels, indicating that interaction with HtrA may be important for proper processing and export of Sip. As seen for β-casein, co-incubation of Sip with HtrA-H6 led to cleavage of Sip into fragments ranging from ~25 to <10 kDa, whereas no fragmentation was visible following co-incubation with HtrA^{S237A}-H6 (**Figure 2.6A**, lane 4 versus 5). Densitometry analysis confirmed the depletion of full-length Sip by HtrA-H6, but not by HtrA^{S237A}-H6 (**Figure 2.6B**). Collectively, these findings demonstrate that Sip is an *in vitro* substrate of GBS' HtrA serine protease.

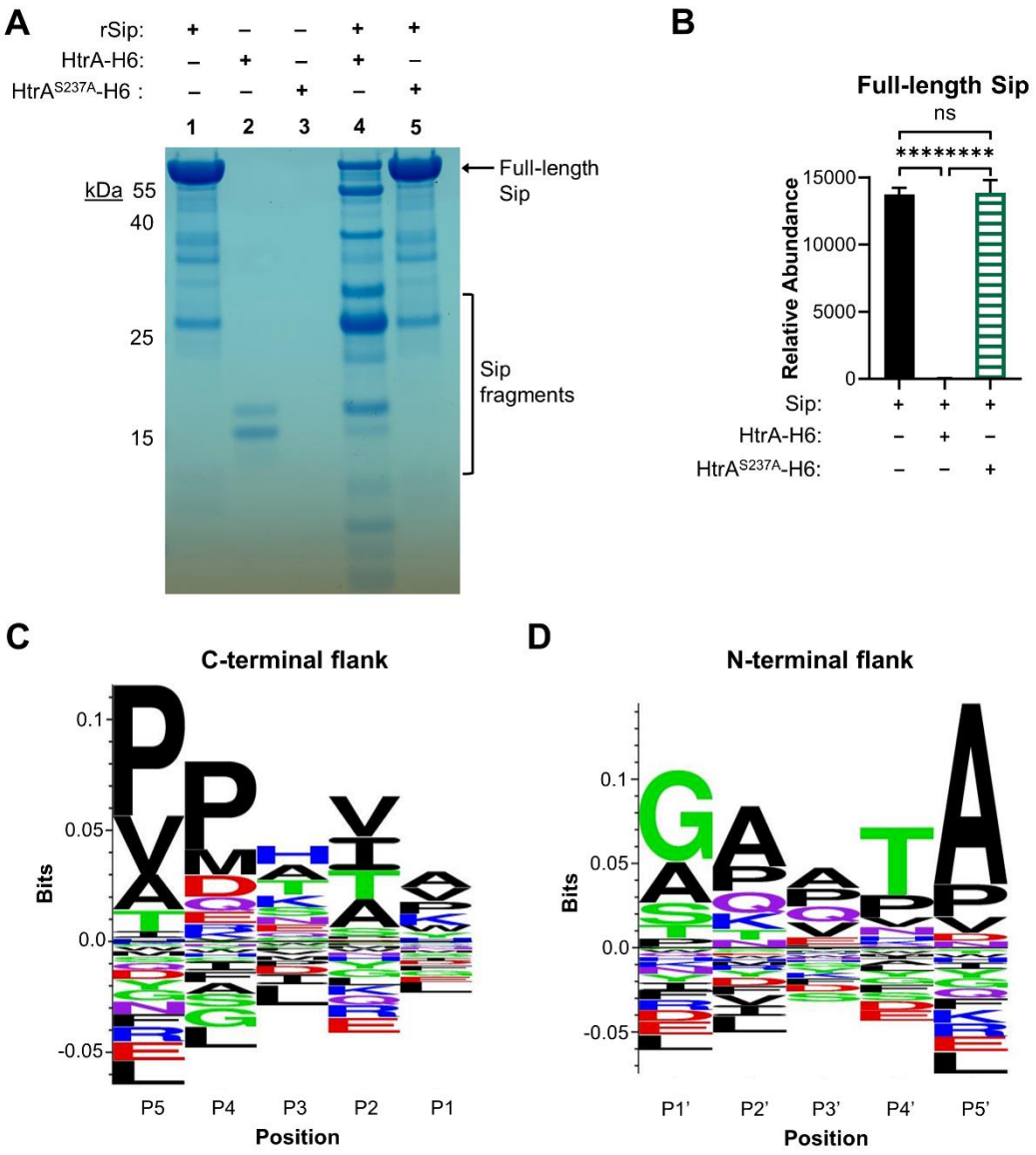

Fig 2.6. HtrA serine protease cleaves Sip. **A)** Cleavage assay assessing HtrA-H6 and catalytically-inactive HtrA^{S237A}-H6 interaction with the putative GBS substrate Streptococcal immunogenic protein (Sip) at 1:1000 (HtrA:Sip). Coomassie stained gel indicates full-length Sip and fragments processed by rHtrA, with relevant controls included to show substrate, HtrA-H6, and HtrA^{S237A}-H6 alone (lanes 1-3). **B)** Densitometry of full-length β-casein was performed in Fiji. Data shows means ± SEM of three replicates, with significance determined by ordinary one-way ANOVA with Tukey's multiple comparison test: **** $P < 0.0001$. **C-D)** N-terminal sequencing was performed on HtrA-cleaved Sip fragments via Edman degradation. Logo plots indicate frequency of amino acids on **C)** C-terminal (P1-P5) and **D)** N-terminal (P1'-P5') side of the proposed cut-site. Underrepresented amino acids are shown as negative bits. Colors indicate amino acid chemical properties. Probability-weighted Kullback-Leibler Logo plots were produced with a Hobohm clustering threshold of 0.8 and default pseudo counts using Seq2Logo 2.0.

To determine which Sip regions are cleaved by HtrA, we performed N-terminal peptide sequencing on the HtrA-cleaved Sip fragments. The N-terminal amino acid sequences of seven

distinct cleaved Sip fragments were mapped against full-length Sip to identify each putative cut site, then extended to obtain the adjacent sequence. Logo plots were produced to display the relative frequency of amino acids within 5 positions from each putative cut site, with P5 to P1 representing the C-terminal side of the cut site and P1' to P5' representing the N-terminal side (**Figure 2.6C-D**). While no perfect consensus HtrA cleavage motif was identified, at the C-terminal flank GBS HtrA appears to prefer proline or another hydrophobic amino acid at P5, while hydrophobic residues of valine, isoleucine, or alanine, or the polar amino acid threonine is preferred at P2. On the N-terminal side of the cut, polar residues were enriched at P1' and threonine was common at P4'. There also appears to be strong preference for alanine at P5'. These results indicate that there is a preference in amino acid sequence composition that directs substrate cleavage by HtrA. Future studies could leverage these patterns to design inhibitors targeted towards the active site of GBS HtrA.

2.3.6 HtrA alters virulence effects of Sip during pregnancy-associated infection

Since Sip is a substrate of HtrA *in vitro*, we next aimed to assess their functions *in vivo*. Additionally, while studies show that Sip is highly immunogenic during GBS infection (195, 196), its impact on virulence has not been examined. To this end, we constructed GBS strains lacking Sip in the presence and absence of HtrA (i.e., COH1Δ*sip* and COH1Δ*htrA*Δ*sip*) and confirmed the absence of *in vitro* growth defects (**Supplemental Figure A-4**). These strains were tested for virulence in the pregnancy-associated ascending infection model, where no significant changes in lower genital tract colonization or ascending infection to the uterus were observed for any strains. Similar to observations for COH1Δ*htrA*, both COH1Δ*sip* and COH1Δ*htrA*Δ*sip* GBS exhibited heightened bacterial burden in pups and placentas compared to the isogenic WT strain. However, GBS burden in pups and placentas was not significantly different between COH1Δ*htrA*, COH1Δ*sip*, or COH1Δ*htrA*Δ*sip* strains (**Figure 2.7A**). Additionally, infections caused by COH1Δ*sip* and COH1Δ*htrA*Δ*sip* GBS resulted in fewer adverse outcomes with rates most similar

to the attenuated COH1Δ*htrA* strain and *p*-values that trended towards significance compared to isogenic WT (**Figure 2.7B**). Since double deletion of HtrA and Sip did not attenuate virulence beyond what was observed for either single deletion strain, we postulate that HtrA and Sip functions may contribute to GBS virulence through an interlinking pathway.

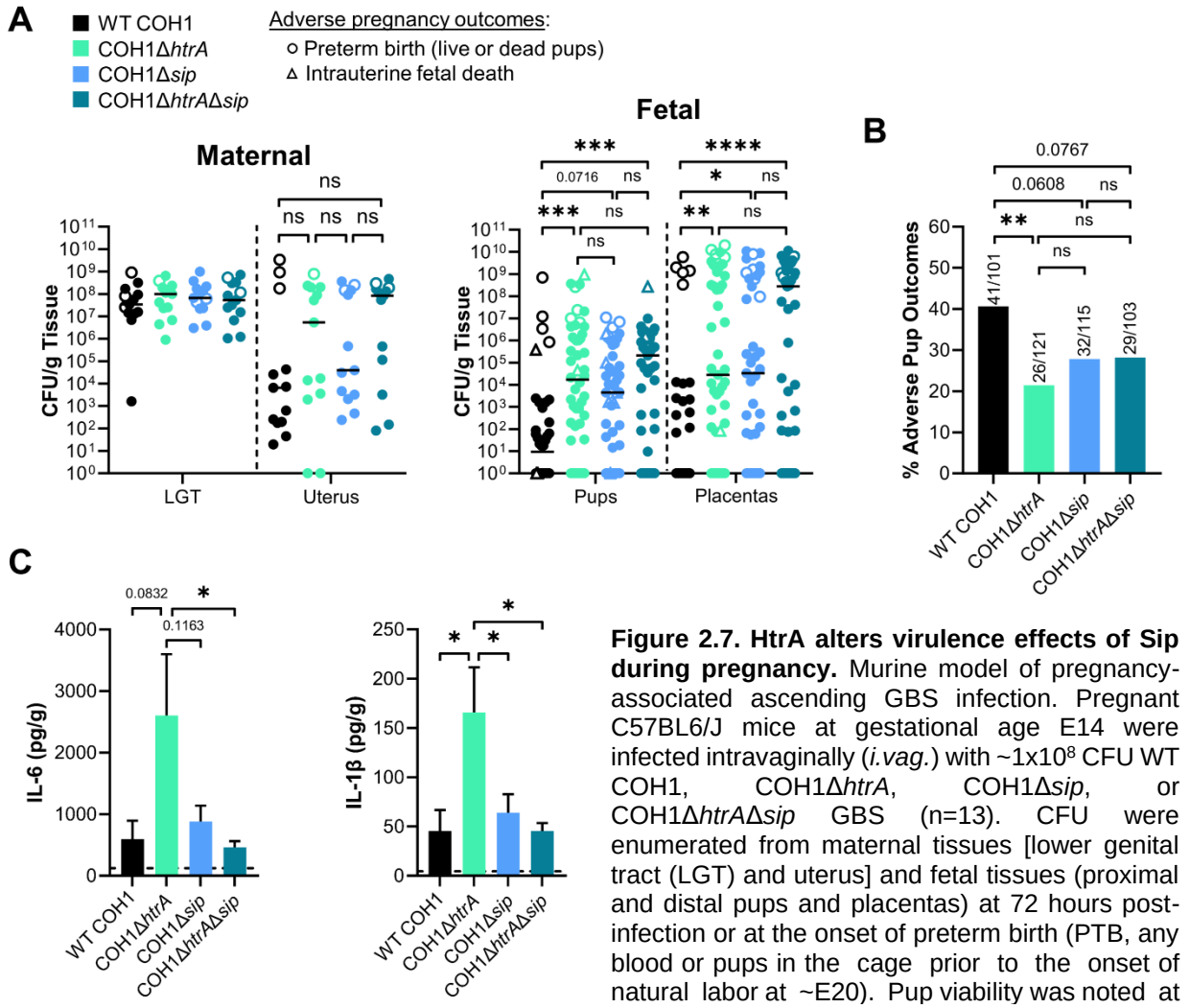

Figure 2.7. HtrA alters virulence effects of Sip during pregnancy. Murine model of pregnancy-associated ascending GBS infection. Pregnant C57BL6/J mice at gestational age E14 were infected intravaginally (*i.vag.*) with ~1x10^8 CFU WT COH1, COH1Δ*htrA*, COH1Δ*sip*, or COH1Δ*htrA*Δ*sip* GBS (n=13). CFU were enumerated from maternal tissues [lower genital tract (LGT) and uterus] and fetal tissues (proximal and distal pups and placentas) at 72 hours post-infection or at the onset of preterm birth (PTB, any blood or pups in the cage prior to the onset of natural labor at ~E20). Pup viability was noted at necropsy. **A)** CFU enumerated were normalized to tissue weight. Graph indicates medians, with PTB and pup deaths are indicated by open symbols. Significant differences were determined using Mann-Whitney test: ns $P > 0.05$, ∗∗ $P < 0.01$, ∗∗∗ $P < 0.001$, ∗∗∗∗ $P < 0.0001$. Data for WT and COH1Δ*htrA* is also shown in Fig 3. **B)** Frequencies of adverse outcomes (including PTB, stillbirth, and intrauterine fetal death) caused by each GBS strain were calculated out of total pups per group. Contingency tables were used to assess significance via Fisher's exact test: ∗∗ $P < 0.01$. **C)** Placental lysates from the above mice were assessed via Luminex to quantify the indicated immuno-analytes. Data was normalized to tissue weight. Graphs show means ± SEM. The dashed line indicates mean analyte levels in PBS-treated control mice. Significant differences were determined using ordinary one-way ANOVA with Tukey's multiple comparison test: ∗ $P < 0.05$. Trending statistics ($P < 0.1$) are also indicated.

A variety of chemokines, pro-inflammatory cytokines, and IL-10 were significantly elevated in placentas infected by COH1Δ*htrA* versus isogenic WT GBS (**Figure 2.3D**); however, the concomitant loss of Sip in the COH1Δ*htrA*Δ*sip* strain resulted in minimal pro-inflammation (**Figure 2.7C**). Concentrations of IL-6 and IL-1β were highest in placentas infected by COH1Δ*htrA* and levels of both cytokines were significantly reduced during COH1Δ*htrA*Δ*sip* infection, suggesting that Sip is important for COH1Δ*htrA*-driven pro-inflammatory responses. Together, these data highlight HtrA and Sip as virulence factors that mediate the generation of adverse pregnancy outcomes during GBS infection and support a model where HtrA-mediated virulence effects may be explained – at least in part – by the combined effects of HtrA and Sip.

2.3.7 HtrA cleavage of Sip modulates Sip-induced NFkB activation

Lastly, we aimed to identify a consequence for HtrA-mediated Sip cleavage that would impact GBS virulence; however, Sip has not been studied in a virulence context prior to our work. Multiple vaccine studies show that Sip is a pro-inflammatory TLR4 ligand (195, 196, 213, 256), so we next assessed whether HtrA cleavage of Sip alters this effect. To this end, we assessed TLR4 activation by full-length versus HtrA-cleaved Sip in HEKBlue cells, which express a secreted embryonic alkaline phosphatase (SEAP) reporter gene under control of the NFkB promoter. According to an endotoxin detection kit, the amount of HtrA-H6 and Sip used in this assay contained ≤ 0.01 EU of LPS. This amount of purified LPS did not activate the NFkB promoter, suggesting that the impact of LPS contamination on our assay is minimal. As expected, full-length recombinant Sip activated TLR4 and induced SEAP activity through the NFkB promoter (**Figure 2.8**). Treatment with HtrA-H6 also resulted in SEAP activity; however, cells that received HtrA-H6 in combination with Sip exhibited no significant increase compared to HtrA-H6 alone, suggesting that the contribution of Sip to TLR4-mediated activation was lost by HtrA activity. Thus, HtrA-

mediated Sip processing may serve as an immune evasion strategy, allowing GBS to eliminate the effects of a protein that could otherwise induce inflammation and immune cell activation.

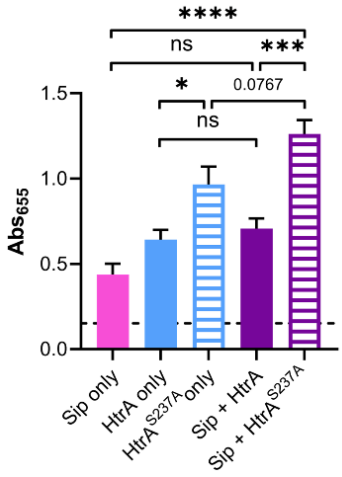

Figure 2.8. HtrA cleavage of Sip modulates Sip-induced NFkB activation. HEKBlue cells expressing human TLR4, its adaptor proteins MD-2 and CD14, and a secreted embryonic alkaline phosphatase (SEAP) reporter under the control of the NFkB promoter were stimulated with Sip and HtrA-H6, Sip and HtrA^{S237A}-H6, or single protein controls at a ratio of 1000:1 (5 µg Sip: 5 ng HtrA). Following 42 hours of stimulation, supernatants were harvested and assessed for SEAP activity. Graph shows means of five biological replicates ± SEM. The dashed line indicates baseline activation in vehicle control treated cells. Statistical significance was assessed using One-way ANOVA with Tukey's multiple comparisons test: ∗ $P < 0.05$, ∗∗∗ $P < 0.001$, ∗∗∗∗ $P < 0.0001$. Trending statistics ($P < 0.1$) are also indicated.

2.4 Discussion

While the HtrA serine proteases have been linked to virulence for many pathogenic bacteria, the impact of this protein on GBS virulence was previously unknown. This study provides evidence that HtrA is important for GBS pathogenesis during systemic and pregnancy-associated infections. Alterations in *htrA* expression caused global changes to GBS proteome content with changes in abundance observed for many previously described GBS virulence factors. We further confirmed that Sip, which exhibited differential abundance in our proteomic screen, was directly cleaved by HtrA. This HtrA processing inhibited Sip-mediated activation of TLR4 and NFkB (**Figure 2.9**). These results provide insight into an alternative mechanism of bacterial virulence regulation and provide a model by which GBS proteases such as HtrA may post-translationally regulate processes that impact virulence.

We show that in the hypervirulent GBS strain COH1 (serotype III, ST-17), deletion of *htrA* attenuates virulence during systemic infection and reduces adverse fetal outcomes during pregnancy-associated infection. This *htrA* deletion strain was also hypersensitive to neutrophil-mediated killing *in vitro*, suggesting that neutrophil responses could partially explain our *in vivo*

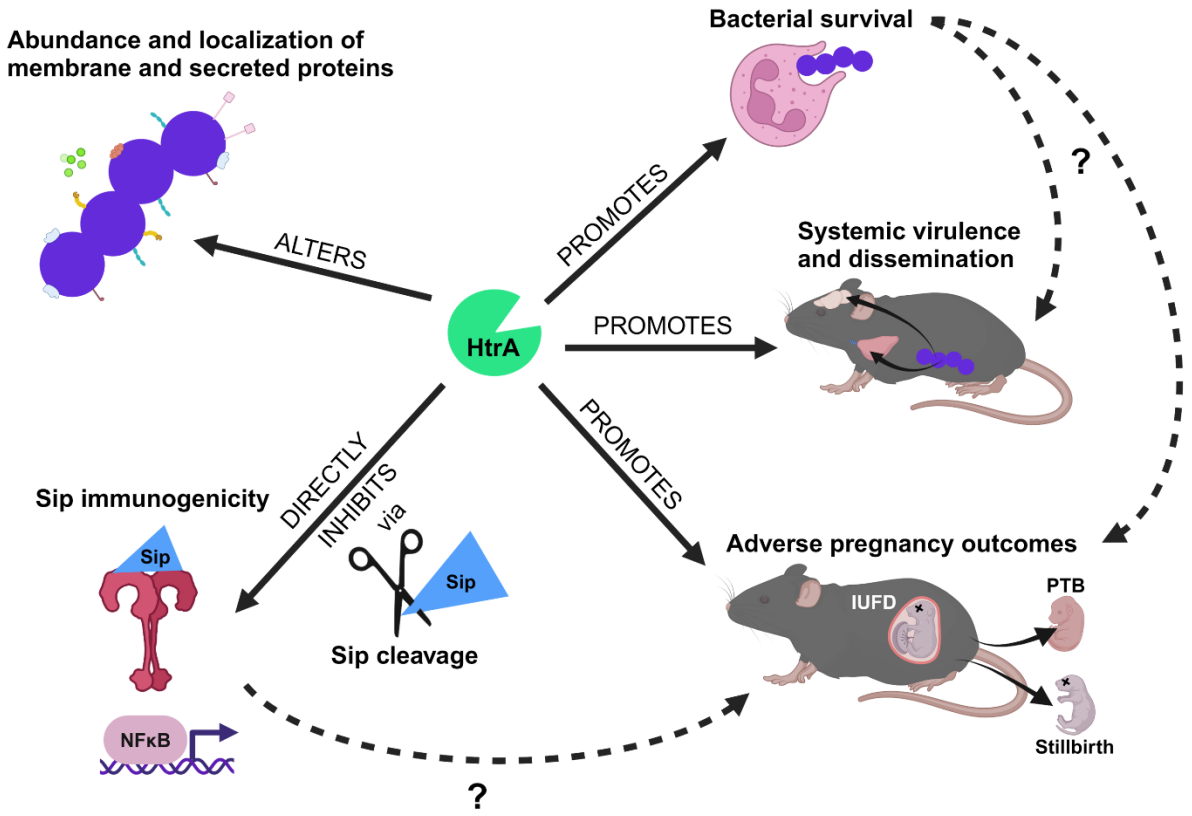

Figure 2.9. Working model for the role of the regulatory protease, HtrA, in GBS virulence. HtrA serine protease regulates virulence through multiple pathways, many of which have yet to be defined. HtrA can modulate the abundance and localization of numerous GBS proteins, including numerous virulence factors that impact pathogenesis. HtrA regulates Sip abundance and proteolytically modifies this novel substrate. Cleavage of Sip by HtrA inhibits activation of TLR4 and NFkB *in vitro*. COH1Δ*htrA* exhibited heightened susceptibility to neutrophils, so HtrA impacts GBS survival. COH1Δ*htrA* were attenuated during systemic infection, resulting in decreased CFU burdens in disseminated tissues. Pregnant dams infected with COH1Δ*htrA* experienced fewer adverse pup outcomes, so HtrA modulates the development of adverse outcomes during pregnancy-associated infection. The effect of HtrA on GBS survival during neutrophil exposure may partially restrain the development of disease *in vivo*. Further, HtrA-mediated Sip cleavage may impact the development of adverse pregnancy outcomes in mice, potentially due to changes in immune signaling.

phenotype. Other bacterial HtrA homologs have been shown to regulate bacterial survival during immune cell-induced stress and exhibit concomitant changes in virulence. For example, a *Bacillus anthracis htrA* deletion mutant was impaired for growth in macrophages and exhibited 6-fold attenuation in a guinea pig infection model (163). Similarly, deletion of *mucD*, the *htrA* homolog in *Pseudomonas aeruginosa*, attenuated virulence and altered neutrophil responses in a corneal infection model. Interestingly, *mucD* deletion led to elevated cytokines and chemokines including IL-1β, Gro-α/KC, and MIP-2 (164), which are strongly associated with neutrophil recruitment and

function (257-259). These factors along with IL-6, MIP-1, and TNF-α were similarly elevated in COH1Δ*htrA* infected placentas. These findings provide evidence that HtrA regulates GBS virulence and the accompanying host innate immune responses through mechanisms that may overlap with other bacterial pathogens. We further speculate that HtrA function could alter neutrophil responses in a way that exacerbates the development of adverse pregnancy outcomes during perinatal GBS infection. The production of placental IL-10 during COH1Δ*htrA* infection may block host damage that might typically occur in the presence of active immune cell recruitment and pro-inflammation (31, 82, 99-101, 103, 205). Whether this immune profile is mediated directly by HtrA or indirectly due to the surface proteome changes associated with *htrA* deletion warrants further study.

In our proteomic survey comparing GBS strains with differing *htrA* expression, we identified 110 proteins whose abundance changed in response to HtrA. The majority of these changes affected proteins localized to the GBS membrane or secreted into the culture supernatant, supporting a role for HtrA in shaping the content of the GBS surface proteome which is in agreement with observations for other pathogenic Streptococci (182-185, 260). Coverage in our proteomic screen accounted for approximately 50% of GBS proteins, suggesting that our analysis may have underestimated proteins subject to processing by HtrA. Nonetheless, we observed over 30 proteins with significantly changed abundance whose functions have been previously linked to virulence. We also noted changes in abundance of multiple moonlighting proteins described in other bacteria to possess unique dual localization-dependent functions. The cytoplasmic chaperone DnaJ has been identified within the surface proteomes of other bacteria, where it can elicit immunomodulatory effects (223, 226). Our GBS proteomics screen observed reduced abundance of DnaJ and DnaK in the membrane of *htrA* deletion mutant or WT GBS compared to an *htrA* over-expressing strain. While this finding suggests that HtrA may directly regulate DnaJ and DnaK abundance at the GBS membrane, their function at the GBS surface remains unknown.

Further, we saw membrane-localized changes in abundance of the glycolytic enzymes glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phosphoglycerate kinase (PGK), which each possess known moonlighting functions at the GBS surface. During infection, GAPDH is immunomodulatory, stimulating IL-10 production that hinders neutrophil recruitment (209). GAPDH can also induce apoptosis in murine macrophages (210) and bind human plasminogen, enhancing GBS interactions with the extracellular matrix (211, 212). Interestingly, GAPDH abundance was highest in the membrane of our HtrA overexpressing strain, suggesting HtrA may indirectly affect GAPDH abundance at the membrane. Consequently, it is unlikely that GAPDH expression was responsible for the increased IL-10 levels seen in placentas infected with the *htrA* deletion strain, which likely arose due to other changes to the GBS surface proteome.

Similarly, surface-localized GBS PGK can function as a plasminogen and actin binding protein *in vitro*, although the relevance of these interactions during GBS infection remains unknown (247, 248). We observed an increase in PGK abundance within the GBS membrane of the *htrA* deletion mutant compared to the *htrA* over-expressing strain, indicating that HtrA may also indirectly regulate PGK surface exposure. Taken collectively, our proteomic findings suggest that HtrA can directly regulate the abundance of a variety of GBS membrane and exported proteins that are linked to virulence. Further, another study notes that *Streptococcus pyogenes htrA* deletion led to altered abundance of the cell wall-localized moonlighting proteins DnaK, enolase, GAPDH, and elongation factor Tu abundance (184), suggesting that a subset of conserved proteins may be targeted by HtrA homologs in Streptococcal pathogens.

A novel protein that our proteomics screen identified to be regulated by HtrA was the Streptococcal surface immunogenic protein (Sip). Although Sip has been extensively studied for use in GBS vaccines, its contribution towards virulence remains unknown. Similar to our findings of the *htrA* deletion strain, perinatal infection by a COH1Δ*sip* mutant also led to fewer adverse fetal outcomes than WT COH1 GBS. From a virulence perspective, if HtrA and Sip contributed to infection through completely divergent pathways, we would expect an additive phenotype from

GBS lacking both proteins. Instead, COH1Δ*htrA*Δ*sip* GBS induced adverse outcome rates that did not significantly differ from either single mutant strain. Thus, we speculate that HtrA and Sip contribute to adverse pregnancy outcomes through a functional interaction. Indeed, protease assays confirmed that HtrA directly cleaves Sip *in vitro*.

Finally, in agreement with published data we observed that GBS Sip is an agonist of human TLR4 (213, 256) and we additionally we show that proteolytic processing by HtrA abolished Sip's immunogenic activity. Our lab has previously demonstrated that GBS can suppress TLR2 and TLR4 signaling *in vivo*, which reduces downstream ROS production by neutrophils. Regulation of Sip immunogenicity via HtrA processing may represent another mechanism that allows GBS to evade host innate immune recognition. As potential evidence for this, we primarily observed pro-inflammatory cytokine production in the placentas of dams infected by HtrA-deficient GBS. Sip cleavage by HtrA cannot occur in our *htrA* deletion mutant, and Sip cleaved by HtrA does not activate NFkB through TLR4. Thus, we speculate that the pro-inflammatory cytokines produced during COH1Δ*htrA* but not COH1Δ*sip* or COH1Δ*htrA*Δ*sip* placental infection may be stimulated in part due to full-length Sip. Precisely how HtrA mediates cleavage of Sip and its other putative targets remains unknown, and whether this processing occurs at the GBS membrane or prior to protein export to affect surface exposure is the subject of future investigation. Further, the specific host-pathogen interactions mediated by Sip remain unknown, and thus further assessment of the biological relevance of HtrA-Sip interaction *in vivo* is challenging. Future studies may aim to characterize the consequences of interaction in greater detail.

Taken together, the data in this study classifies HtrA as a novel serine protease that regulates GBS virulence during systemic and pregnancy-associated infections. Our findings show that HtrA can modulate the abundance of a variety of surface or exported GBS proteins including multiple proteins with immunomodulatory properties or roles in pathogenesis, which may explain the importance of HtrA *in vivo.* We identified Sip as a novel virulence factor during pregnancy-

associated infection and as a substrate that is cleaved by HtrA. Further, we propose that HtrA-mediated Sip cleavage may allow GBS to evade TLR4-mediated immune responses. HtrA inhibitors have been identified for *Escherichia coli*, *Helicobacter pylori*, *Chlamydia trachomatis*, and *Listeria monocytogenes* HtrA homologs, suggesting that this protease family is a tractable drug target (169, 261-263). Future studies evaluating GBS HtrA tractability could inform the development of a small molecule inhibitor that could synergize with antibiotics and vaccines to prevent and treat a wide range of GBS clinical manifestations.

2.5 Methods

2.5.1 Ethics statement

Written, informed consent was obtained from adult patients for donation of human blood, in accordance with the principles in the World Medical Association Declaration of Helsinki and the Department of Health and Human Services Belmont Report and as approved by the Seattle Children's Research Institute Institutional Review Board (PIROSTUDY11117).

All mouse experiments were approved by the Seattle Children's Research Institute's Institutional Animal Care and Use Committee (protocol #00036) and were performed in strict accordance with recommendations from the *Guide for the Care and Use of Laboratory Animals, 8th edition.*

2.5.2 Chemicals

All chemicals were purchased from Sigma-Aldrich unless otherwise noted.

2.5.3 Bacterial strains

The GBS clinical isolate COH1 used for this study is a serotype III, sequence type 17 clone originally isolated from an infant with bacteremia (264). GBS was cultured using tryptic soy agar or broth (TSA/TSB, Difco Laboratories) at 37°C with 5% CO_2. Overnight cultures were diluted

1:10 and grown to an optical density at 600 nm (OD_{600}) of 0.3 for infections and *in vitro* assays, or 0.6 for protein fractionation.

When necessary for strain selection, antibiotics were used at the following concentrations for *E. coli* (kanamycin 50 µg/ml, erythromycin 300 µg/ml, spectinomycin 100 µg/ml, chloramphenicol 10 µg/ml, or ampicillin 100 µg/ml) or for GBS (kanamycin 1000 µg/ml, erythromycin 5 µg/ml, spectinomycin 300 µg/ml, or chloramphenicol 5-10 µg/ml).

2.5.4 Construction of htrA and sip mutant strains

E. coli DH5α (New England Biolabs) was used for cloning unless otherwise noted. Primers are listed in **Table 2.2** (pg. 52) in the order of use.

The *htrA* allelic exchange mutant COH1Δ*htrA* was constructed using previously described methods (82). In short, 1 kb chromosomal DNA flanking the upstream and downstream regions surrounding *htrA*, plus a kanamycin resistance cassette were PCR amplified from COH1 genomic DNA (*htrA* upstream F/R; *htrA* downstream F/R) or pCIV2 plasmid (*kanR* F/R). The temperature-sensitive allelic exchange vector pHY304 was PCR linearized (pHY304 F/R) and the four fragments were combined via Gibson assembly (cloning kit, New England Biolabs) to generate the plasmid pHY304-Δ*htrA* which was subsequently introduced into COH1 GBS by electroporation (265). Selection of allelic exchange mutants was performed as described (266). Deletion of *htrA* was confirmed by PCR and DNA sequencing.

To complement *htrA* in trans, *htrA* was amplified (*htrA* comp F/R) and combined with PCR linearized pDC123 (pDC123 F/R) via Gibson assembly. The resulting vector pDC123*htrA* was introduced into COH1Δ*htrA* GBS by electroporation to produce COH1Δ*htrA*/pDC123*htrA*.

To generate the *sip* allelic exchange mutant COH1Δ*sip*, 1 kb chromosomal DNA flanking the upstream and downstream regions surrounding *sip* and a spectinomycin resistance cassette were PCR amplified from COH1 genomic DNA (*sip* upstream F/R; *sip* downstream F/R) or pLZ12-spec plasmid (*specR* F/R). These fragments were first ligated into PCR linearized pBluescript

(pBluescript F/R) via Gibson assembly, then the resulting insert was cloned within the BamHI and XbaI sites of pHY304. The resulting plasmid pHY304-Δ*htrA* was introduced into COH1 GBS by electroporation, and allelic exchange was selected as described above.

The construction of a COH1Δ*htrA*Δ*sip* mutant strain was achieved by electroporating COH1Δ*htrA* with pHY304-Δ*sip*, followed by selection of allelic replacement as described above.

Table 2.2. Primers used for strain construction

Primer name	Primer sequence
htrA upstream F	GAA TTC CTG CAG CCC AGT ACT CTT AAT ATT ATA TCA GAA AAA AAA G
htrA upstream R	TTT AGC CAT AAA TAG CTA CCT CCG TAA ATT TTT ATA C
htrA downstream F	ATT GTT TTA GTC AAT TCT AGT TAT TTT AAC TAA GAT ATA GTA AAA TTA AA
htrA downstream R	ACT AGT GGA TCC CCC TAA AGT TTT CCA CAG ACT GTG
kanR F	GTA GCT ATT TAT GGC TAA AAT GAG AAT ATC AC
kanR R	CTA GAA TTG ACT AAA ACA ARR CAT CCA GTA AAA TAT AAT ATT TTA TTT TC
pHY304 F	GGG GGA TCC ACT AGT TCT AG
pHY304 R	GGG CTG CAG GAA TTC GAT
htrA comp F	GAA AAA CAA ACA GAG GTT TTA CGG AGG TAG CTA TTT ATG GTG AAA A
htrA comp R	CGG GCC CGG GTA CCG CAT GCT TAG TTA TTT GCT CGT TGT TTA G
pDC123 F	AAC CTC TGT TTG TTT TTC GCC GC
pDC123 F	GCA TGC GGT ACC CGG GC
sip upstream F	ATA GGG CGA ATT GGG TAC CGG GCC GGA TCC GTA TGG TTT TAT CTG CGG
sip upstream R	GTA CCT ATT TTA TAT CCA TAG TTG TGA ATT CTG CTT CAA TTC CTA TTT CC
sip downstream F	AGC CAA ATA GCT TCC TTT TTT ATA CCA TTG CCC TGA CGA AAG TCT ATG
sip downstream R	GCT GGA GCT CCA CCG CGG TTC TAG AGA TAC TCA TAA CTA TTC CCC
specR F	AAA AAG GAA ATA GGA ATT GAA GCA GAA TTC ACA ACT ATG GAT ATA AAA TAG GTA C
specR R	AGC CAA ATA GCT TCC TTT TTT ATA CCA TTG CCC TGA CGA AAG TCT ATG
pBluescript F	AAA AAG GGG AAT AGT TAT GAG TAT CTC TAG AAC CGC GGT GGA GCT CCA G
pBluescript R	CGG GAA CCC GCA GAT AAA ACC ATA CGG ATC CGG CCC GGT ACC CAA TTC G
htrA prot F	ATA CCC ATG GCT TTT GTC ATG AAT CAT AAT GAC AAT ATT C
htrA prot R	AGT GCG GCC GCG TTA TTT GCT C
SDM*htrA*^{S237A} F	GTG CTC CAC CTG CAT TAC CAG GAT TGA TAG CAG CA
SDM*htrA*^{S237A} R	TGC TGC TAT CAA TCC TGG TAA TGC AGG TGG AGC AC

All primers were ordered from IDT.

2.5.5 GBS growth curves

GBS overnight cultures were diluted 1:20 (or 1:15 for COH1Δ*htrA*Δ*sip*) in TSB and incubated at 37°C with 5% CO_2. At 0, 1-, 2-, 4-, and 6-hours post-inoculation, cultures were serially diluted and plated on TSA to enumerate viable CFU/ml. For each experiment, strains were tested in biological triplicate.

2.5.6 GBS Gram stains

GBS overnight cultures were spread onto sterile, pre-warmed glass slides. Once dried, smears were Gram stained: 30 seconds crystal violet, water rinse, 30 seconds Gram's iodine, water rinse, decolorizer rinse, water rinse, 30 seconds safranin (Epredia). Images were acquired on a BZ-X800 microscope (Keyence) using 100x oil immersion.

2.5.7 GBS protein fractionation

GBS cultures (60 ml) grown to OD_{600} 0.6 were pelleted at 3,000 x g at 4°C for 10 minutes. For analysis of secreted proteins, culture supernatants were filtered using a Millex-GP syringe filter (0.22 µm, Millipore) and concentrated to ~1/15th volume via an Amicon Ultra centrifugal filter device (10 kDa, Millipore) prior to storage at -20°C for subsequent Western blot analysis. For sample preparation for proteomics analysis, two cOmplete protease inhibitor cocktail tablets (Roche) were added to filtered supernatant prior to Amicon concentration and precipitated overnight in 10 mM sodium chloride and 4x volume of acetone at -20°C. Precipitated proteins were pelleted via centrifugation at 16,000 *x g* at 4°C for 20 minutes and after evaporation of residual acetone, pellets were solubilized in Dulbecco's PBS and applied to a PD-10 desalting column (Cytivia). Secreted protein fractions were quantified (BCA Protein Assay Kit, Pierce), adjusted to a final concentration of 1 mg /mL, and stored at -80°C until use.

For isolation of GBS membrane and cytosol fractions, GBS pellets were washed in PBS (Corning) and resuspended in lysis buffer [20 mM Tris-HCl, 10 mM magnesium chloride, 500 U

DNase, 50 ng/mL RNase A, cOmplete mini protease inhibitor tablet/10 ml (Roche)]. GBS cells were lysed by a bead beater (PreCellys 24, Bertin Technologies) using 30 second pulses at power 6 with 5 minutes on ice between steps. Lysates were pelleted at 5,000 x *g* at 4°C for 5 minutes and the supernatant was carefully transferred to an OptiSeal ultracentrifuge tube (Beckman Coulter) and pelleted at 60,000 x *g* at 4°C for 45 minutes to separate cytosolic and membrane fractions. The supernatant containing cytosolic proteins was concentrated using an Amicon Ultra centrifugal filter (10 kDa, Millipore) and the remaining pellet of membrane proteins was resuspended (20 mM Tris pH 7.5, 2 mM EDTA, 10% glycerol). Both fractions were quantified (BCA Protein Assay Kit, Pierce), adjusted to a final concentration of 1 mg/ml, and stored at -80 until use. These fractions were used for both Western blot and proteomic analysis.

2.5.8 Western blot analysis

Proteins were denatured in 1x LDS buffer (Invitrogen) for 10 minutes at 95°C prior to SDS-PAGE separation (4-20% bis-tris with MOPS-SDS, Invitrogen), and transferred onto nitrocellulose membrane (Invitrogen). Membranes were blocked for 1 hour in Intercept PBS blocking buffer (LI-COR), followed by incubation overnight at 4°C with rabbit polyclonal anti-HtrA antiserum (Lampire) diluted 1:750 in Intercept antibody diluent reagent (LI-COR). Membranes were washed twice in PBS/0.5% Tween-20, and probed with IRDye 680RD-conjugated goat anti-rabbit IgG (LI-COR) for 45 minutes. Fluorescent signal was imaged using an Odyssey CLx (LI-COR) and analyzed using Odyssey v3.0 software. Images shown are representative of one western blot performed in biological triplicate.

2.5.9 Neutrophil-mediated killing assay

Primary neutrophils were isolated from fresh human blood using the MACSxpress Whole Blood Neutrophil Isolation kit (Miltenyi Biotec). In a flat-bottom 96-well plate, $9x10^5$ neutrophils were combined with GBS in RPMI (Corning) at an MOI of 1, then incubated for 1 hour at 37°C

with 5% CO_2. Mock wells without GBS and wells containing GBS without neutrophils were included as controls. Following incubation, neutrophils were lysed with 0.1% (v/v) Triton-x, serially diluted, and plated to enumerate viable GBS CFU/ml. Percent GBS killing was determined by comparing GBS CFU in the presence of neutrophils divided by GBS CFU in the absence of neutrophils. Neutrophils were isolated from 9 human donors each was tested with a separate biological replicate of GBS.

2.5.10 Murine systemic infection model

6–8-week-old C57BL/6J male and female mice (Jackson Labs) were infected intraperitoneally (*i.p.*) with ~5x10^7 CFU WT COH1 or COH1Δ*htrA* GBS. At 24 hours post-infection, mice were euthanized and blood (cardiac), peritoneal fluid, lungs, spleen, and brain were collected. Solid organs were homogenized and samples were serially diluted and plated on TSA to enumerate CFU, which was normalized according to tissue weight or volume.

2.5.11 Murine pregnancy-associated ascending infection model

6–8-week-old C57BL/6J female mice (Jackson Labs) were paired 1:1 with isogenic male breeders for 48 hours. At approximately embryonic day 15, pregnant mice were infected intravaginally (*i.vag.*) with ~1x10^8 CFU WT COH1, COH1Δ*htrA*, COH1Δ*sip*, or COH1Δ*htrA*Δ*sip* GBS using a gel-loading pipette tip (Rainin). Following infection, mice were monitored for signs of preterm labor (vaginal bleeding or pups in cage). At preterm labor onset or 72 hours post-infection, dams were euthanized and maternal cardiac blood, lower genital tract, and uterus were collected. The proximal and distal pups and placentas in both uterine horns were also collected, and careful notes were taken regarding pup viability. Solid organs were homogenized and samples were serially diluted and plated on TSA to enumerate CFU. Although rare, any questionable colonies were patched onto ChromAgar StrepB (ChromAgar) and colony counts were adjusted to eliminate contaminants when necessary. GBS CFU were normalized according

to tissue weight in grams. Adverse outcomes were calculated out of the total number of pups born from all dams within the infection group. When pups were delivered overnight and pup carcasses were destroyed, the total number of pups born was assumed to be the average litter size for the corresponding infection group.

Tissue homogenates were incubated 1:1 overnight at 4°C in tissue lysis buffer [150 mM sodium chloride, 15 mM tris, 1 mM magnesium chloride, 1 mM calcium chloride, 1% (v/v) Triton-x] containing cOmplete mini protease inhibitor tablets (1 tablet per 5 ml, Roche). Tissue debris was pelleted by centrifugation at 3,000 x *g* for 15 minutes and supernatants were stored at -80 for later analysis.

2.5.12 Luminex analysis of murine tissue lysates

Tissue lysates reserved from murine infections were thawed and centrifuged at 11,000 x *g* for 15 minutes to eliminate residual tissue debris. Luminex assays were performed according to manufacturer's instructions (Mouse ProcartaPlex Multiplex immunoassay, ThermoFisher), using 25 µl of tissue lysates to quantify the levels of 11 immuno-analytes (Gro-α/KC, IFN-γ, IL-1β, IL-10, IL-12p70, IL-6, MCP-1, MIP-1α, MIP-1β, MIP-2α, and TNF-α). Plates were run on an xMAP 200 Luminex machine (BioRad) and data was processed using BioPlex Manager (BioRad). Analyte concentrations were normalized according to tissue weight in grams.

2.5.13 Sample preparation for TMT proteomics

100 µg of GBS protein fractions (1 µg/µl) in 100 mM ammonium bicarbonate were reduced by tris (2-carboxyethyl) phosphine to (final concentration 5 mM) for 15 minutes at room temperature, then alkylated by 10 mM 2-chloroacetamide in the dark for 30 minutes at room temperature. Excess 2-chloroacetamide was quenched by dithiothreitol (10 mM final concentration) at room temperature for 30 minutes. Proteins were digested by 1 µg rLys-C

(Promega) for 4 hours at 37°C, followed by 1 µg of trypsin (Promega) at 37°C overnight. The peptides were desalted over Oasis HLB C18 1 cc cartridges (Waters) and dried in a SpeedVac.

Next, the peptides were TMT-labelled to allow multiplexing. To this end, 800 µg TMT10plex isobaric tag labeling reagents (Pierce) were prepared in 30 µl acetonitrile and added to the digested peptide solution [30% (v/v) final organic concentration]. Each biological replicate for each GBS strain was given a unique isobaric tag to allow pooled fractions to be analyzed in 3 runs (9 experimental samples per run). A standard comprised of equal amounts of each sample's un-tagged peptide mixture was labeled with the final isobaric tag to allow normalization across each run. The peptide mixtures were incubated with their isobaric tag for 1 hour at room temperature. To check labeling efficiency, 2 µg from each sample was combined, dried, run over a C18 ZipTip (Millipore), and analyzed via LC/MS to determine equalization amounts. The sufficiently labeled reactions were quenched with 0.3% (v/v) hydroxylamine for 15 minutes, then the TMT-labeled samples were pooled by fraction (1:1 ratio based on equalization amounts), and concentrated on a SpeedVac to remove acetonitrile. A 20% fraction of the dried pool was desalted over an Oasis HLB C18 1cc cartridge (Waters) and dried with a SpeedVac.

For basic reverse phase (bRP) fractionation, each dried and de-salted TMT-labeled pool was resuspended in 100 µl 10 mM ammonium bicarbonate (pH 8) and acetonitrile (95:5, v/v), and loaded onto a Zorbax Extend-C18 column (2.1 mm x 150 mm, 5 µm; Agilent) connected to a Vanquish Horizon UHPLC (ThermoFisher). The sample was gradient-eluted at a flow-rate 250 µl/min over 55 minutes with a combination of 10 mM ammonium carbonate (solvent A) and acetonitrile (B) using the following gradient: 0 to 5 minutes B was held at 1%, 5 to 55 minutes B varied from 5% to 40%, 55-60 minutes B gradually increased to 90% B, then held at 90% B from 60-65 minutes. The UV signal was monitored at 210 nm. Ninety-six 50 second fractions were collected and combined into 24 pools by concatenation, where every 24th fraction was combined into a pool. The final pools were vacuum centrifuged to near-dryness and resuspended in 45 µl of 2% acetonitrile in 0.1% formic acid for LC-MS analysis.

2.5.14 Liquid chromatography-mass spectrometry

1.5 µl of each TMT-labeled concatenated pool were loaded for liquid chromatography/electrospray ionization-mass spectrometry (LC/ESI-MS/MS) using an Easy1200 nLC system (Thermo Scientific) coupled to a Orbitrap Eclipse Tribrid mass spectrometer with FAIMS Pro interface (Thermo Scientific). In-line de-salting was accomplished using a reversed-phase trap column (100 µm x 20 mm) packed with Magic C18AQ resin (5 µm, 200 Å; Michrom Bioresources) and peptides were separated by a reversed-phase column (75 µm x 270 mm) packed with ReproSil-Pur C18AQ (3-µm, 120 Å resin; Dr. Maisch HPLC) directly mounted on the electrospray ion source. A 180-minute gradient from 4% to 44% B at a flow rate of 300 nl/minute was used for chromatographic separations. A spray voltage of 2300 V was applied to the electrospray tip in-line with a FAIMS Pro source using varied compensation voltage (-40 V, -60 V, -80 V) while the instrument was operated in data-dependent mode. MS survey scans were performed in the Orbitrap (normalized AGC target value 300%, resolution 120,000, max injection time auto) with a 3 second cycle time. MS/MS spectra were detected in the linear ion trap (normalized AGC target value 100%, max injection time 50 ms) by collision-induced dissociation (CID) activation with a normalized collision energy (NCE) of 35% using turbo speed scan. Selected ions were dynamically excluded for 60 seconds after a repeat count of 1.

Following MS2 acquisition, spectra were searched in real time against a *Streptococcus agalactiae* COH1 protein database (HG939456_1_StrepAgalactiae_COH1_NCBI_102521.fasta) using COMET (267). Searches were performed for trypsin specificity with maximum missed cleavages set to 1. Oxidation (+15.9949 Da on M) was set as a dynamic modification. Static modifications included TMT (+229.1629 Da on K) and carbamidomethyl (+57.0215 Da on C). Maximum search time was 35 ms. Scoring thresholds were set to the following: Xcorr 1.4, dCn 0.1, precursor PPM 10, and charge state 2. Synchronous precursor selection (SPS)-MS3 was collected on the top 10 most intense ions detected in the MS2 spectrum, and SPS-MS3 precursors

were subjected to higher energy CID fragmentation with NCE of 65% and analyzed using the Orbitrap (normalized AGC target value 400%, resolution 50,000, maximum injection time 86 ms).

2.5.15 TMT proteomics data analysis

Quantitative proteomic data analysis was performed using Proteome Discoverer 2.5 (Thermo Scientific) and searched against a *Streptococcus agalactiae* COH1 protein database (HG939456_1_StrepAgalactiae_COH1_NCBI_102521.fasta) appended to include common contaminants (Contaminant Repository for Affinity Purification, cRAPome) (268). Searches were performed for trypsin specificity with maximum missed cleavages set to 2. Precursor ion tolerance and fragment ion tolerance were set to 10 ppm and 0.6 Da, respectively. Dynamic peptide modifications included oxidation (+15.995 Da on M) and dynamic modifications on the protein terminus included acetyl (+42.-11 Da on N-terminus), Met-loss (-131.040 Da on M), and Met-loss+Acetyl (-89.030 Da on M), plus static modifications TMT (+229.1629 Da on any N-terminus), TMT (+229.1629 Da on K), and carbamidomethyl (+57.0215 on C). Database searches were performed by SEQUEST HT and results were run through Percolator for peptide validation and false discovery rate calculation.

Raw data were normalized by Proteome Discoverer and intensities were adjusted by adding a value of 1 to each intensity to avoid missing values. *P*-values for pairwise comparisons with adjusted intensity were calculated by t-test. Fold-change ≥ 1.5 and P-value ≤ 0.05 were used to identify significant changes in abundance. Data was log-transformed (Log_2FC or $-Log_{10}P$) to produce volcano plots in GraphPad Prism (version 10.1.0).

Kyoto Encyclopedia for Genes and Genomes (KEGG) functional annotations were assigned to the *Streptococcus agalactiae* COH1 genome (GBCO_p1) using FACoP.v2 (Functional Annotation and Classification of Proteins of Prokaryotes). An additional virulence category was produced through to PubMed database searches.

2.5.16 Purification of recombinant HtrA

A C-terminal His6-tagged HtrA variant was constructed using the expression vector pET32CK as follows (269). Primers noted in parentheses are referenced in **Table 2.2** (pg. 51). NcoI and NotI linearized pET32CK and *htrA* open reading frame amplified from COH1 genomic DNA (*htrA* prot F/R) were combined via Gibson assembly to generate pET32CK-*htrA*-H6. An HtrA^{S237A} catalytic mutant was achieved by replacing Ser237 with Ala by site directed mutagenesis using the QuikChange II kit (Agilent) (SDM*htrA*^{S237A} F/R), producing pET32CK-*htrA*^{S237A}-H6. The resulting vectors were sequence validated and transformed directly into BL21(DE3) gold cells (Agilent).

For induction of His6-tagged HtrA^{S237A} (HtrA^{S237A}-H6), *E. coli* was grown at 37°C in LB supplemented with ampicillin. At OD_{600} of 0.6, cells were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 hours at 37°C. Cells were harvested by centrifugation for 20 minutes at 6,500 x *g* at 4°C and resuspended in urea solubilizing buffer [8 M urea, 0.1 M sodium phosphate (pH 8), 500 mM sodium chloride, 30 mM imidazole] and solubilized overnight at 4°C. Lysates were clarified by centrifugation at 23,000 x *g* for 30 minutes at 4°C and HtrA^{S237A}-H6 was purified under denaturing conditions by affinity chromatography using Ni-NTA resin (Qiagen). The resin was washed in urea solubilizing buffer containing decreasing amounts of urea (8 to 0 M) to allow protein re-folding. Following 0.5 M imidazole elution, HtrA^{S237A}-H6 was reconstituted in 50 mM sodium phosphate buffer (pH 8) and stored at -80°C until use. HtrA^{S237A}-H6 was used for production of HtrA-specific polyclonal rabbit antiserum as a service by Lampire Biologicals.

Due to rapid self-cleavage of HtrA (**Supplemental Figure 2.4**), an alternative purification strategy was necessary for purification of HtrA-H6. In brief, *E. coli* was induced by 0.1 mM IPTG at an OD_{600} of 0.6 for 1.5 hours at 37°C. The cell pellet was resuspended in BugBuster protein extraction reagent (Millipore) and lysed according to manufacturer instructions. HtrA-H6 protein was then purified under native conditions by affinity chromatography using Ni-NTA resin at 4°C.

HtrA-H6 was washed [0.1 M sodium phosphate (pH 8), 500 mM sodium chloride, 30 mM imidazole], eluted as described above, and stored at -80°C until use.

2.5.17 HtrA quantification

Rapid self-cleavage of HtrA-H6 during and after purification typically led protein yields below the detection limits of Bradford and BCA quantification methods. Therefore, quantitative Western blot was employed to quantify HtrA-H6 for downstream enzymatic analysis. In short, 20 ng HtrA^{S237A}-H6 [as quantified by BCA (Pierce)] was separated alongside 1 µL of HtrA-H6 by SDS-PAGE. Subsequent Western blot analysis used rabbit polyclonal HtrA antiserum as described above. Densitometry analysis was performed using Fiji imaging analysis software, and HtrA-H6 concentration was calculated based on signal intensity of HtrA^{S237A}-H6 protein standard (270).

2.5.18 Protease cleavage assays

To assess the activity of HtrA variants against a model protease substrate, 10 µg of β-casein was mixed with 10 ng of HtrA-H6 or HtrA^{S237A}-H6 in 50 mM tris-HCl (pH 7.5) overnight at 37°C. Reactions were inactivated via denaturation by boiling in 1x lithium dodecyl sulphate buffer (LDS sample buffer, Invitrogen). Reactions containing only β-casein, only HtrA-H6, or only HtrA^{S237A} -H6 served as controls. For analysis of the resulting banding pattern, 10 µl of each reaction was separated by SDS-PAGE and stained overnight with PageBlue protein staining solution (Thermo Scientific). Band sizes were determined by comparison to PAGERuler pre-stained protein ladder (ThermoFisher). Assay was repeated in biological triplicate and a representative image is shown. Fiji imaging analysis software was used to quantify the relative abundance of full-length versus cleaved β-casein by densitometry (270).

To assess HtrA activity against an endogenous GBS protein, the assay above was modified to instead use Sip (MyBioSource) as a substrate. All other assay parameters were unchanged.

2.5.19 N-terminal sequencing of cleaved Sip peptides

Cleavage assays were prepared as described above using 50 of µg Sip and 10 ng of HtrA-H6 or HtrAS237A-H6. 40 µl of the inactivated reactions were separated by SDS-PAGE and transferred to PVDF (Immobilon-P, Millipore). The membrane was rinsed, stained with Coomassie-R250, and de-stained in 10% (v/v) acetic acid and 40% (v/v) methanol. The membrane was imaged on a light box and bands representing cleaved Sip peptides were excised.

Washed PVDF membrane slices were sequenced on a PPSQ-53A Protein Sequencer (Shimadzu) at the Iowa State University Protein Facility. The highest likelihood amino acids for 5 consecutive cycles were used to identify all possible N-terminal sequences, which were searched against the Sip amino acid sequence. Sites matching 4/5 or 5/5 possible amino acids were used to generate a probability-weighted Kullback-Leibler Logo plot (Hobohm clustering threshold of 0.8, default pseudocounts) in Seq2Logo 2.0 (271). The sequence was then extended 5 amino acids from the putative cut site in the opposite direction, producing another Logo plot of the resulting C-terminal motif.

2.5.20 Endotoxin quantification in recombinant GBS proteins

Recombinant GBS proteins produced in *E. coli* were assessed for endotoxin contamination using the Pierce Chromogenic Endotoxin Quant Kit according to manufacturer recommendations (Thermo Scientific).

2.5.21 Toll-like receptor stimulation and NF-kB SEAP reporter assay

To assess activation of TLR2 and TLR4 by cleaved and uncleaved Sip, HEK293 cells expressing hTLR2, CD14 co-receptor, and secreted embryonic alkaline phosphatase (SEAP) (HEK-Blue hTLR2 cells, InvivoGen) and HEK293 cells expressing hTLR4, adaptor proteins CD14 and MD2, and SEAP (HEK-Blue hTLR4 cells, InvivoGen) were cultured according to manufacturer's recommendations. An NF-kB SEAP reporter assay was performed as described

with some adjustments (213). Cells were seeded at $1x10^5$ in a 96-well plate and grown overnight in serum-free media. Cells were stimulated with 5 µg of Sip co-incubated for 48 hours with 5 ng of HtrA-H6 or HtrAS237A-H6 in 50 mM tris-HCl (pH 7.5). Control wells were treated with an equal amount of full-length Sip only, either HtrA variant alone, or buffer. Standard curves of LPS-EK (InvivoGen) were used as a positive control. After 48 hours of stimulation, SEAP assay was performed using 20 µl cell supernatants and 180 µl QUANTI-Blue solution (InvivoGen). Wells were developed for 15 minutes at 37°C and absorbance at 655 nm was recorded using a SpectraMax i3D plate reader (Molecular Devices).

2.5.22 Statistical analysis

Statistical tests are noted in methods sections and figure legends. In general, use was determined based on the number of conditions being compared and the data distribution (i.e., parametric versus non-parametric). For all statistical tests, $p < 0.05$ was used for determining significance, although some figures show trending values when the sample rage is broad or when many groups are being compared. For proteomics analysis, an additional cut-off of fold-change ≥ 1.5 was also used. Tests were performed using GraphPad Prism, version 10.1.0.

2.6 Acknowledgements

We thank Erin Sweeney for assistance with COH1Δ*sip* cloning. Andrew Frando shared valuable insights and general molecular biology support throughout the study, and Sean Windle provided opinions for molecular biology and bioinformatic optimization. We thank Seattle Children's Research Institute's Office of Animal Care staff for their diligent support of animals and animal researchers, and Joel Nott and the Iowa State University Protein Facility staff for providing N-terminal sequencing workflow and analysis. This study was funding by NIH grants R01AI167421, R01AI112619, R01AI133976, R01AI145890, R01AI152268 to LR; NIH training grant T32AI007509 to AB (PI: Lee Ann Campbell); and Seattle Children's Research Institute seed

funds to LR. Protein domain cartoon, experimental schematics, and model figures were created using BioRender.com under the Seattle Children's Research Institute Center for Global Infectious Disease Research's institutional license.

2.7 Author contributions

AB, RS, AO, PRG, and LR designed the experiments. AB, GW, SN, RS, and AO performed experiments with assistance from KS, HJK, MC, RZ, and PQ. Data was analyzed by AB, GW, SN, RS, AO, and CL. The manuscript was written by AB, RS, and LR with some excerpts provided by GW, LAJ, CW, and PRG. All authors provided edits prior to final submission. Funding for this project was secured by LR and AB.

CHAPTER 3. A recombinant alpha-like protein subunit vaccine (GBS-NN) provides protection in murine models of Group B Streptococcus infection

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Brokaw, A., Nguyen, S., Quach, P., Orvis, A., Furuta, A., Johansson-Lindbom, B., Fischer, P. B., & Rajagopal, L. (2022). A Recombinant Alpha-Like Protein Subunit Vaccine (GBS-NN) Provides Protection in Murine Models of Group B Streptococcus Infection. *Journal of Infectious Diseases*, 226(1), 177–187. DOI: 10.1093/infdis/jiac148

3.1 Abstract

Group B Streptococcus (GBS) transmission during pregnancy causes preterm labor, stillbirths, fetal injury, or neonatal infections. Rates of adult infections are also rising. The GBS-NN vaccine, engineered by fusing N-terminal domains of GBS alpha C and Rib proteins, is safe in healthy, non-pregnant women, but further assessment is needed for use during pregnancy. Here, we tested GBS-NN vaccine efficacy using mouse models that recapitulate human GBS infection outcomes. Following administration of GBS-NN vaccine or adjuvant, antibody profiles were compared by ELISA. Vaccine efficacy was examined by comparing infection outcomes in GBS-NN vaccinated versus adjuvant controls during systemic and pregnancy-associated infections, and during intranasal infection of neonatal mice following maternal vaccination. Vaccinated mice had higher GBS-NN-specific IgG titers versus controls. These antibodies bound alpha C and Rib on GBS clinical isolates. Fewer GBS were recovered from systemically challenged vaccinated mice versus controls. Although vaccination did not eliminate GBS during ascending infection in pregnancy, vaccinated dams experienced fewer in utero fetal deaths. Additionally, maternal vaccination prolonged neonatal survival following intranasal GBS challenge. These findings demonstrate GBS-NN vaccine efficacy in murine systemic and perinatal GBS infections and suggests that maternal vaccination facilitates the transfer of protective antibodies to neonates.

3.2 Introduction

The gram-positive bacterium Group B *Streptococcus* (GBS, *Streptococcus agalactiae*) is a leading cause of fetal and neonatal morbidity and mortality (11). Systemic GBS infections in non-pregnant adults are also rising in frequency (15). GBS commensally resides in the rectovaginal tract in ~25% of women (11, 12). During pregnancy, ascending GBS infection leads to preterm labor, stillbirth, and fetal injury (8, 11). Alternatively, aspiration of GBS-infected fluids during labor and delivery leads to neonatal pneumonia, sepsis, and meningitis (9, 145). Perinatal GBS colonization is the primary risk factor for adverse pregnancy outcomes and neonatal invasive disease (8), and thus strategies that inhibit GBS transmission are needed.

Intrapartum antibiotic prophylaxis (IAP) lowers the risk of GBS transmission when administered to GBS-colonized mothers during labor and delivery, preventing cases of early onset disease within the first week of life. However, varied screening and IAP policies contribute to the disproportionate burden of GBS (16). Further, IAP is administered too late to target *in utero* transmission and too early to target postnatal transmission. Thus, IAP cannot prevent GBS-associated preterm labor, stillbirth, and fetal injury, or late-onset disease (occurring after 7 days of age). A GBS vaccine would lead to larger reductions in disease prevalence (11).

Currently, there is no licensed vaccine to prevent GBS colonization or infection (272). Most vaccine efforts have focused on serotype-variable capsular polysaccharide (CPS), although multivalent formulations have variable efficacy in preclinical models (137, 273-280) and human trials (138, 281-288). In humans, type III CPS vaccination failed to eradicate GBS from pre-colonized women, but reduced rates of new acquisition by about 40% for serotype III GBS. There was no effect on non-vaccine serotypes (287). While maternal vaccination permits CPS-specific antibody transfer to the infant (283-285), only 50-80% of vaccinated mothers transferred protective levels (281, 283, 284, 289). A major challenge is that an optimal formulation would incorporate of all 10 CPS to provide full coverage against all encapsulated GBS strains. GBS serotype prevalence varies geographically (9, 12, 15, 289, 290), so prevalent CPS variants may

suffice but may not be universally applicable. Additionally, suboptimal vaccines may select for strains expressing non-vaccine CPS (290-292). Conserved GBS surface proteins provide alternative strategies which may generate improved antibody potency against clinically relevant GBS strains.

MinervaX's GBS-NN vaccine incorporates structural components of GBS alpha-like proteins (Alps). This protein family includes six allelic and chimeric variants, alpha C (αC), Rib, and Alp1-4 which are associated with particular GBS serotypes and clonal complexes (293). Adjacent to a C-terminal cell wall-anchoring motif, Alps contain tandem repeats that vary in number and sequence. The N-terminus contains domains that bind to epithelial cells (69-72) and αC N-terminus deletion attenuated virulence in neonatal mice (294), emphasizing this domain's role in GBS virulence.

In humans, low naturally occurring αC- or Rib-specific titers in maternal and neonatal serum correlated with neonatal disease progression (295). Analysis of individual Alp domains revealed that repeat-specific antibodies are immunodominant (295, 296) and that tandem repeat number is inversely related to protection (297). Alp N-termini are more immunogenic in the absence of tandem repeats, and N-terminal vaccination extended murine survival following lethal challenge (296). Consequently, MinervaX's GBS-NN vaccine was engineered to contain fused N-termini from αC and Rib (**Figure 3.1**) (296). This prototype vaccine has completed phase I trials in non-pregnant women (ClinicalTrials.gov NCT02459262), and was safe, well-tolerated, and highly immunogenic (141). While trials to evaluate GBS-NN safety during pregnancy are underway, efficacy has not been tested.

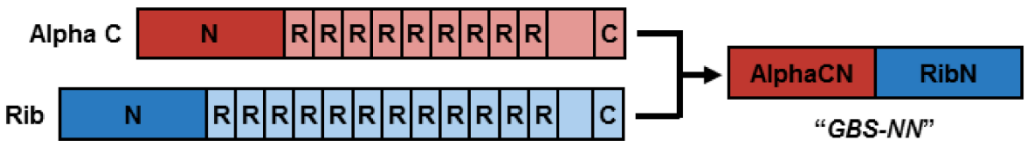


Figure 3.1. Schematic of GBS-NN vaccine antigen composition. The N-terminal (N) domains from the prototype alpha-like proteins (Alps), Alpha C and Rib, were fused to create the GBS-NN peptide antigen. This antigen does not contain tandem repeat (R) or C-terminal (C) domains from either protein. This figure is reused with permission (2) (CCC license # 5774460237872).

We sought to examine GBS-NN vaccine efficacy using GBS mouse models which emulate human disease outcomes. We observed robust production of vaccine-specific antibodies that recognized native forms of these antigens on clinical isolates. These antibodies were associated with reduced GBS recovery from disseminated sites following systemic infection. While GBS-NN vaccination did not confer differences in GBS CFU in maternal and fetal tissues following vaginal challenge, pregnant immunized dams had reduced rates of intrauterine fetal death (IUFD), and maternally vaccinated dams delivered neonates that experienced improved survival following intranasal GBS challenge. These findings demonstrate that, in mice, GBS-NN vaccination elicits the production of antibodies that can protect against multiple GBS serotypes and disease outcomes. Combined with human clinical trial data (141, 143), these findings support the need for further assessment of GBS-NN efficacy during pregnancy in human clinical trials.

3.3 Results

3.3.1 GBS-NN immunization induces a strong vaccine-specific antibody response in mice

To interrogate GBS-NN immunogenicity in mice, male and female C57BL/6J mice were administered three doses of GBS-NN vaccine (alum + GBS-NN) or adjuvant (alum + PBS) every two weeks. Blood was collected prior to each dose and at the study's endpoint (day 48) (**Figure 3.2A**). Serum titers were compared between vaccinated and control mice who received adjuvant. We expected that vaccination would elicit vaccine-specific antibodies, which would increase in abundance over time. Hence, we performed ELISA to quantify GBS-NN-specific IgG titers from serum collected throughout the course of vaccination. As expected, GBS-NN vaccinated mice produced GBS-NN-specific IgG. As represented by area under the ELISA curves, abundance of these antibodies significantly increased in the vaccinated sera over time, while no changes were observed in control sera (**Figure 3.2B**). Overall, immunized mice exhibited a substantial (580-fold) increase in GBS-NN-specific IgG titers versus controls (**Figure 3.2C**). Together, these results indicate that the GBS-NN vaccine is highly immunogenic in mice.

3.3.2 GBS-NN-specific antibodies bind to GBS strains of multiple CPS serotypes

A major benefit of a GBS-NN vaccine is its potential for broader coverage of GBS clinical isolates. GBS-NN-specific antibodies should bind both αC- and Rib-expressing GBS, rendering protection against clinically relevant serotypes. We assessed reactivity of GBS-NN-specific antibodies against αC- and Rib-expressing strains representing serotype Ia (strain A909) and serotype III (strain BM110) by performing whole GBS ELISA. Serum from GBS-NN vaccinated mice bound to GBS A909 and BM110 with modest 1.5-fold difference between strains. In contrast, limited binding was observed with adjuvant control sera (**Figure 3.2D**). These data suggest that GBS-NN vaccination may protect against multiple serotypes expressing either αC or Rib.

3.3.3 GBS-NN immunization reduces bacterial burden during GBS systemic challenge

The studies above indicate that GBS-NN elicited a strong vaccine-specific antibody titer in our mice, confirming previous observations (296). We next evaluated how GBS-NN immunization affects GBS dissemination in adult mice. Male and female C57BL/6J mice (n=16-17/group) were vaccinated as described above, then infected intraperitoneally (*i.p.)* with $1x10^8$ CFU of A909 GBS a week later. Mice were euthanized 48 hours post-infection, and blood, peritoneal fluid (Pf), lungs, spleen, and brain were collected and processed for CFU enumeration (**Figure 3.3A**). Following GBS challenge, vaccinated mice exhibited significantly reduced bacterial burden in the spleen, lungs, and brain versus controls, with lower CFU (albeit not significant) in the Pf and blood (p=0.2842 and 0.1877). Some mice in the control group were moribund or dead at necropsy (n=4), whereas all GBS-NN vaccinated mice survived exhibiting no signs of morbidity or mortality (**Figure 3.3B**). To correlate vaccine-specific antibodies with protection against systemic infection, we compared GBS CFU versus serum IgG titer from the

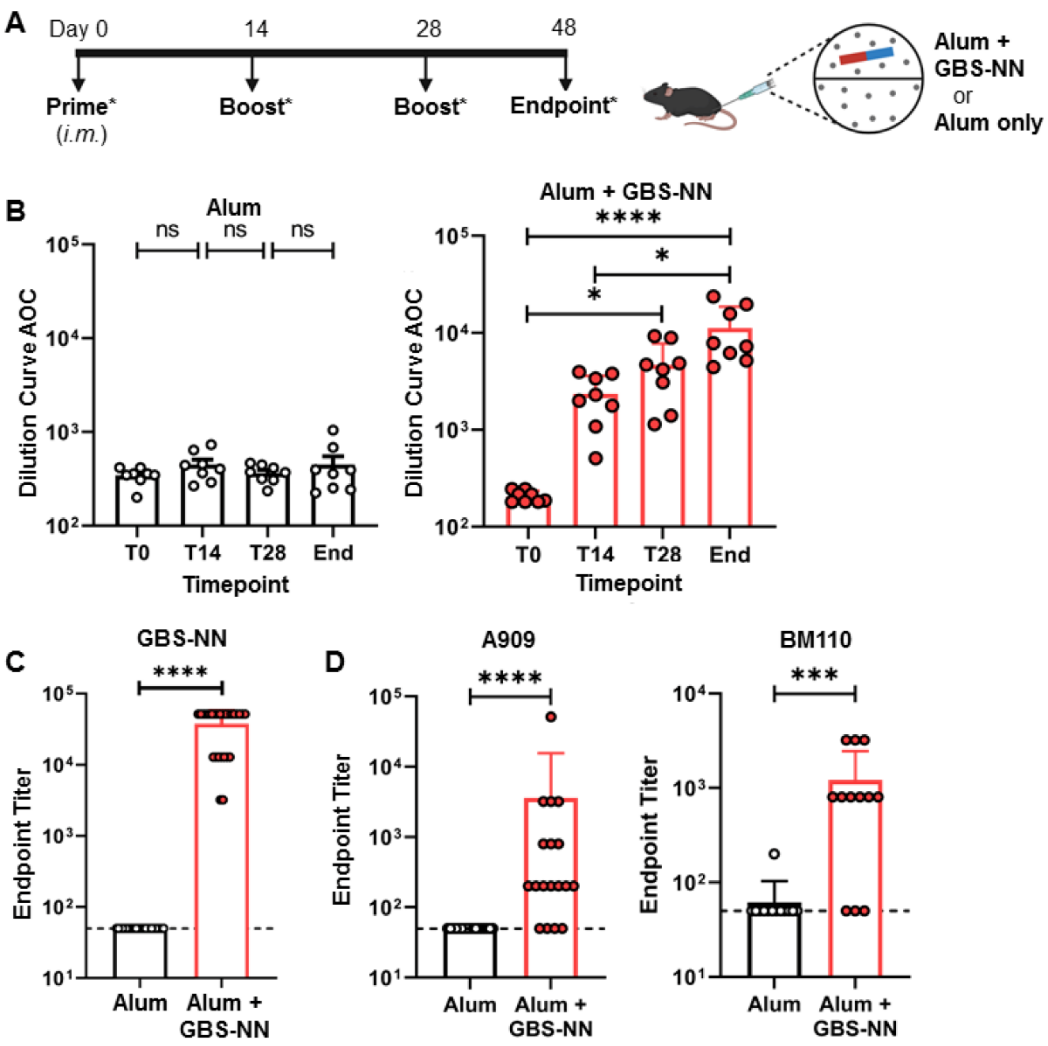


Figure 3.2. GBS-NN immunization elicits a strong vaccine-specific antibody response. **A)** Timeline for *i.m.* administration of full GBS-NN vaccine regimen, comprising of an initial prime followed by 2 boosts. Mice were stratified into alum + GBS-NN or an alum-only control group. Asterisks indicate serum collection. **B)** Serial serum samples isolated from alum control (left) or alum + GBS-NN (right) mice were analyzed by ELISA to measure GBS-NN–specific IgG. Graphs depict AUC calculated from dilution curves for each sample. Data represent the mean ± SD. Statistical differences were determined by Friedman test with Dunn multiple comparisons post hoc test (sample sizes: n = 8 alum, n = 8 alum + GBS-NN). **C)** An ELISA dilution curve for each mouse serum sample at T28 was used to quantify an end point titer of GBS-NN–specific IgG. Data represent the geometric mean ± SD. The dashed line denotes the limit of detection. Statistical differences were determined by Mann-Whitney test (sample sizes: n = 25 alum, n = 27 alum + GBS-NN). **D)** T28 serum samples isolated from alum control or alum + GBS-NN mice were analyzed by ELISA following adsorption to whole GBS (strains A909 and BM110) to quantify vaccine-specific IgG that bind native αC or Rib. Figure shows ELISA end point titers and data represent geometric mean ± SD. The dashed line denotes the limit of detection. Statistical differences were determined by Mann-Whitney test (sample sizes: A909 n = 17 alum, n = 18 alum + GBS-NN; BM110 n = 13 alum, n = 12 alum + GBS-NN): ns $P > .05$, * $P < .05$, *** $P < .001$, **** $P < .0001$. This figure is reused with permission (2) (CCC license # 5774460237872).

same mouse. Association of these variables was assessed by Spearman correlation. Having a higher GBS-NN-specific antibody titer was moderately and significantly associated with having

less GBS CFU in the spleen (p=0.0425, Spearman coefficient=-0.3792), and this relationship trended towards significance in the lungs and brain (p=0.0926 and 0.1004) (**Supplemental Figure B-1**). Thus, although sterilizing immunity (GBS clearance) did not occur, GBS-NN immunization was associated with low bacterial burden; thus, vaccination promoted protection against systemic GBS. We did not perform a survival study following vaccination, as this was reported previously (296).

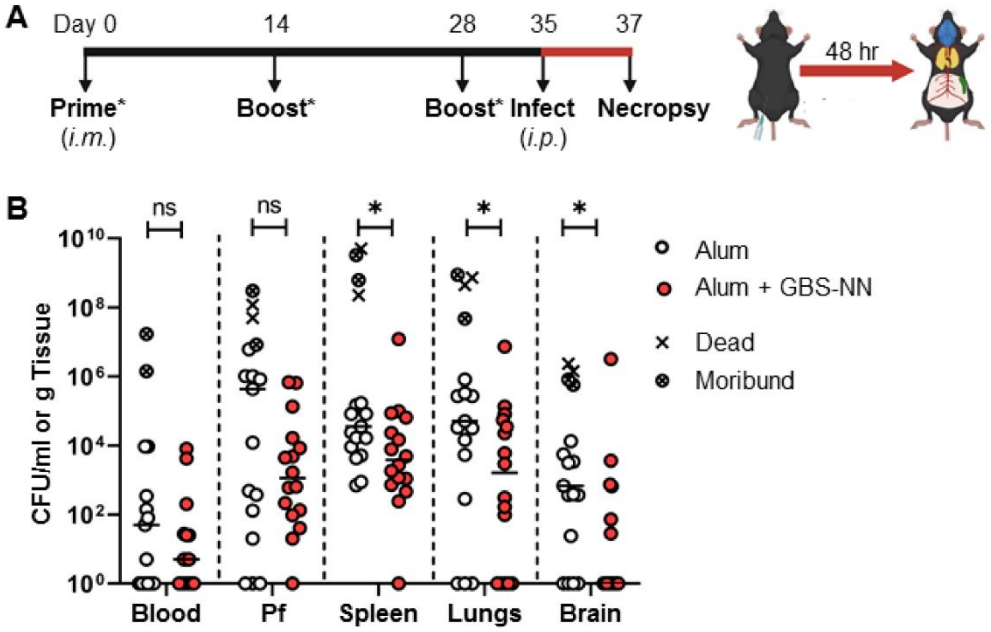


Figure 3.3. GBS-NN immunization diminishes GBS systemic infection. A) Timeline for systemic challenge of GBS following administration of GBS-NN vaccine regimen. Asterisks indicate serum collection. **B)** One week after the final boost, mice were systemically challenged (*i.p.*) with 10^8 CFU of GBS (strain A909). Bacterial burden was evaluated in blood, peritoneal fluids, lungs, spleen, and brain at 48 hours after GBS infection or earlier if mice exhibited morbidity. Data are shown as medians with circles representing values from individual mice. The Mann-Whitney test was used for comparison between groups (sample sizes: n = 17 alum, n = 16 alum + GBS-NN): ns $P > .5$, $* P < .05$. This figure is reused with permission (2) (CCC license # 5774460237872).

3.3.4 GBS-NN maternal immunization prevents in utero fetal demise

Since GBS-NN vaccination protected our mice against systemic GBS challenge, we hypothesized that it may also protect against ascending infection during pregnancy. To test this, vaccinated or adjuvant control female mice (n=9/group) were mated for pregnancy. On day 45 (embryonic day 14), pregnant dams were infected intravaginally (*i.vag.*) with 1x10^8 CFU of A909

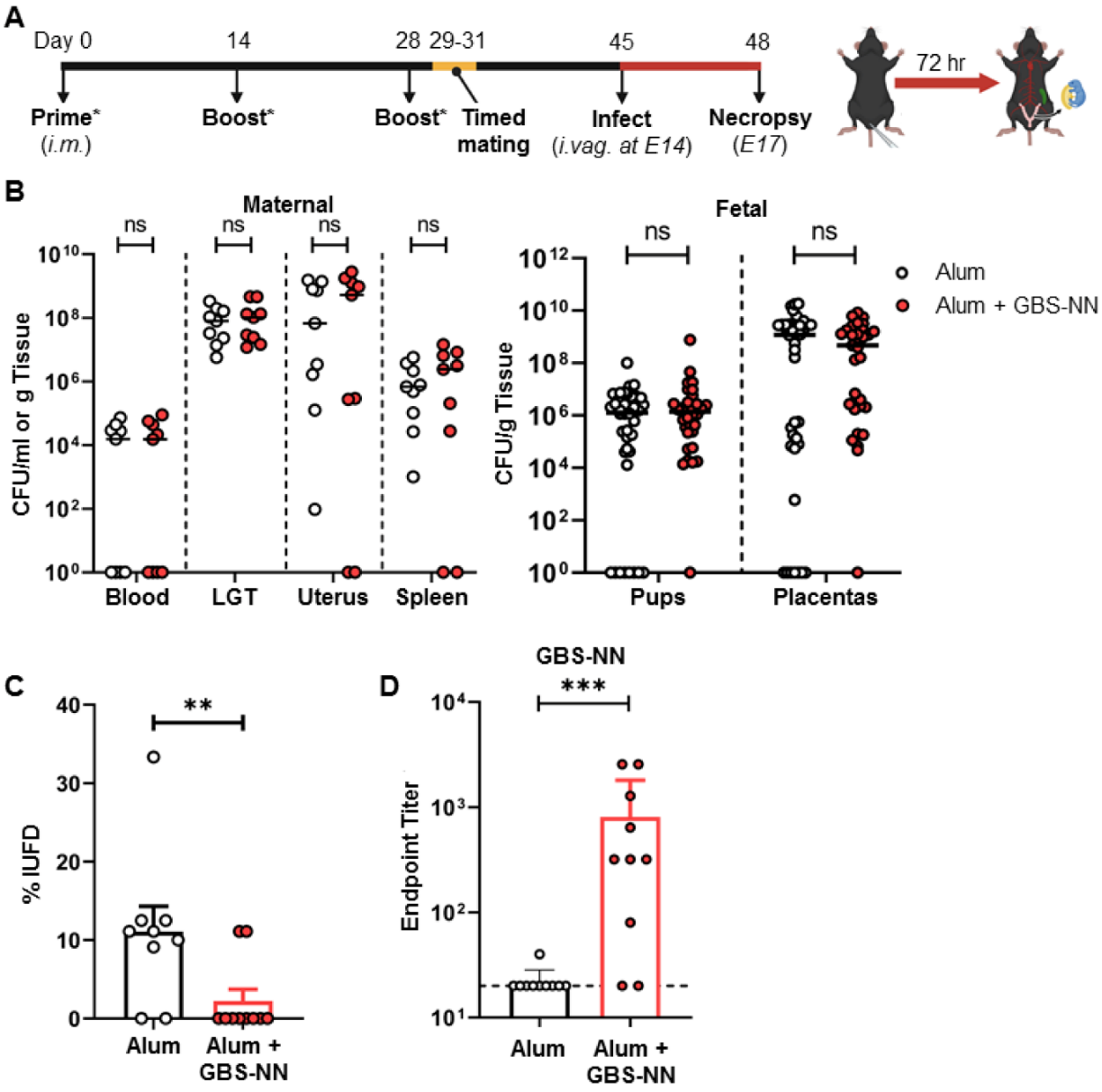


Figure 3.4. GBS-NN immunization diminishes fetal demise. A) Timeline for pregnancy-associated vaginal GBS challenge following administration of full GBS-NN vaccine regimen. After their final boost, mice were mated and monitored for pregnancy. On day 14 of pregnancy, mice were intravaginally challenged with 10^8 CFU of GBS (strain A909). Dams were euthanized 72 hours post-infection and blood, lower genital tract, uterus, spleen, and proximal and distal pups and their placentas were collected, homogenized, and plated for CFU enumeration. Asterisks indicate serum and vaginal lavage collection. **B)** Bacterial CFU obtained from maternal (left) or fetal (right) tissues. Medians are indicated with circles representing values from individual mice. Statistical differences were determined by Mann-Whitney test (sample sizes: n = 9 alum, n = 9 alum + GBS-NN). **C)** Upon necropsy, fetal viability was noted to calculate percent IUFD for each dam. Data are shown as mean ± SD and statistical differences were determined by Mann-Whitney test (sample sizes: n = 9 alum, n = 9 alum + GBS-NN). **D)** An ELISA dilution curve for each T28 mouse vaginal lavage sample was used to quantify an end point titer of GBS-NN-specific IgG. Graph shows geometric mean values ± SD. The dashed line denotes the limit of detection. Statistical differences were determined by Mann-Whitney test (sample sizes: n = 10 alum, n = 10 alum + GBS-NN): ns $P > .05$, ** $P < .01$, *** $P < .001$. This figure is reused with permission (2) (CCC license # 5774460237872).

tract (LGT), uterus, and spleen), plus fetal tissues (proximal and distal placenta and pups from the left and right uterine horns) were collected and processed for CFU enumeration (**Figure 3.4A**). Surprisingly, we observed no differences in GBS CFU from maternal or fetal tissues between vaccinated and adjuvant control mice (**Figure 3.4B**). However, GBS-NN vaccination led to significantly reduced IUFD rates (**Figure 3.4C**). Although GBS-NN-specific IgG titers were 15-fold higher in vaginal lavage fluids from vaccinated mice versus controls, they were nearly 100-fold lower than titers in vaccinated mouse serum (**Figure 3.4D** and **Figure 3.2C**). GBS-NN-specific mucosal IgA were not detected (data not shown). We assessed the relationship between vaccine-specific antibody titer and IUFD outcomes using Spearman correlation as described above, comparing each dam's IUFD rate versus their matched serum and vaginal lavage titers. Interestingly, reduced IUFD rates were significantly and moderately associated with having higher GBS-NN-specific IgG titers in the serum (p=0.0432, Spearman coefficient=-0.4562), but not in vaginal lavage (p=0.2676) (**Supplemental Figure B-2**). Under the conditions tested, although maternal GBS-NN immunization did not lead to significant CFU reductions in reproductive or fetal tissues, vaccine-specific serum IgG helped prevent fetal demise.

3.3.5 Maternal GBS-NN immunization results in vertical transmission of GBS-NN-specific antibodies to neonatal mice

To determine if GBS-NN maternal vaccination permits transfer of maternal vaccine-specific antibodies to neonates, vaccinated and adjuvant control female mice were mated for pregnancy as described. Dams were monitored until delivery (days 48-51), and half of each litter was euthanized on postnatal day 0 for serum (pooled by liter) (**Figure 3.5A**). GBS-NN-specific antibodies were quantified by ELISA, and significantly higher titers were noted from neonates born to vaccinated versus adjuvant mothers (**Figure 3.5B**). Thus, vaccine-specific maternal IgG transfer vertically to neonatal mice following maternal GBS-NN vaccination.

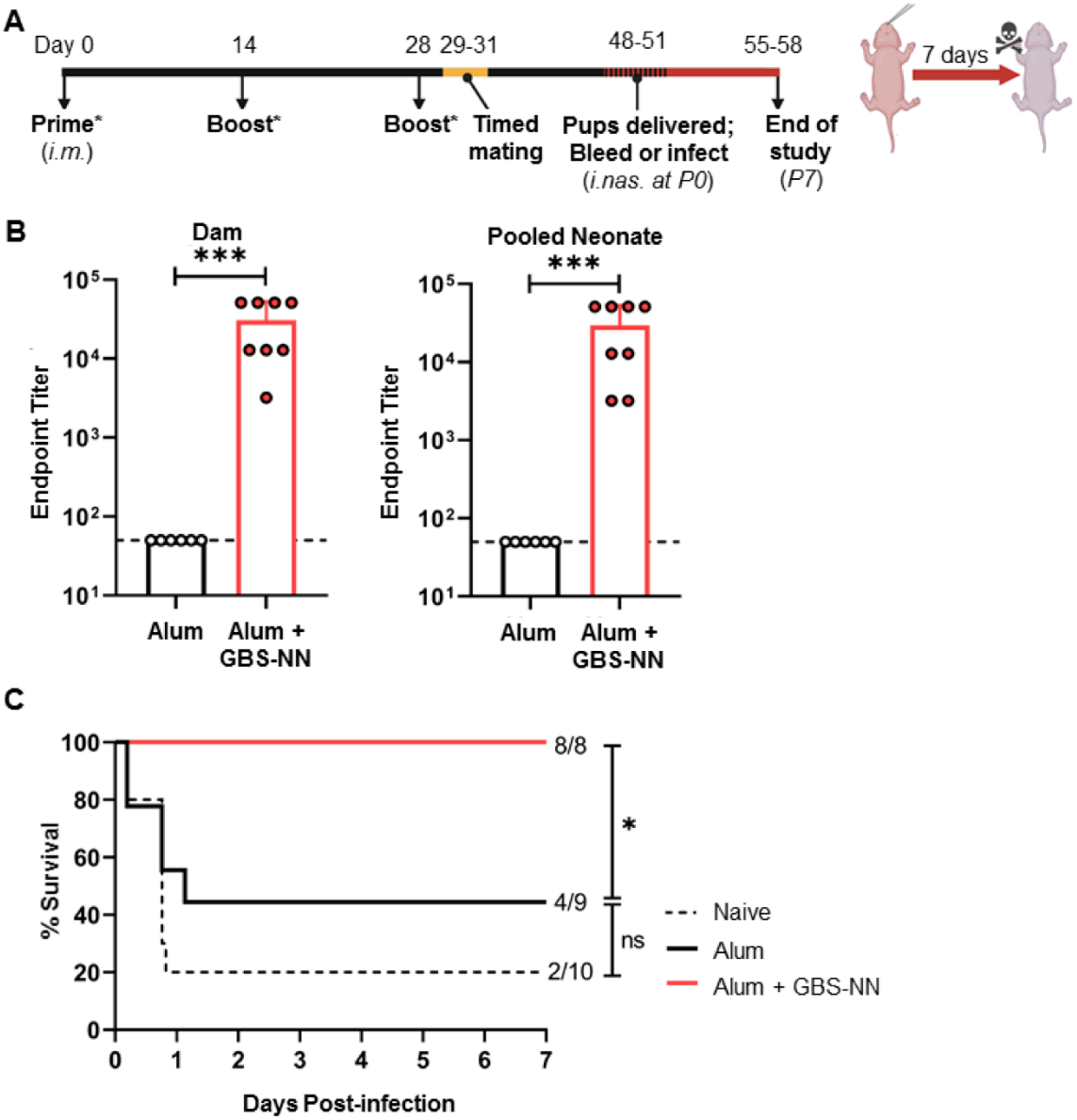


Figure 3.5. Maternal GBS-NN immunization confers neonatal protection against intranasal GBS challenge. A) Timeline for neonatal intranasal challenge following maternal GBS-NN immunization. After their final boost, mice were mated and monitored for pregnancy. Upon delivery, neonates from each litter were either euthanized for serum or intranasally challenged with 5×10^6 CFU of GBS (strain A909). Asterisks indicate maternal serum collection. **B)** GBS-NN–specific serum IgG end point titers from dam and neonate pairs. Data are depicted as geometric means ± SD. The dashed line denotes the limit of detection. Statistical differences were determined by Mann-Whitney test (sample sizes: n = 4 alum, n = 3 alum + GBS-NN). **C)** Survival of intranasally infected neonates was monitored for a week. Kaplan-Meier curve indicates survival of pups born to naive, alum control, and alum + GBS-NN immunized dams and survival ratios are shown. Statistical differences were determined using Log-rank test (sample sizes: n = 10 naive, n = 9 alum, n = 8 alum + GBS-NN): ∗ $P < .05$; ∗∗∗ $P < .001$. This figure is reused with permission (2) (CCC license # 5774460237872).

3.3.6 Maternal GBS-NN immunization confers neonatal protection against intranasal GBS challenge

To examine the functional consequence of maternal GBS-NN-specific antibodies, neonatal mice were challenged intranasally (*i.nas.*) with $5x10^6$ CFU of A909 GBS on postnatal day 0. Neonates were monitored for survival and euthanized after one week (**Figure 3.5A**). Neonates born to GBS-NN immunized dams experienced significantly improved survival following intranasal challenge, as 100% of these pups survived versus only 44% of those born to adjuvant controls (**Figure 3.5C**). Overall, these data suggest that maternal transfer of GBS-NN-specific antibodies contributes to protection against subsequent GBS neonatal infection.

3.4 Discussion

GBS is a significant cause of fetal and neonatal morbidity and mortality (11), and additionally causes invasive infections in healthy, non-pregnant adults which are increasing in prevalence (15). Current prophylactic strategies include IAP (16), although this strategy can only restrict transmission occurring during delivery. The development and licensing of a GBS vaccine would prevent GBS transmission by other routes, resulting in greater reductions in disease burden (272).

MinervaX's protein-based GBS-NN vaccine is promising and may confer broad protection against GBS. The formulation tested in this study contains a fusion peptide derived from αC and Rib N-termini combined with aluminum hydroxide, an adjuvant approved for use during pregnancy (298). Phase I immunogenicity and safety trials exhibit GBS-NN safety in healthy non-pregnant women, eliciting robust antibody production with minimal side-effects (141). Similar trials are underway in pregnant women but due to the disproportionate global burden of GBS and differences in screening and IAP policy, it will be difficult and expensive to pursue the corresponding efficacy trials (11, 16, 272). Here, we evaluated GBS-NN vaccine efficacy in mice

using three relevant GBS infection models that recapitulate common human disease manifestations.

Murine GBS-NN immunization elicited high vaccine-specific serum antibody titers, which increased with additional doses. These findings mirror human clinical trial findings (141). GBS-NN-specific antibodies generated through vaccination bound both purified GBS-NN and GBS strains representing canonical αC- and Rib-associated serotypes (299). Detection by GBS-NN-specific antibodies was similar between strains. While these results suggest that GBS-NN-specific antibodies may confer similar levels of protection against these and other GBS isolates, a previous study noted heightened protection against BM110 compared to A909 for mice that were either actively or passively immunized (296).

GBS-NN vaccination reduced burden of systemic GBS upon intraperitoneal challenge. This model recapitulates dissemination profiles and disease manifestations that occur in neonates and non-pregnant adults, including GBS bloodstream invasion during sepsis, pneumonia, and meningitis. In our systemic model, GBS-NN reduced overall bacterial dissemination but did not fully clear GBS. Despite this lack of clearance, our reduced dissemination is in line with previous observations that GBS-NN vaccination led to significantly extended survival following systemic infection of mice by a lethal GBS dose (296). In our vaginal challenge model during pregnancy, GBS burdens did not vary between vaccinated and control mice in the maternal reproductive tissues, placentas, and fetuses that were sampled. Despite this, it is exciting that fetal survival was improved for GBS-NN vaccinated dams, who experienced significantly reduced IUFD rates versus controls. Overall, these findings suggest that the GBS-NN vaccine is partially effective in these models.

The findings in our pregnancy-associated ascending infection model contrast with observations following systemic infection; however, this reflects the disparate GBS-NN-specific antibody responses detected in serum versus vaginal fluid. To determine whether improved vaccine-specific antibody responses are achievable in the murine vaginal tract, and to test

whether this may permit reductions in vaginal colonization, vertical transmission, or adverse outcomes, future studies should compare alternative formulations containing other adjuvants, such as CpG which stimulates mucosal immunity (300). Alternately, GBS-NN-elicited immunity may be too slow to impede murine ascending infection and vertical transmission given the short distance between the vaginal tract and placenta, thus resulting in modest protection. It is possible that GBS-NN immunization may more effectively inhibit ascension and transmission in humans, where trafficking to the uterus and invasion of fetal tissues likely takes longer.

Finally, maternal GBS-NN vaccination permitted maternal vaccine-specific antibody transfer to neonatal mice and enhanced neonatal survival upon GBS intranasal challenge, highlighting a protective role for maternally derived GBS-NN-specific antibodies. The intranasal route used in this study is biologically-relevant, and recapitulates human transmission through aspiration of GBS-infected fluids during delivery.

Overall, these data demonstrate MinervaX's GBS-NN vaccine efficacy in three distinct murine models that recapitulate varying aspects of human GBS disease. The GBS-NN prototype vaccine contains two of six Alps variants, but αC and Rib expression is strongly associated with serotypes Ia/Ib/II and II/III, respectively (301, 302), which comprise >50% of maternal colonizations (12) and most cases of neonatal invasive disease (9). Alps expression also varies by GBS sequence type (ST). Rib is well-associated with ST17 isolates that cause strikingly high rates of neonatal sepsis and meningitis (302); αC is common to ST12, among which serotype Ib isolates are multidrug resistant and cause fatal bloodstream infections (302). That GBS-NN may protect against these hypervirulent strains is promising. However, MinervaX's final formulation would include a second peptide (GBS-NN2) containing Alp1 (serotype 1a/V/VI and ST1/ST23) and Alp2/3 (III/V and ST1/ST23) N-terminal fusions (301, 302). While these associations undergo geographical variability, GBS-NN/NN2 is predicted to target strains expressing αC, Rib, Alp1, Alp2, or Alp3. Strains expressing non-vaccine Alp4 and Alps-deficient GBS are rarely observed in geographically-diverse surveillance studies (299, 301, 302). Thus, GBS-NN/NN2 represents a

promising alternative to CPS-based vaccines, which require more than two vaccine antigens to achieve similar coverage. GBS-NN/NN2 has completed phase I assessment (ClinicalTrials.gov NCT03807245), but future studies should similarly assess efficacy of the full vaccine in mice. This formulation will soon undergo a booster follow-up study (NCT05005247), and phase II trials in pregnant women and their babies in Denmark, United Kingdom, South Africa, and Uganda (NCT04596878, NCT05154578). These clinical trials may finally determine whether Alps-based vaccine strategies may facilitate global reductions in GBS disease burden; however, combined with our observations of murine efficacy, the future of this vaccine looks promising.

3.5 Methods

3.5.1 Ethics statement

Animal experiments were approved by Seattle Children's Research Institute's Institutional Animal Care and Use Committee (protocol IACUC00036), and performed per the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 8th ed.

3.5.2 Bacterial strains and growth conditions

The GBS clinical isolates A909 (303) and BM110 (304) were used for this study. GBS was cultured on tryptic soy broth agar or broth (Difco Laboratories) at 37°C with 5% CO2. Overnight cultures were diluted 1:10 and grown to an optical density at 600 nm (OD_{600}) of 0.3 for infections, or 1.0 for enzyme-linked immunosorbent assay (ELISA).

3.5.3 GBS-NN vaccine production

The GBS-NN antigen, composed through fusion of A909 αC and BM110 Rib N-termini, was generated as described (296) and stored in phosphate-buffered saline (PBS; pH 7.2) at 1.75 mg/mL. On immunization day, fresh formulations of vaccine [10 µg GBS-NN and 100 µg

aluminum hydroxide (Alhydrogel, InvivoGen) in PBS] and adjuvant (100 µg aluminum hydroxide in PBS) were prepared and stored on ice until administration.

3.5.4 Murine vaccination

Six- to 8-week-old male and female wildtype (WT) C57BL/6J mice were used for vaccination studies. On day 0, 50 µL of formulated vaccine or adjuvant were injected intramuscularly. Boosters were provided on days 14 and 28.

3.5.5 Mouse serum and vaginal lavage collection

To assess antibody responses, retro-orbital blood was collected on days 0, 14, and 28 prior to administration of vaccine or adjuvant. Unchallenged mice were euthanized on day 48 and cardiac blood was collected. Pooled or individual blood samples were transferred to serum separator tubes (gold tubes; BD), centrifuged (6000 x *g*, 90 seconds), and sera were stored at −80°C.

Vaginal lavage fluid was obtained by performing PBS washes with a micropipette tip (P10; Rainin). Lavage fluid was clarified by centrifugation (6000 x *g*, 2 minutes), and samples were stored at −80°C.

3.5.6 ELISA and endpoint titer calculation

High-binding, flat-bottom 96-well polystyrene plates (Costar) were incubated with GBS-NN (0.5 µg/mL in PBS; room temperature, 2 hours). Wells were washed with buffer (PBS + 0.05% Tween-20) and blocked with PBS + 1% bovine serum albumin (BSA; 37°C, 1 hour). Mouse serum was serially diluted 1:4 (1:50 to 1:51 200) in antibody diluent (PBS + 1% BSA + 0.05% Tween-20), and incubated (4°C, overnight). For vaginal lavage, serial 1:2 dilutions (1:20 to 1:640) were used. Wells were washed and biotinylated goat anti-mouse IgG (H + L) secondary antibody (1:40 000; Southern Biotech) was added (37°C, 1 hour). Wells were incubated with streptavidin-horseradish

peroxidase (1:200, room temperature, 45 minutes; R&D Systems), washed, and tetramethylbenzidine peroxidase substrate and solution B were added (1:1; KPL). Sulfuric acid stop solution was added (approximately 333 mM final concentration) upon sufficient color change. Absorbances were obtained at 450 nm using a plate reader (SpectraMax i3x; Molecular Devices), and dilution curves were generated. For each plate, a cutoff curve was made by calculating the average absorbances of the pre-immune (T0 or naive) serum plus 3 × the standard deviation of the controls at each dilution. The end point titer is the last dilution where a sample's absorbance is greater than that of the cutoff curve.

To quantify binding of GBS-NN–specific antibodies to whole GBS, methods were adapted for whole GBS ELISA (305). GBS (A909, BM110) at 5×10^8 CFU/mL in PBS was coated at 4°C overnight. The next day, wells were washed in wash buffer containing 0.02% sodium azide and remaining steps were performed as described above.

3.5.7 Murine systemic challenge model

Vaccinated or control male and female mice were challenged intraperitoneally as described (35). Mice were infected with approximately 1×10^8 CFU GBS (strain A909) on day 35 (relative to the vaccine schedule). At 48 hours post-infection or earlier (if exhibiting morbidity), mice were euthanized, and blood, peritoneal fluid, lungs, spleen, and brain were collected. Organs were homogenized. All samples were serially diluted and plated to enumerate GBS CFU.

3.5.8 Murine pregnancy-associated vaginal challenge model

Vaccinated or control mice were challenged intravaginally during pregnancy as described (82). The day following their final boost, female mice underwent timed pairing with isogenic males for 48 hours. Mice were monitored for signs of pregnancy including weight gain and palpation to detect pups. Pregnant mice were infected intravaginally on day 45 (relative to the vaccine schedule) with 1×10^8 CFU GBS (strain A909) using a micropipette tip (P10; Rainin). Infected

mice were monitored twice daily for signs of preterm labor (vaginal bleeding or pups in cage). At 72 hours post-infection, dams were euthanized and pup viability was noted. Maternal blood, lower genital tract, uterus, and spleen were collected. The 2 left and right proximal and distal pups and placentas were also collected. Organs were homogenized. All samples were serially diluted and plated to enumerate GBS CFU.

3.5.9 Maternal vaccination and neonatal intranasal challenge model

Vaccinated and control pregnant mice were monitored for litters between days 48 and 51 (relative to the vaccine schedule). Within 24 hours of birth (postnatal day 0), half of each litter was randomized into a bleed group to assess antibody transfer. Following decapitation, blood was collected into serum separator tubes, processed as described, and pooled by litter. The remaining neonates were infected intranasally with 5×10^6 CFU GBS (strain A909, 1 µL per nare). Infected pups were monitored for survival twice daily. Survivors were euthanized at postnatal day 7 and maternal blood was collected by cardiac bleed. Time-matched naive litters were bled for ELISA control serum.

3.5.10 Statistics

Statistical tests used are noted in the corresponding figure legend and a *P* value of <0.05 was considered significant. Tests were performed using GraphPad Prism, version 8.4.3.

3.6 Acknowledgements

We thank Lucas Senatore, Jessica Spaulding, and Timothy Gervasi for technical support throughout the study, the Seattle Children's Research Institute's Office of Animal Care staff for their tireless contribution to husbandry and vivarium maintenance (especially during the COVID-19 pandemic), and Connie Hughes for administrative support. This work was funded by NIH grants R01AI145890, R01AI152268, and R01AI133575 to LR; NIH training grants T32AI007509

to AB (PI, Lee Ann Campbell) and T32AI055396 to AF (PI, Ferric Fang); Seattle Children's Research Institute seed funds to LR; and funds provided by MinervaX A/S (to PF and BJ-L). Experimental timeline figures were created using BioRender.com under the Seattle Children's Research Institute Center for Global Infectious Disease Research's institutional license.

3.7 Author contributions

AB and LR designed the experiments. AB and SN performed the experiments with assistance from PQ, AO, and AF. Data analysis was performed by AB, SN, and LR, and AB and LR wrote the manuscript with input from all authors. Funding for this project was secured by LR, AB, AF, BJ-L, and PF.

CHAPTER 4. Conclusions and Future Directions

4.1 Summary of findings

The studies summarized in this dissertation emphasize the importance of membrane proteins in Group B Streptococcal biology. Proteins within this compartment are poised to facilitate important host-pathogen interactions that may be crucial to pathogenesis, but this comes at a cost. The regulation of protein trafficking to the membrane is tightly coordinated and mis-localization can be problematic for the cell. Further, membrane proteins that face the extracellular environment can be detected by the host.

In Chapter 2, I aimed to assess the impact of the GBS HtrA serine protease on virulence. We found that HtrA is important during both systemic and pregnancy-associated ascending infection of mice, specifically noting that HtrA-deficient GBS cause significantly fewer adverse pregnancy outcomes despite achieving high CFU burden in fetal tissues. We also identified numerous changes to the GBS proteome in response to altered levels of HtrA. Like observations for other Streptococci (182-185, 260, 306), this HtrA-dependent mechanism of regulation appears to primarily impact GBS membrane and secreted proteins, and also affected multiple GBS virulence factors. The changes in abundance of these virulence factors may help to explain the attenuation we observed *in vivo*. In particular, the abundance of Streptococcal surface immunogenic protein (Sip) was reduced in secreted fractions and exhibited dose-dependence with regard to *htrA* expression, leading us to identify Sip as a substrate that is directly cleaved by HtrA. While we do not fully understand the biological relevance of this interaction, it appears that HtrA-mediated Sip degradation may modulate its immunogenicity. This may serve as a novel and HtrA-dependent immune evasion strategy for GBS. Despite the mechanistic insights gained by this work, a variety of next steps will be highlighted in section **4.2**.

In Chapter 3, I validated efficacy of MinervaX's GBS-NN protein-based vaccine using murine models of GBS infection. In mice, GBS-NN immunization resulted in robust production of

GBS-NN-specific antibodies in the serum compared to adjuvant controls. In addition to binding the GBS-NN antigen, these vaccine-specific antibodies also bound native alpha-like proteins on the surface of GBS clinical isolates. Mice that were GBS-NN immunized also exhibited significant reductions in GBS bacterial burden upon systemic challenge versus those that received only adjuvant. Immunization had no effect on GBS burden in our pregnancy-associated ascending infection challenge model, but immunized dams experienced healthier pregnancies than adjuvant controls that resulted in significantly fewer cases of intrauterine fetal death upon GBS challenge. It is likely that GBS-NN immunization did not limit GBS levels in this model due to the more variable levels of GBS-NN-specific antibodies in vaginal fluids compared to serum. Maternal immunization of dams prior to pregnancy resulted in vertical transfer of GBS-NN-specific antibodies to their offspring, and pups born to immunized mothers exhibited significantly higher concentrations of vaccine-specific antibodies compared to those born to adjuvant controls. Finally, these maternal antibodies protected pups against GBS intranasal challenge. These findings validate partial efficacy of the GBS-NN vaccine in mice and suggest that maternal vaccination is likely a feasible strategy of neonatal protection against GBS. Further, we support the advancement of the GBS-NN vaccine in human clinical trials aimed to more systematically assess efficacy in people. The future directions outlined in **Table 4.1.2** will be described in section **4.3**.

4.2 Future directions for Chapter 2

Given our observations that HtrA regulates GBS virulence through multiple distinct mechanisms, there are many avenues left to explore. An obvious next step would be to interrogate the interactions between HtrA and other putative substrates identified in our proteomics screen**.** These and other follow-up experiments are listed in **Table 4.1**.

Table 4.1. Future directions for Chapter 2: "The serine protease HtrA cleaves surface immunogenic protein (Sip) and regulates Group B *Streptococcus* virulence."

Research topic	Experimental strategy
Mechanistic contributions of HtrA-mediated proteomic changes to attenuated virulence	– Construct GBS strains with deletions of various proteins that underwent HtrA-dependent changes in abundance. – Test the putative substrates above using HtrA cleavage assays. – Assess virulence phenotypes *in vivo*, and dissect mechanisms of attenuation using various methods.
Identification of additional GBS HtrA substrates	– *In vitro* assessment of motif-screened putative substrates identified in our proteomic screen. – Proximity-dependent biotinylation labelling mass spectrometry to identify GBS and host substrates in conditions relevant during infection.
Phenotyping the host placental immune response during infection by HtrA- and Sip-deficient GBS strains	– Assess immune cell infiltration in GBS infected placentas. – Interrogate association between novel immune cell phenotypes and rates of GBS-induced adverse pregnancy outcomes.
Screening and design of a GBS HtrA-specific inhibitor	– Test known bacterial HtrA inhibitors and a panel of similar compounds for effects against GBS HtrA function. – Design an irreversible mimetic peptide that targets the GBS HtrA active site.

Although we have identified many putative GBS protein substrates through my proteomic screen, it is unclear which are directly targeted by HtrA and which effects are indirect. Our use of N-terminal sequencing did not produce a specific motif for HtrA-mediated Sip cleavage, but still provides insight into the biochemical constrains that may regulate HtrA-substrate interactions. Thus, the sequences in our Logo plots which represent fragments of Sip could be queried in BLAST searches against the sequences of our other putative HtrA substrates. Proteins with high similarity could then be tested for HtrA-mediated cleavage *in vitro*, and the validated substrates could undergo mechanistic studies to determine how their modification by HtrA affects GBS virulence. Beyond assessment of endogenous bacterial proteins, multiple host targets have been identified for bacterial HtrA homologs (169, 307-313). Direct assessment of these substrates through *in vitro* cleavage assays may uncover other mechanisms through which HtrA mediates GBS pathogenesis. However, it would remain unclear whether these interactions are biologically relevant. Thus, an alternative method could utilize proximity-dependent biotin labelling mass spectrometry system (BioID) to agnostically identify high likelihood HtrA interactors (314, 315). This method can be adapted to a more complex system that is more representative of GBS

interactions in a host. For example, targets can be identified during GBS infection of human vaginal epithelial cells, neutrophils, or even during GBS infection of a host organism (316, 317). This method could simultaneously identify bacterial and host substrates of HtrA, especially if this protease is exported. The identification of additional substrates for GBS HtrA would improve our understanding of how this protease affects pathogenesis and may also reveal new host-pathogen interactions that could be targeted therapeutically.

In our *in vivo* assessment of HtrA and Sip function, we also observed significant differences in host immune responses during placental infection. HtrA-deficient GBS resulted in elevated levels of chemokines and pro-inflammatory cytokines, but also high levels of immunosuppressive IL-10. Sip-deficient GBS did not induce pro-inflammatory responses but stimulated production of chemokines and high IL-10, while responses caused by GBS deficient for both HtrA and Sip generally matched isogenic WT or Sip-deficient infections. Thus, future work could utilize flow cytometry to characterize the intricacies of these divergent immune responses. For example, it is likely that the immune cell subsets may vary between these infections – especially due to their differences in chemokine production. Further, differences in IL-10 production suggest that immune cell activation and function may vary between infections (318-320). Despite these differences observed by Luminex, all strains deficient for HtrA and/or Sip resulted in attenuated levels of adverse pregnancy outcomes. While these attenuation phenotypes likely depend on HtrA-mediated changes to virulence factor function and on the immune response itself, it would be interesting to disentangle the mechanisms behind attenuation for each strain. Additionally, if IL-10 production in the placenta results acts to prevent a potent and pro-inflammatory immune response, this may also prevent the development of adverse pregnancy outcomes. It may however come at the cost of exacerbated bacterial replication, as was observed during HtrA- or Sip-deficient placental infections. Due to the multiple mechanisms at play, answering these immunological questions would certainly be an endeavor. However, any findings in this arena could provide critical insights to inform the development of improved

diagnostic tools or novel host-directed therapies which could be used to prevent the devastating effects of GBS-induced adverse pregnancy outcomes.

Finally, HtrA inhibitors have been identified for *Escherichia coli*, *Helicobacter pylori*, *Chlamydia trachomatis*, and *Listeria monocytogenes* using a variety of methods (261, 263, 321-327). These inhibitors appear to be quite specific for each bacterial HtrA homolog (261), and thus may represent a novel family of antimicrobials. Modified pyrazolo[1,5-α]-1,3,5-triazines and tetrahydrobenzothiophene derivatives display efficacy against *E. coli* and *H. pylori* HtrA homologs, respectively (321, 322). Thus, to identify inhibitors specific to GBS HtrA, a library of structural analogs and known serine protease inhibitors could be used in a functional screen. To increase our throughput, a fluorescence resonance energy transfer (FRET) probe could be designed based on our Sip cleavage site sequences and tested against recombinant HtrA-H6 in the presence and absence of the inhibitor analog panel in a well-plate assay. Molecules with inhibitory function could undergo structural optimization to improve half-life or potency. Lead compounds may also be tested against whole GBS to assess target availability and identify their bacteriostatic or bactericidal effects. An alternative strategy used to design an HtrA inhibitor for *H. pylori* was ligand-based design. Since N-terminal sequencing of cleaved Sip provided information about amino acid biochemical properties at sites processed by HtrA, this information could be used to design a competitive mimetic peptide that could irreversibly bind within the active site of GBS HtrA. While this strategy is more targeted and may result in the design of a more potent inhibitor, either method could identify an inhibitor that could be used in combination with current antibiotic strategies to treat invasive GBS infections.

4.3 Future directions for Chapter 3

We noted that the GBS-NN vaccine is highly immunogenic and that it displayed partial efficacy in mice, which was exciting given that mice are not the natural host for GBS. **Table 4.2.** describes some future directions that would further inform the use of this vaccine in humans.

Research topic	Experimental strategy
Full GBS-NN/NN2 formulation and cross-protection	– Assess immunogenicity of GBS-NN/NN2 vaccine using the methods described in Chapter 2. – Interrogate cross-reactivity through challenge with GBS strains that represent multiple serotypes and sequence types.
Placental- versus breast milk-mediated antibody transfer	– Perform cross-feeding studies where pups born to vaccinated dams are fostered by adjuvant dams (and vice versa).

Table 4.2. Future directions for Chapter 3: "A recombinant alpha-like protein subunit vaccine (GBS-NN) provides protection in murine models of Group B *Streptococcus* infection."

MinervaX's leading GBS vaccine formulation is comprised of GBS-NN (representing αC and Rib N-termini) and GBS-NN2 (representing Alp1 and Alp2/3 N-termini). Thus, our study that utilized only the GBS-NN peptide could be repeated to assess the immunogenicity and efficacy of the full vaccine, GBS-NN/NN2. Since this formulation includes more Alpha-like protein variants, it should also elicit protection against a wider range of GBS strains. To assess this cross-reactivity, we would perform ELISA to determine the vaccine-specific antibody responses against both GBS-NN and GBS-NN2 and would next test for binding to a broader panel of GBS isolates that represent diverse serotypes and sequence types (STs). This panel would include A909 (used in our study, serotype Ia/ST-7, expressing αC), BM110 (serotype III/ST-17, expressing Rib), NCTC12906 (serotype Ia/ST-XX, expressing Alp1), and NEM316 (serotype III/ST-XX, expressing Alp2), plus additional strains with known serotypes but unknown Alps expression including DK23 (serotype II), NCTC10/84 (serotype V), CJB111 (serotype V), and JM9 (serotype VIII). A subset of these strains could also be used for GBS murine challenge. The GBS-NN/NN2 vaccine is more representative of the vaccine formulation currently undergoing human clinical trials, and as such its assessment would be informative.

Given that GBS is a major perinatal pathogen that impacts both mothers and their infants, another hot topic in GBS vaccine research is mechanisms of vertically transmitted protection.

Maternal vaccination represents an attractive prophylactic strategy to protect both the mother and the infant. In our study, we were excited to see that female mice immunized before pregnancy did indeed transmit vaccine-specific antibodies to their pups. Further, these antibodies were protective against subsequent neonatal intranasal challenge. To dissect the mechanisms of maternal antibody transmission, a follow-up study could utilize cross-fostering to isolate the protective effects mediated by placental versus breastmilk antibody transfer. Specifically, adjuvant dams do not have vaccine-specific antibodies and thus cannot transfer protective antibodies across the placenta, but their cross-fostered pups may receive breastmilk antibodies from vaccinated dams. Conversely, immunized dams with high titers of vaccine-specific antibodies could transmit protection to their pups *in utero*, but their pups cannot receive breastmilk antibodies when cross-fostered by adjuvant dams. If both breast milk antibodies and placentally-transferred antibodies are important for protection, we would expect to see reduced survival of cross-fostered pups upon GBS intranasal challenge compared to pups born and raised by immunized dams. Pup serum and milk band contents could also be collected and the vertically transferred vaccine-specific antibodies in these compartments could be quantified by ELISA. While the underlying mechanisms of vertical antibody transfer are different between mice and humans, upcoming clinical trials for GBS-NN/NN2 should also seek to dissect these contributions in humans to better understand what is required for protection of the infant. Mothers in the placebo group would serve as controls for both placenta- and breastmilk-mediated antibody transfer, while infants born to immunized mothers could be stratified into breastmilk-fed versus formula-fed groups to assess the impact of breastmilk-mediated transfer. Data supporting vertical transfer of GBS-specific antibodies are essential to support maternal vaccination as a method to prevent GBS infection in the infant in addition to the mother.

4.4 Concluding remarks

Nearly a century after Rebecca Lancefield's discovery of GBS, we have made great strides towards understanding GBS biology and pathogenesis. However, even with these advances GBS remains a sizeable global health concern. Researchers across the world are diligently studying GBS, and these global perspectives will be essential to one day eliminate GBS as a major perinatal pathogen. Still, many gaps remain in our understanding of the global epidemiology of GBS and – given its disproportionate burden and major variability in screening guidelines – filling these data gaps remains an important goal that will require international collaboration. With MinervaX's GBS-NN/NN2 and Pfizer's GBS6 vaccines quickly progressing in human clinical trials, we may be running short on time to improve our baseline understanding of GBS epidemiology. While the development of these vaccines is obviously a huge win for the GBS field (and for mothers and babies everywhere!), we expect that vaccine rollout will alter GBS strains and serotypes prevalence. Gaining a more complete and representative understanding of today's pre-vaccine epidemiology will allow us to better understand how these strategies may shift GBS evolution and phylogeny following the application of these and other interventions.

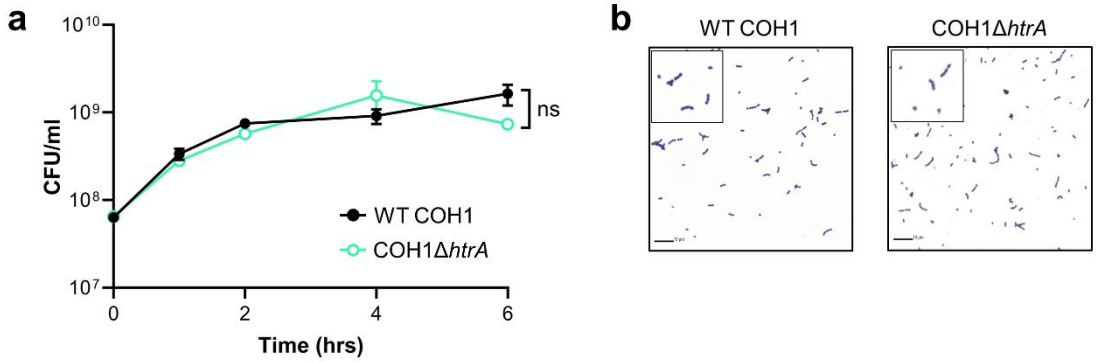
While it is easy to celebrate the progress of these vaccines, it is also important to remember that the immunogenicity and safety profiles observed in human clinical trials do not guarantee success in human populations. Massive multi-site trials will soon address vaccine efficacy in at-risk human populations. These studies will be expensive, and in order to show efficacy they must capture sufficient rates of adverse pregnancy events to allow comparisons of outcomes between vaccine and placebo groups. Alongside ethical concerns that could arise regarding the use of study populations that may not receive IAP, the effects of this prophylaxis in other regions where its use is commonplace will certainly be an important confounder that must be considered during study design and analysis.

If MinervaX's GBS-NN/NN2 and Pfizer's GBS6 vaccines prove effective against GBS, vaccine production must also be financed, and manufacturing and roll-out must consider many

factors in order to produce a final product that can be implemented in a variety of social and economic settings. GBS researchers, economists, and medical professionals are already working to raise interest in GBS as a global health issue, but women's health is not perceived as particularly lucrative to investors. Since we all begin as neonates, perhaps re-framing the GBS burden of disease in this manner may help to persuade stakeholders that this pathogen causes a sizeable problem that is worthy of investment. We must also remember that pregnancy is a high stress and scary time for many women. Working now to understand global acceptability of vaccination during pregnancy may help to improve GBS vaccination rates after FDA approval. We have recently learned that – especially in the United States – vaccines can be politicized. Improving the accessibility of vaccine safety information and raising public awareness about GBS may also have positive impacts on the success of future vaccination campaigns.

Finally, current progress for vaccines should not be considered the end of the road. Most vaccines do not result in disease eradication, and thus GBS research will remain valuable even upon reaching a post-vaccine era. There is still so much that we do not understand about GBS, and the development of alternative treatment and prophylactic strategies could complement antibiotics and vaccination to further reduce the global GBS burden of disease. Future GBS research should aim to better understand: 1) where and how GBS colonization occurs (e.g. contribution of various body sites, host factors that impact susceptibility), 2) why carriage is so dynamic and variable (e.g. factors that drive lack of colonization versus persistent or recurrent colonization), 3) why GBS only sometimes shifts from an asymptomatic colonizer to an invasive pathogen (e.g. host factors that impact susceptibility or dissemination, bacterial factors associated with virulence and their regulation), and 4) why invasive disease rates are increasing in all ages (e.g. epidemiology of comorbidities, increased carriage rates, evolution of hypervirulent GBS isolates). Collaborative and creative studies that bridge multi-omics screens and molecular techniques, that connect questions from host and pathogen perspectives, and that compare findings from animal models and human samples will be important tools moving forward.

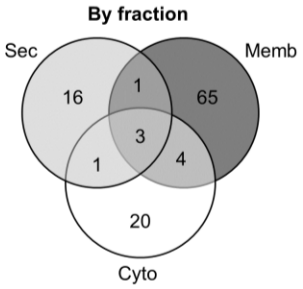
APPENDIX A. Supplemental Figures: Chapter 2



Supplemental Figure A-1. Deletion of *htrA* does not impact GBS growth *in vitro*. a) Viable counts were used to generate a growth curve of WT COH1 and COH1Δ*htrA* GBS grown in TSB. Means of three biological replicates ± SEM were assessed for significance using two-tailed unpaired t-tests with Holm-Šídák multiple comparisons test: ns $P > 0.05$. **b)** Representative Gram stains of WT and COH1Δ*htrA* stationary phase cultures imaged at 100x. Scale bar indicates 10 µm and inset shows a zoomed version of representative chain forming cocci.

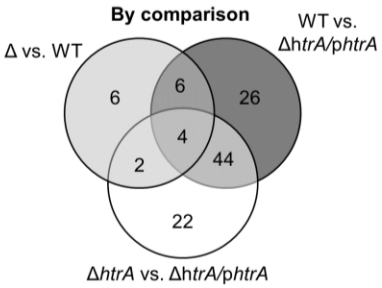
a

	Identified	Significant	Unique within Fraction
Secreted	98	35	21
Membrane	518	95	73
Cytosol	446	36	28



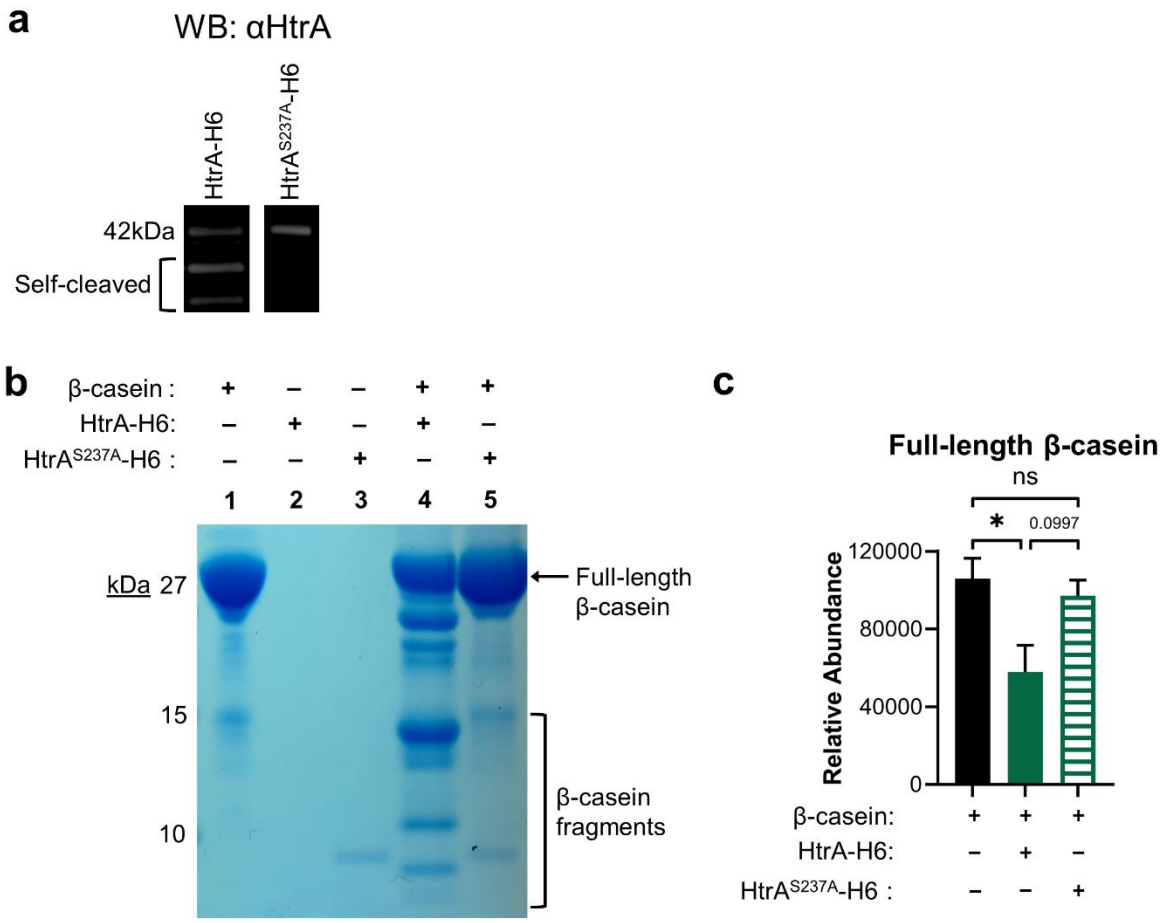
b

	Identified	Significant	Unique within Comparison
COH1Δ*htrA* vs. WT COH1	1,059	19	18
WT COH1 vs. COH1Δ*htrA*/p*htrA*	1,058	86	80
COH1Δ*htrA* vs. COH1Δ*htrA*/p*htrA*	1,061	82	72



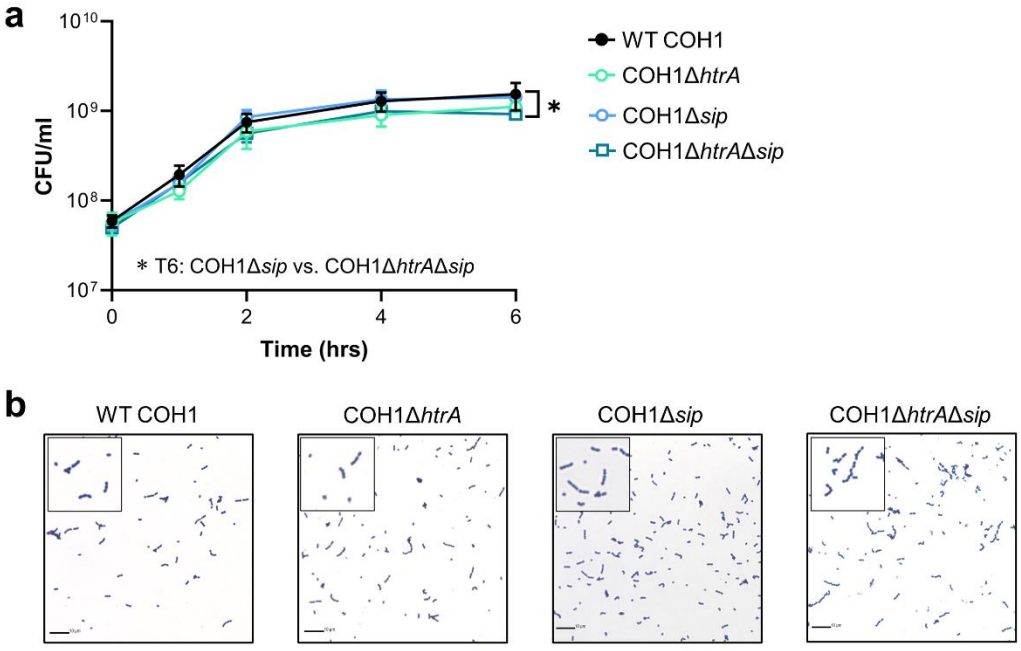
Supplemental Figure A-2. Overview of proteomic changes by strain comparison or by fraction. a) Table indicates total number of proteins identified by LC-MS/MS, number of significant changes in abundance, and number of unique proteins undergoing changes of abundance within each protein fraction. Venn diagram summarizes overlap between fractions. **b)** Table indicates total number of proteins identified by LC-MS/MS, number of significant changes in abundance, and number of unique proteins undergoing changes of abundance within each strain comparison. Venn diagram summarizes overlap between strain comparisons.

APPENDIX A. Supplemental Figures: Chapter 2



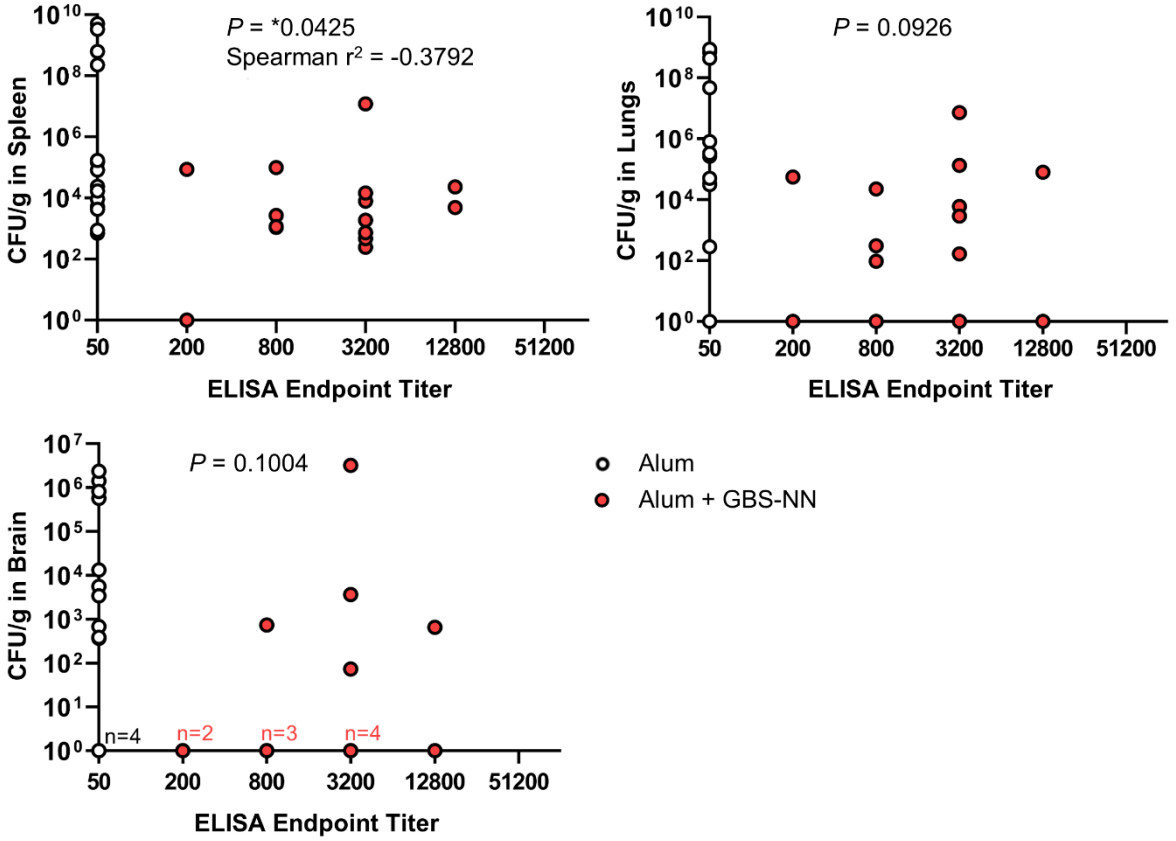
Supplemental Figure A-3. Purification of proteolytically active recombinant HtrA proteins and assessment in protease activity assays. a) Recombinant HtrA-H6 or catalytically-inactive HtrA^{S237A}-H6 purified from *E. coli* BL21(DE3) gold via Ni-NTA resin. Eluted fractions were assessed via Western blot using rabbit serum raised against recombinant HtrA^{S237A}-H6 protein. Panel shows representative blot with catalytically active HtrA-H6 undergoing self-cleavage. Catalytically-inactive HtrA^{S237A}-H6 did not self-cleave. **b)** Protease activity HtrA-H6 and catalytically-inactive HtrA^{S237A}-H6 was assessed using the model protease substrate β-casein at 1:1000 (HtrA:β-casein). Controls included β-casein substrate alone (lane 1), HtrA-H6 alone (lane 2), and HtrA^{S237A}-H6 alone (lane 3). Coomassie stained gel indicates full-length β-casein and its fragments due to rHtrA protease activity. **c)** Densitometry of full-length β-casein was performed in Fiji. Data shows means ± SEM of three replicates, with significance determined by ordinary one-way ANOVA with Tukey's multiple comparison test: * $P < 0.05$. Trending ($P < 0.1$) statistics are shown.

APPENDIX A. Supplemental Figures: Chapter 2



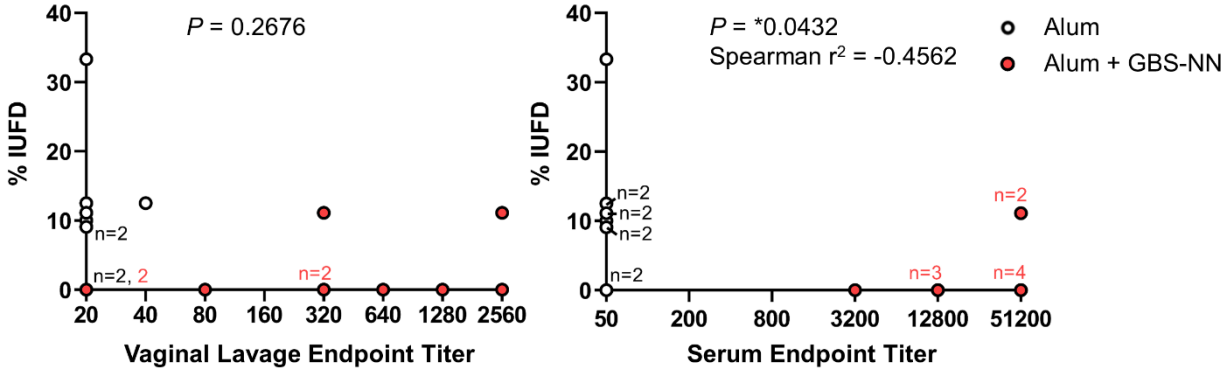
Supplemental Figure A-4. Deletion of *sip* does not impact GBS *in vitro* growth. a) Viable counts were used to generate a growth curve of WT COH1, COH1Δ*htrA*, COH1Δ*sip* and COH1Δ*htrA*Δ*sip* grown in TSB. Means of three biological replicates ± SEM were assessed for significance using two-tailed unpaired t-tests with Holm-Šídák multiple comparisons test: ∗ $P < 0.05$. **b)** Representative Gram stains of WT, COH1Δ*htrA*, COH1Δ*sip,* and COH1Δ*htrA*Δ*sip* stationary phase cultures imaged at 100x. Scale bar indicates 10 μm and inset shows a zoomed version of representative cocci chains.

APPENDIX B. Supplemental Figures: Chapter 3



Supplemental Figure B-1. GBS-NN-specific IgG in serum are associated with reductions in bacterial burden at disseminated sites. To detect relationships between GBS-NN-specific IgG titers and CFU recovered from the spleen, lungs, and brain following systemic GBS challenge, paired values for CFU at these sites were plotted (as y-values) with the mouse's matched serum titer (x-values) and association was measured by Spearman correlation. Positions with multiple overlapping datapoints are indicated by "n=X" in the color corresponding to the treatment group with X representing the number of mice whose data overlaps (sample sizes: n=14 alum, n=15 alum + GBS-NN): ∗ $P<0.05$, $P\leq0.1$ trending towards significance. This figure is reused with permission (2) (CCC license # 5774460237872).

APPENDIX B. Supplemental Figures: Chapter 3



Supplemental Figure B-2. GBS-NN-specific IgG in the serum, but not in vaginal lavage fluid, are associated with improved fetal outcomes. To detect relationships between GBS-NN-specific IgG titers and rates of adverse pregnancy outcomes induced by vaginal GBS challenge during pregnancy, paired values for percent intrauterine fetal death (IUFD) were plotted (as y-values) with the dam's matched vaginal lavage (left) or serum (right) titer and association was measured by Spearman correlation. Positions with multiple overlapping datapoints are indicated by "n=X" in the color corresponding to the treatment group with X representing the number of mice whose data overlaps (sample sizes: n=10 alum, n=10 alum + GBS-NN): ∗ P<0.05. This figure is reused with permission (2) (CCC license # 5774460237872).

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