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Analyzing Group B Streptococcal and Host Factors Influencing Vaginal Colonization and Exploring Therapeutic Interventions

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy

in

Biology

by

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2015

This Dissertation of Kathryn Ann Patras is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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DEDICATION

To my best friend and husband J: my source of encouragement, emotional support, and eager assistant for colony counting, tip filling, and plate pouring, and to my son Will: the cutest distraction from dissertation writing.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SUPPLEMENTAL MATERIALS

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ABSTRACT OF THE DISSERTATION

Analyzing Group B Streptococcal and Host Factors Influencing Vaginal Colonization and Exploring Therapeutic Interventions

by

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Streptococcus agalactiae (Group B Streptococcus, GBS) is a Gram-positive bacterium which colonizes the cervicovaginal tract in 20-30% of healthy women. Colonization is asymptomatic; however, during pregnancy, GBS can cause several complications such as chorioamnionitis and urinary tract infections, or alternatively, can be vertically transmitted to newborns peripartum causing pneumonia, sepsis or

meningitis. Current prophylaxis, consisting of late gestation screening and intrapartum antibiotics, has failed to completely prevent transmission, and GBS remains the leading cause of bacterial neonatal meningitis in the United States. Unfortunately, little is known about the host and bacterial factors that promote or permit GBS vaginal colonization. For this PhD dissertation project, I examined the host innate and adaptive immune responses during GBS vaginal colonization and identified several key bacterial factors, such as toxin production and strain differences, that elicited a strong immune response or altered persistence in the vaginal tract. This was accomplished using immortalized human cervical and vaginal cell lines *in vitro*, as well as utilizing an established mouse model of GBS vaginal colonization. Secondly, I identified GBS factors that contribute to successful vaginal colonization, including determinates controlling interactions with host tissues and other normal flora. GBS has multiple two component regulatory systems that have been previously shown to regulate bacterial gene expression, some of which control factors that promote host cell adherence and production of a putative bacteriocin-like inhibitory substance (BLIS). I utilized molecular techniques to both study contributions of specific two component systems to vaginal colonization as well as confirmed BLIS activity. Lastly, I explored therapeutic intervention strategies to remove GBS from the vaginal tract including treatment with a novel immunostimulatory peptide or administration of a probiotic microbe to limit GBS vaginal colonization. Altogether, this dissertation furthered our understanding of the GBS-host interaction within the vaginal environment, which will lead to potential therapeutic targets to control maternal vaginal colonization during pregnancy and prevent transmission to the vulnerable newborn.

CHAPTER 1

INTRODUCTION

Streptococcus agalactiae (Group B Streptococcus, GBS) is an encapsulated Gram-positive bacterium that colonizes the human lower gastrointestinal tract, and in females, the lower reproductive tract, of 20-30% of healthy adults[1]. GBS has also been readily been isolated in both commensal and pathogenic forms from a variety of mammals and fish, and is a frequent cause of mastitis in dairy cows[2]. Little is known about the dissemination of GBS across species; however, a cross-sectional cohort study revealed cattle exposure was a predictor of human GBS colonization suggesting interspecies transmission can occur[3]. Diversity of the GBS polysaccharide capsule may in part allow for its broad range of hosts, as the capsule has recently been established as important in biofilm formation[4]. 10 different variants of the capsule have been described (Ia, Ib, II-IX), with serotypes Ia, Ib, II, III, and V most commonly isolated from humans[5]. GBS has more recently also been classified by sequence type based on an allelic profile of seven different loci, with sequence types (ST) with the majority of GBS human isolates being ST-1, ST-17, ST-19, or ST-23[6]. Although primarily considered an asymptomatic colonizer, in immune compromised individuals, such as neonates, pregnant women, and the elderly, GBS may transition to an invasive pathogen, resulting in pneumonia, sepsis, urinary tract infections, and meningitis[7].

GBS vaginal colonization during pregnancy and neonatal disease

In the 1970s, GBS emerged as the leading agent of infectious neonatal mortality, with over 7,000 cases of neonatal disease annually[8]. Early-onset GBS disease (EOD) occurs in the first hours or days of life arising as pneumonia or respiratory distress and

often develops into sepsis, whereas late-onset disease (LOD) ensues after several months and presents with bacteremia which frequently advances to meningitis[7]. By the mid-1990s, the Centers for Disease Control and Prevention issued the recommendation for intrapartum antibiotic prophylaxis (IAP) to GBS-positive mothers and in 2002, further recommended universal screening in late gestation[8]. Despite the reduction of early-onset disease to approximately 1000 cases in the United States annually with IAP, GBS remains the leading cause of early-onset neonatal sepsis and late-onset occurrence remains unaffected[8]. Whether *in utero*, or during labor, or even in late-onset cases, neonatal exposure to GBS requires survival, transversal through a number of host environments and barriers, immune evasion, and in the case of meningitis, crossing of the highly regulated blood-brain barrier[7]. Upstream of these virulent interactions within the neonate, is the initial colonization of the maternal vaginal tract.

Maternal vaginal GBS colonization rates appear similar in developed and developing countries across all 6 inhabited continents, ranging from 8-18% with an overall estimated colonization of 12.7%[9,10]. GBS colonization of the vaginal tract during pregnancy may be constant, intermittent, or transient in nature among individual women[11]. Interestingly, maternal age >36 years of age is associated with persistent colonization[12]. Numerous risk factors for GBS vaginal colonization have been identified both biological and socio-economic in nature. Biological factors include gastrointestinal GBS colonization and absence of *Lactobacillus* within the gut; however, ethnicity, obesity, hygiene and sexual activity have also been associated with GBS vaginal carriage[13].

The majority of GBS-positive women are stably colonized during the peripartum period, however changes in serotype or sequence type or subsequent loss of specific sequence types have been documented[12]. Many studies have examined the most common serotypes of colonizing strains in the United States and are in agreement that serotypes Ia, III, and V are the most represented serotypes[12,14,15]. However, given the possibility of capsular switching, a more recent study has examined GBS sequence types and found sequence types 1, 23 and 19 the most abundant colonizing strains[12].

Although notorious for causing neonatal infections, GBS also causes a variety of maternal infections both peripartum and postpartum. GBS carriage is increased in women presenting with vaginitis[16] and in some cases, may even be the disease entity[17]. Additionally, GBS ascension of the reproductive tract during pregnancy may result in intra-amniotic infection or chorioamnionitis[18]. Moreover, in up to 3.5% of pregnancies, GBS disseminates to the urinary bladder to cause a urinary tract infection or asymptomatic bacteriuria[19]. GBS bacteriuria during pregnancy is associated with increased risk of intrapartum fever, chorioamnionitis, preterm delivery, and premature rupture of membranes[20]. Even postpartum, GBS can cause symptomatic or asymptomatic mastitis which has been proposed as a possible infection route for late-onset neonatal disease[18]. Taken together, the presence of GBS within the vaginal tract is linked to infections of multiple host tissues, and the ability to eliminate GBS from this niche is imperative for both maternal and neonatal health.

GBS interactions with vaginal epithelium

The transient nature of GBS vaginal colonization is likely dependent on vaginal pH, normal flora, pregnancy, immune responses, and estrous cycle, among many other

constituents. Increased adherence to vaginal epithelial cells has been observed *in vitro* as pH shifts from acidic to neutral[21], however factors that favor persistence of GBS in this complex biological niche are not well understood. Although so much is still unknown, a few GBS determinants have been shown to contribute to vaginal cell adherence and colonization, including surface Serine Rich Repeat (Srr) proteins, Srr-1 and Srr-2[22], pili protein, PilA of GBS Pilus Island (PI)-2a[22], bacterial surface adhesion of GBS (BsaB)[23], as well as alpha-like proteins and capsular serotypes[24].

The ability of bacteria to form biofilms may also support the colonization of mucosal surfaces such as the vaginal tract. One study found that increasing pH from a vaginal pH (pH 4.2) to a more neutral pH (pH 6.5) resulted in more GBS strains forming biofilms both in rich media and simulated vaginal fluid[25]. However, since then, two other studies have observed contradictory results, demonstrating the acidic conditions such as the vaginal tract promote the formation of GBS biofilms[26,27]. Recently, PI-2a and FbsC have been implicated in the formation of biofilms *in vitro*[28-30]. These pili, and other GBS surface proteins, also facilitate adherence to extracellular matrix (ECM) constituents such as collagen[31], fibrinogen[32], fibronectin[33], and laminin[34], all of which have been identified in multiple vaginal proteome studies[35] suggesting potential importance in this environment. Furthermore, GBS possesses metallopeptidases capable of cleaving all four of these ECM proteins[36], which may aid in tissue invasion or niche establishment.

Potential impact of GBS regulatory systems on vaginal colonization

GBS has several regulatory systems in place that may control the transition from a commensal niche (e.g. vaginal tract) to invasive niches (e.g. blood, lung, brain, and other organs). Bacteria respond to changes in environmental stimuli using two-component

systems (TCS) to alter gene expression. TCS typically consist of a membrane-associated histidine kinase and a cytoplasmic transcriptional regulator[37]. Sequence analysis has revealed that GBS may have as many as 20 TCS[38]. Probably the most well-characterized GBS TCS is the sensor histidine kinase CovS (Cov, control of virulence), and a response regulator CovR, which coordinately down-regulate the expression of fibrinogen-binding proteins A, B and C (FbsA, FbsB, FbsC), genes involved in iron uptake, and repression of *cylE*, a gene involved in the production of the GBS β-hemolysin toxin (β-H/C) which has recently been shown to be a hemolytic ornithine lipid [28,39,40]. Another GBS TCS, CiaR/H enhances resistance to oxidants and antimicrobial peptides, promotes intracellular survival, and regulates the production of several putative peptidases[41]. A recent study discovered that certain GBS strains have as many as 21 TCS, and identified TCS-16 as playing a role in GBS vaginal colonization perhaps through regulation of fructose metabolism[42]. Although it is established that these TCS regulate virulence factors, stress response, and potential antimicrobial activity, little is about their specific contributions to host colonization.

Host responses to GBS vaginal colonization

The type, magnitude, and duration of the host immune response is an additional factor that likely impacts GBS vaginal colonization. Host responses to GBS have been well characterized during pathogenesis in a variety of *in vitro* and animal models. One of the main measurements of immune induction is the production of cytokines, which are intracellular molecules that act as growth factors for immune cells or are pro-inflammatory or anti-inflammatory in nature, and chemokines, which organize the migration of immune cells from the blood into other tissues[43]. Immune cytokine

stimulation has been examined in many human immune, epithelial, and endothelial cells that comprise host barriers and defenses including: dendritic cells[44], monocytes[45], lung epithelial cells[46], urinary bladder epithelial cells[47], brain microvascular endothelial cells[48], and coronary artery endothelial cells[49]. Intravenous or intraperitoneal GBS elicits robust innate immune responses in mice including production of TNF-α, IL-1β, and IL-6[50,51]. In agreement, intraperitoneal GBS stimulates IFN-γ, TNF-α, IL-1β, IL-6, IL-8, and IL-10 in a humanized mouse model[52].

The mechanisms and roles of these immune components are not fully known but have been the focus of recent studies. GBS RNA and β-H/C were found to induce IL-1β in macrophages through activation of the NLRP3 inflammasome[53]. In turn, IL-1β contributes to CXCL1 and CXCL2 chemokine signaling resulting in neutrophil recruitment to GBS-infected tissues[54]. GBS GAPDH has also been linked to host immune modulation through induction of IL-10[55]. Alternatively certain pathways have been ruled out, as NOD2 receptor signaling does not appear to be a critical host intracellular defense against GBS[56].

GBS can also stimulate adaptive immune responses during pathogenesis. In hallmark studies, Dr. Rebecca Lancefield demonstrated that GBS elicits antibody production in the host in a type-specific manner[57]. For decades, we have known that the human host can generate serum antibodies against the GBS polysaccharide capsule, and these antibodies are specific to a particular serotype[58]. Recent work has attempted to identify all major GBS proteins that generate human serum antibodies[59]. The antibodies mounted to GBS may in fact be protective, as infants born to women with higher levels of anti-GBS IgG were at lower risk for early-onset disease than women with low levels of

anti-GBS IgG[60]. Moreover, both IgG and IgM type antibody responses have been observed in infants surviving meningitis suggesting that the neonatal immune response may also serve some function in protection[61].

In contrast, studies examining potential host immune responses resulting from asymptomatic GBS vaginal colonization, or from other tissues of the female reproductive tract, are much fewer in number. One study comparing phagocyte engulfment of GBS in colonized and non-colonized pregnant women observed that monocytes from colonized women engulfed significantly more GBS but released more superoxide extracellularly suggesting impaired or insufficient immune function may contribute to GBS vaginal persistence[62]. Another work demonstrated that vaginally colonized women possessed elevated levels of IgG and IgA antibodies to GBS in cervical secretions compared to non-colonized women[63]. Additionally, increased levels of maternal serum IL-1β have been associated with increased risk of GBS neonatal infection and early term birth[64]. Experimentally, rectal inoculation of GBS in mice stimulated vaginal secretion of IgA[65]. Until the start of this dissertation project, GBS induction of host responses in the human vaginal epithelium had not been examined and is addressed in Chapter 2.

Since GBS is capable of crossing placental barriers *in utero*, several groups have also begun to examine the host responses to GBS within these tissues. Like other host tissues, GBS stimulates HBD-2, IL-1β, IL-8 and TNF-α in human extraplacental or chorioamniotic membranes *ex vivo*[40,66,67]. In a new *in utero* infection model, GBS β-H/C was implicated in GBS-mediated fetal injury through both NLRP3 inflammasome-dependent and independent pathways[68]. Furthermore, recent work suggests that not only can GBS cause placental dysfunction, but additionally, maternal inflammation may affect

offspring brain development and neurobehavioral traits[69]. Altogether, there is much information lacking in female reproductive tract responses to GBS, and this must be addressed in future research in maternal and neonatal health.

GBS interactions with the human vaginal microbiota

Currently, the healthy human vaginal microbiome has been clustered into five different communities. Four clusters are dominated by *Lactobacillus* species which are believed to lower the environmental pH through lactic acid production and thus protect the host from a variety of microbial infections[70]. Whether or not GBS should be considered a native component of the vaginal microbiota is still debated. Some studies have identified no differences between dominant microbial populations such as *Lactobacillus*, *Bifidobacterium*, and *Candida* species in GBS-positive individuals suggesting that GBS does not perturb native flora[11]. In contrast, others have demonstrated a relative reduction of *Lactobacillus* populations in GBS-positive women[71], and this inverse relationship between *Lactobacillus* and GBS has also been observed in cows with subclinical mastitis[72]. As can be anticipated, certain *Lactobacillus* strains have inhibited GBS adherence to vaginal epithelial cells[73], and antimicrobial activity of *Lactobacillus* against GBS has been documented *in vitro*[74] and reduction of colonization seen *in vivo*[75].

Although the complexity of the vaginal microbiome is only now being characterized, preliminary *in vitro* studies have begun to characterize GBS communication with other microbial organisms. Interestingly, GBS culture supernatant increases production of toxic shock syndrome toxin 1 in *Staphyloccus aureus*[76]. Recently, GBS was found to possess antimicrobial resistance against nicin, a lantibiotic produced by *Lactococcus lactis*[77]. Moreover, others have investigated exchange of quorum sensing

molecules between GBS and other *Streptococcus* species *in vitro*[78]. Nevertheless, the molecular mechanisms governing GBS vaginal persistence including host immune response, epithelial and extracellular matrix adherence, and competition with normal microbiota, remain to be elucidated.

Current and emerging therapeutic interventions

As mentioned previously, the current recommendations for GBS neonatal disease prevention consists of universal maternal screening for GBS in the 35-37th week of gestation, with IAP given to GBS-positive mothers during labor[8]. Unfortunately, even with screening women just prior to full term is still not a completely accurate depiction of colonization status at delivery. In one study, over 20% of 37th week GBS-positive women were GBS-negative at the time of delivery[79]. Additionally, hospital compliance with CDC guidelines confounds the efficacy of IAP with as little as 65% of GBS carriers receiving IAP[80]. When given correctly, IAP reduces GBS vaginal colonization to 47% after 2 hours of administration, and 12% after 4 hours of administration[81]. 4 hours of IAP with a beta-lactam has been shown to be highly effective in preventing early-onset disease[82] yet there is evidence to support treating for longer than 4 hours when possible[83]. Another benefit of IAP is that neonates are less likely to be colonized hindering the propagation of GBS[84]. Whether or not IAP alters infection rates of other pathogens or increases GBS antibiotic resistance in still unclear. Some studies have observed these negative effects of IAP including increased infections with Gram-negative bacteria such as ampicillin-resistant *E. coli*[85,86], whereas others have not[87]. As an alternative to antibiotic treatment, intrapartum chlorhexidine vaginal washes have been considered, but

resulted in no significant reduction of EOD, but did significantly lower neonatal colonization[88].

Although IAP has greatly reduced the incidence of EOD, GBS remains a global disease burden for maternal and neonatal health. Alarmingly, EOD incidence has increased significantly in African American neonates between 2003-2006, occurring 2.8 times more frequently than white neonates among preterm infants[89]. The most commonly discussed alternative, or addendum to prophylaxis, is maternal immunization. Work performed by Dr. Carol J. Baker in the mid-1970s which examined production of maternal IgG to GBS capsular polysaccharide (CPS) types laid the foundation for the development of a GBS vaccine[90]. The vaccine strategy that has progressed the furthest is a trivalent GBS conjugate vaccine (against serotypes Ia, III, and V) which has completed Phase II clinical trials in pregnant women[91]. Moreover, Phase III trials of this conjugate vaccine have been proposed[92]. Besides GBS CPS vaccines, several other GBS surface proteins have been proposed targets including pilus proteins[93], alpha C protein[94], Sip, and ScpB[95]. Finally, analytic models have determined the GBS vaccine has comparable cost-effectiveness to other pediatric vaccines[96]. Although this strategy has been sought after for over 40 years, it appears its completion is on the horizon.

In an age where "natural" medicine is popular, a more recent approach to GBS therapies is the use of a probiotic cocktail to modify the vaginal flora to control GBS colonization. A frequent concern with IAP is that early bacterial colonization of the neonate with be altered, perhaps even detrimentally; however, there are only of few studies that have examined this impact thus far. One group found that *Clostridium* is reduced in the gut of infants from mothers receiving IAP[97]. Excitingly, some of these

affected gut flora may serve as the alternative solution to IAP or a vaccine. Potential treatments with probiotic species such as *Bifidobacterium* species, which are also reduced in the neonatal gut after IAP, have antibacterial activity against GBS[98]. Thus far, only one small study examined use of oral prebiotics to control GBS vaginal colonization[99]. An alternative probiotic microbe to control GBS vaginal colonization is the topic of Chapter 6.

SPECIFIC AIMS

The main purpose of this dissertation is to further our understanding of the GBS-host interaction within the vaginal environment, which will lead to potential therapeutic targets to control maternal vaginal colonization and prevent transmission to the vulnerable newborn. *I hypothesize that the host immune response, GBS gene regulation by two component systems, and the vaginal microbial community, are the key factors that govern GBS vaginal colonization.* Specifically, I will examine these three areas using our established mouse model of GBS vaginal colonization and molecular techniques as outlined in the following aims:

Aim 1: Identify host immune components that respond to GBS presence within the vaginal tract.

a. Investigate the role of innate immune pathways in GBS vaginal persistence.
b. Identify IL-17 immune responses to GBS strains exhibiting long-term vaginal persistence.

Aim 2: Identify bacterial factors that promote vaginal persistence and effective competition with vaginal flora.

a. Examine the role of GBS two component regulatory systems in establishing vaginal colonization.
b. Characterize the role of a CiaR-regulated putative protease in GBS colonization ability.

Aim 3: Explore novel therapeutic strategies to reduce GBS vaginal colonization.

a. Characterize a novel immunostimulatory peptide, EP67, for ability to reduce GBS vaginal colonization.
b. Examine the impact of *Streptococcus salivarius* on GBS colonization *in vivo.*

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CHAPTER 2

Group B Streptococcus CovR regulation modulates host immune signaling pathways to promote vaginal colonization

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PREFACE TO CHAPTER 2

Chapter 2 in full is an article published in *Cellular Microbiology*. The work in Chapter 2 addresses Aim 1a: Investigate the role of innate immune pathways in GBS vaginal persistence. This was accomplished through a microarray and subsequent qPCR and Western blot analysis of human vaginal epithelial cells *in vitro*. Furthermore, we quantified several innate cytokines present in vaginal lavage fluid after GBS vaginal colonization in a mouse model, and found that cytokine production in part relies on signaling through the CXCL2 receptor. Chapter 2 also addresses Aim 2a: Examine the role of GBS two component regulatory systems in establishing vaginal colonization. This was achieved by comparing an isogenic mutant of the two component system (CovR) to wild type in the ability to persist in the vaginal tract, stimulate cytokine production, and interact with vaginal epithelial cells. This work is the first to our knowledge to experimentally examine innate immune components and two component system influence on GBS vaginal colonization.

ABSTRACT

Streptococcus agalactiae (Group B Streptococcus, GBS) is a frequent commensal organism of the vaginal tract of healthy women. However, GBS can transition to a pathogen in susceptible hosts, but host and microbial factors that contribute to this conversion are not well understood. GBS CovR/S (CsrR/S) is a two component regulatory system that regulates key virulence elements including adherence and toxin production. We performed global transcription profiling of human vaginal epithelial cells exposed to wild type, CovR deficient, and toxin deficient strains, and observed that insufficient regulation by CovR and subsequent increased toxin production results in a drastic increase in host inflammatory responses, particularly in cytokine signaling pathways promoted by IL-8 and CXCL2. Additionally, we observed that CovR regulation impacts epithelial cell attachment and intracellular invasion. In our mouse model of GBS vaginal colonization, we further demonstrated that CovR regulation promotes vaginal persistence, as infection with a CovR deficient strain resulted in a heightened host immune response as measured by cytokine production and neutrophil activation. Using CXCr2 KO mice, we determined that this immune alteration occurs, at least in part, via signaling through the CXCL2 receptor. Taken together, we conclude that CovR is an important regulator of GBS vaginal colonization and loss of this regulatory function may contribute to the inflammatory havoc seen during the course of infection.

INTRODUCTION

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is an encapsulated Gram-positive bacterium that commensally colonizes the lower gastro-intestinal tract, and in females, the lower reproductive tract, of 20-30% of healthy adults[1]. However, in immune compromised individuals, such as neonates, pregnant women, and the elderly, GBS may transition to an invasive pathogen, resulting in pneumonia, sepsis, urinary tract infections, and meningitis[2]. Despite currently recommended intrapartum antibiotic prophylaxis for GBS-positive mothers, GBS remains the leading cause of early-onset neonatal sepsis[3]. Whether *in utero* or during labor, neonatal exposure to GBS requires survival, transversal through a number of host environments and barriers, immune evasion, and in the case of meningitis, crossing of the highly regulated blood-brain barrier[2]. Upstream of these virulent interactions within the neonate, is the initial commensal colonization of the maternal vaginal tract.

Vaginal colonization by GBS is believed to be somewhat transient, and likely dependent on vaginal pH, normal flora, pregnancy, and estrous cycle, among many other constituents. Factors that favor the persistence of GBS in this complex biological niche are not well understood. GBS biofilm formation has been demonstrated in simulated vaginal fluids, with bacterial survival and growth improving as pH approaches neutral[4,5]. However, optimal pH for GBS biofilm formation is unclear, as one group reported biofilm production increased as pH rose from 4.2 to 6.5[4], and another group, that GBS biofilm formation is enhanced at pH 4.5 compared to pH 7.0[5]. Although much is still unknown, a few GBS determinants have been shown to contribute to vaginal cell adherence and colonization, including surface Serine Rich Repeat (Srr) proteins, Srr-1

and Srr-2, and pili protein, PilA of GBS Pilus Island (PI)-2a[6]. Interestingly, it has been recently observed that GBS PI-1 does not mediate attachment to vaginal cells *in vitro*[7]. Others have also begun exploring bacteriocin-like inhibitory substances produced by native vaginal species that negatively impact GBS growth[8]. Nevertheless, the molecular mechanisms governing GBS vaginal persistence and competition with normal microbiota remain to be elucidated.

GBS has several regulatory systems in place that may control the transition of the organism from a commensal niche (e.g. vaginal tract) to invasive niches (e.g. blood, lung, brain, and other organs). Bacteria respond to changes in environmental stimuli using two-component systems (TCS) to alter gene expression. TCS typically consist of a membrane-associated histidine kinase and a cytoplasmic transcriptional regulator[9]. In GBS, a TCS consisting of a sensor histidine kinase CovS (Cov, control of virulence), also known as CsrS (Csr, capsule synthesis regulator), and a response regulator CovR (CsrR) down regulates the expression of 27 genes and activates the expression of 3 genes in all GBS strains examined[10,11,12]. The conserved regulatory functions of CovR in GBS include repression of fibrinogen-binding proteins A and B (FbsA, FbsB), genes involved in iron uptake, and in particular, repression of *cylE*, the gene encoding GBS toxin β-hemolysin/cytolysin (β-H/C)[13]. CovR-deficient strains exhibit increased hemolytic activity[10,11,12,14]. The β-H/C toxin is a well-established virulence factor known to promote GBS cellular interactions, and to provoke host innate immune responses in human epithelial and endothelial cell models by activating transcription of host genes encoding the chemokines IL-8, CXCL1, and CXCL2 for neutrophil recruitment[15,16]. *In vivo* studies have further indicated that the β-H/C contributes to the development and

severity of meningitis[16], pneumonia[17], arthritis[18], and sepsis[19]. The cytotoxic and proinflammatory properties of the β-H/C toxin work to the detriment of the host. The CovR regulatory system itself has also been shown to contribute to disease progression[12]. GBS CovR/S mutation has been observed in clinical isolates[20] and CovR/S mutations are frequently observed in *Streptococcus pyogenes* (Group A *Streptococcus*)[21,22].

We hypothesized that the CovR/S TCS plays an important role in modulating GBS colonization and virulence, and that loss of CovR regulation in the vaginal environment will impact host response and GBS persistence. In this study, we examine for the first time the acute response of vaginal epithelium to GBS using microarray, real-time RT-PCR, and protein analysis. We show that human vaginal epithelial cells respond to GBS with the increased production of genes involved in the immune response and tissue remodeling compared to a native vaginal bacterium, *Lactobacillus crispatus*. Infection with GBS deficient in CovR induces a much more aggressive inflammatory response than does wild-type (WT) GBS, which is at least partially due to increased β-H/C production. Differentially induced genes were primarily proinflammatory chemokines such as those involved in neutrophil activation and recruitment. Experiments with isogenic GBS mutants lacking CovR, β-H/C, or both factors demonstrate that other CovR regulated factors, independent of the β-H/C toxin, contribute to GBS adherence and invasion. Using an *in vivo* model of GBS vaginal colonization, we demonstrate that functional CovR regulation dampens cytokine production and promotes bacterial persistence in the vaginal tract. Our studies suggest that the host vaginal epithelium plays an active role in immune surveillance and that GBS precisely modulates gene expression to promote survival and colonization.

MATERIALS AND METHODS

Bacterial strains and growth conditions

Streptococcus agalactiae (GBS) strains were grown in Todd-Hewitt broth (THB) (Hardy Diagnostics) at 37°C. The wild-type (WT) clinical isolates used in this study are A909 (serotype Ia)[23] and COH1 (serotype III)[24]. Both A909 and COH1 Δ*cylE* mutants were constructed previously[13], as were the A909Δ*covR* and Δ*covR*/Δ*cylE* mutants[12,14]. The COH1Δ*covR* was derived using methods described[14]. The A909Δ*fbsB*/Δ0956 strain was constructed using methods described[25], where the gene encoding kanamycin (Ω*km*-2) was used for allelic replacement. The complemented *covR* strain (p*covR*) was generated using methods as described previously[26]. Briefly, the gene encoding CovR was amplified using primers 5'GCGCGGAGCTCTTGTTAAGTAAAGAATAAG 3' and 5'GCGCGAGGATCCTTTATTTTTCACGAATCAC 3' and the PCR products were digested and ligated into the complementation vector pDC123[27] downstream to the tetracycline promoter, P_{tet} followed by electroporation into the GBS A909Δ*covR* mutant. When necessary, mutants were maintained and grown in antibiotics at the following concentrations: spectinomycin (300 μg/mL), chloramphenicol (2 μg/mL), and kanamycin (500 μg/mL). *Lactobacillus crispatus* (LC) (Strain # 33820, ATCC) was grown in Lactobacilli MRS broth (BD Biosciences) at 37°C.

Cell lines

Immortalized human vaginal epithelial cell (HVEC) line, VK2/E6E7, and ectocervical epithelial cell line, Ect1/E6E7, were obtained from the American Type Culture Collection (ATCC CRL-2616 and ATCC CRL-2614 respectively)[28]. Passages 5-

20 were used for all cell assays. Cells were maintained at 37°C in a 5% CO_2 atmosphere in keratinocyte serum-free medium (KSFM) (Invitrogen) with 67.419 pg/mL human recombinant epidermal growth factor and 65 μg/mL bovine pituitary extract as described previously[6].

Vaginal cell infection and microarray analysis

HVEC were grown to confluency in 24 well tissue culture treated plates and washed prior to bacterial exposure. Bacteria were grown to mid-log phase and then added to cells at a multiplicity of infection (MOI) of 50. After an infection period of 4 hours, total RNA was extracted (Macherey-Nagel) and microarray analysis (HumanHT-12_v4, Illumina) was performed at BIOGEM at the University of California, San Diego. HVEC microarrays were performed with two independent biological replicates of each strain (A909, Δ*covR*, and Δ*cylE*) and media only controls, and one replicate of *L. crispatus*. Heatmap analysis was performed using Cluster 3.0 and TreeView (Eisen Laboratories), and the Venn Diagram calculated with the assistance of Area-Proportional Venn Diagram (BioInfoRx).

***In vitro* cell assays**

GBS adherence and invasion assays of HVEC were conducted as described previously[6] with minor modifications. Concisely, cells were grown to confluency in 24 well tissue culture treated plates and washed prior to bacterial addition. Bacteria were grown to mid-log phase and added at an MOI of 1 for adherence assays. After 30 minutes of incubation, cells were washed 6 times with PBS and lysed by adding trypsin-EDTA and Triton X-100. Lysate was serially diluted and plated on THB agar plates to

enumerate bacterial cfu. Total adherent cfu was calculated as (total cfu recovered/total cfu of original inoculum)×100%. To quantify intracellular bacteria, cells were incubated at MOI of 10 for 2 hours, monolayers washed, treated with antibiotics, and incubated for an additional 2 hours[6]. Cells were washed 3 times with PBS, lysed as described above, and intracellular GBS determined by serial dilution plating and total intracellular bacteria quantified as above.

RT-qPCR and ELISA

For analysis of gene expression induction, HVEC were grown to confluency in 24 well tissue culture-treated plates and washed prior to bacterial exposure. Bacteria were grown to mid-log phase and added at an MOI of 10 and incubated for 4 hours. Cells were then lysed, total RNA extracted (Macherey-Nagel), and qPCR performed (Quanta Biosciences). Primers and primer efficiencies for IL-8, CXCL1, CXCL2, CCL20 and GAPDH were utilized as previously described[29]. For ELISA assays, HVEC were infected as described above with several modifications. Bacteria were added at an MOI of 10-50 as listed in figure legends, and cells were incubated with bacteria for 1.5-2 hours as indicated. After initial incubation, cells were washed with once with PBS, and fresh KSFM added containing 5 μg/mL penicillin and 50 μg/mL gentamicin. Cells were incubated an additional 3.5-4 hours in the presence of antibiotics and cell supernatants were analyzed for chemokine secretion using human IL-8 (R&D Systems), CXCL1 (R&D Systems), and CXCL2 (Antigenix America) ELISA kits according to manufacturer's instructions.

***In vitro* HVEC viability assay**

To determine viability of HVEC infected with GBS *in vitro*, adherence and invasion assays were performed as described above. Afterwards, supernatant was aspirated and placed into microfuge tubes. To dislodge adherent cells, 100 μL of 0.25% trypsin-EDTA (Gibco) was added to each well and incubated at 37°C for 5 minutes. Trypsin activity was halted by adding 200 μL of DMEM F12 (Cellgro) with 10% fetal bovine serum (Invitrogen). Cells were gently removed from wells by pipetting up and down several times and suspended cells were added to supernatant. To distinguish live cells from dead cells (with permeable membranes), 0.4% Trypan Blue (Life Technologies) was added at 1:10, and live cells (unstained) and dead cells (stained) were quantified using a hemocytometer. Percent of live cells per sample was calculated as (number of live cells/total cells counted)×100%. Two grids per sample were counted and results averaged.

***In vivo* mouse model of GBS vaginal colonization**

All mouse work was approved by the Office of Lab Animal Care at San Diego State University and conducted under accepted veterinary standards. Female CD1 and BALB/c mice (8-17 weeks old) were obtained from Charles River Laboratories and used for colonization assays adapted from previous work[6]. Breeding pairs of CXCr2 (CXCL2 receptor) knock out (KO) mice (formerly IL8r KO mice), were originally purchased (C.129S2(B6)-Cxcr2^{tm1Mwm}/J, Jackson Laboratories). The mutation was crossed onto a BALB/c background prior to being deposited at Jackson Laboratories. We established a homozygous x homozygous breeding colony at the UCSD VA Hospital using mice that were maintained on water containing co-trimoxazole (200 μg/mL sulfamethoxazole and 40 μg/mL trimethoprim). For the 17 week old females used in this study, antimicrobial

treatment was terminated 48 hours prior to inoculation with GBS. To synchronize estrus and promote bacterial colonization[30,31], mice were injected intraperitoneally with 0.5mg 17β-estradiol suspended in sesame oil (Sigma) 24 hours prior to inoculation. Mice were inoculated with ~1×10^7 cfu (in 10 μL PBS) GBS in the vaginal lumen. Immediately prior to inoculation, vaginal lavage was performed by pipetting the lumen with 20 μL of PBS several times to collect cells and cytokines as described elsewhere[32,33]. On successive days, the vaginal lumen of each mouse was first lavaged for cytokine analysis and then swabbed with ultrafine calcium alginate-tipped swabs. Bacterial load was determined by serial dilution plating of swab samples. WT or mutant GBS strains were identified as mauve or light pink-pigmented colonies on CHROMagar Strep B agar (DRG International Inc.)[34]. For tissue collection, mice were sacrificed using CO_2 asphyxiation and reproductive tracts excised from mid-uterine horn to just proximal of the vulva. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. ELISA assays were performed on vaginal lavages for KC (R&D Systems), MIP-2 (R&D Systems) and IL-1β (eBiosciences) as described by manufacturer.

Myeloperoxidase Assay

Neutrophil recruitment and activation was determined by quantifying the neutrophil enzyme, myeloperoxidase (MPO), as described previously[35], but modified for murine vaginal samples. Cells were collected from swabs as described above and suspended in 100 μL PBS. 50 μL of the swab sample was added to 100 μL of 0.05% hexadecyltrimethylammonium bromide (HTAB) buffer and MPO was released from neutrophils by bead-beating with 1.0 mm diameter zirconia beads (BioSpec) for 1 minute using a Mini BeadBeater (BioSpec). Samples were centrifuged at 13,000 rpm for 15

minutes at 4°C, and 10 μL of supernatant was added to 190 μL phosphate buffer containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. Absorbance was measured at 450 nm in 96-well flat bottom plates and MPO activity calculated as milliunits per volume of homogenate supernatant (mU/mL of supernatant).

Microscopy

Vaginal cell monolayers were propagated on glass cover slips within 24 well plates. Following a standard adherence assay (MOI = 100), monolayers were washed 6 times with DPBS and the cover slips were then removed from the trays. Cover slips were air-dried and heat fixed then subjected to a standard Gram stain protocol as described previously[6]. Paraffin embedded reproductive tracts were sectioned on a Leica RM 2125 microtome at 5 μm and stained with a standard H&E staining protocol. All images were taken on a Zeiss upright microscope with attached Axiocam Icc3 camera at indicated magnification.

Statistical Analysis

GraphPad Prism version 5.04 was used for statistical analyses. Differences in *in vitro* assays including adherence, invasion, qPCR, ELISA and cell viability were calculated using unpaired Student's *t* test analysis. Differences in bacterial loads recovered from mouse vaginal tracts were calculated using Kruskal-Wallis test (nonparametric) with Dunn's multiple comparisons post-test, or Fisher's exact test analysis as indicated in figure legends. Differences in cytokine and MPO levels from vaginal lavage were calculated using either Mann-Whitney test (nonparametric) or Fisher's exact test analysis as indicated in figure legends. Statistical significance was determined at a $p<0.05$.

RESULTS

Differential gene expression profile of vaginal epithelium induced by GBS

To determine the response of vaginal epithelium to GBS colonization, we performed global transcriptional profiling on human vaginal epithelial cells (HVEC) during infection with WT GBS (strain A909, serotype Ia) or isogeneic Δ*covR* and Δ*cylE* mutant strains. As a control, we exposed HVEC to a nonpathogenic vaginal organism, *L. crispatus*[36]. As can be expected, exposure to the native flora species, *L. crispatus*, did not induce an inflammatory response compared to media alone (Fig. 1A and Suppl. Table 1). However, when HVEC were infected with WT GBS, numerous immune pathways were activated including proinflammatory cytokines IL1-α and IL1-β, and chemokines involved in leukocyte recruitment and activation such as CCL20, IL8, CXCL1, and CXCL2 (Fig. 1A and Suppl. Table 1). A similar global pattern of gene induction was observed during infection with another WT (serotype V) GBS strain (data not shown). Of particular interest, infection with the Δ*covR* mutant induced higher levels of expression of several of these genes, including IL-8 (2-fold) and CXCL2 (5-fold) when compared to WT. GBS β-H/C has previously been shown to activate neutrophil recruitment in other models of infection[16], and here we show that infection with the Δ*cylE* mutant resulted in lower induction of genes required for leukocyte recruitment and activation as a whole, including 4-fold lower transcription of IL-8 and CXCL2, indicating that β-H/C mediates similar responses in the vagina. Moreover, infection with the Δ*covR* mutant resulted in the upregulation of 708 genes (>2-fold) in vaginal epithelial cells as compared to 310 or 125 genes induced by WT or Δ*cylE* infection respectively (Fig. 1B). These results illustrate the impact of β-H/C alone in the vaginal tract by and demonstrate

a tremendous shift in vaginal epithelium response to GBS when CovR regulation is removed.

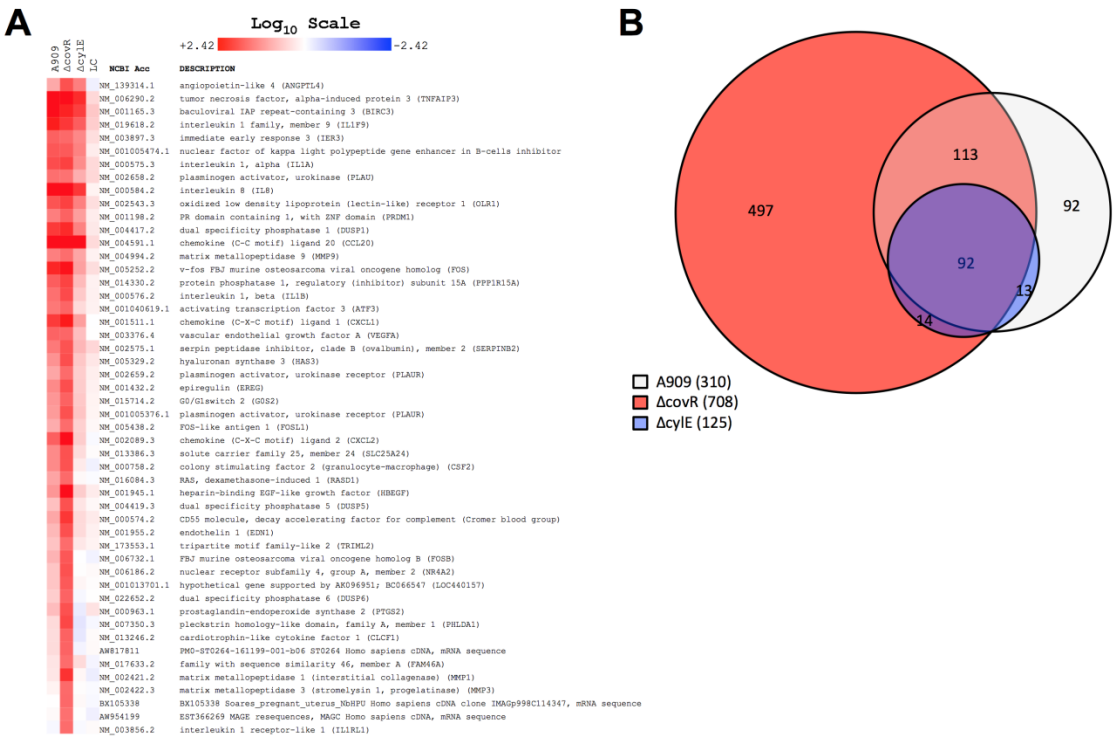


Figure 2.1. Microarray of gene expression levels in HVEC upon infection with A909, Δ*covR*, Δ*cylE* or *Lactobacillus crispatus* (LC). (A) Each column represents the mean of two biological replicates (one replicate for LC) of a microarray experiment. Shown are the top 50 genes upregulated by the Δ*covR* mutant. Red and blue coloring indicates induced or down regulated genes respectively. Values expressed are on a Log_{10} scale and clustering was performed as described in Experimental Procedures. (B) Venn Diagram of all genes upregulated >2 fold. Circumferences and overlap were calculated as described in Experimental Procedures.

To verify the microarray data *in vitro*, we examined differences in transcript and protein levels of highly induced genes involved in neutrophil recruitment. Additionally, we examined the effect of infection with a Δ*covR*/Δ*cylE* double mutant to determine the proportion of increased inflammatory response contributed by increased β-H/C

production in Δ*covR*. We observed that infection with the Δ*covR* mutant resulted in increased transcription of IL-8 and CXCL2 compared to WT infection, while transcription of CXCL1 and CCL20 was not significantly different (Fig. 2A). When comparing the response to WT and Δ*cylE* infection, loss of β-H/C reduced chemokine transcription of IL-8, CXCL1, CXCL2, and CCL20. Interestingly, transcription of all chemokines was also down regulated in the Δ*covR*/Δ*cylE* mutant compared to WT, highlighting the inflammatory role of β-H/C in human vaginal cells.

We further analyzed protein secretion of the most potent and differentially induced neutrophil chemokines during infection with WT, Δ*covR*, Δ*cylE*, and Δ*covR*/Δ*cylE* mutant stains. Production of IL-8 and CXCL2 protein was drastically increased in cells infected with A909 WT and Δ*covR* compared to infection with commensal bacterium, *L. crispatus* or to the media control (Fig. 2B,C). As was observed at the transcriptional level, IL-8 and CXCL2 secretion was significantly lower in cells infected with Δ*cylE* and Δ*covR*/Δ*cylE* strains as compared to WT (Fig. 2B,C). Similar results were observed for CXCL1 production (Suppl. Fig. 1A). We observed evidence of toxicity in HVEC during infection with the hyper-hemolytic A909 Δ*covR* strain at the length of time required to detect protein production (data not shown), which may explain why protein levels of IL-8 and CXCL2 are not higher during A909 Δ*covR* infection compared to that observed during WT A909 infection. However, differential IL-8 secretion was detected during infection of ectocervical cells (Fig. 2D). Furthermore, we observed that infection of HVEC with a less hemolytic GBS strain, COH1, a serotype III clinical isolate which is a sequence type (ST)-17 strain, and its isogenic Δ*covR* mutant

resulted in increased protein secretion of IL-8, CXCL1 and CXCL2 after exposure to the COH1 Δ*covR* mutant strain compared to WT COH1 (Suppl. Fig. 1B).

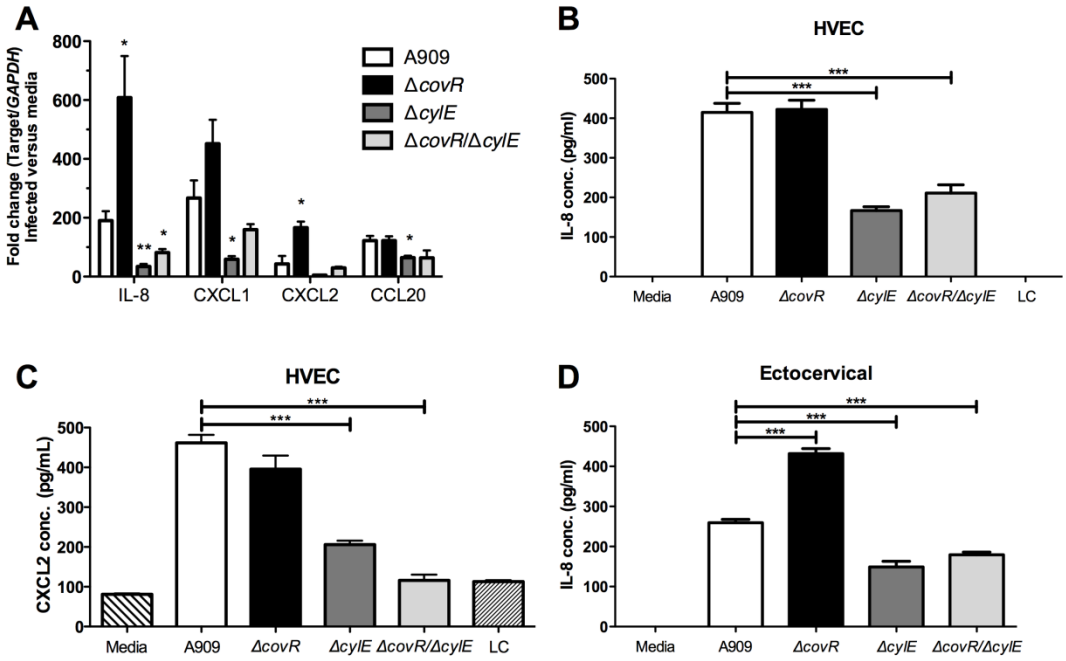


Figure 2.2. mRNA expression of IL-8, CCL20, CXCL1 and CXCL2 and protein production of IL-8 in HVEC and ectocervical cells upon infection with WT and mutant strains. (A) mRNA expression levels of IL-8, CCL20, CXCL1 and CXCL2 in HVEC infected with either A909, Δ*covR*, Δ*cylE* or Δ*covR*/Δ*cylE* using quantitative RT-PCR. Fold change was calculated using GAPDH. Data is one representative experiment of at least 3 independent experiments performed in 5 replicates. Protein expression of IL-8 (B) or CXCL2 (C) in HVEC supernatants 5 hours post-infection with A909, Δ*covR*, Δ*cylE*, Δ*covR*/Δ*cylE* or *L. crispatus* (LC) at MOI of 50. (D) Protein expression of IL-8 in ectocervical supernatants 5 hours post-infection with A909, Δ*covR*, Δ*cylE* or Δ*covR*/Δ*cylE* at MOI of 50. Experiments were performed at least two times with at least four replicates, and one representative experiment is shown. Data was analyzed by unpaired Student's *t* test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

CovR moderates adherence and invasion in vaginal epithelial cells

Several GBS WT strains, including A909, have already been shown to readily attach to HVEC *in vitro*[6,7]. To determine the effect of CovR regulation on GBS interaction with vaginal epithelium, we characterized attachment and invasion of WT,

Δ*covR*, Δ*cylE*, and Δ*covR*/Δ*cylE* strains in HVEC using standard methods as previously described[6,37]. After 30 minutes of incubation, and multiple washes to remove nonspecific binding, adherent GBS was quantified from monolayer lysates. We observed that both Δ*covR* (p = 0.0068) and Δ*covR*/Δ*cylE* (p = 0.0136) mutants adhered significantly more than WT, with Δ*covR* approaching 100% adherence of original inoculum (Fig. 3A). Adherence of the Δ*cylE* mutant did not significantly differ from that observed for WT GBS. Adherent GBS in association with vaginal epithelium were visualized by microscopy following multiple wash steps and Gram staining (Fig. 3C). Additionally, we quantified viable intracellular bacteria from cell lysates following a 2 hour incubation period and a 2 hour antibiotic treatment to kill any extracellular bacteria. Our data show that, when compared to WT, Δ*covR* and Δ*covR*/Δ*cylE* mutants exhibited reduced invasive capability (Fig. 3B). We recovered a relatively low level of intracellular bacteria (0.5% of the initial inoculum of WT GBS), but we were able to recover only 0.002% of the Δ*covR* mutant. Thus approximately 0.021% and 0.000016% of the adherent WT and Δ*covR* GBS, respectively, had invaded the intracellular compartment. Similar results were observed when CovR was deleted from serotype III GBS strain, COH1 (Suppl. Fig. 2A,B). Interestingly, the A909Δ*cylE* mutant had significantly more intracellular colony forming units than WT, indicating expression of β-H/C may decrease intracellular survival in vaginal epithelial cells, however, this effect was not seen in Δ*covR*/Δ*cylE*, suggesting that additional genes regulated by CovR impact GBS intracellular survival.

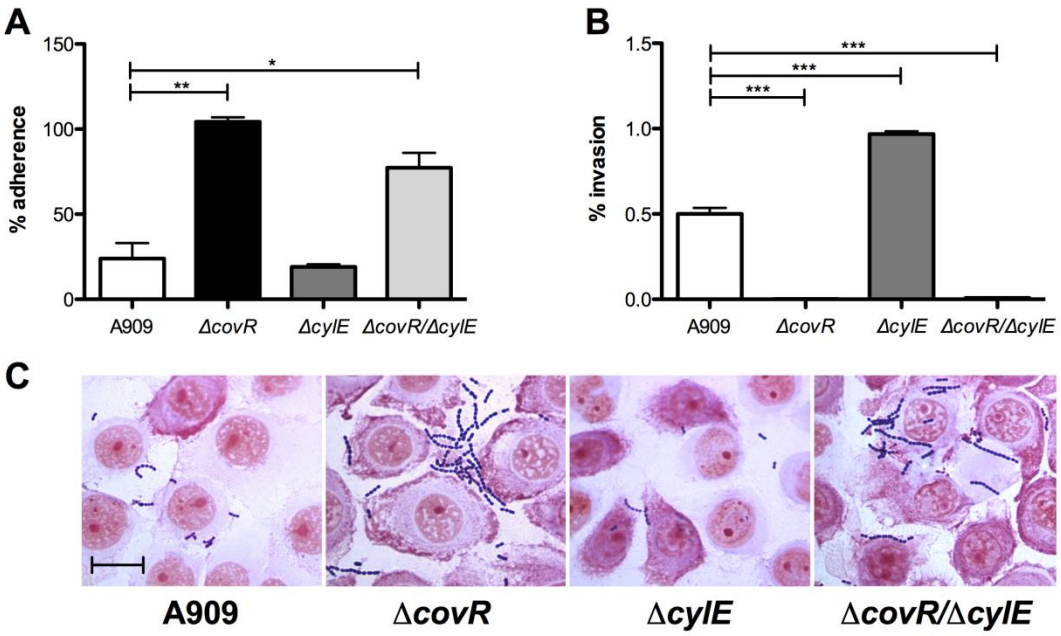


Figure 2.3. GBS interaction with vaginal epithelium *in vitro*. Adherence (A) and invasion (B) of GBS A909 and mutant strains to HVEC. Values are expressed as the total of cell-associated cfu (A) or total intracellular cfu (B) recovered compared to original inoculum. Assays were performed at an MOI of 1 (A) or MOI of 10 (B). (C) Gram-stain of HVEC infected with WT and mutant strains. Magnification = 1000X, scale bar = 20 μm. Experiments were repeated at least 3 times in triplicate and data from a representative experiment is shown. Data was analyzed by unpaired Student's *t* test. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

To verify that the Δ*covR* mutant phenotype could be complemented, we cloned the A909 *covR* sequence into a GBS expression vector and transformed this plasmid (p*covR*) into the Δ*covR* mutant as described in Experimental Procedures. GBS strains expressing more β-H/C typically produce more red pigment[38], such as our Δ*covR* mutants, and we observed a loss of pigmentation when the Δ*covR* mutant was complemented with p*covR* (Suppl. Fig. 3C). Additionally the complemented strain exhibited WT levels of β-H/C activity (data not shown). In both adherence and invasion assays, the complemented p*covR* strain differed significantly from the Δ*covR* strain, exhibiting significantly lower adherence (Suppl. Fig. 3A) and significantly greater

invasion (Suppl. Fig. 3B), approaching WT levels. Additionally, because we had observed toxicity during infection with the Δ*covR* mutant at higher MOIs and longer incubation times, we sought to confirm that increased toxin production of Δ*covR* was not compromising HVEC integrity during our invasion assays. As described in Experimental Procedures, we stained cells with Trypan Blue under the same conditions used in the invasion assay. We observed no difference in cell viability during incubation with WT or any of the mutant strains, and all treatment groups were >95% viable (Suppl. Fig. 3D).

Role of CovR in vaginal persistence *in vivo*

Our *in vitro* data indicates that CovR regulation alters both vaginal epithelial interactions (Fig. 3) as well as host immune response (Fig. 1, 2). To confirm these results *in vivo*, we used a mouse model of GBS vaginal colonization adapted from previous work by our lab[6] and others[31,39]. In rodents, normal flora load and novel bacterial colonization ability appear to peak at estrus[30,40,41]. We found this to be true in our model of GBS colonization (Suppl. Fig. 4A,B). Consequently, we treated 8-week old CD1 mice with 17β-estradiol one day prior to bacterial inoculation. We inserted ~1×10^7 cfu GBS into the vagina, and on successive days, the vaginal lumen was swabbed and recovered bacteria quantified on agar plates to determine changes in bacterial load over time. The Δ*covR* mutant exhibited decreased persistence in the vaginal tract by day 2 post-inoculation, as significantly more mice treated with Δ*covR* had cleared GBS when compared to WT (p = 0.0108) (Fig. 4A) in the combined results of 4 independent experiments. Similarly, a significant reduction was observed when analyzing the isogenic Δ*covR* mutant in the COH1 background (Suppl. Fig. 2C). We also examined the colonization of other GBS mutants deficient in genes highly regulated by CovR in the A909 strain: Δ*cylE*,

Δ*covR*/Δ*cylE,* and a double mutant in *fbsB* (SAK_0955) and adjacent gene (SAK_0956) encoding a hypothetical protein. Although in all instances, the mean number of mutant bacterial cfu recovered from mice decreased during colonization compared to the WT strain over the course of 5 days, the differences were not significant largely due to the high level of variation observed in this model (Suppl. Fig 4C). However, at day 5 there was an 8.0-fold decrease in mean bacterial load recovered from mice inoculated with Δ*covR* compared to only a 1.6-fold reduction in WT bacteria recovered.

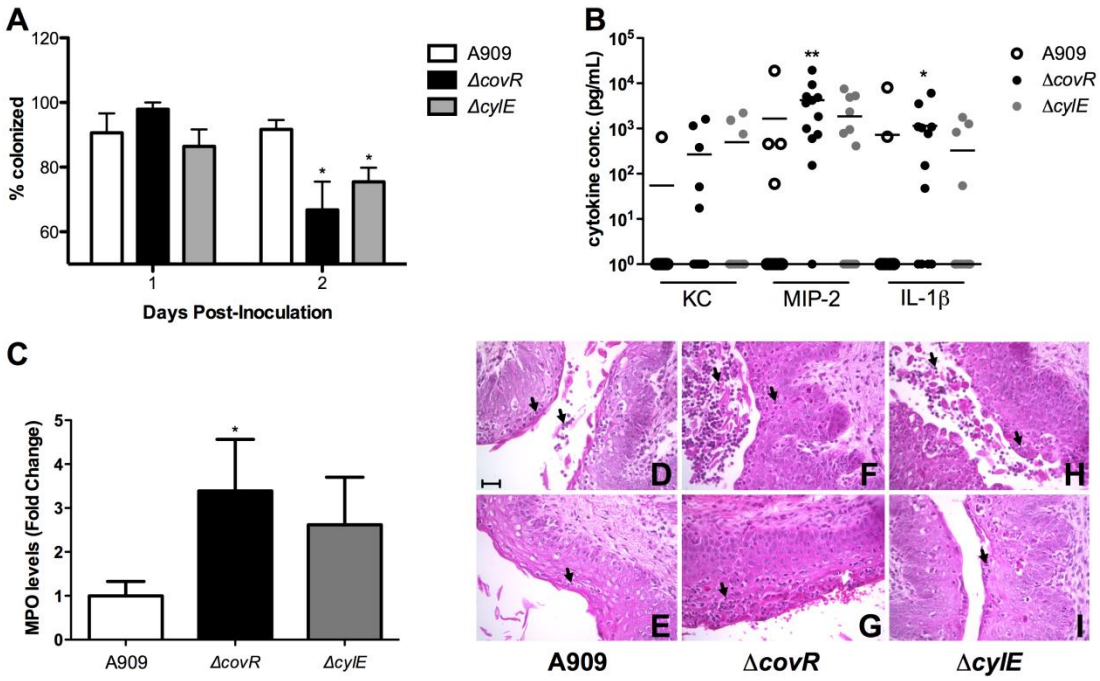


Figure 2.4. CovR regulation affects host immune response *in vivo* soon after inoculation. (A) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 8 week old CD1 mice in 4 independent experiments and combined data at day 2 post inoculation is shown (n = 39 total per group). (B) KC, MIP-2 and IL-1β levels in vaginal lavage fluid from mice on day 2 post inoculation were quantified by ELISA (n = 12 per group). Lines represent mean cytokine concentration. (C) In three independent experiments, 8 week old CD1 mice were inoculated with $1x10^7$ cfu GBS (n = 30 total per group), and neutrophils collected by swabbing the vagina at day 2 post-inoculation. MPO levels were quantified, fold changes of mutants normalized to WT, and combined data is shown. Mice that did not have detectable MPO levels were excluded. (D-I) Vaginal epithelium histology of CD1 mice inoculated as in (A) at day 2 post inoculation stained with H&E. Two representative images are shown per bacterial strain. (D,E) Mice inoculated with WT show some neutrophils present in the vaginal epithelium and lumen (black arrows). (F,G) Mice inoculated with Δ*covR* show increased neutrophil infiltration (black arrows) and hemorrhaging (white arrow) compared to WT. (H,I) Mice inoculated with Δ*cylE* show variability in neutrophil infiltration (black arrows). Magnification = 400X, scale bar = 50 μm. Data was analyzed by Fisher's exact test for colonization assays and ELISA's, and Mann-Whitney test for MPO assays. * $p<0.05$, ** $p<0.01$.

Loss of CovR regulation heightens host immune response *in vivo*

Since we observed an increase in numerous innate immune factors in vaginal cells exposed to Δ*covR in vitro* (Fig. 1, 2), as well as a decreased vaginal persistence (Fig. 4A),

we sought to characterize host immune response *in vivo*. Vaginal lavage from CD1 mice was collected for cytokine analysis by gently pipetting PBS in the vaginal lumen multiple times as described[32] prior to GBS inoculation and then throughout GBS colonization. Cytokine levels of murine chemokines KC and MIP-2, as well as IL-1β, were measured by ELISA. We observed large variation in cytokine concentrations between individual mice, which has been seen previously[32]. Even so, by day 2 post-inoculation, significantly more mice inoculated with Δ*covR* secreted MIP-2 (11/12 vs. 4/12, $p = 0.0094$) and IL-1β (8/12 vs. 2/12, $p = 0.0361$) compared to WT (Fig. 4B). Additionally, more Δ*covR* treated mice secreted chemokine KC than WT treated mice (5/12 vs. 1/12), but this difference was not significant.

Since MIP-2 and KC are both key chemoattractants involved in the recruitment of neutrophils to the vaginal epithelium[32,42], we assessed neutrophil activation and influx into the vaginal lumen by measuring neutrophil enzyme myeloperoxidase (MPO) during GBS colonization. MPO serves as an effective indication of neutrophil infiltration[43]. We observed that mice inoculated with Δ*covR* expressed significantly higher fold levels of MPO than mice treated with WT (Fig. 4C). To visualize neutrophil infiltration, we collected vaginal tissues 2 days post GBS inoculation for histopathologic analysis. Consistent with our previous results there were fewer mice colonized with Δ*covR* at day 2 post inoculation compared to WT (data not shown). At this time point, neutrophils were present in most of the mice, however, mice treated with Δ*covR* showed greater inflammation, epithelial rearrangement and hemorrhaging than WT and Δ*cylE* treated mice, with two representative images shown (Fig. 4D-I). Mice inoculated with Δ*cylE* exhibited more variation within the epithelium than either of the other groups (Fig. 4H-I),

in combination with significantly fewer cfu recovered compared to WT (Fig. 4A), which may be explained by individual immune system differences between mice in response to a less virulent form of GBS.

The CXCr2 receptor contributes to host immune response *in vivo*

Thus far, we have demonstrated that infection with the Δ*covR* mutant results in a heightened inflammatory response *in vitro* and *in vivo*. Moreover, we have observed a decreased persistence of the Δ*covR* mutant in the vaginal environment compared to WT, and we hypothesized that enhanced immune response during infection with Δ*covR* is responsible for this difference. Because neutrophil chemokines were differentially induced by the Δ*covR* mutant, both *in vitro* and *in vivo*, we used mice that lack a receptor for these chemokines. We inoculated CXCr2 KO mice, formerly known as IL-8r KO mice, and control BALB/c mice with either WT GBS or the Δ*covR* mutant as described in Experimental Procedures. Mice were swabbed and lavage fluid collected daily to enumerate bacterial load and cytokine production respectively. GBS persisted longer in BALB/c mice than in CD1 mice (data not shown), but by day 5, mean cfu values, although not significant, were distinctly different with fewer Δ*covR* (4.1×10^5) recovered compared to WT (1.6×10^6) (Fig. 5A). However, we did not observe a difference in recovered bacteria between the WT and Δ*covR* over time in CXCr2 KO mice (Fig. 5A). Our results revealed that, like CD1 mice, WT BALB/c exhibited significantly increased MIP-2 ($p = 0.0372$) and KC ($p = 0.0328$) levels in mice receiving Δ*covR* as compared to WT by day 2 post-inoculation (Fig. 5B,C). At no time point examined did we observe significant differences in KC and MIP-2 levels in CXCr2 KO mice in either group (Fig. 5B,C). Furthermore, we did not observe neutrophils in vaginal lavage fluid of CXCr2 KO

mice at any time point examined (Suppl. Fig. 5). In combination, this data suggests that increased host inflammatory response to Δ*covR* occurs at least in part by signaling through the CXCL2 receptor.

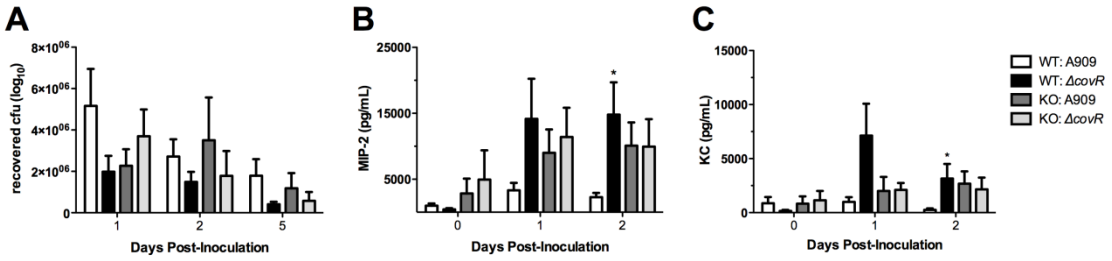


Figure 2.5. Role of the CXCL2 receptor during GBS infection *in vivo.* (A) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 16-17 week old WT BALB/c and CXCr2 KO mice. GBS persistence was measured by swabbing the vagina and enumerating recovered bacteria as described in Experimental Procedures. For all groups, n = 10, or n = 9 for WT BALB/c mice inoculated with WT GBS. (B) MIP-2 and (C) KC levels in vaginal lavage from same mice as (A) measured by ELISA. Data was analyzed by Kruskal-Wallis test for persistence and Mann-Whitney test for cytokine production. * $p<0.05$.

DISCUSSION

GBS poses a severe threat to newborn infants worldwide. GBS propagates through vertical transmission as 50-70% of infants born to colonized mothers become colonized[44]. A better understanding of mechanisms important for GBS colonization of the vaginal tract, identification of host and bacterial factors contributing to colonization, and genetic and environmental stimuli that promote GBS colonization and subsequent transmission is essential. Through combining our *in vitro* and *in vivo* models, we have continued to identify host and microbe features that impact GBS persistence in the vaginal environment. Herein, we have demonstrated that the CovR/S regulatory system is necessary for limiting the expression of virulence factors during vaginal colonization, thereby reducing the host innate immune response to promote colonization.

GBS CovR/S has been shown to positively and negatively regulate a variety of genes, of which, *cylE* (β-H/C) is one of the most highly repressed[10,11,12]. Host global transcriptional profiling during infection with WT, Δ*covR*, or Δ*cylE* GBS revealed the β-H/C toxin as a key mediator in provoking an acute inflammatory response in the vaginal epithelium, which was further confirmed by analysis of specific gene transcripts during infection with a Δ*covR*/Δ*cylE* mutant. Most dramatic was the shift in gene induction profiles when CovR regulation was absent, including chemokines IL-8 and CXCL2, which are involved in neutrophil activation and recruitment[45]. Additionally, vaginal epithelial cells responded to GBS infection with increased production of proinflammatory factors such as IL-1β, which promotes neutrophil recruitment and bacterial clearance[46], GM-CSF (CSF2), which controls the production, differentiation, and function of granulocytes, macrophages and other leukocytes[47], and oxidized low density lipoprotein (lectin-like) receptor 1 (OLR1), a leukocyte cell adhesion molecule[48]. Furthermore, GBS infection stimulated an increased production of extracellular matrix modifiers, including those associated with inflammation and epithelial disruption such as VEGF, MMP1, MMP3, MMP9, plasminogen activators and HAS3[49,50,51,52,53]. While these proteins are associated with inflammation and neutrophil migration, their expression may also allow GBS access to underlying tissues and bloodstream by disruption of the epithelial barrier.

CovR regulates many secreted or cell wall and envelope-associated GBS factors[10,12]; thus logically, CovR regulation may impact host cell interactions. We have shown previously that the A909 CovR deficient strain exhibits increased adherence and decreased invasion in brain endothelium and lung epithelium[12]. Although the exact mechanism is not known, we reveal that loss of CovR in two different GBS serotypes, Ia

and III, promotes adherence to and limits invasion of vaginal epithelial cells independent of *cylE* expression. Additionally, we observed that WT GBS adherence and invasion levels can be restored by complementing the CovR deficient strain. However, the invasion phenotype was not completely rescued, which may suggest a more complex regulation of this function. Consistent with our results, a recent study demonstrated that a CsrR/S (CovR/S) deficient strain exhibited increased adherence to epithelial and abiotic surfaces[54]. Environmental signals such as vaginal pH may alter CovR regulation as neutral, and not acidic, pH enhanced GBS binding to vaginal epithelial cells, and this was partially dependent on CsrR/S (CovR/S)[54]. Furthermore, CovR/S down regulates virulence factors such as *cylE* and *scpB* (a C5a peptidase) in acidic pH[55]. The enhanced adherence observed in the CovR-deficient strain may prove detrimental to colonization, as increased host-microbe interaction may provoke the increased immune activation and cytokine production that we observed. Additionally, host intracellular responses may also explain the decreased invasion seen in Δ*covR* and Δ*covR*/Δ*cylE* mutants (Fig. 3B), because it has been demonstrated that, although a CovR deficient mutant was phagocytosed more efficiently by macrophages, it exhibited decreased ability to survive intracellularly[56]. Therefore, we cannot exclude the possibility that CovR regulation effects both bacterial invasion and intracellular survival, which consequently may impact colonization.

Other CovR-regulated factors, FbsA and FbsB, which are fibrinogen binding proteins, have been shown to contribute to bacterial attachment and invasion of other host cells[57,58,59]. In the A909Δ*covR* mutant, these genes are highly expressed; transcription of FbsA (SAK_1142) was increased 25-fold, and FbsB (SAK_0955) and an adjacent gene

(SAK_0956) encoding a hypothetical protein was increased 151-fold and 157-fold respectively[12]. We constructed a mutant lacking FbsB and the adjacent gene (Δ*fbsB*/Δ*0956*), and subsequent analysis revealed that it exhibited similar levels of vaginal cell adherence and invasion to the WT strain (data not shown), and further, it was not cleared from the mouse vagina as readily as the Δ*covR* mutant (Suppl. Fig. 4C). This suggests that these factors are not responsible for the altered adherence and invasion phenotype of the CovR deficient strain. However, fewer Δ*fbsB*/Δ*0956* bacteria were recovered from the mouse vaginal tract compared to WT (Suppl. Fig. 4C) indicating fibrinogen binding may contribute to GBS colonization *in vivo*.

Murine chemokines KC (CXCL1) and MIP-2 (CXCL2) are both functional homologs of human CXCL8 (IL-8) and bind to CXCr2 (IL-8 receptor homolog) on neutrophils, resulting in their migration to the site of chemokine production[60]. To determine the functional role of chemokine signaling during GBS colonization, we utilized CXCr2 KO mice in our murine vaginal model and observed no difference in cytokine levels between GBS WT or Δ*covR* groups, starkly contrasting our results in CD1 and BALB/c mice. Cytokine levels in CXCr2 KO mice steadily increased in vaginal lavage over the time course examined when compared to BALB/c mice, indicating that chemokine production was not directly affected by the lack of the receptor. Steady increase of MIP-2 levels in CXCr2 KO mice has been observed in other models of pathogenic infection and is believed to occur because of lack of effective down-regulation and/or continued stimuli[61]; KC was not examined in this particular study. Of note, CXCr2 KO mice did not have neutrophils visible in vaginal lavage fluid either prior to or during the experimental period when viewed under the microscope, whereas

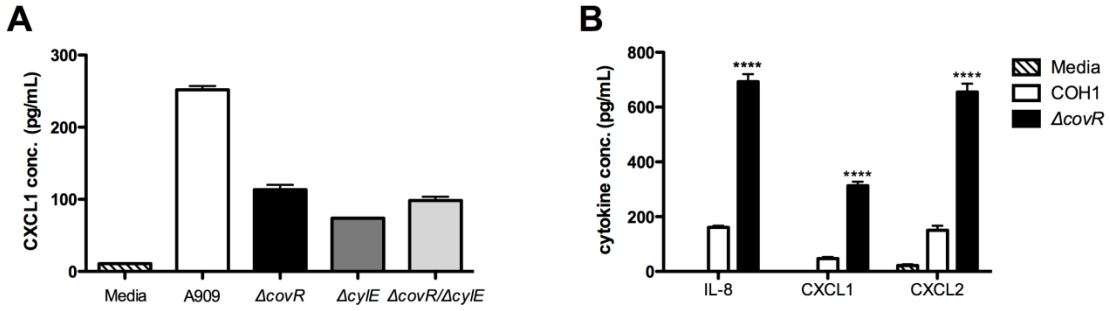
neutrophils were present in the control mice, both prior to inoculation as part of the estrous cycle, and following GBS inoculation (Suppl. Fig. 5). This supports previous work showing MIP-2 expression is necessary to recruit neutrophils to the vaginal lumen during the normal estrous cycle[32]. This data also suggests that the increased cytokine levels observed in BALB/c mice treated with Δ*covR* may be explained by paracrine signaling and secretion by neutrophils that have infiltrated the vaginal epithelium.

Within the vaginal tract, mucosal epithelial immunity is tightly controlled by the estrous cycle, with sex steroids affecting all aspects of innate and adaptive immunity, whether directly or indirectly[62]. In human vaginal fluid samples, IL-8 and IL-1β production positively correlate with pattern-recognition proteins, and these cytokines levels vary with stage of estrous cycle[63]. In the mouse, neutrophils are recruited after the estrogen peak in each estrous cycle with MIP-2 (CXCL2) controlling the majority of neutrophil migration[32]. KC (CXCL1) and IL-1β are also implicated in this process[42]. In our mouse model of vaginal colonization, we have observed GBS persist for several weeks[6], or up to several months (Patras, Doran, unpublished data), depending on the stage of estrus, estradiol treatment regime, or specific mouse strain used. We have observed nearly 100% colonization of mice within the first 24 hours post-inoculation, and subsequently, GBS bacterial strain, immune response, and normal flora determine the course of persistence (Patras, Doran, unpublished data). Furthermore, we have also observed that mice were either chronically or intermittently colonized, mimicking human GBS persistence[64]. We recognize that there are many differences between humans and our murine model counterparts including vaginal pH, normal flora, length of estrous cycle, and immune repertoire. However, given the success of other vaginal disease mouse

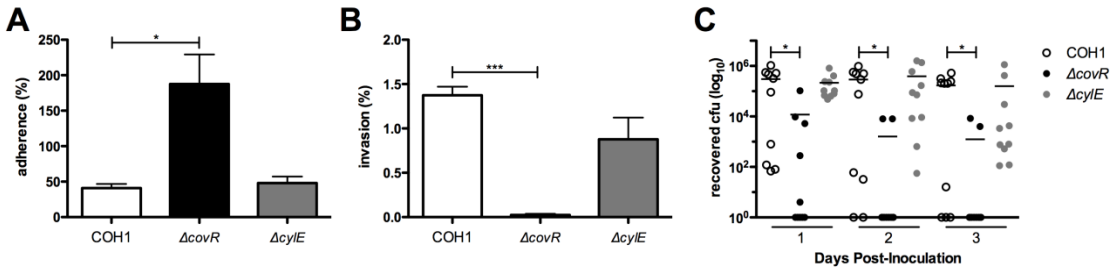
models[30,65,66], and our findings thus far, we believe this model has useful applications in studying host-GBS interactions in the vaginal environment. Herein, we show CovR deficiency hinders GBS persistence *in vivo*, coinciding with increased innate immune markers known to recruit neutrophils, which have been described as first responders in other GBS infection models[16]. To our knowledge, this is the first time vaginal immune response to GBS has been characterized *in vivo*.

In summary, we have shown that CovR deficiency provokes increased inflammatory response both *in vitro* and *in vivo*, as well as increased adherence to a vaginal epithelial cell line. This heightened response may contribute to the decreased persistence observed in our mouse model of colonization. Finally, a functional CXCr2 receptor may contribute to the differential increase in host immune signaling pathways in response to loss of CovR regulation *in vivo*. Collectively, our work indicates that GBS virulence regulation by the CovR/S two-component system is critical for niche establishment and maintaining a commensal state in the female vaginal tract.

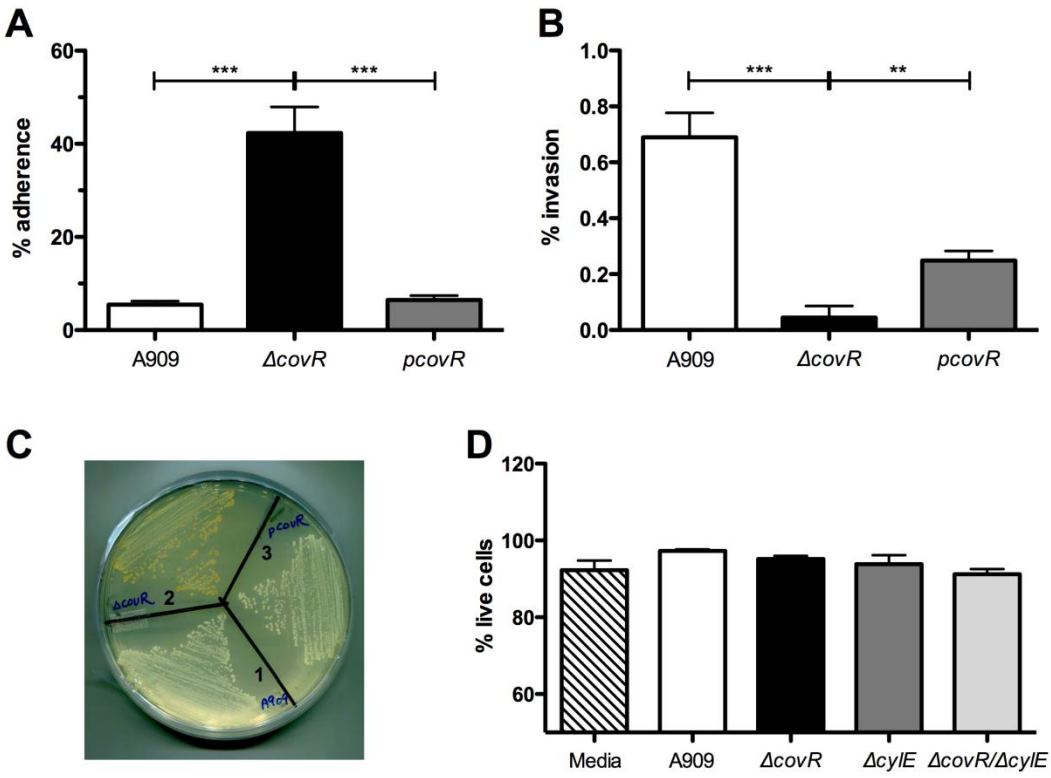
SUPPLEMENTAL MATERIALS



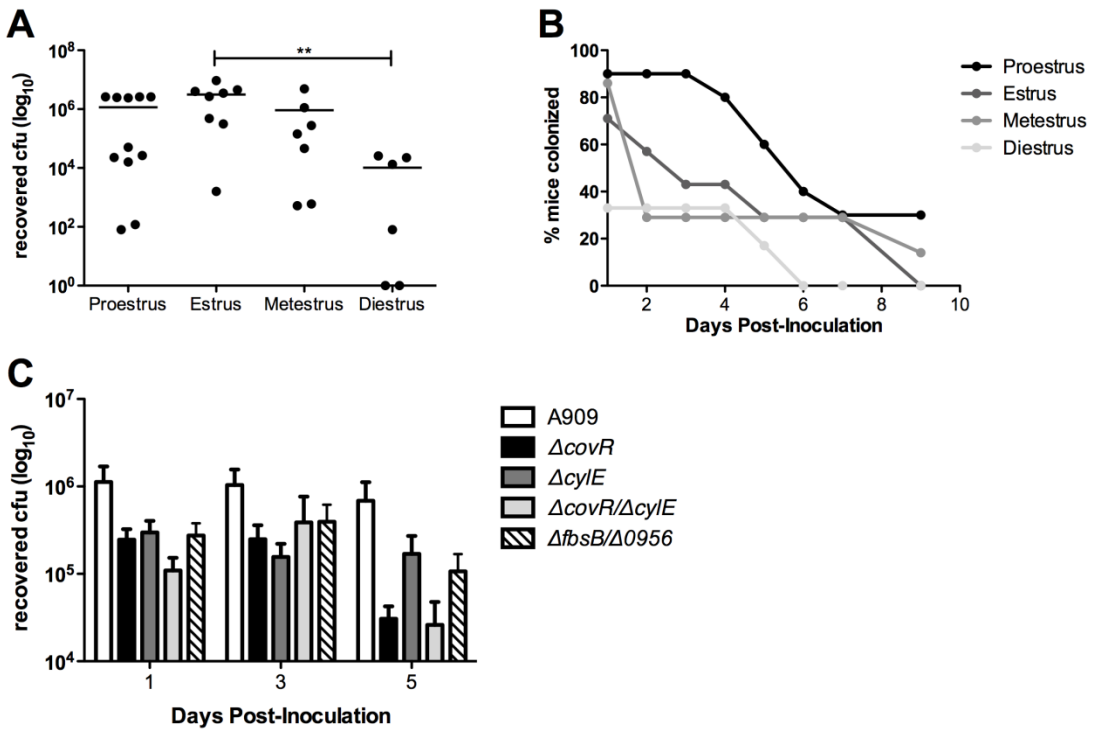
Supplemental Figure 2.1. Protein production of CXCL1, CXCL2, and IL-8 in HVEC upon infection with either A909 or COH1 WT and mutant strains. Protein expression of CXCL1 (A) in HVEC supernatants 5 hours post-infection with A909, Δ*covR*, Δ*cylE* or Δ*covR*/Δ*cylE* at MOI of 10. (B) Protein expression of IL-8, CXCL1, and CXCL2 in HVEC supernatants 6 hours post-infection with COH1 or Δ*covR* at MOI of 10. Values are expressed as absorbance at 450 nm. Experiments were performed at least two times with at least three replicates, and one representative experiment is shown. Data was analyzed by unpaired Student's *t* test. **** $p<0.0001$.



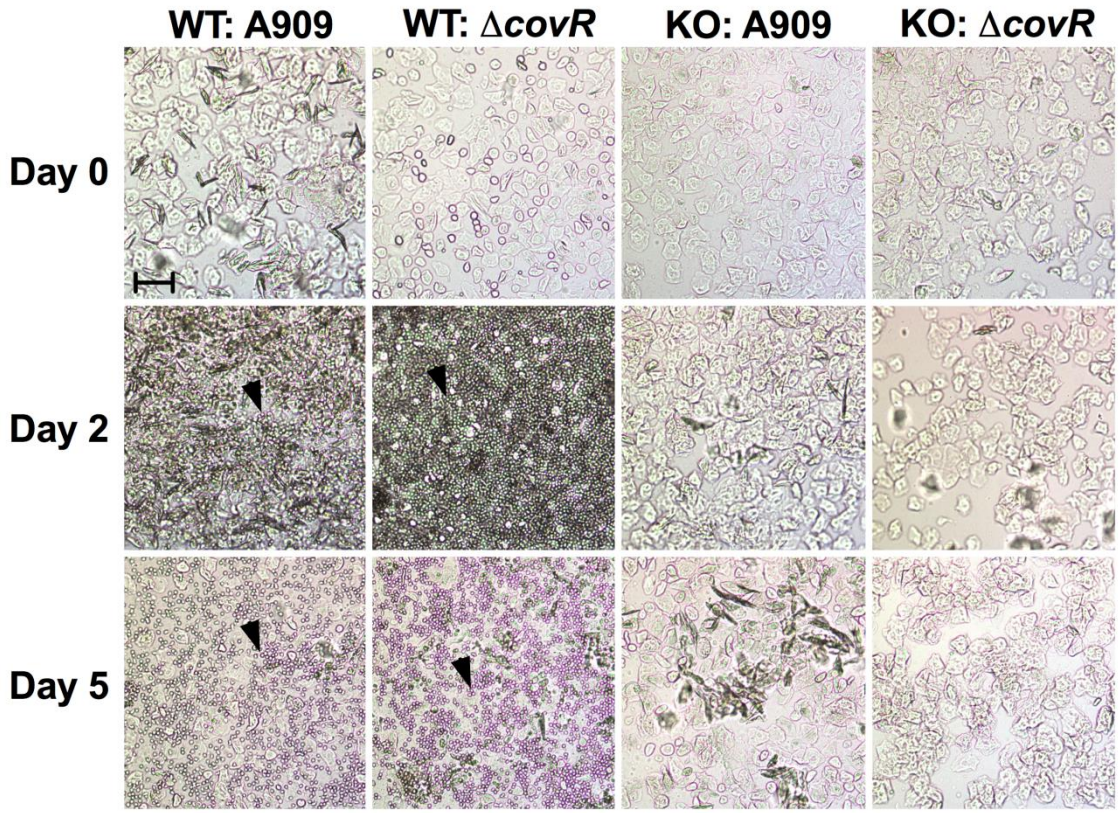
Supplemental Figure 2.2. GBS COH1 interaction with vaginal epithelium *in vitro* and persistence *in vivo.* Adherence (A) and invasion (B) of GBS COH1 and mutant strains to HVEC. Values are expressed as the total of cell-associated cfu (A) or total intracellular cfu (B) recovered compared to original inoculum. Assays were performed at an MOI of 1. (C) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 8 week old CD1 mice (n = 10 per group). GBS persistence was measured by swabbing the vagina and enumerating recovered bacteria. Experiments were repeated at least 2 times in triplicate (for cell assays) and data from a representative experiment is shown. Data was analyzed by unpaired Student's *t* test for cell assays and Kruskal-Wallis test for persistence. * $p<0.05$, ** $p<0.01$, *** $p<0.001$.



Supplemental Figure 2.3. Restoration of WT phenotypes *in vitro* following *covR* complementation, and cell viability assays. Adherence (A) and invasion (B) of GBS A909, Δ*covR*, and complemented p*covR* strains in HVEC. Values are expressed as the total of cell-associated cfu (A) or total intracellular cfu (B) recovered compared to original inoculum. Assays were performed at an MOI of 1 (A) or MOI of 10 (B). (C) Pigmentation of A909 (1), Δ*covR* (2), and p*covR* (3) on THB agar. (D) Percent of viable HVEC subjected to invasion assay with A909 or mutant strains at an MOI of 10. Values are expressed as the percent of cells unstained with Trypan Blue compared to total cells. Experiments were repeated at least twice with four replicates and data from a representative experiment is shown. Data was analyzed by unpaired Student's *t* test. ** $p<0.01$, *** $p<0.001$.



Supplemental Figure 2.4. Estrous stage impacts normal flora load and both estrous stage and CovR regulation affects GBS vaginal persistence *in vivo.* (A) Estrous stage of 8 week old CD1 mice was determined by vaginal lavage. Total aerobic normal flora load was calculated by swabbing the vagina and enumerating recovered bacteria on Tryptic Soy agar similar to as described in Experimental Procedures. Aerobic flora load was significantly higher at estrus compared to diestrus (p = 0.0074). Lines represent mean cfu recovered. (B) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of same CD1 mice as (A). GBS persistence was measured by swabbing the vagina and enumerating recovered bacteria. Mice were placed in groups, by staging estrus just prior to inoculation, and sample size was as follows: proestrus (n = 11), estrus (n = 8), metestrus (n = 7), and diestrus (n = 6). (C) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 8 week old CD1 mice as in (B) (n = 12 per group). All *in vivo* experiments were conducted independently at least twice and data from one representative experiment is shown. Data was analyzed by Kruskal-Wallis test. ** p<0.01.



Supplemental Figure 2.5. Neutrophil infiltration into the vaginal lumen requires a functional CXCr2 receptor *in vivo*. Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 16-17 week old WT BALB/c and CXCr2 KO mice. Vaginal lavage fluid was collected and wet-mounted unstained onto slides. For all groups, n = 10, or n = 9 for WT BALB/c mice inoculated with WT GBS. One representative image from each treatment group per given day is shown. Prior to bacterial inoculation (Day 0) only stratified squamous vaginal epithelial cells were observed. On subsequent days, free neutrophils were observed in the vaginal lumen (Black arrows) in WT mice only. Magnification = 100X, scale bar = 100 μm.

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CHAPTER 3

Characterization of host immunity during persistent vaginal colonization by Group B *Streptococcus*

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PREFACE TO CHAPTER 3

Chapter 3 in full is an article published in *Mucosal Immunology*. The work in Chapter 3 addresses Aim 1b: Identify IL-17 immune responses to GBS strains exhibiting long-term vaginal persistence. Using ELISA, flow cytometry, and a recombinant IL-17 protein, we characterized the role of IL-17 in vaginal colonization with a persistent GBS strain and found that IL-17 is produced only after several weeks of colonization, and that IL-17 production corresponds to clearance of GBS. Moreover, treatment with recombinant IL-17 resulted in reduced GBS vaginal load. This work is the first to our knowledge to demonstrate that IL-17-mediated immunity may counter GBS at the mucosal surface. Additionally, this work also includes novel findings of the rapid ascension of GBS to the cervix and uterus in mice and for the first time compares the ability of wild type GBS strains of different serotypes to colonize the murine vaginal tract.

ABSTRACT

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a Gram-positive bacterium, which colonizes the vaginal tract in 10-30% of women. Colonization is transient in nature, and little is known about the host and bacterial factors controlling GBS persistence. Gaining insight into these factors is essential for developing therapeutics to limit maternal GBS carriage and prevent transmission to the susceptible newborn. In this work, we have used human cervical and vaginal epithelial cells, and our established mouse model of GBS vaginal colonization, to characterize key host factors that respond during GBS colonization. We identify a GBS strain that persists beyond a month in the murine vagina, whereas other strains are more readily cleared. Correspondingly, we have detected differential cytokine production in human cell lines after challenge with the persistent strain versus other GBS strains. We also demonstrate that the persistent strain more readily invades cervical cells compared to vaginal cells, suggesting that GBS may potentially use the cervix as a reservoir to establish long-term colonization. Furthermore, we have identified IL-17 production in response to long-term colonization, which is associated with eventual clearance of GBS. We conclude that both GBS strain differences and concurrent host immune responses are crucial in modulating vaginal colonization.

INTRODUCTION

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is a Gram-positive bacterium that is frequently isolated from the gastrointestinal and genitourinary tracts of healthy adults[1], and has also been isolated from human breast milk[2]. However, GBS is associated with neonatal invasive disease such as sepsis, pneumonia, and meningitis, affecting approximately 2000 live births per year in the United States alone[3]. Maternal vaginal colonization rates appear similar in developed and developing countries across all 6 inhabited continents, ranging from 8-18% with an overall estimated colonization of 12.7%[4,5]. Current recommendations for neonatal disease prevention consist of late gestation screening and intrapartum antibiotic prophylaxis, and while these practices have reduced early-onset disease, they have had no effect on late-onset disease or maternal colonization[6]. Alarmingly, GBS adult infections such bacteremia, pneumonia, arthritis, and urinary tract infections are on the rise as well[7,8]. Furthermore, there is currently no vaccine available for GBS.

Vaginal colonization by GBS may be transient and intermittent, and likely dependent on vaginal pH, normal flora, pregnancy, and estrous cycle, among many other constituents. Increased adherence to vaginal epithelial cells has been observed *in vitro* as pH shifts from acidic to neutral[9], however, factors that favor persistence of GBS in this complex biological niche are not well understood. GBS determinants that have been shown to contribute to vaginal cell adherence and colonization include the two component system CovRS, surface Serine Rich Repeat (Srr) proteins, Srr-1 and Srr-2, and pili protein, PilA of GBS Pilus Island (PI)-2a[10-12]. These, and other GBS surface proteins, also facilitate adherence to extracellular matrix (ECM) constituents such as

collagen[13], fibrinogen[14], fibronectin[15], and laminin[16], all of which have been identified in vaginal proteome studies[17] suggesting potential importance in this environment. Furthermore, GBS possesses metallopeptidases capable of cleaving all four of these ECM proteins[18], which may aid in tissue invasion or niche establishment. Within the vaginal environment, GBS invokes innate immune responses including neutrophil recruitment[10] and production of multiple inflammatory cytokines[19]. A recent study found that GBS β-hemolysin/cytolysin expression is critical for fetal disease and preterm birth in a murine pregnancy model[20]. Nevertheless, the molecular mechanisms governing GBS vaginal persistence remain to be elucidated.

In this study, we compare the ability of 3 different GBS strains to colonize the murine vaginal tract, and elicit a host immune response, as well as characterize interactions with human vaginal and cervical epithelial cell lines. Here, for the first time, we examine GBS presence and host immune responses in the cervix and uterus of non-pregnant mice. We further establish key host immune responses including a previously unidentified GBS stimulation of the IL-17 immune response and the effect on vaginal persistence. We conclude that both GBS strain differences and concurrent host immune responses are crucial in modulating vaginal colonization.

MATERIALS AND METHODS

Bacterial Strains

Streptococcus agalactiae (GBS) wild-type clinical isolates A909 (serotype Ia)[49], COH1 (serotype III)[50], and CJB111 (serotype V) (ATCC BAA-23) were grown aerobically in Todd-Hewitt broth (THB) (Hardy Diagnostics, Santa Maria, CA) at 37^0C.

Human cell lines

Immortalized human vaginal (VK2/E6E7), ectocervical (Ect1/E6E7), and endocervical (End1/E6E7) epithelial cell lines were acquired from American Type Culture Collection (ATCC CRL-2616, ATCC CRL-2614, and ATCC CRL-2615, respectively). Cell lines (passage 5-25) were cultured in keratinocyte serum-free medium (KSFM) (Life Technologies, Carlsbad, CA) with 0.5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract at 37°C with 5% CO_2.

***In vitro* cell assays**

GBS adherence, invasion, and intracellular survival assays of cell lines were conducted as described previously[12]. Briefly, cells were grown to confluency in 24-well tissue culture plates and washed prior to bacterial infection. Bacteria were grown to mid-log phase and added at a multiplicity of infection (MOI) of 10 for adherence and invasion assays and an MOI of 1 for intracellular survival assays to prevent the possibility of cellular toxicity over time. For adherence assays, after 2 hours of incubation, cells were washed 6 times with PBS. Cells were lifted from plates by adding trypsin-EDTA and then lysed with 0.025% Triton X-100. Lysate was serially diluted and plated on THB agar plates to quantify adherent colony forming units (CFU). Total adherent CFU was calculated as (total CFU recovered/total CFU of original inoculum)×100%. To quantify invading bacteria, cells were incubated with GBS for 2 hours, monolayers washed 3 times with PBS, treated with media containing antibiotics, and incubated for an additional 2 hours for invasion assays, or as given for survival assays. Cells were washed 3 times with PBS, lysed as described above, and viable intracellular GBS determined by serial dilution plating as quantified above.

RT-qPCR, ELISA, and Western Blot of cell lines

To quantify gene expression induction, human vaginal, ectocervical, and endocervical cells were grown to confluency in 24-well tissue culture plates, washed prior to bacterial infection, and given fresh media. Bacteria were grown to mid-log phase, added to cells at an MOI of 10, and incubated for 4 hours. Cells were lysed, then total RNA was extracted, and reverse transcription (Macherey-Nagel, Düren, Germany) and qPCR were performed (Quanta Biosciences, Gaithersburg, MD). Human primer sequences utilized are as follows: GAPDH; Forward sequence 5'-3': GAA GGT GAA GGT CGG AGT GAA; Reverse sequence 5'-3': TCC TGG AAG ATG GTG ATG GGA, IL-1β; Forward sequence 5'-3': GCC CTA AAC AGA TGA AGT GCT C; Reverse sequence 5'-3': GAA CCA GCA TCT TCC TCA G, IL-6; Forward sequence: 5'-3': GGA GAC TTG CCT GGT GAA AA, Reverse sequence 5'-3': CAG GGG TGG TTA TTG CAT CT, IL-23 (α subunit); Forward sequence 5'-3': GCT TCA AAA TCC TTC GCA G, Reverse sequence 5'-3': TAT CTG AGT GCC ATC CTT GAG, and IL-36γ; Forward sequence 5'-3': GAA ACC CTT CCT TTT CTA CCG TG; Reverse sequence 5'-3': GCT GGT CTC TCT TGG AGG AG.

For ELISA and Western blot assays, human cell lines were infected as described above with several modifications. Bacteria were added at an MOI of 10 and cells were incubated with bacteria for 6-10 hours, washed, and cell lysates were analyzed for cytokine production using human IL-1β (R&D Systems, Minneapolis, MN) ELISA kit according to manufacturer's instructions. Cell lysates were also analyzed via Western blot as described[13]. Membranes were probed with antibodies against human GAPDH (1:150,000; EMD Millipore, Billerica, MA) or human IL-36γ (1:400, R&D Systems), and analyzed using ImageJ version 1.46r (National Institutes of Health, Bethesda, MD).

Mouse model of GBS vaginal colonization

All animal work was authorized by the Office of Lab Animal Care at San Diego State University and conducted using approved veterinary standards. 8-12 week-old female CD1 mice (Charles River Laboratories, Wilmington, MA) were injected intraperitoneally with 0.5 mg 17β-estradiol (Sigma Aldrich, St. Louis, MO) in 100 μL sesame oil on Day -1[10,12]. On Day 0, mice were vaginally inoculated with 1×10^7 CFU GBS in 10μL of PBS (or PBS as a control for some experiments), and on subsequent days, each vaginal lumen was swabbed with a sterile ultrafine swab, and recovered GBS were enumerated by light pink or mauve colonies on CHROMagar StrepB agar (DRG International, Springfield, NJ)[10].

Tissue dissection, homogenization, and ELISA

For tissue collection, mice were sacrificed using CO_2 asphyxiation and reproductive tracts excised from mid-uterine horn to just proximal of the vulva. Tissues were visually separated by sterile razor blade to prevent bacterial cross-contamination between tissues. Tissues were placed in PBS with a protease inhibitor cocktail and homogenized with 1.0mm zirconia beads using a mini beadbeater (BioSpec Products, Bartlesville, OK). For initial screening, tissues were analyzed with the Mouse Pro-inflammatory Panel 1 V-PLEX Kit (Meso Scale Discovery, Rockville, MD) per manufacturer's instructions. ELISA assays were performed on tissue homogenates for KC and MIP-2 (R&D Systems), as well as IL-1β, IL-23, and IL-17 (eBioscience, San Diego, CA) as described by manufacturer.

Tissue digestion and flow cytometry

Conversely, murine reproductive tract tissues were obtained as described above and subjected to enzymatic digestion. Tissues were finely diced with a sterile razor blade and incubated for two hours at 37^0C in RPMI 1640 containing 10% FBS, 0.4 mg/mL collagenase, and 1:1000 Brefeldin A (BD Biosciences, San Jose, CA). During this incubation, tissues were vigorously pipetted through 1000 μL and then 200 μL pipette tips to aid in tissue digestion. Following digestion, samples were placed in fresh RPMI 1640 with 10% FBS and 1:1000 Brefeldin A and incubated for an additional 4 hours at 37^0C. Samples were surfaced-stained with fluorochrome-conjugated antibodies CD11b-PE and Ly6G-FITC clone 1A8 (BD Biosciences), as well as antibody CD4-PE-Cy7 (eBioscience). Samples were then fixed and permeabilized using BD Cytofix/Cytoperm™ per manufacturer's instructions, and stained with IL-17-AlexaFluor647 (BD Biosciences). Prior to performing flow cytometry, samples were passed through 40 μm filter tips to remove larger tissue debris. Samples were analyzed with an Accuri C6 Cytometer (BD Biosciences) and cell populations were assessed for percent of fluorescent staining and staining brightness using Accuri analysis software.

Immunohistochemistry

Whole reproductive tract tissues were collected as described above and were fixed with paraformaldehyde and embedded in paraffin. For immunohistochemistry, sections were deparaffinized, rehydrated, and microwave heated in citrate buffer for antigen retrieval. Tissues were incubated with rabbit polyclonal anti-IL-17 (ab91649, Abcam) at 5μg/mL overnight, then incubated with goat anti-rabbit IgG-HRP, and developed with diaminobenzidine chromogen (Sigma Aldrich). Tissues were counterstained with

haematoxylin and visualized on a Zeiss upright microscope with attached Axiocam Icc3 camera at 200X magnification.

Recombinant protein treatment

For rIL-17 treatment experiments, mice were first colonized with 1×10^7 CFU of CJB111 as described above. One day later, mice were swabbed to determine colonization status, and were then treated with an intravaginal dose of 50-100 pg of recombinant mouse IL-17 (eBioscience) in 10 μL of diluent, or only diluent as a control. Mice were swabbed and treated daily for the duration of the experiment.

Statistical analysis

GraphPad Prism version 5.04 was used for statistical analyses. Differences in recovered bacteria for intracellular survival, cytokine transcripts, and protein from *in vitro* assays were evaluated using two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test. One-way ANOVA with Bonferroni's multiple comparisons post-test was used for all other *in vitro* assays. *In vivo* results for recovered bacteria and ELISA experiments were analyzed using Kruskal-Wallis with Dunn's multiple comparisons post-test. *In vivo* flow cytometry and recombinant protein experimental data were analyzed with Mann-Whitney. Statistical significance was accepted at $P<0.05$.

RESULTS

Differential persistence of GBS strains within the murine vaginal tract

We have established a robust murine model of GBS vaginal colonization using a variety of wild type GBS clinical isolates including A909[10], CJB111[21], and NCTC 10/84[11]. In this model, we have observed transient or intermittent colonization similar to

that seen in humans[22]. Although GBS is not a native murine vaginal species, it is eventually cleared from the vaginal tract in the majority of mice in a range of several weeks to several months. The length of GBS persistence not only depends on mouse strain and duration of 17β-estradiol treatment (data not shown), but likely also differs among GBS strains. Thus, we examined the ability of different GBS strains representing various serotypes to establish persistent vaginal colonization. Interestingly, GBS strains A909 (serotype Ia) and COH1 (serotype III) exhibited similar colonization profiles with the majority of mice clearing the bacterium below detection limits in 1-2 weeks, while GBS strain, CJB111 (serotype V), persisted beyond several weeks in >50% of mice (Fig. 1A). Furthermore, although these 3 strains achieved similar levels of bacterial load within the first 3 days, CJB111 maintained higher bacterial load over time while A909 and COH1 both decreased (Fig. 1B). At 1 month post-inoculation, CJB111 was readily isolated from the vagina, cervix and uterus; whereas A909 and COH1 were not detected (Fig. 1 C-E).

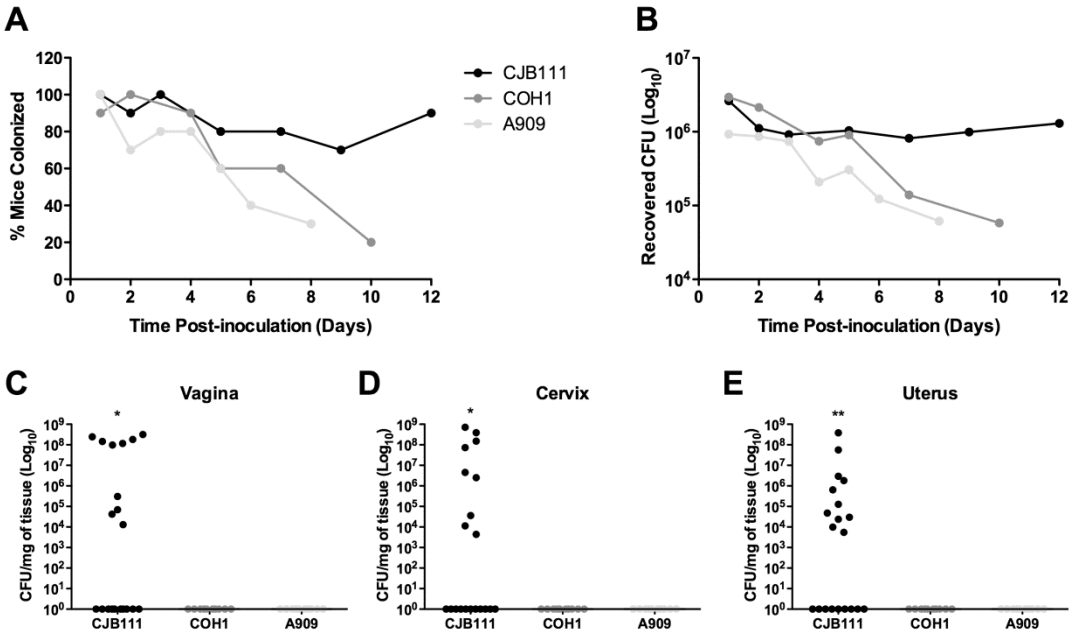

Figure 3.1. GBS strain persistence in the murine vaginal tract. CD1 mice (n=10/group) were colonized with 1×10^7 CFU of GBS clinical isolates CJB111, COH1, or A909 in the vaginal lumen. GBS persistence was determined by swabbing the vagina and plating recovered bacteria. (A) Percentage of mice with detectable GBS within the vaginal lumen via swabbing. Limit of detection is 50 CFU. (B) Mean recovered GBS CFU of same mice as in (A). (C-E) Reproductive tract tissues were isolated 4 weeks post-inoculation with GBS strains. Bacterial load was quantified by plating serially diluted tissue homogenates. *In vivo* experiments were conducted independently at least twice and data from one representative experiment is shown. Data was analyzed using Kruskal-Wallis with Dunn's multiple comparisons post-test. *, $P<0.05$; **, $P<0.01$.

Differential interaction of GBS strains with human vaginal and cervical epithelial cells

To gain more insight and to establish that the observed differences between GBS strains observed in murine colonization could be reciprocated *in vitro* with human cell lines, we performed cellular adherence, invasion, and intracellular survival assays with human vaginal, ectocervical, and endocervical epithelial cell lines. Although we have previously shown that A909 and COH1 are capable of adhering to and invading these 3

cell types[12], interactions of CJB111 with the female reproductive epithelium have not yet been characterized. For adherence assays, GBS strains were incubated with cells for 2 hours, and non-adherent bacteria washed away prior to quantification. We observed that GBS strains exhibited variable range of ability to attach to vaginal and cervical epithelial cells with CJB111 being the most adherent strain, whereas A909 was the least adherent overall (Fig. 2A-C). We did note increased adherence of COH1 over A909 to vaginal cells, corresponding with increased vaginal epithelial adherence of serotype III strains over serotype Ia strains noted previously[24]. Additionally, CJB111 exhibited increased ability to invade certain cell lines compared to COH1 and A909 (Fig. 2D-F). To access invasive capability, we recovered and quantified viable intracellular bacteria from cell lysates after a 2 hour infection and a 2 hour antibiotic treatment to kill all extracellular bacteria. CJB111 showed significantly increased invasion over COH1 and A909 in both vaginal and cervical cell lines (Fig. 2D-F). Similarly, for intracellular survival, cell monolayers were infected for 2 hours, and then cell lysates analyzed for viable intracellular GBS following antibiotic treatment of extracellular bacteria at indicated time points. Here, CJB111 also exhibited a significantly increased ability to survive within cervical cell lines at all time points tested compared to COH1 and A909, but no striking differences were observed in vaginal cells (Fig. 2G-I). These results highlight that CJB111 acts discretely from COH1 and A909 in its interaction with host epithelium, consonant with its long-term colonization phenotype *in vivo* (Fig. 1). Furthermore, these results demonstrate that CJB111 more readily invades and/or survives within cervical epithelium, which may be beneficial in niche establishment and long-term cervico-vaginal persistence.

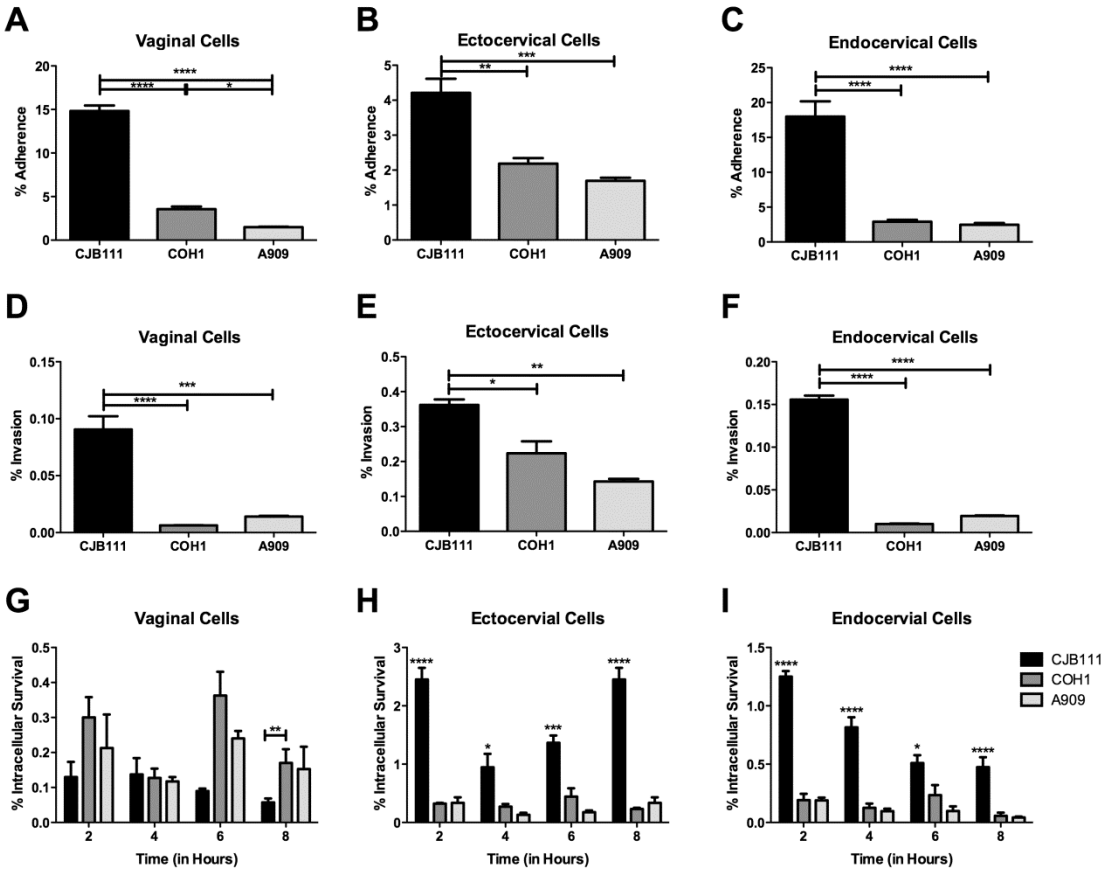

Figure 3.2. Interaction of GBS strains with human female reproductive epithelial cells. (A-C) Adherence, (D-F) Invasion, or (G-I) Intracellular survival of GBS CJB111, COH1, or A909 with indicated epithelial cells, MOI = 10 for A-F and 1 for G-I. For H-I, significance given is CJB111 in comparison to both COH1 and A909. Experiments were repeated at least twice with four replicates and data from a representative experiment is shown. Data was analyzed by one-way ANOVA with Bonferroni's multiple comparisons post-test for (A-F) and two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test for (G-I). *, P<0.05; **, P<0.01; ***,P<0.001; ****,P<0.0001.

GBS cytokine induction in human vaginal and cervical epithelial cells

We next sought to determine whether CJB111 stimulates a distinct immune profile within the female reproductive tract compared to other GBS strains. Our previous studies with human vaginal epithelial cells using microarray, RT-PCR and protein analysis revealed increased transcription and production of multiple pro-inflammatory cytokines and chemokines including IL-8, CCL20, CXCL1 and CXCL2 following

exposure to GBS[10]. The microarray data also suggested additional innate components such as IL-1β, IL-6, IL-23, and IL-36γ were also stimulated in vaginal epithelium in response to GBS. These cytokines have been implicated in adaptive immunity, such as the T_H17 cell response, which contributes to neutrophil homeostasis and sustained inflammatory signaling[25,26].

We sought to verify and extend the earlier microarray data by analyzing transcript abundance in vaginal, ectocervical, and endocervical cells after infection with GBS strains. These experiments revealed IL-1β, IL-6, IL-23, and IL-36γ mRNA transcripts were up-regulated in all three cell lines compared to media controls (Fig. 3A-C). Interestingly, COH1 and A909 invoked more dramatic up-regulation of IL-36γ in all three cell types, IL-23 in vaginal and endocervical cells, IL-1β in vaginal and ectocervical cells, and IL-6 in endocervical cells when compared to stimulation with CJB111. We next examined cytokine production at the protein level to confirm biological relevance. In this study, we observed that IL-1β production significantly increased in all three cell lines in response to COH1 and A909 at 6 and 8 hours post-infection compared to media controls, however, CJB111 did not illicit this same response (Fig. 3D-F). Additionally, we were unable to detect IL-23 under any of the tested conditions (data not shown). We quantified IL-36γ production via Western blot, and detected significantly heightened production in ectocervical cells treated with A909 after 8 and 10 hours, and in endocervical cells treated with A909 at 6 hours (Fig. 3H-I). Although we did note increased IL-36γ production in vaginal cells in response to CJB111 at 6 hours (Fig. 3G), we also observed lower levels of intracellular CJB111 in the vaginal cell assays (Fig. 2). Taken together, the inverse relationship between cytokine production and intracellular

viable CJB111 in the reproductive tract epithelium is of great interest and the topic of future study.

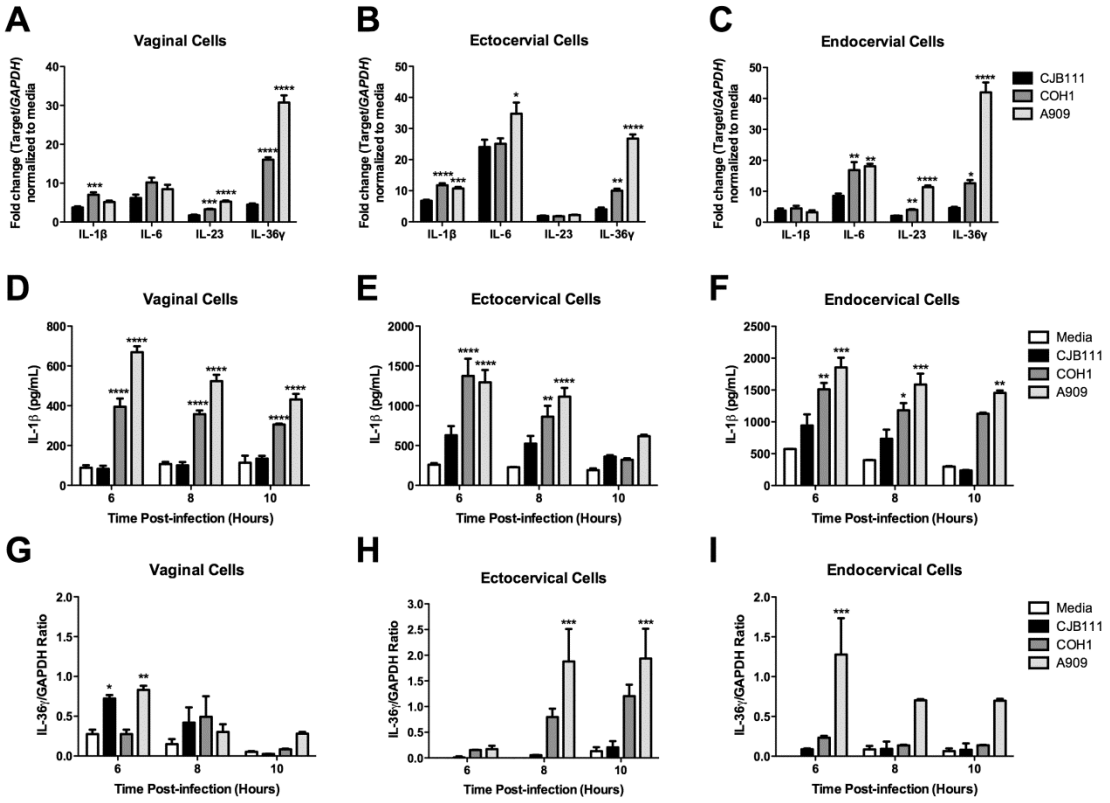

Figure 3.3. GBS cytokine induction in human female reproductive epithelial cells. (A-C) Transcript abundance of IL-1β, IL-6, IL-23, and IL-36γ in human epithelial cells was determined using quantitative RT-PCR following infection with CJB111, COH1, or A909 (MOI = 10). Fold change was calculated using GAPDH and then normalized to media controls as described Methods. Statistical values are in reference to CJB111. (D-F) Protein expression of IL-1β and (G-I) IL-36γ in human epithelial cell lysates was determined as described in Materials and Methods 4 hours post-infection with CJB111, COH1, or A909 at an MOI = 10. Statistical values are in reference to media controls. Data is one representative experiment of at least 2 independent experiments performed in 4 replicates at minimum. Data was analyzed by one-way ANOVA with Bonferroni's multiple comparisons post-test for (A-C) and two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test for (D-I).*, $P<0.05$; **, $P<0.01$; ***,$P<0.001$; ****,$P<0.0001$.

GBS colonization and cytokine production

Although GBS is a frequent colonizer of both pregnant and non-pregnant healthy women, the human host response to GBS presence within the vaginal tract remains to be fully described. Thus far, this response has been preliminarily characterized using *in vitro* cell based assays[10], and murine models[10,19]. To better define early immune responses to GBS strains A909, COH1, and CJB111 *in vivo* within the murine reproductive tract, a multiplexed electrochemiluminescence detection assay was used to ascertain presence of INF-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, KC/GROα, and TNF-α in the vagina, cervix, and uterus 2 days post-inoculation with GBS. We observed stimulation of IL-1β, IL-6, and KC, whereas the other cytokines on the panel were detected at much lower levels (data not shown). To confirm this preliminary screen, mice were colonized with CJB111, COH1, and A909, and 2 days post-inoculation, we recovered reproductive tract tissues for protein analysis and bacterial quantification. In vaginal tissues, GBS colonization resulted in significantly higher levels of KC and IL-6, and elevated levels of IL-1β and MIP-2, but no increased production of IL-23 (Fig. 4A-E). No GBS strain differences in cytokine profiles were noted upon *in vivo* infection in contrast to *in vitro* results. When bacterial loads were quantified from these same mouse tissues, more CJB111 CFU were recovered than the other strains (Fig. 4G-I), particularly in the cervix, suggesting that although CJB111 stimulates the host immune response similarly to other GBS strains, it possesses a unique set of factors allowing it to thrive within this environment to promote longer vaginal persistence. Given that several early cytokines linked to the T_H17 response were present in the murine reproductive tract, we measured IL-17A production after four weeks of colonization to allow time for development of an

adaptive immune response. Interestingly, we observed significantly more IL-17A in mice colonized with CJB111 compared to PBS controls, but not in mice colonized with COH1 or A909 (Fig. 4F). Additionally, we analyzed IL-17A levels at days 2, 5, and 10 post-inoculation with A909, COH1, or CJB111 and did not observe any increase in IL-17A production over PBS controls at these earlier time points (data not shown).

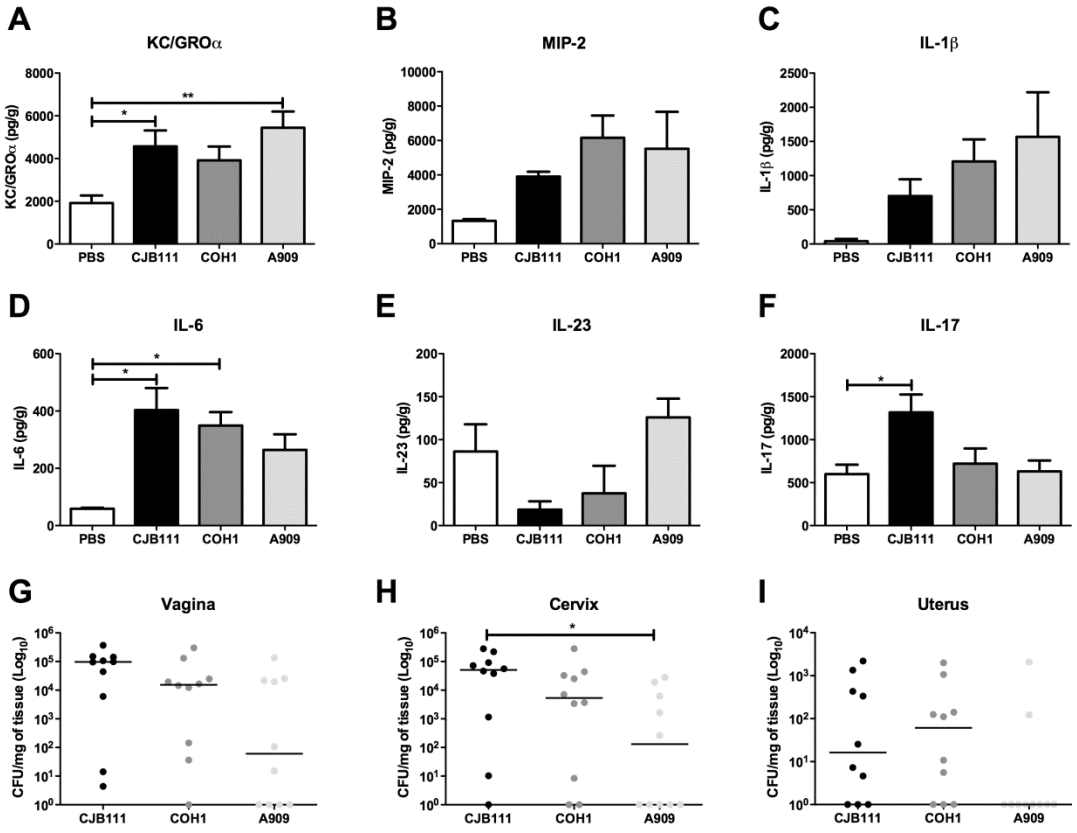

Figure 3.4. GBS colonization and cytokine production in the murine reproductive tract. KC/GROα (A), MIP-2 (B), IL-1β (C), IL-6 (D), and IL-23 (E) levels in vaginal homogenates from mice 2 days post-inoculation, or IL-17A (F) levels at 4 weeks post-inoculation, were quantified by ELISA. Data is the combined results of two independent experiments (n = 10-20 per group). (G-I) GBS load in reproductive tract tissue homogenates collected from mice 2 days post-inoculation (n = 10 per group). Lines represent median values of each group. Data was analyzed by Kruskal-Wallis with Dunn's multiple comparisons post-test.*, P<0.05; **, P<0.01.

Presence of IL-17 within the reproductive tract is associated with clearance of the persistent GBS strain CJB111

Several groups have demonstrated that the T_H17 response is activated in response to *Candida albicans*[27] and *Neisseria gonorrhoeae*[28] within the vaginal tract, and one group identified elevated, but not significant, IL-17 in the total reproductive tract of GBS-colonized mice[19]. However, the role of IL-17A production in response to GBS pathogenesis or colonization has not yet been recognized.

Consistent with the production of IL-17A in the reproductive tract after 4 weeks of GBS colonization, we observed the presence of IL-17+ cells beneath the epithelium, within the lamina propria, in both vaginal and uterine tissues (Fig. 5I-J). To determine the outcome of IL-17A production during long-term GBS colonization, we colonized mice with CJB111 and collected tissues at 2 weeks and 4 weeks post-inoculation for bacterial quantification and evaluation of IL-17A+ cells. We separated mice into two groups to analyze the data: mice that remained colonized at the time of sacrifice, and mice that had cleared CJB111 to below our limit of detection. By two weeks post-inoculation, mice that had cleared CJB111 possessed significantly higher abundance of total IL-17A+ cell populations in the vagina (3.1%) than colonized mice (0.98%, P=0.0320). This same trend of increased total IL-17A+ cells was present in the cervix (P=0.0666) and uterus (P=0.0979) (Fig. 5A). The average basal levels of total IL-17A+ cells in uninfected mice were 0.80% in the vagina, 0.6% in the cervix, and 1.1% in the uterus. We further identified that IL-17A+ cells expressed surface markers Ly6G, CD11b, or CD4, and observed increased levels in cleared mice in all tissues, but particularly in Ly6G+/IL-17+ and CD11b+/IL-17+ cells the uterus (Fig. 5B-D). At four weeks post-inoculation, we

determined that the primary location of IL-17+ cells was in the cervix, with distinctive differences between colonized mice and those that had cleared GBS. Interestingly, of these IL-17+ cells, those expressing either Ly6G or CD4 were significantly elevated in the cervix of cleared mice versus those that remained colonized (Fig. 5G). This effect was not seen in the vagina or uterus, both of which contained much lower quantities of total IL-17A+ cells (Fig. 5E, F, H).

To test whether IL-17A presence within the vagina is sufficient to result in successful GBS clearance, we colonized mice with CJB111 for 24 hours, and then began daily vaginal administration of rIL-17 as described in Materials and Methods. We found that after two days of rIL-17 treatment, treated mice had significantly lower bacterial load than non-treated controls (Fig. 5K). Taken together, these experiments suggest that in mice colonized with CJB111, a more persistent strain of GBS, production of IL-17A corresponds with the eventual ability to clear GBS.

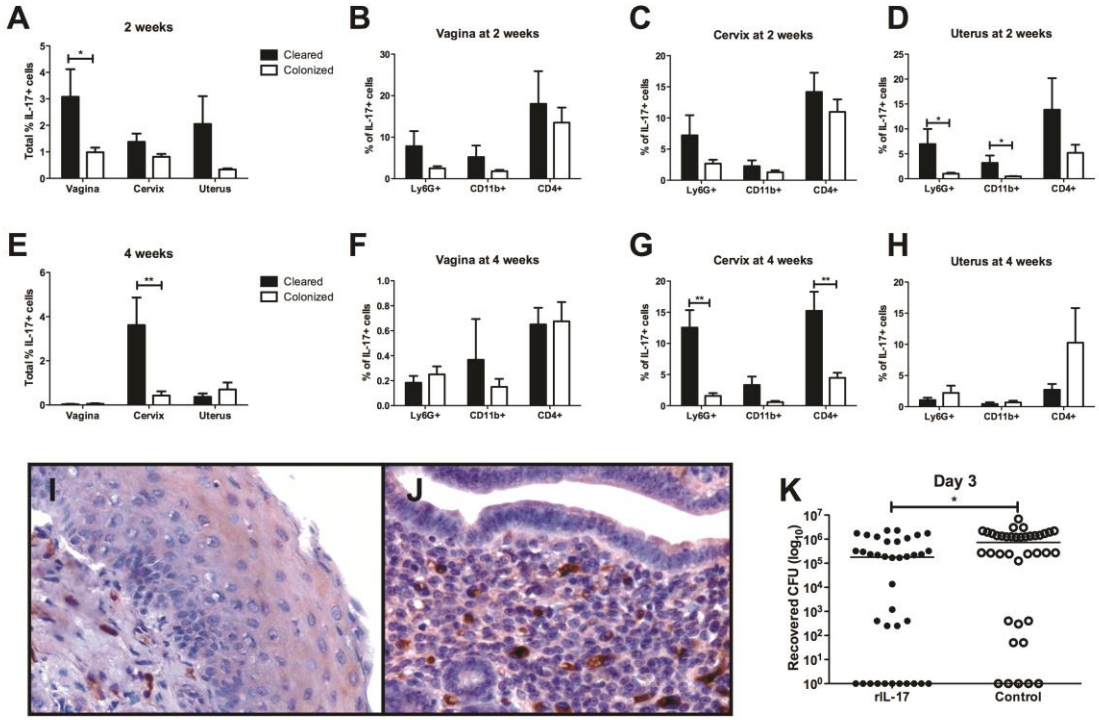

Figure 3.5. Presence of IL-17 within the reproductive tract is associated with reduced GBS CJB111. (A,E) Total IL-17A+ cells present in reproductive tract tissues collected from mice (n = 4-6 per group) at indicated time points post-inoculation with CJB111 were quantified by flow cytometry as described in Methods. Cleared mice (black bars) were separated from colonized mice (white bars) if GBS counts were below the limit of detection at the time of sacrifice (50 CFU/tissue). (B-D,F-H) Surface markers Ly6G, CD11b, and CD4 present on populations identified within total IL-17A+ cells from (A,E). Representative data from one of two independent experiments is shown. Immunohistochemistry of the vagina (I) and uterus (J) of GBS-colonized mice with IL-17+ cells visualized with DAB (brown) and counter-stained with hematoxylin (blue). Magnification = 200X. (K) Mice were colonized with CJB111, and one day later, received daily treatment of rIL-17 within the vaginal lumen. Data shown is from 3 days post-inoculation with CJB111, and is the result of 3 independent experiments combined (total n = 38 per group).Lines represent median values of each group. Data was analyzed by Mann-Whitney.*, $P<0.05$; **, $P<0.01$.

DISCUSSION

GBS continues to be a leading cause of neonatal disease including sepsis and meningitis in many developed nations[2], yet the scope of our understanding of host response to GBS vaginal colonization, and subsequent preventative measures to control

vertical transmission of GBS from mother to newborn, remain limited. In this study, we observed two distinct factors controlling GBS interactions with the female reproductive tract: variable determinants present among GBS strains and host immune profiles. GBS strains displayed differential abilities to adhere to and survive intracellularly among multiple epithelial cell types, as well as varying lengths of vaginal persistence. Concurrently, increased host innate cytokine production corresponded to decreased intracellular GBS *in vitro*, in line with increased IL-17+ cell populations in mice that had successfully cleared GBS. These data substantiate that both GBS strain differences and concurrent host immune responses are crucial in modulating vaginal colonization.

One of the most widely recognized GBS virulence factors, the polysaccharide capsule, has also been used to separate GBS strains into 10 unique serotypes based on chemical structure (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX)[29]. Epidemiological studies have revealed that serotype III strains are significantly more prevalent in neonatal meningitis[30], whereas serotype V is dominant in adult cases of GBS infections, with serotypes Ia, Ib, II, III, and V representing the vast majority of both neonatal and adult cases[7]. Elements driving the prevalence of serotypes in various disease states are currently unknown, but likely depend on bacterial virulence and fitness factors common within serotypes as well as concurrent host responses. In this study, we characterized distinct phenotypes of strains representing serotypes Ia, III, and V, with the serotype V strain CJB111 displaying greater intracellular survival and lesser cytokine stimulation in cervical cells, and increased persistence in the mouse vaginal tract. Although all 3 of these strains have been fully or partially sequenced[23], the molecular machinery determining length of GBS vaginal colonization is unknown. Possible explanations for

this phenomenon include either differences in their repertoire of cellular adhesins/invasins, ability to outcompete normal flora, and/or dialogue with the host immune system. CJB111 possesses a set of 20 unique genes compared to other fully sequenced GBS strains[23], and is adept at forming biofilms *in vitro*[31]. Others have previously noted serotype V strain antigens (from CJB111) invoke an alternative IgM immune response compared to other serotypes, which conjure an IgG response[32]. Future work seeks to identify the genetic constituents of CJB111 that allow for increased perpetuation within the host, both intracellularly, and at the mucosal surface.

Another aim of this study was to expand our understanding of female reproductive tract responses to GBS. Clinical studies have demonstrated that vaginal IL-1α levels correlate to GBS colonization status in non-pregnant women[33], while increased levels of maternal serum IL-1β are associated with increased risk of GBS infection and early term birth in pregnancy[34]. Using murine models of GBS, we have previously observed increased vaginal cytokine levels of MIP-2 and IL-1β in response to hyper-hemolytic GBS[10], and another group has noted increased reproductive tract levels of TNF-α and IL-1α after several weeks of GBS colonization[19]. However, global cytokine changes over time, and the subsequent effect on vaginal colonization, have yet to be established.

We utilized human vaginal and cervical epithelial cells to examine potential human immune responses to infection with several strains of GBS. Based on earlier microarray data of vaginal cells infected with GBS, we pursued several enhanced innate components: IL-1β, IL-6, IL-23, and IL-36γ. IL-1β is a potent inflammatory cytokine that in conjunction with IL-6 and TGF-β induce T_H17 cell maturation[35]. IL-23 promotes T_H17

cell maintenance and function, but has also been linked to T cell-independent inflammation in mucosal surfaces[36]. IL-36γ, a recently identified IL-1 family member produced by internal epithelium and keratinocytes, is a potent inducer of IL-6, and heightens cytokine production of activated CD4+ T cells[37]. Past work has identified spontaneous production of IL-6 and IL-8 in these vaginal, ectocervical and endocervical cells lines, and production of IL-1β upon cytokine stimulation[38]. We have previously demonstrated GBS induction of IL-8 from vaginal epithelial cells[10]. In this work, we established that these three cell lines produced IL-1β and IL-36γ protein in response to GBS, but were unable to detect IL-23. A former study done with this exact vaginal cell line was also unable to detect IL-23 production either spontaneously, or with lactic acid stimulation[39], so it is possible that these cell lines are incapable of generating functional IL-23. To our knowledge, this is the first recorded incidence of IL-36γ production from these 3 cells lines. Overall, IL-36γ production in the female reproductive tract in response to pathogens has not been well characterized, with the exception of HIV infection in vaginal cells[40].

Alternatively *in vivo*, we observed increased production of several innate immune cytokines in response to A909, COH1 and CJB111 over PBS controls; however, no differences were seen between GBS strains suggesting that early host responses to GBS are similar independent of strain or serotype. Even so, CJB111 was able to persist beyond these early immune responses in the majority of mice in contrast to A909 and COH1. A distinctive host immune response we identified in this work was increased production of IL-17A within the vaginal tract of mice persistently colonized with CJB111. The T_H17/IL-17 response has been associated with control of mucosal pathogens in multiple

host tissues including the lung[41] and gut[42]. T_H17 cells and their associated responses are very closely linked to the microbiota living upon the mucosal surface of a given tissue, considering germ-free mice possess drastically reduced IL-17+ cell populations in the small intestine[43]. Specifically within the human vaginal tract, detection of at least one dominant *Lactobacillus* species has been associated with increased presence of vaginal IL-17 and VEGF[44]. Moreover, other clinical work has revealed that percentages of vaginal *Lactobacillus* species are reduced in pregnant women that are GBS-positive[45]. These human studies, combined with our *in vivo* work here, suggest that vaginal IL-17, stimulated by either healthy normal flora or vaginal inflammation in response to GBS, may be an important immune response for controlling GBS colonization and maintaining a beneficial vaginal microbiota.

In this work, we identified at least two cellular populations that produced reproductive tract IL-17A including neutrophils and CD4+ T cells. Whether both sources are effective in reducing GBS in our system remains to be determined. However, previous studies have noted influx of both IL-17A producing CD4+ T cells and neutrophils in mucosal models of pathogenesis[27,46]. Additionally, the timing of IL-17A production at mucosal surface varies across pathogenic agent and host tissues, with responses occurring within 2-3 days or up to 6 weeks in the lung[46,47], or approximately 2 weeks in the vaginal tract[27]. In our model, we observed higher numbers of IL-17A+ cells in the vagina and uterus at 2 weeks, and higher numbers in the cervix at 4 weeks (Fig. 5). Interestingly, ELISA analysis identified increased IL-17A in the vaginal tract at 4 weeks (Fig. 4), even though flow cytometry displayed very low populations of IL-17A+ cells at this time point (Fig. 5). It is possible that cervical IL-17A+ cells were the source of

vaginal IL-17A at this time, as cervical cytokines are thought to be critical in protecting against reproductive tract pathogens[48]. Another group studying the murine reproductive tract response to GBS in mice sustained in estrus observed increased levels of IL-17 in the murine reproductive tract 30 days post-inoculation, although this difference was not significant[19]. Even though this work was done in a different murine background with another strain of GBS, this corroborates our results that induction of IL-17 within the vaginal tract requires GBS persistence beyond several weeks.

In our model, only ~40% of mice were able to clear CBJ111 from the vaginal tract by 4 weeks, and these mice demonstrated increased IL-17A+ cells compared to mice that remained colonized. The host and microbial factors controlling development of the IL-17 response to GBS remain unknown and require further investigation. However, our experiments with exogenous IL-17A treatment suggest that regardless of the source of IL-17A, it may contribute to the reduction of GBS vaginal colonization. Prolonged treatment of rIL-17 (2 weeks) resulted in enhanced clearance of GBS from the vaginal tract with 75% clearance observed in treated mice compared to 45% in controls, although this difference was not statistically significant (data not shown). We should note that this level clearance in control mice is consistent with the 50% clearance we observed at later time points in an earlier experiment (Fig. 1C), thus we do not believe that the diluent impacted these results. This information combined with further studies may be useful for developing pharmacological interventions such as vaccine development for controlling GBS within the vaginal tract. Additionally, because strains A909 and COH1 were effectively cleared from the murine vaginal tract without the stimulation of an IL-17 response, future studies should also seek to identify additional host immune responses

and presence or absence of GBS constituents that allow for shorter persistence within the vaginal tract.

In summary, we have continued to demonstrate that murine models of vaginal colonization can be correlated with *in vitro* human cells to further deepen our understanding of GBS-host interactions within the reproductive tract. GBS strains differentially engage host innate and adaptive immune responses, an element that combined with future work, will lead to successful elimination of GBS as a global concern of neonatal and even geriatric disease.

DISCLOSURE

The authors declare no conflict of interest.

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CHAPTER 4

The Group B Streptococcal two-component system CiaR/H regulates a functional peptidoglycan hydrolase that promotes vaginal colonization

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PREFACE TO CHAPTER 4

Chapter 4 is a manuscript in preparation to a journal yet to be determined. The work in Chapter 4 addresses Aim 2a: Examine the role of GBS two component regulatory systems in establishing vaginal colonization. Using our mouse model, we determined that the two component system CiaR/H is critical for establishing vaginal colonization. Chapter 4 also addresses Aim 2b: Characterize the role of a CiaR-regulated putative protease in GBS colonization ability. We used molecular techniques to both create a protease-deficient mutant of GBS in two GBS strain backgrounds, as well as generated purified protease using an *E. coli* expression system. With these tools, we were able to characterize this putative protease which has previously been undescribed. We termed this protease zoocin A, and demonstrated that this GBS factor plays roles in vaginal colonization and interactions with other microbes. This work needs additional experimentation prior to publication, but lays the foundation for future strategies to identify GBS constituents that effect vaginal colonization.

ABSTRACT

Streptococcus agalactiae (Group B Streptococcus, GBS) is a Gram-positive bacterium, which colonizes the reproductive tract of up to 30% of healthy women. Although colonization itself is asymptomatic, during pregnancy, GBS can cause severe complications such as chorioamnionitis and pre-term birth, or alternatively, can be vertically transmitted to newborns peripartum, leading to pneumonia, sepsis or meningitis. Proximal to neonatal disease is the successful colonization of GBS within the volatile vaginal tract. Gaining insight into GBS factors permitting vaginal colonization is essential for developing novel therapeutics to limit maternal GBS carriage and prevent transmission to the vulnerable newborn. Our lab has previously identified a GBS two-component system, CiaR/H, involved in stress responses and intracellular survival in a variety of host cell types. We have since established that the Δ*ciaR* mutant displays impaired ability to persist in the vaginal tract using a murine model of GBS vaginal colonization. To further investigate the role of CiaR regulation during GBS-host interactions, we have begun examination of key genes regulated by CiaR as determined by microarray analysis. One such gene is a putative peptidase with high similarity to a known bacteriocin-like inhibitory substance, zoocin A, produced by *Streptococcus equi* ssp. *zooepidemicus* which hydrolyzes peptidoglycan of other streptococcal species. The GBS gene homologue, which we designate as *zooA*, is present and highly conserved among all sequenced GBS genomes. We have generated a *zooA*-deficient mutant in two GBS strains and have cloned and purified recombinant GBS zoocinA protein using an *E. coli* expression system. In our established mouse model of GBS vaginal colonization, *zooA*-deficient mutants are less able to colonize the murine vaginal tract. Similar to

zoocin A, the purified GBS protein displays antibacterial activity against a variety of bacteria including *Streptococcus pyogenes* and *Micrococcus luteus*. Future work seeks to determine the molecular mechanisms of zoocin A regulation and activity, and to examine the contribution of both host immune response and other microbes to the decrease in the colonization observed by the GBS Δ*zooA* mutant. We conclude that GBS zoocin A may play an important role in both GBS-microbe and GBS-host interaction during vaginal niche establishment.

INTRODUCTION

Conventionally, the vaginal microbiota is thought to consist primarily of beneficial *Lactobacillus* species which lower environmental pH through lactic acid production and protect the host against pathogens that cause bacterial vaginosis, yeast infections, and other urogenital diseases. More recent work has begun to reveal the complexity of the vaginal microbiome via large cohorts and culture-independent 16S rRNA sequencing techniques[1,2]. Within this environment, there are microbes with inherent virulence properties that do not cause disease in colonized tissues; a phenomenon which may be largely controlled by both host immunity and other microbial members within that specific environment[3]. *Streptococcus agalactiae* (Group B Streptococcus, GBS) is a Gram-positive, encapsulated bacterium that is harbored in the vaginal tract of approximately 25% of healthy women, but can be a pathogen in immune-compromised individuals[4]. GBS is one such organism which is frequently isolated from asymptomatic individuals, but rarely causes disease despite its myriad of virulence factors, which gives evidence of regulation[3]. However, the specific microbial mechanisms governing this regulation are currently unknown. Furthermore, the genetic attributes that GBS requires to aptly colonize the vaginal tract is not well understood.

Bacterial two component regulatory systems (TCS) are common and are frequently observed in pathogenic and nonpathogenic bacteria alike. TCS function through phosphotransfer signals from a membrane-bound sensor histidine kinase, which senses environmental changes, to subsequent activation of a cytoplasmic response regulator, with downstream transcription modulation[5]. GBS genome sequence analysis suggests multiple putative TCSs, but most of these remain currently undescribed[6]. One

recent study has found as many as 21 TCS in one strain of GBS[7]. Established GBS TCSs include DltR/S, which maintains constant levels of ɒ-alanylation in GBS LTA[8]; RgfA/C, which represses the expression of C5a peptidase[9]; CovR/S global regulatory system, which controls β-H/C expression along with other virulence factors[10]; LiaFSR, which regulates cell wall stress and pilus expression[11]; FspSR, which regulates fructose-6-phosphate metabolism[7]; and CiaR/H, which promotes survival in the host intracellular environment[12].

CiaR/H is not fully characterized in GBS, but it has been linked to stress tolerance and host defense resistance similar to the role of CiaR/H in *S. mutans*[13] and *S. pnuemoniae*[14]. CiaR-deficient GBS display decreased intracellular survival in neutrophils, macrophages, and brain microvascular endothelial cells and are more susceptible to killing by antimicrobial peptides and reactive oxygen species suggesting CiaR/H as a vital element for environmental stress tolerance[12]. Furthermore, CiaR has been attributed colonization and biofilm formation of the nasopharynx in *S. pnuemoniae*[14].

We recently discovered that the GBS two component system, CiaR/H, regulates a putative bacteriocin-like inhibitory substance (BLIS)[12], suggesting that CiaR may also regulate interactions with other microbes. Bacteriocins are small antimicrobial peptides generated by numerous bacteria that target and kill other microbes with high potency and specificity[15]. Originally characterized in Gram-negative bacteria, bacteriocin-like inhibitory substances (BLIS) have been isolated from Gram-positive species as well, and are currently under robust investigation[16]. Previous work has established that GBS possesses *in vitro* inhibitory activity against groups A, B, C, and G streptococci,

Lactobacillus and *Gardnerella vaginalis* and that GBS is inhibited by certain staphylococci and enterococci[17]. Additionally, others have suggested that vaginal colonization between GBS and other β-hemolytic *Streptococcus* is mutually exclusive[18]. CiaR/H regulates a putative peptidase (SAN_0039) exhibiting a high degree of homology (70% similarity, 56% identity) to a BLIS termed zoocin A (*zooA*)[12].

Zoocin A is produced by *S. zooepidemicus* (Group C *Streptococcus*) which has a bacteriolytic effect on several other Streptococcal species[19]. Zoocin A has two functional domains, an N-terminal catalytic domain and a C-terminal substrate-binding or target recognition domain[20,21]. Zoocin A was recently determined to act as a D-alanyl-L-alanine endopeptidase which hydrolyses the cross bridge of peptidoglycan of certain *Streptococcus* species[22]. Although the catalytic domain of zoocin A is very similar to other bacteriocin-like inhibitory substances like lysostaphin from *Staphylococcus aureus*[23], the target recognition domain is unlike any other conserved bacterial domains[22].

Our initial hypothesis is that GBS CiaR may be an important regulator of the colonizer-to-pathogen transition and function to promote a colonization state in response to vaginal environmental cues. In this work, we examine the impact of CiaR regulation on the establishment of GBS vaginal colonization using our murine model. To elaborate on this hypothesis, we further investigate the contribution of a CiaR-regulated gene, here named *zooA*, to vaginal colonization. We find that CiaR is important in GBS persistence within the vaginal tract, and *zooA* partially contributes to this phenomenon. Moreover, we determine that purified zoocin A possesses lytic activity both to GBS and other Gram-positive species including *S. pyogenes.* Overall, this study increases our knowledge of

GBS factors that regulate vaginal colonization both through interactions with the host and competition with other microbes.

MATERIALS AND METHODS

Bacterial Strains

Streptococcus agalactiae strains used in this study include A909 (ATCC BAA-1138)[24]and COH1 (ATCC BAA-1176)[25]. The isogenenic mutant COH1Δ*ciaR* was generated previously[12], and isogenenic COH1Δ*zooA* and A909Δ*zooA* mutants were constructed as described below. *S. pyogenes* strain NZ131 was used in this study[26]. *Micrococcus luteus* strain T-18 was used as a standard indicator strain[27]. *Escherichia coli* strains DH5α and M15 were used for molecular cloning and were cultured in Luria-Bertani broth with appropriate antibiotics when required. All Gram-positive strains were grown aerobically in Todd-Hewitt broth (THB) (Hardy Diagnostics, Santa Maria, CA) or on THB agar plates at 37ºC. When antibiotic selection was needed, 5 μg/mL erythromycin or 2 μg/mL chloramphenicol (Cm) was used. *E. coli* strains were grown in Luria-Bertani broth (LB; Hardy Diagnostics), and 500 μg/mL erythromycin or 10 μg/mL Cm was added when required.

Human cell lines

Immortalized human vaginal (VK2/E6E7), ectocervical (Ect1/E6E7), and endocervical (End1/E6E7) epithelial cell lines were purchased from American Type Culture Collection (ATCC CRL-2616, ATCC CRL-2614, and ATCC CRL-2615, respectively)[28]. Cell lines (passage 5-25) were cultured in keratinocyte serum-free medium (KSFM) (Life Technologies, Carlsbad, CA) with 0.5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract at 37°C with 5% CO_2.

Allelic exchange mutagenesis

In-frame allelic exchange of GBS *zooA* (COH1 SAN_0039 and A909 SAK_0064) with the chloramphenicol acetyltransferase (*cat*) gene were performed as previously described[12]. In short, primers *zooA*-upF (5′-CCAACAGACTACTCAATCGCTTCAGC-3′), *zooA*-upR (5′-AGAATTAATATAATGAAGTGCTCAAACACTTG-3′), *zooA*-downF (5′-TCCAGTAAAGTGTGATATTATAGTCTC-3′), and *zooA*-downR (5′-CGTAGTCACAGGAACTGCTGG-3′) were used to amplify a 1038-bp and 887-bp fragment immediately upstream and downstream, respectively, of the *zooA* gene. *zooA*-upF (5′-AGCACTTCATTATATTAATTCT-3′) and *zooA*-downR (5′-GTCCGAGACTATAATATCA-3′) primers were constructed with 36-bp extensions homologous to 5′ and 3′ ends of the *cat* gene, respectively. In a successive PCR, upstream and downstream *zooA* flanking PCR fragments were combined with a 701-bp amplicon of the full *cat* gene using nested *zooA*-upF and *zooA*-downR primers. The ensuing PCR amplicon, containing an in-frame replacement of the *zooA* gene with *cat*, was subcloned into the temperature-sensitive pHY304 vector. Allelic-exchange mutagenesis and screening were conducted as described[29], and the COH1 Δ*zooA* mutant was confirmed by PCR analysis.

Co-culture assays

For co-culture experiments, overnight cultures were subcultured to mid-log phase (OD_{600} = 0.4). Log phase cultures (100 μL each) were inoculated into 3 mL of fresh THB either in single culture or in co-culture with another strain as indicated in figures. At 24 hours post-inoculation, cultures were serially diluted and plated on THB agar plates with appropriate antibiotics.

***In vitro* cell assays**

GBS adherence and invasion of hVEC, Ect1, and End1 were performed as described previously[30]. Briefly, cells were grown to confluency in 24-well tissue culture plates, washed, and supplemented with fresh media prior to infection. Bacteria were grown to mid-log phase and added to cells at a multiplicity of infection (MOI) of 1. For adherence assays, monolayers were infected for 30 minutes and washed 6 times with PBS. Cells were lifted from plates with trypsin-EDTA and lysed with 0.025% Triton X-100. Cell lysate was serially diluted and plated on THB agar plates to quantify adherent CFU. Total adherent CFU was calculated as (total CFU recovered/total CFU of original inoculum)×100%. To assess invading bacteria, monolayers were infected for 2 hours and washed 3 times with PBS. Cells were given fresh media containing antibiotics and incubated for another 2 hours. Monolayers were washed 3 times with PBS, lysed as described above, and viable intracellular bacteria quantified by serial dilution plating as with adherence assays.

Protein expression and purification

Recombinant protein expression was carried out as described previously[20]. In brief, PCR was used to amplify the full length *zooA* gene, a his-tag was added, and the subsequent PCR product was ligated into the pQE80L vector and transformed into electro-competent *E. coli* M15. The recombinant *E. coli* M15 producer strain was grown to OD_{600nm} = 0.7 and IPTG and 200 μl of [1-14C]glycine (1.25 ml, 250 μCi, 56.0 mCi mM−1 [1-14C]glycine; Amersham Pharmacia Biotech, UK) was added. Recombinant proteins were purified by Ni–NTA affinity purification (Qiagen) according to the

manufacturer's instructions and yield and purity analyzed as described[20]. Purified recombinant proteins were stored at −20°C until use.

Mouse model of GBS vaginal colonization

All animal work was authorized by the Office of Lab Animal Care at San Diego State University and carried out using accepted veterinary standards. 8-12 week-old female CD1 mice (Charles River Laboratories, Wilmington, MA) were injected intraperitoneally with 0.5 mg 17β-estradiol (Sigma Aldrich, St. Louis, MO) in 100 μL sesame oil on Day -1[30,31]. On Day 0, mice were vaginally inoculated with 1×10^7 CFU GBS. On Day 1, and all subsequent days, the vaginal lumen was swabbed with a sterile ultrafine swab, and recovered GBS were enumerated by plating on the differential media CHROMagar[32]. GBS colonies were considered light pink or mauve.

Statistical analyses

GraphPad Prism version 5.04 was utilized for statistical analyses. Differences in recovered bacteria for co-culture assays were evaluated using two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test. Student's *t*-test was used for *in vitro* adherence and invasion assays. *In vivo* results for recovered bacteria were analyzed using Kruskal-Wallis with Dunn's multiple comparisons post-test for individual days, and two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test for analyses over time. Statistical significance was accepted at $P<0.05$.

RESULTS

The two-component system CiaR/H promotes vaginal colonization

We have previous demonstrated that the two-component system CiaR/H is important for promoting intracellular survival and protection against multiple host

defenses[12]. To determine if the response regulator CiaR played a role in establishment of vaginal colonization, we utilized our mouse model. CD1 were inoculated with 1×10^7 CFU of COH1 or the isogenic mutant Δ*ciaR* into the vaginal tract, and the vaginal lumen of each mouse was swabbed on successive days to monitor colonization. By Day 3 post-inoculation, the Δ*ciaR* mutant was recovered at significantly lower levels than wild-type (P = 0.0336), and this trend continued on Day 4 (P = 0.0203) and Day 5 (P = 0.0048, Fig. 1). At later time points, differences were no longer significant due to large sample variance since mice began clearing GBS in both groups (data not shown).

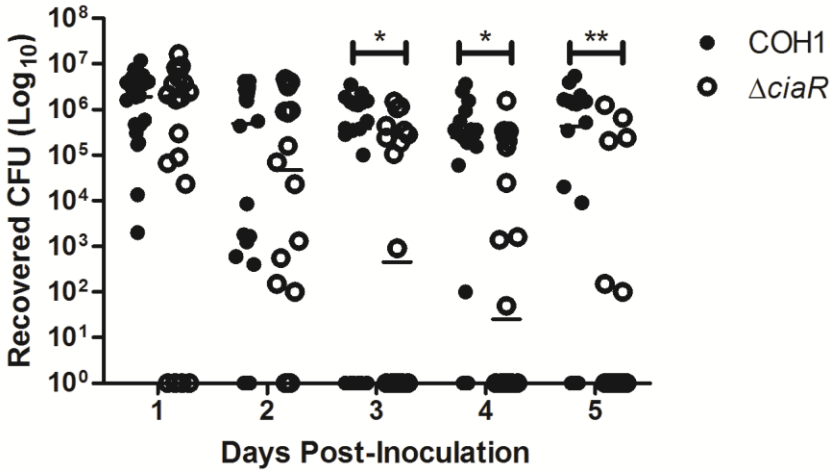

Figure 4.1. GBS CiaR promotes vaginal colonization. CD1 mice (n=20/group) were colonized with 1×10^7 CFU of GBS COH1 or COH1Δc*iaR* in the vaginal lumen. GBS persistence was determined by swabbing the vagina and plating recovered bacteria. *In vivo* experiments were conducted independently at least twice and data from two combined experiments is shown. Data was analyzed using Kruskal-Wallis with Dunn's multiple comparisons post-test. *, P<0.05; **, P<0.01.

GBS zoocin A promotes vaginal colonization

CiaR is responsible for the regulation of 5 genes in the COH1 strain, and the potential GBS homologue of zoocin A is the second most down-regulated gene in COH1

following SAN_2180[12]. To determine the contribution of GBS zoocin A to the Δ*ciaR* phenotype, we generated zoocin A-deficient strains in two backgrounds of GBS: COH1 (serotype III) and A909 (serotype Ia). We replaced the *zooA* gene, SAN_0039 and SAK_0064, in COH1 and A909 respectively with the chloramphenicol acetyltransferase (*cat*) gene as described in Materials and Methods. The growth of these Δ*zooA* mutants was very similar to wild type in both A909 (Fig. 2A) and COH1 (Fig. 2B) in rich media suggesting that the overall metabolism of GBS is not affected by the absence of *zooA*. Complete absence of the gene was confirmed using PCR and primers specific for *zooA* (Fig. 2C).

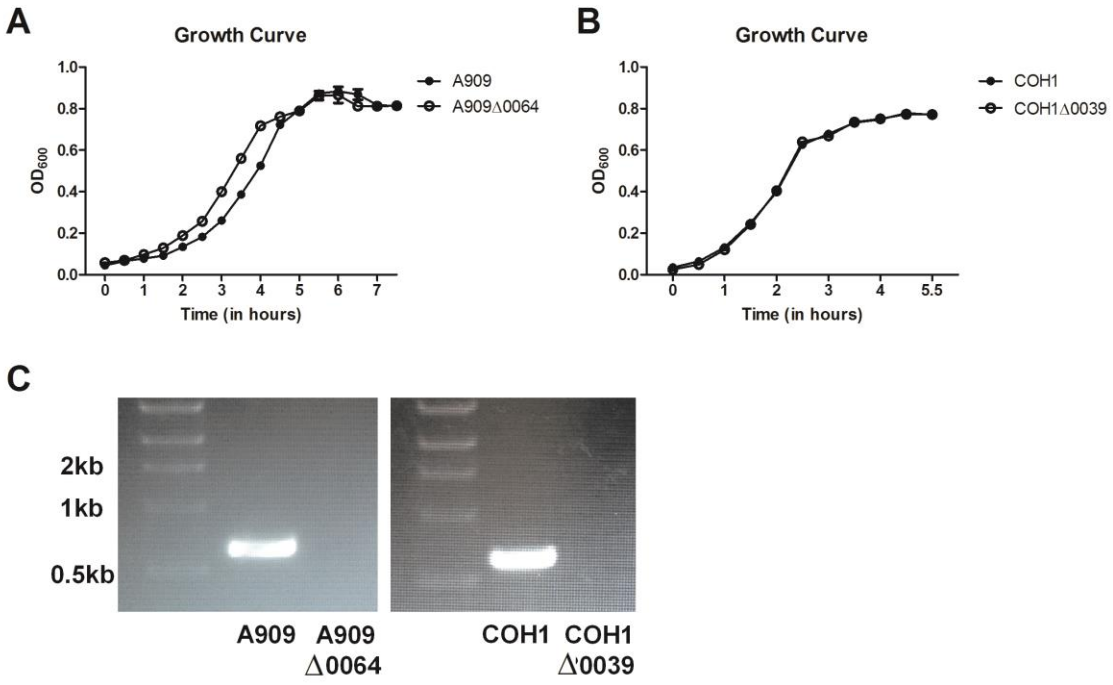

Figure 4.2. Generation of *zoo*A-deficient GBS in COH1 and A909 strains. Growth curves of mid-log phase A909 (A) and COH1 (B) wild type and isogenic mutants in Todd-Hewitt broth as described in Materials and Methods. *In vitro* experiments were conducted independently at least twice and data from one representative experiment is shown. (C) Gel electrophoresis of PCR products with *zooA* primers in A909, COH1, and respective isogenic mutants. Expected PCR product size is 649bp.

We tested the fitness for Δ*zooA* mutants to colonize the murine vaginal tract by performing a colonization trial as done with Δ*ciaR* in Figure 1. We noted in both GBS strain background that Δ*zooA* mutants were less able to colonize the vaginal tract. In the COH1 background, Δ*zooA* also appeared less able to persist in the vaginal tract with significantly fewer bacteria recovered on Day 6 compared to wild type ($P = 0.0277$, Fig. 3A). In the A909 background, the effect was more subtle, but still significant, with recovered Δ*zooA* CFU being lower than wild type over the first six days of colonization ($P = 0.0401$) although no specific day achieved significance (Fig. 3B). We also tested the contribution of SAN_2180, the most down-regulated gene in the absence of *ciaR*[12], to GBS vaginal colonization. We recovered significantly fewer Δ2180 CFU on days 4 and 5 post-inoculation than wild type (Fig. 3C). Since Δ*ciaR* was impaired in colonization ability by Day 3 (Fig. 1) and this phenotype repeated in Fig. 3A and 3C, it appears that regulation of *zooA* and SAN_2180 only partially explain the Δ*ciaR* colonization phenotype.

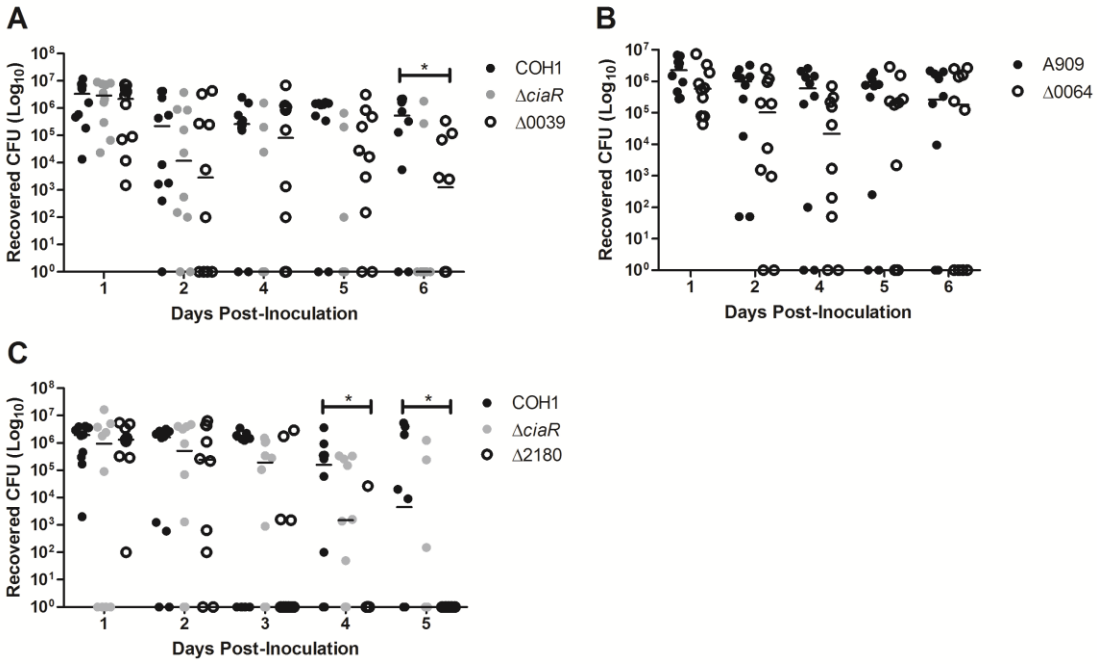

Figure 4.3. GBS zoocin A promotes vaginal colonization. CD1 mice (n=10/group) were colonized with 1×10^7 CFU of GBS COH1 (A,C) or A909 (B) or respective Δ*ciaR*, Δ*zooA*, or Δ2180 mutants in the vaginal lumen. GBS persistence was determined by swabbing the vagina and plating recovered bacteria. *In vivo* experiments were conducted independently at least twice and data from one experiment is shown. Data was analyzed using Mann-Whitney test for individual days and Kruskal-Wallis with Dunn's multiple comparisons post-test. *, $P<0.05$.

GBS zoocin A impacts intracellular bacteria in the reproductive tract epithelium

Based on our previous observations that CiaR regulation promotes GBS survival within human brain microvascular endothelial cells[12], and our murine data here revealing roles of both CiaR and zoocin A in vaginal colonization (Fig. 1, 3), we next examined the contribution of *zooA* in GBS interactions with the female reproductive tract epithelium. We performed adherence and invasion assays with wild type and *zooA* mutants in both GBS backgrounds with human vaginal, ectocervical, and endocervical epithelial cells. To quantify bacterial adherence, epithelial cells were infected with GBS for 30 minutes, non-adhering bacteria was washed away, and then cells were removed and lysed, and adherent

bacteria quantified by serial dilution plating. We did not observe any differences in adherence between wild type and Δ*zooA* in either A909 or COH1 backgrounds in any of the three cell lines (Fig. 4A-C). Additionally, we performed invasion assays where cells were infected with GBS for 2 hours and then treated with antibiotics for an additional 2 hours so that only intracellular GBS would be quantified. Interestingly, the A909 Δ*zooA* mutant was significantly more invasive than wild type in vaginal and endocervical cells, and was also increased in ectocervical cells (P = 0.068, Fig. 4D-F). This phenotype was not as pronounced in the COH1 background, with significance only achieved in endocervical cells (Fig. 4D-F).

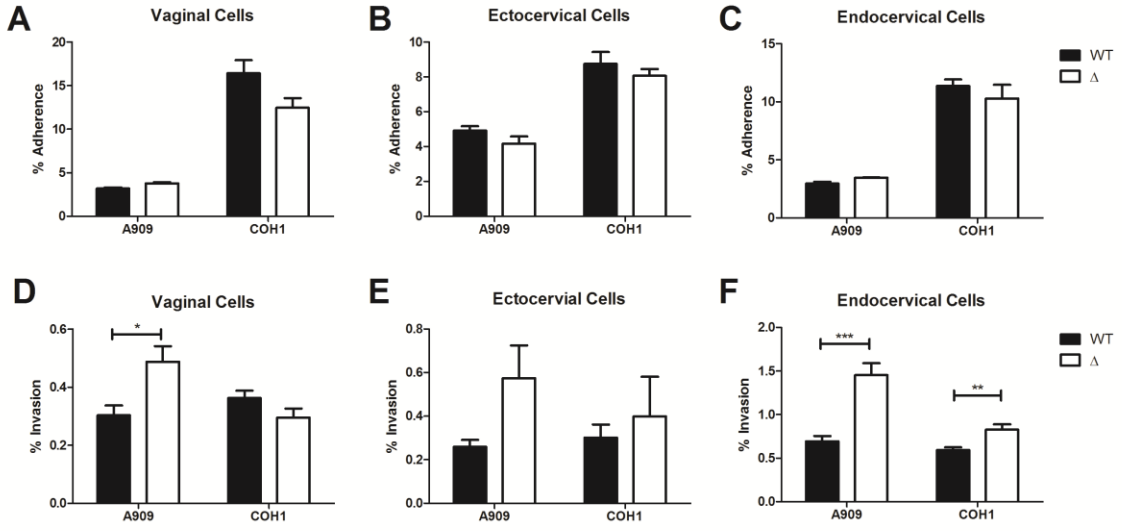

Figure 4.4. Contribution of *zooA* to GBS interactions with human female reproductive epithelial cells. (A-C) Adherence and (D-F) Invasion of A909, COH1 and isogenic Δ*zooA* mutants with indicated epithelial cells, MOI = 1. Experiments were repeated at least twice with four replicates and data from a representative experiment is shown. Data was analyzed by Student's *t*-test. *, P<0.05; **, P<0.01; ***,P<0.001.

Both CiaR and zoocin A alter GBS interactions with GAS

The two component system CiaR/H appears in several *Streptococcus* species including *S. pneumoniae*[33] and *S. agalactiae*[12], thus it may be important in interactions across *Streptococcus*. Furthermore, *S. zooepidemicus* zoocin A displays activity on peptidoglycans of multiple *Streptococcus* species[23]. To test whether GBS CiaR and zoocin A also are involved in GBS interactions with other *Streptococcus*, we performed co-cultures with GBS and GAS *in vitro*. Wild type COH1 and isogenic Δ*ciaR* and Δ*zooA* and wild type GAS strain, NZ131, were grown either in single or co-culture for 24 hours in rich media and plated to determine viable CFU. Viability of wild type and Δ*zooA* were not affected in the presence of NZ131, however, we recovered significantly fewer CFU of Δ*ciaR* when grown in the presence of NZ313 (Fig. 5A). Moreover, viability of NZ131 was drastically reduced in the presence of both wild type GBS and Δ*ciaR*, yet Δ*zooA* was not as effective in inhibiting NZ131 (Fig. 5B).

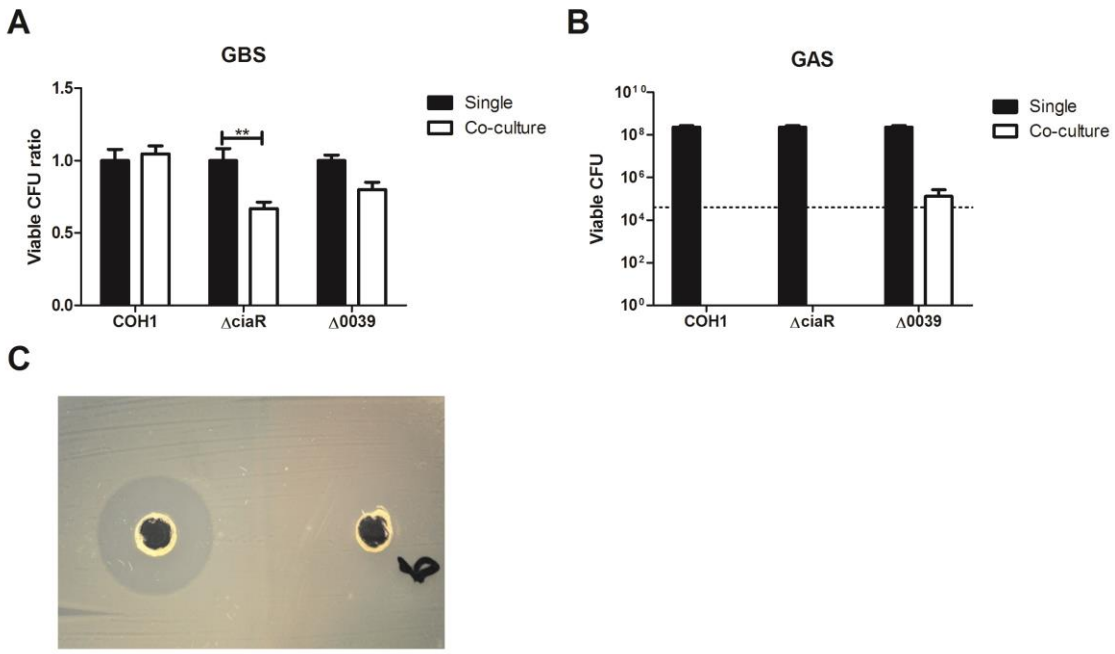

Figure 4.5. CiaR and zoocin A impact GBS interactions with GAS. Viable GBS CFU ratio (A) or GAS CFU (B) after 24 hours post-inoculation either in single culture or in co-culture. (C) Image of purified protein fractions from *E. coli zooA* (left) or vector only (right) on a lawn of GAS as described in Materials and Methods. Experiments were carried out at least twice independently with three replicates, and one representative experiment is shown. Data was analyzed using two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test. **, $P<0.01$.

Purified GBS zoocin A possesses lytic activity

Our *in vitro* co-culture experiments suggested that GBS *zooA* is important in controlling the growth of another *Streptococcus* species. To determine if GBS zoocin A demonstrates lytic activity, we purified zoocin A using an *E. coli* expression system as described in Materials and Methods. Purified zoocin A, or a vector-only control, were spotted on to lawns of GAS. Exposure to zoocin A, but not the control, resulted in a zone of clearance of GAS where the protein was applied (Fig. 5C). Additionally, purified zoocin A also generated zones of clearance on lawns of GBS and *Micrococcus luteus* (data not shown).

DISCUSSION

Although primarily considered an asymptomatic mucosal colonizer, GBS may cause disease in immune compromised individuals, such as neonates, pregnant women, and the elderly, resulting in pneumonia, sepsis, urinary tract infections, and meningitis[4]. GBS has several regulatory systems in place that may control the transition from a commensal niche (e.g. vaginal tract) to invasive niches (e.g. blood, lung, brain, and other organs). Here, we investigated the roles of the two component system response regulator CiaR and a key regulated gene, zoocin A, in vaginal colonization and GBS interactions with other microbes. We found that CiaR is a critical regulator in establishment of vaginal colonization, with dramatically reduced recovery of CiaR-deficient GBS by 3 days post-inoculation (Fig. 1) compared to wild type. Previous work had revealed the response regulator CovR was critical for successful colonization by 2 days post-inoculation[31]. Others have noted that CiaR is required for mucosal colonization in *S. pnuemoniae*[14]. Together these studies highlight the importance of two components systems in bacterial persistence in mucosal environments, although more work needs to be done to identify the specific regulated genes involved in colonization.

In this study, we divulged that one CiaR-regulated gene, which in this work we term *zooA,* contributes to successful vaginal colonization. *zooA* is highly conserved across sequenced GBS strains, and the strains A909 (SAK_0064) and COH1 (SAN_0039) shared 100% gene identity, including flanking regions. In our original CiaR study, we noted that COH1 CiaR had regulated 3 identical copies of the *zooA* gene: SAN_0039, SAN_0058, and SAN_0059[12]. However, allelic exchange mutagenesis of SAN_0039 done here confirmed that the two other copies of the gene were the result of

errors in genome assembly and are not actual genes. The COH1 genome has since been corrected.

Herein, we have observed that GBS zoocin A contributes to vaginal colonization but significant differences between zoocin A-deficient and wild type strains were not observed until after 6 days of colonization. Therefore, zoocin A regulation only partially explains the more dramatic phenotype of CiaR-deficient GBS. Furthermore, we determined that GBS zoocin A is active against GBS itself, as well as *S. pyogenes* and *Micrococcus luteus*. It was previously known that *S. zooepidemicus* zoocin A is active against other *S. zooepidemicus* species as well as *S. pyogenes*, *S. mutans*, and *S. gordonii*, but not *S. rattus*, *S. oralis*, or *Lactococcus* spp.[23]. *S. pyogenes* strain NZ131 was dramatically inhibited by GBS in our *in vitro* co-culture assays. Previous studies have found *S. pyogenes* extremely sensitive to *S. zooepidemicus* zoocin A at the ng/mL level[19], so it is possible that *S. pyogenes* is also highly sensitive to GBS zoocin A although future studies must be conducted to quantify the minimum inhibitory concentrations. Since numerous *Streptococcus* species including *S. anginosus*, *S. salivarius*, and *S. mutans* have been isolated from the human vaginal tract[34], we hypothesize that GBS zoocinA may be a key factor in GBS competition with other strains in this environment and this will be the subject of future research.

S. zooepidemicus produces an adjacent zoocin A immunity factor (zif) which acts by lengthening the peptidoglycan cross-bridge to inhibit both the binding of the target recognition domain, and hydrolyzing by the catalytic domain of zoocin A[22]. Interestingly, GBS does not possess an immunity factor to its own zoocin A rendering it susceptible to peptidoglycan hydrolysis by this enzyme. The fact that we were unable to create a viable

zooA over-expression mutant in GBS (data not shown) provides further evidence for GBS susceptibility and this stresses the importance of tight regulation by CiaR and other regulatory systems.

Lastly, we observed that zoocin A also impacts interactions with the host epithelium in the reproductive tract environment. In this work, we did not observe any contribution of zoocin A to cellular adherence; however, we did note that intracellular recovery of GBS was greater in the absence of zoocin A. We did observe differences in GBS strain backgrounds, with A909 Δ*zooA* consistently being increased over wild type in all three human cell lines. We speculate that this phenomenon is the result of zoocin A being produced when GBS has invaded human cells. Since GBS is sensitive to its own zoocin A, the thicker capsule present on the COH1 strain may help protect it, masking any phenotype in zoocin-A deficient mutants. Interestingly, the endocervical cell line revealed the most pronounced effect with the absence of *zooA*, and though not addressed in this study, should be the subject of future experiments.

In summary, we have identified the two component system CiaR/H as being vital to GBS vaginal colonization. Part of CiaR regulation includes a peptidoglycan hydrolase, zoocin A, which promotes vaginal colonization through bacteriolytic activity against other Gram-positive organisms. Because GBS is so frequently recovered from the human vaginal tract, and poses a risk to neonatal and elderly health subsequent to colonization, more studies must be conducted as we gain better understanding of GBS factors regulating microbial and human interactions.

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CHAPTER 5

A novel C5a-derived immunobiotic peptide reduces *Streptococcus agalactiae* colonization through targeted bacterial killing

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PREFACE TO CHAPTER 5

Chapter 5 in full is an article published in *Antimicrobial Agents and Chemotherapy.* The work in Chapter 5 addresses Aim 3a: Characterize a novel immunostimulatory peptide, EP67, for ability to reduce GBS vaginal colonization. This project was a collaborative effort between San Diego State University and the University of Nebraska Medical Center. Herein, we demonstrated that EP67 effectively reduced GBS within the murine vaginal tract and peritoneal cavity in the matter of days and hours respectively. We further established that the mechanism of action occurred not through immune stimulation as originally anticipated, but rather through targeted bacterial killing of GBS in *in vitro* minimum inhibitory concentration and killing assays. This work is the first of its kind to identify a peptide therapy to control GBS vaginal colonization. This work is dedicated to our friend and colleague Dr. Edward Morgan.

ABSTRACT

Streptococcus agalactiae (Group B Streptococcus, GBS) is a Gram-positive bacterium that colonizes the cervicovaginal tract in approximately 25% of healthy women. Although colonization is asymptomatic, GBS can be vertically transmitted to newborns peripartum, causing severe disease such as pneumonia and meningitis. Current prophylaxis, consisting of late gestation screening and intrapartum antibiotics, has failed to completely prevent transmission, and GBS remains a leading cause of neonatal sepsis and meningitis in the United States. Lack of an effective vaccine and emerging antibiotic resistance necessitate exploring novel therapeutic strategies. We have employed a host-directed immunomodulatory therapy using a novel peptide, known as EP67, derived from the C-terminal region of human complement component C5a. Previously, we have demonstrated *in vivo* that EP67 engagement of the C5a receptor (CD88) effectively limits staphylococcal infection by promoting cytokine release and neutrophil infiltration. Here, using our established mouse model of GBS vaginal colonization, we observed that EP67 treatment results in rapid clearance of GBS from the murine vagina. However, this was not dependent on functional neutrophil recruitment or CD88 signaling, as EP67 treatment reduced the vaginal bacterial load in mice lacking CD88 or the major neutrophil receptor CXCr2. Interestingly, we found that EP67 inhibits GBS growth *in vitro* and *in vivo* and that antibacterial activity was specific to *Streptococcus* species. Our work establishes that EP67-mediated clearance of GBS is likely due to direct bacterial killing, rather than enhanced immune stimulation. We conclude that EP67 may have potential as a therapeutic to control GBS vaginal colonization.

INTRODUCTION

Streptococcus agalactiae (Group B Streptococcus, GBS) is currently a leading pathogen responsible for early-onset neonatal sepsis in the United States[1]. However, GBS predominantly exists as a commensal colonizer of the lower gastrointestinal and vaginal tract of approximately 25% of healthy adults[2]. Neonatal exposure to this pathobiont occurs *in utero* or peripartum through contact with vaginal fluids[3]. More recently, several clinical cases suggest transmission also occurs post-partum through consumption of breast milk[4]. Early-onset septicemia typically results in pneumonia and respiratory failure and the late-onset form, occurring up to 7 months after birth, presents with bacteremia and meningitis[3]. Current guidelines recommend by the Centers for Disease Control include intrapartum antibiotic prophylaxis (IAP) to mothers who are GBS-positive based on rectovaginal cultures at 35-37 weeks gestation. While IAP has reduced incidence of early-onset infections by 80%, late onset incidence has remained unaffected[1] and adult GBS infections are on the rise[5]. Lack of an effective vaccine for GBS and emerging antibiotic resistance impel development of novel treatment strategies to control GBS vaginal colonization and abrogate transmission to the vulnerable newborn.

One promising strategy is to exploit or modulate the innate immune system to enhance the host's ability to combat microbial infection while limiting inflammation-induced tissue injury. Accordingly, we have engineered a conformationally-biased, response-selective analogue of the biologically active C-terminal region of human complement component $C5a_{65\text{-}74}$[6]. This analogue, termed EP67, has the sequence Tyr-Ser-Phe-Lys-Asp-Met-Pro-(N-methylLeu)-D-Ala-Arg or YSFKDMP(MeL)aR, and was designed to bind C5a receptors (CD88) on antigen presenting cells (APCs) such as

macrophages, but limit inflammatory properties by not engaging C5aRs on polymorphonuclear cells (PMNs)[6,7]. EP67 has shown adjuvant potential in vaccine designs against ovalbumin[6] and *Coccidioides*[8] by enhancing activation of a T_H1 response via engagement of C5aR-bearing APCs. Additionally, our previous work has demonstrated that prophylactic EP67 treatment limited infection with community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA) in a mouse model of dermonecrosis where we observed that EP67 treatment reduced skin lesion size, an effect dependent both on PMN recruitment and signaling through CD88[9]. EP67 has also been shown to lessen MRSA biofilm infection[10].

Considering these previous results, we hypothesized that EP67 may similarly exhibit immunostimulatory properties through enhancing innate immune signaling pathways to prevent GBS colonization. In this study, we demonstrate that EP67 treatment promotes rapid clearance of GBS using a murine model of vaginal colonization. In contrast to our studies with Staphylococcal infections, however, this effect is largely independent of neutrophil receptor CXCr2 and CD88 signaling. Interestingly, *in vitro* assays reveal that EP67 possesses bacteriocidal activity against GBS and other *Streptococcus* species. Our results indicate that EP67 exhibits protective activity against GBS infection and colonization primarily through targeted bacterial killing, rather than immune stimulation.

MATERIALS AND METHODS

Peptide synthesis and preparation

EP67 (YSFKDMP(MeL)aR) and an inactive scrambled sequence, sEP67 ((MeL)RMYKPaFDS), were generated by solid-phase methods using the Fmoc method

of orthogonal synthesis, purified by analytical and preparative reverse-phase HPLC, and characterized by electrospray mass spectrometry according to previously published methods[11]. For *in vitro* and *in vivo* assays, peptides were suspended in 1X PBS (CellGrow) at given concentrations.

Bacterial strains and growth conditions

Streptococcus agalactiae clinical isolates A909 (serotype Ia)[12], 515 (serotype Ia)[13], NCTC 10/84 (1169-NT1; ATCC 49447) (serotype V)[14], COH1 (serotype III)[15], and *Streptococcus pyogenes* (GAS) serotype M1T1 clinical isolate 5448[16], *Streptococcus equi* (ATCC 6580), *Streptococcus salivarius* (ATCC 13419), *Streptococcus gordonii* strain M99[17] were grown aerobically in Todd-Hewitt broth (THB) (Hardy Diagnostics) at 37°C. *Streptococcus pneumoniae* (SPN) serotype 2 strain D39 (NCTC 7466)[18] was grown in THB supplemented with 1.5% yeast extract (THY) at 37°C with 5% CO_2. *Staphylococcus aureus* USA300 isolate (TCH1516-HOU-MR, ATCC accession number BAA-1717)[19] was grown aerobically in tryptone soy broth (TS) (Oxoid) at 37°C. *Salmonella enterica* serovar Typhimurium 14028S 1/9[20] was grown aerobically in Luria broth (LB) (Criterion) at 37°C. *Proteus mirabilis*, *Ruminococcus albus*, and *Enterococcus faecalis* (murine vaginal isolates from our lab; verified with 16S sequencing at Eton Biosciences Inc.) were grown in LB at 37°C.

Murine strains

All mouse work was approved by the Office of Lab Animal Care at San Diego State University and conducted under accepted veterinary standards. Female CD1 and BALB/c mice (8-20 weeks old) were obtained from Charles River Laboratories. Breeding pairs of CXCr2 (CXCL2 receptor) knock out (KO) mice (formerly IL8r KO mice), were

originally purchased (C.129S2(B6)-Cxcr2^{tm1Mwm}/J, Jackson Laboratories) and a homozygous × homozygous breeding colony established at the UCSD VA Hospital. Mice were maintained on water containing co-trimoxazole (200 μg/mL sulfamethoxazole and 40 μg/mL trimethoprim) and for the 17-week-old females used in this study, antimicrobial treatment was stopped 48 hours prior to inoculation with GBS. Female C5aR1 (CD88) KO mice, 8 weeks of age, were purchased from Jackson Laboratories (C.129S4(B6)-*C5ar1*^{tm1Cge}/J).

***In vivo* model of GBS vaginal colonization**

Female CD1, BALB/c, CXCr2 KO and CD88 KO mice were used for colonization assays adapted from previous work[21,22]. To synchronize estrus and promote bacterial colonization[23,24], mice were injected intraperitoneally with 0.5mg 17β-estradiol suspended in sesame oil (Sigma) 24-72 hours prior to inoculation. Mice were inoculated with 1×10^7 cfu (in 10 μL PBS) GBS A909 in the vaginal lumen. Immediately prior to inoculation, vaginal lavage was performed by pipetting 20 μL of PBS several times in the vaginal lumen to collect cells and cytokines as described elsewhere[25,26]. Bacterial load was determined by swabbing the vaginal lumen with ultrafine calcium alginate-tipped swabs and serial dilution plating of swab samples. GBS and native *Enterococcus* strains were identified by mauve or blue-pigmented colonies respectively on CHROMagar Strep B agar (DRG International Inc.)[27]. GBS was allowed to colonize for at least 24 hours prior to peptide dosage. After colonization was established, 250 μg (in 12.5 μL PBS) of EP67 or sEP67 was administered into the vaginal lumen every 24 hours after first collecting lavage and swab samples. ELISA assays were performed on vaginal lavage fluid for MIP-2 and KC (R&D Systems) as described by manufacturer.

***In vivo* model of GBS peritoneal infection**

Female BALB/c, CXCr2 KO and CD88 KO mice were used for peritoneal infection assays adapted from previous work[28]. Mice were injected intraperitoneally (IP) with 1×10^7 cfu GBS A909 suspended in 100 μL PBS, or 100 μL PBS only for uninfected controls. Immediately following infection, 750 μg of EP67, sEP67, or an equivalent volume of PBS (100 μL) for uninfected controls, was injected IP. After 2 hours of incubation, all the mice were euthanized by CO_2 asphyxiation and peritoneal cavities lavaged with 5 mL PBS. To quantity percentage of original inoculum recovered, lavage fluid was serially diluted and plated on THB agar to enumerate viable bacteria. Additionally, total leukocytes present in lavage fluid were quantified using a hemocytometer loaded with 10 μL of unstained lavage fluid.

Flow cytometry

Vaginal lavage from BALB/c and CD88 KO mice and peritoneal lavage from BALB/c mice were subjected to flow cytometry to identify specific immune cell populations present. Cells from lavage were incubated for 30 minutes in RPMI containing 30% serum (mouse and FBS) to block nonspecific binding. Samples were stained with fluorochrome-conjugated antibodies specific for CD11b-AlexaFluor647, CD11c-PECy7, and NK1.1-PE (eBioscience), as well as antibodies specific for Ly6G-FITC and B220-PE (BD Biosciences). Neutrophils were identified as small cells, by forward scatter analysis, and were Ly6G+CD11b+. Macrophages were identified as large cells that were CD11c+CD11b+ and Ly6G-B220-. Dendritic cells were determined by being highly CD11c+ and B220-. Natural killer cells were determined to be small cells that were Ly6G- and NK1.1+CD11c+. Samples were analyzed with an Accuri C6

Cytometer (BD Biosciences) with 20,000 events recorded per sample. Due to presence of cell debris and large cells in vaginal samples, immune cell populations were determined from a population gated for cell size. Cell populations were assessed for percent of fluorescent staining and staining brightness using Accuri analysis software.

Antimicrobial Assays

Minimal inhibitory concentrations (MIC) were determined during aerobic growth in a 96-well plate format as described previously[29] with several modifications. Briefly, bacterial strains were grown to mid-exponential phase (approximately 1×10^8 cfu/mL). The cultures were diluted 1:100, and 140 μL was added to each well. 10 μL of EP67 or sEP67 was added to each well in 2-fold increment concentrations, from 0 to 403.2 μM (500 μg/mL). Bacterial growth was measured by optical density (OD = 600 nm) after a 24 hour incubation at 37°C. MIC was expressed as the lowest concentration of peptide that inhibited microbial growth completely. Assays were performed in triplicate and were repeated at least 3 times. Minimum bactericidal concentrations (MBC) were determined by subculturing bacteria from wells onto an agar plate using a 48-prong replicator. MBC was determined by the lowest peptide concentration exhibiting no visible growth on the agar. Kinetic killing assays were performed as described above for MIC assays with the following modifications: mid-exponential phase cultures were diluted 1:100 and 1.4 mL was combined with 100 μL of 201.6 μM (250 μg/mL) EP67 or sEP67 and incubated at 37°C. Samples were collected at indicated time points and plated on THB agar to determine viable cfu.

Live/Dead Bacterial Viability Stain

GBS viability during EP67 treatment was determined using the Live/Dead *Bac*Light bacterial viability kit (Molecular Probes, Inc.) per manufacturer's directions with several modifications. Briefly, mid-exponential phase GBS cultures were diluted 1:100, and 1.4 mL was combined with 250 μg EP67, sEP67 or an equivalent volume of PBS (100 μL) and incubated for 2, 5, or 24 hours at 37°C. Bacteria were pelleted and resuspended in 100 μL of diluted THB (equal parts THB and 0.85% NaCl), 0.25 μL of SYTO 9, and 0.25 μL of propidium iodide. Suspensions were incubated in the dark at room temperature for 15 minutes and 10 μL was wet-mounted onto slides. Fluorescent images were collected at 1000X magnification (Zeiss Axio Observer D1 with an attached Zeiss MRc camera). Green and red channel images were merged using AxioVision software (Zeiss).

Statistical analyses

GraphPad Prism version 5.0f was used for statistical analyses. Differences in *in vivo* peritoneal assays including recovered cfu, total leukocyte counts, and flow cytometry cell populations were calculated using unpaired Student's *t* test analysis or one-way ANOVA with Tukey's multiple comparisons test. Differences in bacterial persistence in mouse vaginal experiments were calculated using log-rank (Mantel-Cox) or Mann Whitney tests. Differences in cytokine levels from vaginal lavage were calculated using two-way ANOVA with Bonferroni post-test. Statistical significance was determined at a $p<0.05$.

RESULTS

EP67 treatment promotes rapid GBS clearance from the murine vaginal tract

Previous work has demonstrated that EP67 is protective in reducing CA-MRSA dermal infection[9] and promoting survival against lethal infection with Influenza A[30]. To determine if EP67 has similar protective activity during GBS colonization, we used our established GBS vaginal colonization mouse model[22]. Briefly, we treated 8-week-old CD1 mice with 17β-estradiol one day prior to bacterial inoculation to promote successful colonization. We inserted 1×10^7 cfu GBS into the vagina and, 24 hours post-inoculation, began administering EP67 or the inactive scrambled control, sEP67, into the vaginal lumen. On successive days, the vaginal lumen was first swabbed to determine changes in bacterial load over time and then dosed with either EP67 or sEP67. Consistent with other infection models, EP67 treatment significantly decreased GBS persistence over the 5 day regimen when compared to treatment with sEP67 ($p = 0.0124$) (Fig. 1). Moreover, we observed this same significant effect with another GBS clinical isolate, COH1 (data not shown).

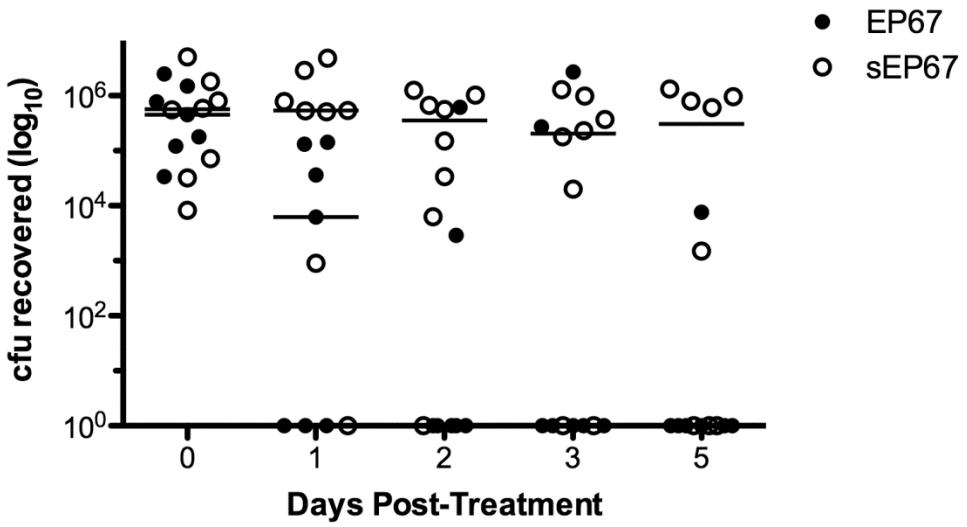


Figure 5.1. EP67 treatment reduces GBS vaginal colonization. GBS, 1×10^7 cfu, was inoculated into the vaginal lumen of 8-week-old CD1 mice (n = 7-8 per group). GBS was allowed to establish colonization for 24 hours prior to initial treatment with EP67 or sEP67. GBS persistence was measured by swabbing the vagina and enumerating recovered bacteria. Lines represent median cfu for each treatment group (median is 0 cfu for EP67 group on days 2, 3, and 5). *In vivo* experiments were conducted independently at least three times and data from one representative experiment is shown. EP67 significantly decreased GBS persistence compared to sEP67 (p = 0.0124) as calculated using log-rank test.

EP67-mediated reduction of vaginal GBS does not require neutrophil recruitment or C5a signaling

In work using EP67 to control CA-MRSA, neutrophils were found to be the key responders limiting dermonecrosis[9]. We have also shown that neutrophil antimicrobial activity corresponds with GBS clearance from the murine vaginal tract early on during colonization[22]. Therefore, we examined the requirement for neutrophil signaling and recruitment during EP67-mediated clearance of GBS in the vaginal tract. Murine chemokine MIP-2 (CXCL2) is a homologue of human CXCL8 (IL-8) and engages CXCr2 (IL-8 receptor homologue) on neutrophils, recruiting them to the site of

chemokine production[31]. Prior work has shown limited neutrophil recruitment in CXCr2 KO mice during several states of pathogenesis including *Escherichia coli* urinary tract infection[32], pneumonic plague[33], and *Streptococcus pneumoniae*-induced pulmonary infection[34]. Consequently, we examined the effect of impaired neutrophil recruitment on EP67 activity during GBS vaginal colonization using CXCr2 KO mice. As seen in CD1 mice (Fig. 1), treatment with EP67 enhanced GBS vaginal clearance in BALB/c mice compared to sEP67 (Fig. 2A, 2C). Surprisingly, we also observed that EP67 significantly accelerated GBS clearance in CXCr2 KO mice ($p = 0.0033$) (Fig. 2B, 2C). Furthermore, 9 days post-inoculation, although MIP-2 levels were fluctuating as expected during the estrous cycle[25] in both BALB/c and CXCr2 KO mice, there were no significant differences observed between groups (Fig. 2D). These results suggest that EP67-mediated GBS reduction in the vagina is occurring independent of neutrophil recruitment through the CXCL2 receptor.

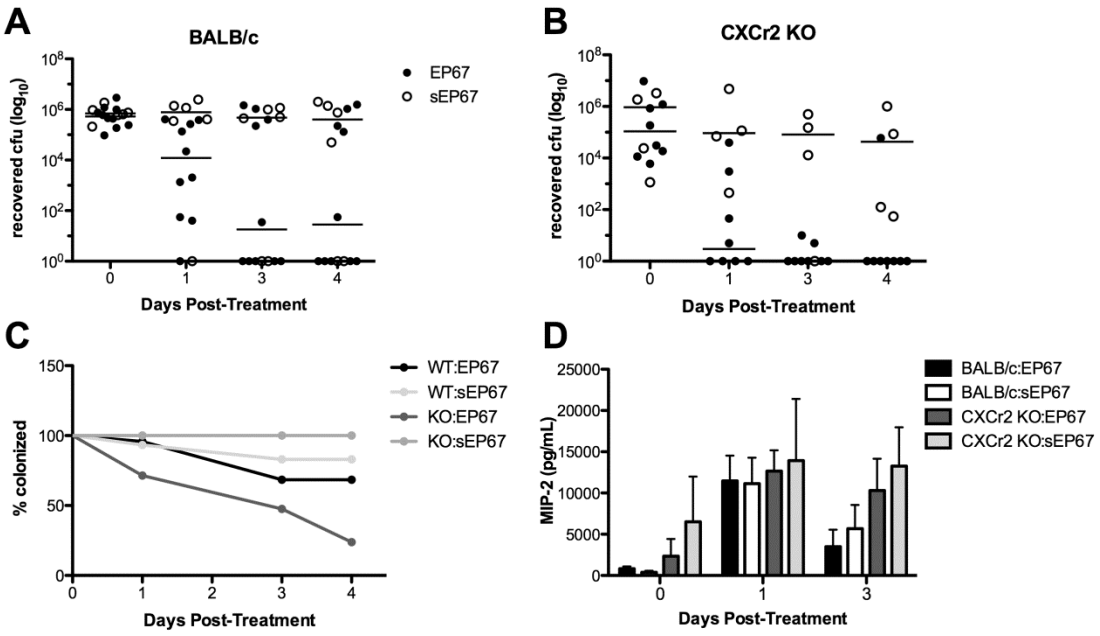


Figure 5.2. GBS vaginal clearance by EP67 does not require signaling through the CXCL2 receptor. (A,B) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 16-week-old BALB/c mice (n = 5-10 per group) (A) or CXCr2 KO mice (n = 3-9 per group) (B). GBS was allowed to colonize for 9 days to ensure colonization following antibiotic removal (see maintenance of CXCr2 KO mice in Materials and Methods) prior to initial treatment with EP67 or sEP67. GBS persistence was measured by swabbing the vagina and enumerating recovered bacteria, lines represent median cfu recovered (A,B). (C) The percent of mice that were colonized over time were calculated following EP67 or sEP67 treatment. (D) Chemokine MIP-2 levels were quantified by ELISA in vaginal lavage fluid collected from mice at indicated time points.

EP67 interacts with C5aR (CD88) resulting in pro-inflammatory signaling cascades from cells such as splenic antigen presenting cells[6] with subsequent recruitment of neutrophils, natural killer cells, and dendritic cells[9,30]. To investigate if CD88 signaling contributes to the EP67-mediated GBS vaginal reduction *in vivo*, we utilized CD88 KO mice. As seen previously (Fig. 2), EP67 treatment significantly accelerated GBS vaginal clearance in BALB/c mice as compared to treatment with the sEP67 control ($p = 0.0404$) (Fig. 3A, 3C). We also observed reduced GBS vaginal persistence in CD88 KO mice treated with EP67 as compared to sEP67, but this difference was not significant (p =

0.1163) (Fig. 3B, 3C). This suggests that CD88 signaling may contribute in part to EP67 action. However, analysis of vaginal lavage fluid from both WT and CD88 KO mice colonized with GBS and treated with either EP67 or sEP67 revealed no differences in Ly6G+CD11b+ cell populations (Fig. 3D) or total CD11b+ leukocyte populations (data not shown). These data are consistent with our results above demonstrating that increased immune signaling is likely not responsible for the observed reduction of bacterial colonization during EP67 treatment.

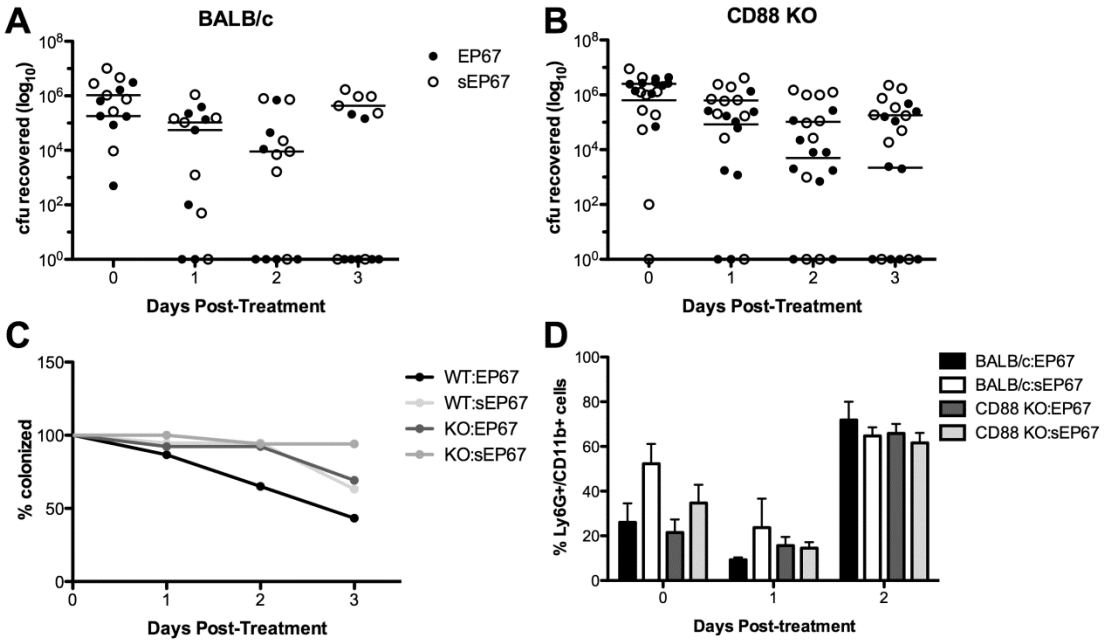


Figure 5.3. Increased GBS vaginal clearance during EP67 treatment does not require signaling through the CD88 receptor. (A,B) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 8-week-old BALB/c mice (n = 7 per group) (A) or CD88 KO mice (n = 10 per group) (B). GBS was allowed to colonize for 2 days prior to initial treatment with EP67 or sEP67. GBS persistence was measured by swabbing the vagina and enumerating recovered bacteria. Lines represent median cfu recovered (A,B). (C) The percent of mice that were colonized over time were calculated following EP67 or sEP67 treatment. (D) Neutrophils (Ly6G+CD11b+) present in vaginal lavage fluid collected from mice at indicated time points were quantified by flow cytometry as described in Materials and Methods.

EP67 exhibits direct antibacterial activity

Since our results thus far suggested that certain immune signaling pathways are not critical to EP67 action, we sought to determine if EP67 exhibited direct antimicrobial activity against GBS. We assessed bacterial growth in the presence of EP67 and observed dramatic growth inhibition of GBS, strain A909, (MIC >100μM), while treatment with sEP67 resulted in no inhibition (Fig. 4A). We also observed that EP67 was bactericidal at concentrations greater than 100 μM, whereas sEP67 was not bactericidal at any concentration tested as determined by MBC assays (data not shown). Other GBS clinical isolates, COH1, NCTC 10/84, and 515 were similarly inhibited (Table 1). Additionally, we tested other *Streptococcus* species and observed growth inhibition in the presence of EP67 (Table 1). However, EP67 did not inhibit growth of other Gram-positive bacteria, including *Staphylococcus*, *Ruminococcus*, and *Enterococcus*, or Gram-negative bacteria such as *Salmonella* and *Proteus* (Table 1).

Table 5.1. EP67 Minimum Inhibitory Concentrations

Streptococcal species; strain	EP67 [μM]
S. agalactiae; A909	100.8
S. agalactiae; COH1	100.8
S. agalactiae; NCTC 10/84	100.8
S. agalactiae; 515	100.8
S. pyogenes; 5448	50.4
S. pneumoniae; D39	12.6
S. gordonii; M99	100.8
S. equi; ATCC 6580	100.8
S. salivarius; ATCC 13419	100.8
Murine vaginal isolates	
Proteus mirabilis	> 404.2
Ruminococcus albus	> 404.2
Enterococcus faecalis	> 404.2
Additional human pathogens	
Staphylococcus aureus; USA300	> 404.2
Salmonella enterica; serovar Typhimurium 14028S 1/9	> 404.2

To evaluate the killing kinetics of EP67 activity against GBS, bacterial survival was assessed over time in the presence of EP67 or sEP67. Our results demonstrated that approximately 95% of both A909 and COH1 strains were killed by EP67 within 3 hours, whereas sEP67 did not inhibit growth of either GBS strain (Fig. 4B). To visually confirm the ability of EP67 to directly kill GBS, we performed a two-color fluorescence assay of bacterial viability. This assay labels all bacteria with a green fluorescent nucleic acid stain (STYO 9) and selectively stains bacteria with permeable membranes using a red fluorescent nucleic acid (propidium iodide). Previous work has established that combining these two dyes at equal ratios can detect membrane damage, as demonstrated by decreased SYTO 9 fluorescence and increased propidium iodide uptake in permeable cells[35,36]. We incubated GBS with EP67, sEP67, or PBS and at multiple time points conducted fluorescent staining. Microscopic analysis revealed that by 2 hours post-EP67

exposure, the majority of bacteria were red indicating membrane permeability (Fig. 4C). As expected, minimal numbers of cells showed membrane permeability with sEP67 or PBS treatment (Fig. 4C). These results further confirm that EP67 treatment results in compromised membrane integrity and corresponding cell death.

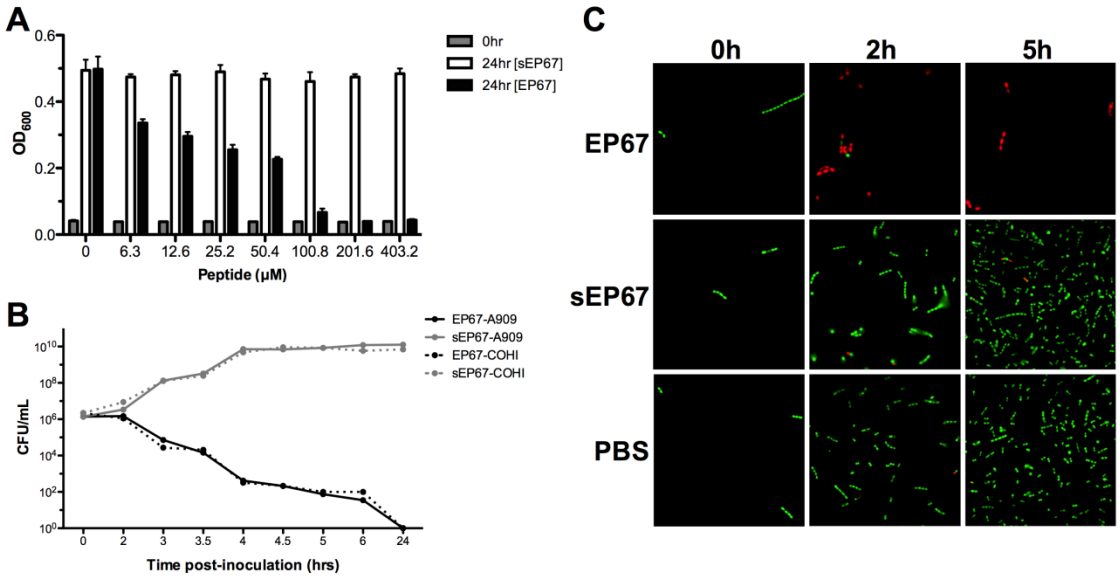


Figure 5.4. EP67 exhibits antibacterial activity against GBS. (A) GBS strain A909 was grown to logarithmic phase and subjected to increasing levels of EP67 or sEP67. After 24 hours, GBS growth was measured by absorbance (OD_{600}). (B,C) Killing kinetics of WT GBS strains A909 and COH1 upon exposure to 200 μM EP67 or sEP67 control as measured by cfu (B) or the Live-Dead stain as described in materials and methods (C). GBS with intact membrane fluoresce green, whereas GBS with damaged membranes fluoresce red. Representative images are shown. Magnification = 1000X. All experiments were conducted independently at least twice and representative data from one experiment is shown.

EP67 treatment reduces GBS bacterial survival *in vivo*

Because EP67 promoted GBS clearance in the vaginal tract amid native flora and estrous cycle influences and *in vitro* assays displayed direct killing capability, we sought to examine this peptide-microbe interaction in a more controlled *in vivo* environment. We selected a mouse model of intraperitoneal (IP) infection adapted from previous work[28].

Immediately following GBS A909 peritoneal infection, BALB/c mice were injected IP with EP67, sEP67, or PBS for uninfected controls. After 2 hours of incubation, mice were sacrificed, the peritoneal cavity lavaged, and bacterial survival was assessed via enumeration of recovered GBS cfu. Viable GBS load was significantly reduced in animals treated with EP67 compared to sEP67 ($p = 0.0003$) (Fig. 5A). We quantified and identified innate immune cells in peritoneal lavage fluid of EP67 and sEP67 2 hours post-infection with GBS using flow cytometry and antibodies to distinguish neutrophils, macrophages, dendritic cells (DC), and natural killer (NK) cells. Macrophages, DCs, and NKs were rare and did not change between treatment groups (Fig. 5B and data not shown). Neutrophils were the predominant innate immune cell present with infected mice having significantly more neutrophils than uninfected controls ($p = 0.0043$) (Fig. 5B). Interestingly, neutrophil levels in the peritoneal cavity did not differ between EP67 and sEP67-treated mice at this early 2 hour time point (Fig. 5B). Thus, these results suggest EP67-mediated GBS reduction may be due to direct antibacterial killing instead of immune activation.

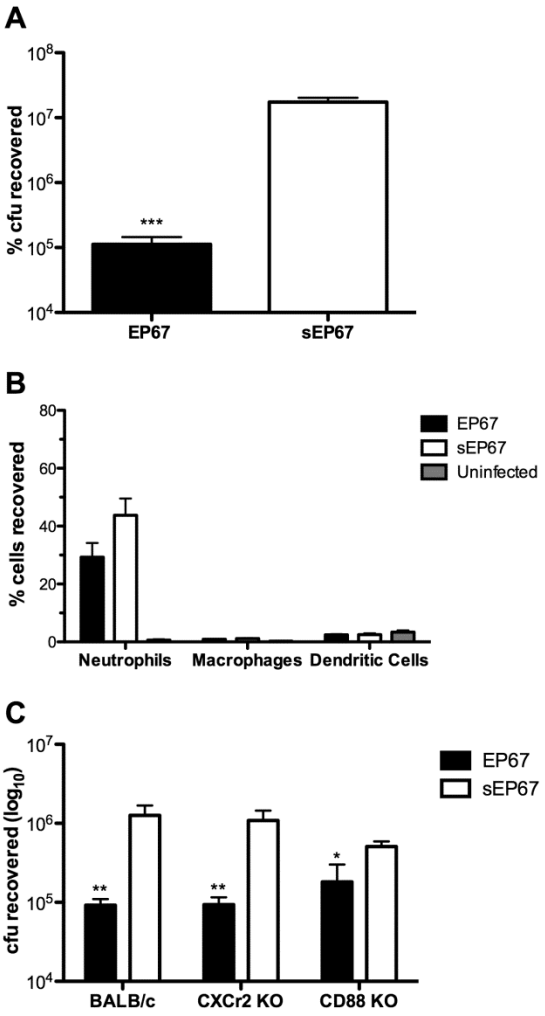


Figure 5.5. EP67 treatment inhibits bacterial load *in vivo* but not immune cell influx and acts independently of CXCr2 and CD88 signaling pathways. (A) Approximately 1×10^7 cfu GBS was injected into the peritoneal cavity of 20 week old BALB/c mice (n = 5 per group). Immediately following GBS injection, mice were treated with EP67 or sEP67 in the peritoneal cavity. Peritoneal lavage was conducted 2 hours post-treatment and GBS survival was enumerated. Peritoneal infection was conducted independently at least twice and data from one representative experiment is shown. (B) Lavage samples from same mice in (A) were analyzed for neutrophil (Ly6G+CD11b+), macrophage (B220-CD11c+), and dendritic cell (B220-CD11c+, B220-CD11c+CD11b+) infiltration by flow cytometry. Data is expressed as percentage of 20,000 cells analyzed per sample. (C) Peritoneal infection and peptide treatment was performed as in (A) using 8-16 week-old BALB/c (n = 5-8 per group), CXCr2 KO (n = 4-8 per group), and CD88 KO (n = 7 per group) mice. Bacterial cfu were recovered from peritoneal lavage 2 hours post-treatment. Data were analyzed by unpaired Student's *t* test. * $p<0.05$, ** $p<0.005$, *** $p<0.001$.

We further performed peritoneal infection in both CXCr2 and CD88 KO mice. Interestingly, we found that EP67 treatment significantly reduced bacterial load compared to sEP67 in the peritoneal cavity of BALB/c (p = 0.0039), CXCr2 KO (p = 0.0023) and CD88 KO mice (p = 0.0440) (Fig. 5C). Additionally, we quantified total leukocytes present in the peritoneal cavity and observed no differences between treatment groups or mouse strains (data not shown). These data align with results seen in our vaginal model (Figs. 2, 3) and suggests that EP67 activity reduces GBS bacterial load independent of immune activation.

DISCUSSION

GBS is a leading cause in neonatal bacterial meningitis and sepsis and current prophylaxis, although dramatically reducing early-onset incidence rates, is insufficient at controlling GBS transmission and infection. Despite such intervention, early-onset GBS infection in the United States remains at 1 in 3,000 live births, corresponding to approximately 1,200 infected infants per year[1]. Thus, the development of novel therapeutics and improving risk factor assessments and treatment strategies is needed. Through our *in vivo* mouse vaginal colonization model and subsequent *in vitro* assays we have identified a response-selective, conformationally-biased C5a agonist, EP67, as a potential candidate for controlling GBS vaginal colonization. The effect of EP67 was independent of anticipated immune signaling and indicated direct killing as the main mechanism of action.

Using our murine model, we have observed GBS persistence in CD1, C57BL/6, and FVB mice for several weeks and in BALB/c mice for several months[22] (Patras, Doran, unpublished data). Contributing factors to GBS vaginal persistence include

estrous stage and continuous treatment with β-estradiol (Patras, Doran, unpublished data), as seen in other murine vaginal colonization/infection models[37,38,39]. Within the mouse vaginal tract, innate immune cell populations are tightly regulated by estrous cycle and steroid hormones directly or indirectly steer immune cell recruitment and activation[40]. CXCr2 functions as the predominant receptor for both KC (CXCL1) and MIP-2 (CXCL2) and previous work has shown that MIP-2 binds with higher affinity to CXCr2 than KC[41]. In our murine vaginal model, we observed similar levels of MIP-2 and KC in both wild type and CXCr2 KO strains, suggesting that neither chemokine production nor neutrophil recruitment account for GBS reduction mediated by EP67. Although CXCr2 deficiency delays neutrophil recruitment, there are alternative pathways to recruit neutrophils, including CC chemokines and complement component C5a, and these pathways are independent of CXCr2[41]. Even so, we did not observe differences in vaginal leukocyte populations in between EP67 and sEP67 groups.

Because previous work has shown that EP67 activates both neutrophil recruitment[9] and T_H1 immune activation[6] through C5aR (CD88) we investigated the role of CD88 signaling during EP67 treatment. Human vaginal epithelial cells (HVECs) possess C5aRs as measured by microarray, however, *in vitro* exposure of HVECs to GBS minimally alters C5aR expression as measured by microarray[22]. Moreover, the murine C5aR1 (NM_007577, Probe ID: 101728) has been determined present in vaginal tissue[42]. Given the presence of this receptor, it is possible that EP67 engages C5aR on the epithelium, yet we still observed reduction of GBS in CD88 KO mice treated with EP67. Of note, GBS possesses a C5a peptidase (SCPB), which cleaves human C5a or $C5a_{desarg}$ between the His and Lys at amino acid positions 67 and 68 very near the C terminus[43].

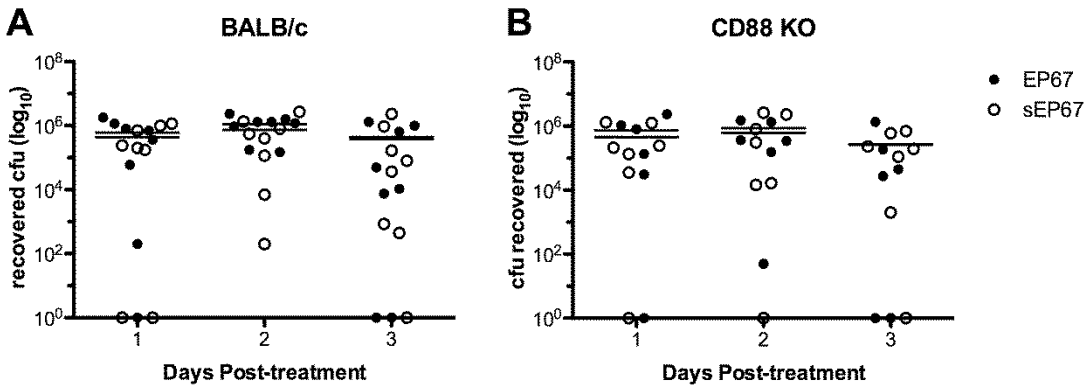
However, SCPB has been shown to have little protease activity with rodent C5a, even though there is high homology[43]. EP67 is only a 10-residue peptide that lacks this His-Lys bond, making sensitivity to the GBS SCPB highly unlikely. Further, using subtractive electrospray (ES) mass spectrometry, we observed no proteolytic cleavage of EP67 following exposure to GBS (unpublished data).

Because EP67-mediated clearance of GBS from the vaginal tract did not appear to require immunostimulatory properties, we investigated if EP67 was alternatively acting as an antimicrobial peptide (AMP) due to its similarity to members of the complement system. Aside from the well-known complement cascade involving C1-C9 proteins, C5a functions as a powerful anaphylatoxin and inflammatory mediator causing leukocyte chemotaxis, cytokines production, and vascular permeability[44]. Furthermore, recent work demonstrated that another complement component, C3a exhibits direct antimicrobial activity by inducing breaks in bacterial membranes[45]. Many AMPs are cationic, which permits binding to negatively charged microbial surfaces[2]. EP67 is also slightly positively charged (+1), which may promote initial contact with GBS cell wall and/or membrane surfaces.

Our *in vitro* assays indicate that EP67 is bacteriocidal to GBS and other *Streptococcus* species. Although not as potent as endogenous C3a and C3a-derived peptides, which kill *E. faecalis* and *S. pyogenes* at <10 μM[45], or the AMP LL-37 that kills GBS at <16 μM[46], EP67 differentially targets GBS *in vivo* without inhibiting or altering native murine vaginal flora (Supp. Fig. 1 and Table 1). AMPs that directly target bacterial membranes, such as LL-37 or its derivatives, can permeabilize Gram-positive and Gram-negative membranes within a matter of minutes[47]. In contrast, EP67 initiates a

microbicidal effect that leads to GBS cell death and membrane damage over a longer time period suggesting the possibility of its interaction with an internal target(s) to exert its antimicrobial effect. Combining our *in vivo* and *in vitro* work, we proposed EP67-mediated direct killing of GBS in a clinically relevant model of GBS vaginal colonization. Although future work must continue to characterize the mechanism of action, here we reveal that EP67 contains unique properties as a potential therapeutic to control GBS colonization and infection.

SUPPLEMENTAL MATERIALS



Supplemental Figure 5.1. EP67 treatment does not impact colonization of murine native vaginal flora *Enterococcus in vivo.* (A) Approximately 1×10^7 cfu GBS was inoculated into the vaginal lumen of 8-week-old BALB/c mice (n = 8 per group). GBS was allowed to establish colonization for 48 hours prior to initial treatment with EP67 or sEP67. During GBS colonization and EP67 or sEP67 treatment, native *Enterococcus* was monitored by swabbing the vagina and enumerating recovered bacteria. (B) Same experiment protocol from (A) utilizing CD88 KO mice (n = 10 per group). Mice in which *Enterococcus* were not isolated were excluded from the data set.

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CHAPTER 6

The probiotic microbe *Streptococcus salivarius* K12 limits Group B Streptococcus vaginal colonization

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Running Title: *S. Salivarius* K12 limits GBS colonization

Keywords: GBS/vaginal colonization/*S. salivarius* K12

PREFACE TO CHAPTER 6

Chapter 6 in full is an article in submission to *Infection and Immunity*. The work in Chapter 6 addresses Aim 3b: Examine the impact of *Streptococcus salivarius* on GBS colonization *in vivo*. This study was a collaborative project between San Diego State University and BLIS Technologies Ltd. of New Zealand. Here, we determined that the probiotic microbe *Streptococcus salivarius* K12 is capable of reducing GBS vaginal carriage in our murine model. In both *in vitro* and animal experiments, we observed a partial dependency of the efficacy of K12 on its megaplasmid which encodes multiple bacteriocin-like inhibitory substances. Using human cell lines, we established that *S. salivarius* K12 is able to interact with vaginal epithelial cells but does not invoke an inflammatory immune response like GBS. This work is the first to identify a probiotic strain that counters GBS both *in vitro* and *in vivo* which highlights its potential as a preventative therapy against GBS.

ABSTRACT

Streptococcus agalactiae (Group B Streptococcus, GBS) colonizes the rectovaginal tract in 20-30% of women and during pregnancy can be transmitted to the newborn, causing severe invasive disease. Current routine screening and antibiotic prophylaxis have fallen short of complete prevention of GBS transmission and GBS remains a leading cause of neonatal infection. We have investigated the ability of *Streptococcus salivarius*, a predominant member of the native human oral microbiota, to control GBS colonization. Comparison of the antibacterial activity of multiple *S. salivarius* strains using a deferred antagonism test showed that *S. salivarius* K12 (K12) exhibited the broadest spectrum of activity against GBS. K12 effectively inhibited all GBS strains tested, including disease-implicated isolates from newborns, and colonizing isolates from the vaginal tract of pregnant women. Inhibition was dependent on the presence of megaplasmid pSsal-K12, which encodes the bacteriocins salivaricin A and salivaricin B; however, in co-culture experiments, GBS growth was impeded by K12 independent of the megaplasmid. We also demonstrated that K12 adheres to and invades human vaginal epithelial cells at levels comparable to GBS. Inhibitory activity of K12 was examined *in vivo* using a mouse model of GBS vaginal colonization. Mice colonized with GBS were treated vaginally with K12. K12 administration significantly reduced GBS vaginal colonization compared to non-treated controls, and this effect was partially dependent on the K12 megaplasmid. Our results suggest that K12 may have potential as a preventative therapy to control GBS vaginal colonization and thereby prevent its transmission to the neonate during pregnancy.

INTRODUCTION

Streptococcus agalactiae (Group B Streptococcus, GBS) is a Gram-positive organism that is currently the leading infectious agent responsible for neonatal morbidity and mortality in the United States[1]. The primary risk factor for newborn disease is maternal GBS colonization within the gastrointestinal or vaginal tract[1]. GBS colonization of the vaginal tract during pregnancy may be constant, intermittent, or transient in nature among individual women[2]. In lieu of an effective vaccine, the current recommendations from the Centers for Disease Control and Prevention include late-gestational screening and intrapartum antibiotic prophylaxis (IAP) to GBS-positive mothers, which has resulted in reduced early-onset GBS disease and infant colonization[1]. However, IAP to GBS-positive mothers has been determined an independent risk factor for *E. coli* early-onset sepsis[3], and has recently been shown to reduce beneficial *Bifidobacterium* spp. in week-old newborn intestinal flora[4]. There are likely to be other unrecognized negative effects of antibiotic exposure on long-term gut health which prompts development of alternative prophylatic strategies. Several studies have proposed the use of probiotic strains to control GBS, and have observed inhibitory activity *in vitro* with *Lactobacillus*[5] and *Bifidobacterium*[4], yet to date, reduction of vaginal GBS populations has not been achieved.

In this present study, we examined the inhibitory capacity of the oral commensal *Streptococcus salivarius* against GBS. *S. salivarius* is an early and prolific colonizer of the human oropharyngeal tract and certain strains have been recently developed for use as oral probiotics due to their production of a diverse assortment of bacteriocins having inhibitory activity that is especially directed against members of other *Streptococcus*

species[6]. Several of these bacteriocins are encoded by megaplasmids which have demonstrated mobility to other *S. salivarius in vivo*[7]. *S. salivarius* strains K12 and M18, developed and marketed by BLIS Technologies Ltd as probiotic strains BLIS K12 and BLIS M18, contain the fully-sequenced megaplasmids pSsal-K12[6] and pSsal-M18[8] respectively. These two megaplasmids show greater than 50% alignment, with pSsal-K12 encoding loci for salivaricins A2 and B and pSsal-M18 containing loci for salivaricins A2, 9, and MPS[6,8]. Because of their known inhibitory effect on *S. pyogenes* and similar *Streptococcus* species[7], we hypothesize that some of these megaplasmid-encoded bacteriocins will also have activity towards GBS.

In this study, we have utilized a deferred antagonism test as well as co-culture assays to demonstrate that *S. salivarius* strains possess varying abilities to inhibit the growth of GBS clinical isolates. Furthermore, we have shown that *S. salivarius* strain K12 is capable of interacting with human vaginal epithelial cells in a similar manner to GBS. Our *in vitro* results are corroborated in a murine model of vaginal colonization in which inoculation with K12 reduces GBS vaginal carriage. Based on our data, we conclude that administration of K12 may have potential as a preemptive treatment to limit GBS vaginal colonization during pregnancy and thereby prevent neonatal transmission.

MATERIALS AND METHODS

Bacterial Strains

Streptococcus agalactiae strains utilized in this study include A909 (ATCC BAA-1138), CJB111 (ATCC BAA-23), COH1 (ATCC BAA-1176), NCTC 10/84 (ATCC 49447), NEM316 (ATCC 12403), 515 (ATCC BAA-1177), 2603 V/R (ATCC BAA-

611), and 6 vaginal isolates obtained from pregnant women[19]. All *S. salivarius* strains used in this study were from the laboratory collection at BLIS Technologies Ltd. and are given in Table 1. All strains were grown aerobically in Todd-Hewitt broth (THB) (Hardy Diagnostics, Santa Maria, CA) at 37°C with 5% CO_2. For co-culture assays, all strains were grown in THB supplemented with 1% (wt/vol) glucose and 4mM calcium chloride. When required, antibiotics were added at the following concentrations: streptomycin (Strep), 1000 μg/mL; spectinomycin (Spec), 100 μg/mL.

Human cell lines

Immortalized human vaginal epithelial cells (hVEC, VK2/E6E7) were attained from American Type Culture Collection (ATCC CRL-2616)[20]. Cell lines (passage 5-25) were cultured in keratinocyte serum-free medium (KSFM) (Life Technologies, Carlsbad, CA) with 0.5 ng/mL human recombinant epidermal growth factor and 0.05 mg/mL bovine pituitary extract at 37°C with 5% CO_2.

Deferred antagonism assays

Deferred antagonism assays were carried out as described previously[21]. In brief, test strains were inoculated as a 1 cm-wide streak across a Columbia agar base (Difco) supplemented with 5% human blood (vol/vol) and 0.1% calcium carbonate (wt/vol). After an 18 hour incubation, visible growth of the test strain was removed with a glass slide and the agar surface was sterilized with chloroform vapors for 30 min. Plates were then inoculated with liquid indicator strain cultures perpendicular to original test strain growth and incubated for 24 hours. Distinct inhibition of indicator strains at the zone of test strain growth was recorded as +. The indicator strain *Micrococcus luteus* was used as

a positive control. All tests were performed in duplicate, and further testing was undertaken if significant discrepancies were detected in the inhibition patterns obtained.

Co-culture assays

For co-culture experiments, overnight cultures were subcultured to mid-log phase (OD_{600} = 0.4). Log phase cultures (100 μL each) were inoculated into 3 mL of fresh THB supplemented with 1% (wt/vol) glucose and 4mM calcium chloride either in single culture or in co-culture with another strain as indicated in figures. At 0 hours and 4 hours post-inoculation, cultures were serially diluted and plated on THB agar plates with appropriate antibiotics.

***In vitro* cell assays**

GBS adherence and invasion of hVEC were performed as described previously[9]. In short, cells were grown to confluency in 24-well tissue culture plates and washed prior to infection. Bacteria were grown to mid-log phase and added at a multiplicity of infection (MOI) of 1. For adherence assays, after 30 minutes of infection, monolayers were washed 6 times with PBS. Cells were removed from plates with trypsin-EDTA and then lysed with 0.025% Triton X-100. Lysate was serially diluted and plated on THB agar plates to quantify adherent CFU. Total adherent CFU was calculated as (total CFU recovered/total CFU of original inoculum)×100%. To quantify invading bacteria, cells were incubated with bacteria for 2 hours, and monolayers washed 3 times with PBS. Cells were given fresh media containing antibiotics, and incubated for an additional 2 hours. Monolayers were washed 3 times with PBS, lysed as described above, and viable intracellular bacteria determined by serial dilution plating as quantified with adherence assays.

ELISA

For ELISA assays, hVEC were infected as described above with several modifications. Bacteria were added at an MOI of 10, and cells were incubated with bacteria for 4 hours. Cell supernatants were analyzed for chemokine secretion using a human IL-8 ELISA kit (R&D Systems) according to manufacturer's instructions.

Microscopy

For light microscopy, hVEC monolayers were propagated on glass cover slips within 24 well plates. Following an adherence assay as described above (MOI = 100), coverslips were washed 6 times with PBS, air-dried and heat fixed, and then subjected to a standard Gram stain protocol as described previously[9]. All images were taken on a Zeiss upright microscope with attached Axiocam Icc3 camera at indicated magnification.

Mouse model of GBS vaginal colonization

All animal work was approved by the Office of Lab Animal Care at San Diego State University and conducted using accepted veterinary standards. 8-12 week-old female CD1 mice (Charles River Laboratories, Wilmington, MA) were injected intraperitoneally with 0.5 mg 17β-estradiol (Sigma Aldrich, St. Louis, MO) in 100 μL sesame oil on Day -1[9,10]. For single challenge experiments, on Day 0, mice were vaginally inoculated with 1×10^7 CFU A909 or K12. For probiotic treatment experiments, on Day 0, mice were vaginally inoculated with 1×10^7 CFU A909 in 10μL of PBS (or PBS as a control for some experiments). On Day 1, and all subsequent days, mice were vaginally inoculated with 1×10^8 CFU K12 or K12ΔpK12 in 10μL of PBS. Control mice were given 10μL of PBS. For all mouse experiments, beginning on Day 1, prior to inoculation with *S. salivarius* or PBS, the vaginal lumen was swabbed with a sterile

ultrafine swab, and recovered GBS or *S. salivarius* were enumerated by plating on THB agar plates with appropriate antibiotics.

Statistical analyses

GraphPad Prism version 5.04 was utilized for statistical analyses. Differences in recovered bacteria for co-culture assays were evaluated using two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test. One-way ANOVA with Bonferroni's multiple comparisons post-test was used for *in vitro* adherence, invasion, and ELISA assays. *In vivo* results for recovered bacteria were analyzed using Kruskal-Wallis with Dunn's multiple comparisons post-test for individual days, and two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test for analyses over time. Statistical significance was accepted at $P<0.05$.

RESULTS

Streptococcus salivarius* inhibits GBS growth *in vitro

To test the inhibitory capacity of *S. salivarius* towards GBS, we utilized an established deferred antagonism test[7]. In all, we tested 9 wild-type *S. salivarius* strains against 13 GBS human clinical isolates (Table 1). We observed a broad range of inhibition from complete inhibition (all 13 GBS strains inhibited) by strains K12 and Tove R, to no inhibition (none of the GBS strains inhibited) by strains MPS, Trev P, and CCHSS3. Additionally, we observed a dependency of inhibition on the presence of the K12 megaplasmid pSsal-K12, as there was no inhibition of GBS by the K12 megaplasmid-deficient strain K12ΔpK12. Furthermore, megaplasmid-dependent inhibitory activity could be transferred to another strain background, with M18pK12

demonstrating complete inhibition (all 13 GBS) compared to partial inhibition (2 of 13 GBS) observed in the parental M18 strain lacking the pK12 plasmid (Table 1).

Table 6.1. Deferred antagonism test with Group B Streptococcus clinical isolates.

Strain inhibited	**Description**	**Number of GBS isolates**
K12	WT *S. salivarius*, human oral isolate	13/13
K12ΔpK12	K12 cured of pSsal-K12	0/13
M18	WT *S. salivarius*, human oral isolate	2/13
M18pK12	M18 cured of pSsal-M18 and replaced with pSsal-K12	13/13
Tove R	WT *S. salivarius*, human oral isolate	13/13
NR	WT *S. salivarius*, human oral isolate	5/13
20P3	WT *S. salivarius*, human oral isolate	1/13
#5	WT *S. salivarius*, human oral isolate	1/13
MPS	WT *S. salivarius*, human oral isolate	0/13
P	WT *S. salivarius*, human oral isolate	0/13
CCHSS3	WT *S. salivarius*, human blood isolate	0/13

Because the deferred antagonism test examines efficacy of passively secreted bacterial products, we also examined inhibitory activity using live cultures. Briefly, diluted log-phase GBS, strain A909, and selected *S. salivarius* strains were grown either in single or co-culture for 4 hours, and the viable CFU determined with antibiotic selection on THB agar plates. After 4 hours of co-culture, there was significant reduction of GBS viability when co-cultured with either K12, K12ΔpK12, M18, or M18pK12 when compared to single culture growth of GBS (Fig. 1A). This reduction in viability was not seen in any of the tested *S. salivarius* strains after 4 hours of culture with GBS (Fig. 1B).

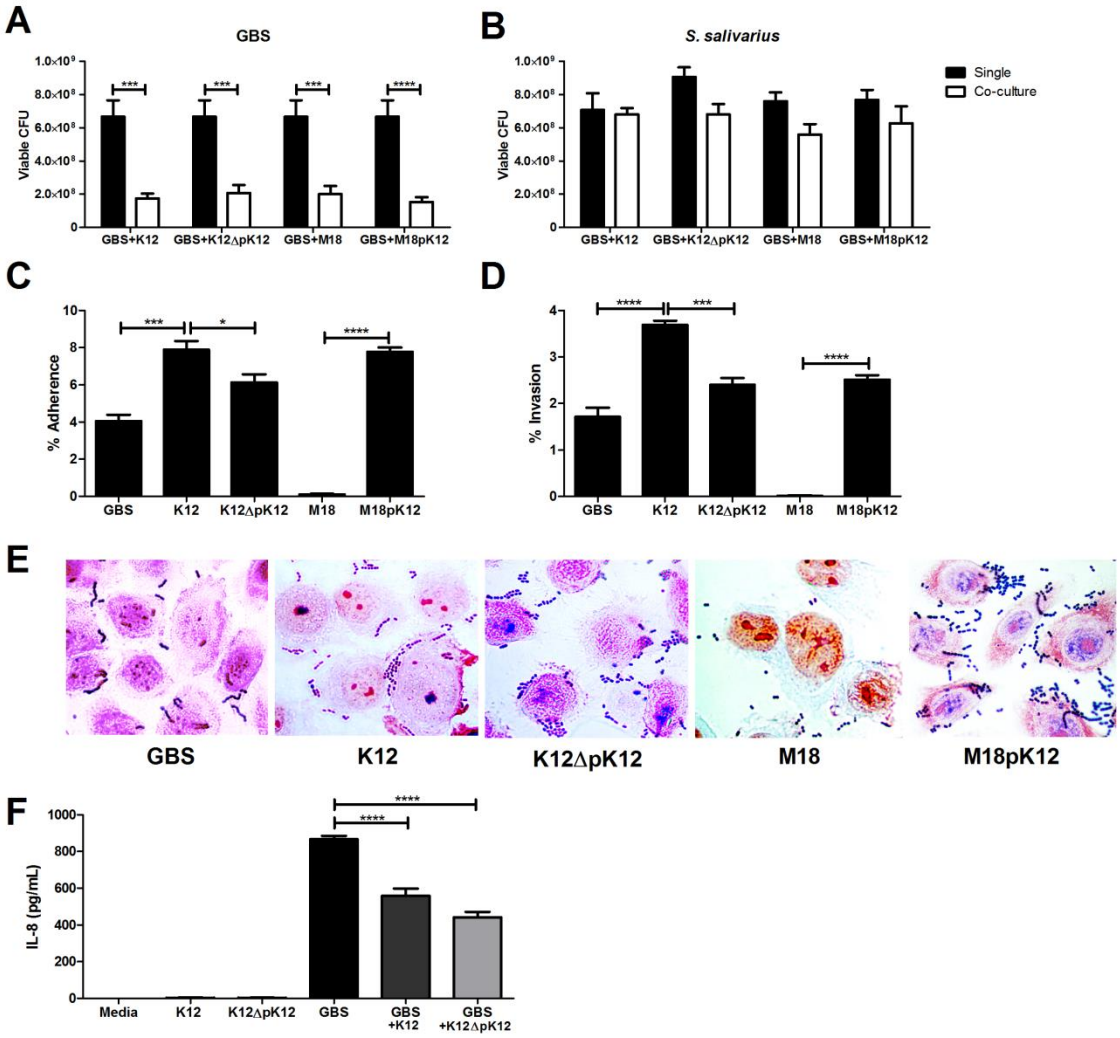

Figure 6.1. *S. salivarius* inhibits the growth of GBS and interacts with human vaginal epithelium *in vitro*. Viable GBS A909 CFU (A) or *S. salivarius* CFU (B) after 4 hours post-inoculation either in single culture or in co-culture. Adherence (C) and invasion (D) of GBS, *S. salivarius* K12, K12ΔpK12, M18, or M18pK12 to human vaginal epithelial cells (hVEC) at an MOI of 1. (E) Gram-stain of hVEC infected for 30 minutes with GBS A909, *S. salivarius* K12, K12ΔpK12, M18, or M18pK12 at an MOI of 100. Magnification = 1000X. (F) Protein expression of IL-8 in hVEC supernatants 4 hours post-infection with GBS, *S. salivarius* K12, K12ΔpK12, M18, or M18pK12 at MOI of 10. Experiments were carried out at least twice independently with three replicates, and one representative experiment is shown. Data was analyzed using two-way repeated measures ANOVA (A-B) and one-way repeated measures ANOVA (C-D, F) with Bonferroni's multiple comparisons post-test. *, $P<0.05$; ***, $P<0.001$; ****, $P<0.0001$.

***Streptococcus salivarius* interacts with human vaginal epithelial cells**

Since *S. salivarius* is predominantly an oral bacterium[6], we tested the ability of strains K12 and M18 to interact with the human vaginal epithelium *in vitro* using adherence and invasion assays. For adherence assays, bacterial strains were incubated with immortalized human vaginal epithelial cells for 30 minutes and non-adherent bacteria removed via washing prior to quantification. We observed that K12 adhered to vaginal cells at significantly higher levels than GBS, whereas M18 demonstrated very little capacity to bind to vaginal cells (Fig. 1C). Furthermore, the ability of *S. salivarius* to adhere to vaginal cells was partially conferred by the pSsal-K12 plasmid, as the K12ΔpK12 strain showed significantly reduced adherence, while M18pK12 achieved adherence levels comparable to wild-type K12 (Fig. 1C). We further visualized bacterial adherence to vaginal cells using Gram-staining and light microscopy (Fig. 1E). Similarly, we performed invasion assays in which we recovered and quantified viable intracellular bacteria from vaginal cell lysates after 2 hours of infection and a 2 hour antibiotic treatment to eliminate extracellular bacteria. The relative invasive capacity of K12 and M18 mimicked their adherence phenotypes, with K12 invading at levels higher than GBS, and M18 showing minimal levels of invasion (Fig. 1D). Invasive capacity may also be partially dependent on genes encoded on the pSsal-K12 plasmid since its expression in M18pK12 conferred significantly increased invasion compared to the M18 parental strain (Fig. 1D). However, it is also possible that successful invasion first requires cellular attachment making factors responsible for adherence versus invasion difficult to distinguish.

Streptococcus salivarius K12 dampens the host innate immune response *in vitro*

Because *S. salivarius*, particularly K12, exhibited a robust ability to attach to the vaginal epithelium, we next examined whether *S. salivarius* elicits a host immune response *in vitro.* To assess immune induction, we quantified the secretion of IL-8 from human vaginal epithelial cells following incubation with either GBS or K12, or both strains together, for 4 hours. As seen previously[10], GBS was a potent inducer of IL-8 secretion from vaginal cells, yet K12, either with or without the pSsal-K12 plasmid, did not induce IL-8 production over media controls (Fig. 1F). Interestingly, when vaginal cells were incubated with both GBS and K12, IL-8 levels were significantly reduced compared to infection with GBS alone, and this reduction was not dependent on expression of pSsal-K12 (Fig. 1F).

Streptococcus salivarius K12 reduces the GBS load in the vaginal tract

To substantiate our *in vitro* findings that K12 is capable of interacting with the human vaginal epithelium, we assessed the ability of K12 to colonize the vaginal tract *in vivo.* Using our previously established model of murine GBS vaginal colonization[9,10], we monitored the bacterial levels of mice either colonized with a single inoculation of GBS or K12 over time. We detected vaginal colonization with K12 in 100% of mice 1 day post-inoculation; however, on subsequent days, mice began clearing the bacterium with approximately half of the animals clearing K12 by 3 days post-inoculation (Fig. 2A). In mice colonized with GBS, we observed similar rates of bacterial load and persistence as seen previously[10]. Additionally the amount of K12 recovered from the vaginal vault was 10-fold lower than what was recovered for GBS (Fig. 2A).

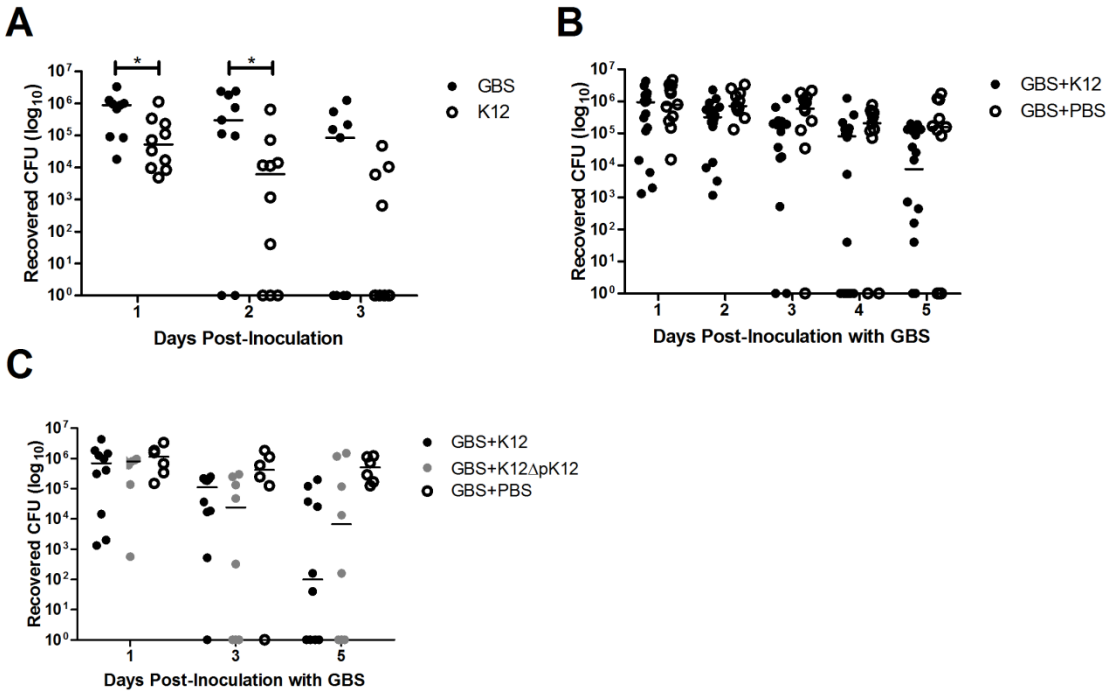

Figure 6.2. *S. salivarius* colonizes the vaginal tract and limits GBS colonization *in vivo*. (A) CD1 mice (n=9-10/group) were colonized with 1×10^7 CFU of GBS A909 or K12 and the vaginal lumen was swabbed daily to determine bacterial load. (B,C) CD1 mice (n=9-18/group) were colonized with 1×10^7 CFU of GBS, and on subsequent days were dosed daily with 1×10^8 CFU *S. salivarius* K12, K12ΔpK12 (C only), or PBS as described in Materials and Methods. Lines represent median values of each group. Data was analyzed using two-way repeated measures ANOVA with Bonferroni's multiple comparisons post-test. *, $P<0.05$.

Based on the inhibitory effect of K12 on GBS growth *in vitro*, we next examined whether K12 presence in the vaginal tract could alter the persistence and/or bacterial levels of GBS. To evaluate this possibility, mice were first inoculated with GBS and allowed 24 hours to establish colonization. On subsequent days, mice were first swabbed to assess bacterial loads, and then inoculated with K12 or given PBS as a control. By day 5 post-inoculation with GBS, mice treated with K12 exhibited significantly lower GBS levels than the control group ($P = 0.0146$) (Fig. 2B). Since CD1 mice begin clearing GBS about one week post-inoculation, we did not observe significant differences between

K12-treated and control groups at later days (data not shown). Furthermore, we examined the impact of the pSsal-K12 plasmid on reduction of GBS vaginal load, and observed that although GBS levels were lower 5 days post-inoculation in mice treated with K12ΔpK12, this effect was not to the same extent as wild-type K12 and did not significantly differ from the control group (Fig. 2C).

DISCUSSION

GBS prevails as a leading cause of global neonatal disease, yet our ability to control GBS vaginal colonization, the primary route of neonatal transmission, remains limited. The host and mucosal microbiota constituents that govern GBS presence in the vaginal tract are not fully known. Several clinical studies have observed an inverse correlation between levels of *Lactobacillus* species and GBS in the human vaginal tract[11,12]. Even so, preliminary human studies with *Lactobacillus* probiotic treatments[12,13], as well as murine models[5], have failed to show significant reduction of GBS vaginal colonization.

In this study, we propose a novel use of the probiotic *S. salivarius* for controlling GBS colonization of the vaginal tract. We observed a spectrum of inhibition of GBS growth by *S. salivarius* strains and this is likely due to differences in repertoires of bacteriocin genes or gene expression across the strains. In deferred antagonism tests, GBS growth inhibition by K12 was completely dependent on the presence of the pSsal-K12 plasmid and the capability for expression of inhibitory activity could be transferred to another cured plasmid negative strain, M18, via the plasmid. However, in co-culture experiments, we did not observe this same dependency, as K12 and M18 strains equally inhibiting GBS growth with or without pSsal-K12. Additionally, the K12ΔpK12 strain

was also capable of reducing GBS bacterial levels within the murine vaginal tract, although not to the same degree as K12. Altogether, these data imply that there are several *S. salivarius* chromosomal and plasmid-based factors which can impact GBS growth depending on interactions with secreted products or direct contact between live bacteria. Furthermore, we observed reduced IL-8 secretion in vaginal cells exposed to both GBS and K12. Possible explanations include downregulation of the host immune response by K12 via the NF-κB pathway as seen previously[14], or a reduction in total GBS CFU over the incubation period through inhibition of growth by K12. The exact mechanisms of GBS growth inhibition or host immune modulation by *S. salivarius*, and these dynamics are the focus of future work.

Although primarily considered a member of the human oral microbiota, *S. salivarius* has been isolated from the vaginal tract in approximately 2% of pregnant women[15]. Our *in vitro* data suggests that the ability of *S. salivarius* to adhere to the vaginal epithelium varies greatly among different strains and adherence may be conferred by genes encoded on megaplasmids such as pSsal-K12. Correspondingly, we were able to demonstrate persistence of K12 within the murine vagina for several days with one inoculation (Fig. 2), however, repeated subsequent doses improved colonization rates (data not shown). Previous work has also demonstrated that *S. salivarius* is capable of surviving in combination with *Lactobacillus* at pH values comparable to the vagina[16]. Importantly, *S. salivarius* K12 has also been found safe and well-tolerated as an oral probiotic in a human randomized placebo-controlled clinical trial[17]. Taken together, these data support that *S. salivarius* K12 is capable of persisting within the human vaginal tract, and may serve as a beneficial microbe in this environment.

In summary, we have demonstrated that the probiotic *S. salivarius* K12 can effectively inhibit the growth of GBS *in vitro*, as well as significantly reduce vaginal GBS levels in a murine colonization model. Although K12 treatment did not completely remove GBS from the vaginal tract in mice, we did observe a 10-fold to 100 fold reduction after 4 days of treatment (Fig. 2). Since high vaginal GBS load has been associated with increased risk for GBS early-onset disease[1] and neonatal colonization[18], we suggest that K12 has therapeutic potential to control GBS vaginal colonization and neonatal transmission in combination with additional preventative measures such as a maternal GBS vaccine. Future work must examine the efficacy of K12 treatment on GBS levels within the human vaginal tract to help establish the potential of this strategy for eradicating GBS as a pervasive element of neonatal pathogenesis.

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CHAPTER 7

CONCLUSIONS

Streptococcus agalactiae (Group B Streptococcus, GBS) remains a global burden of both neonatal and adult pathogenesis in developed and developing countries alike. Although current recommendations of intrapartum antibiotic prophylaxis have reduced GBS early-onset disease to approximately 1000 cases in the United States annually[1], GBS still takes an economic toll on US healthcare costs. The estimated cost of neonatal intensive care stemming from GBS infection is over $30,000 with a potential for increased lifetime healthcare cost of more than $250,000 in 2001 dollars[2]. Aside from economic factors, GBS disease and subsequent sequelae, such as lethargy, need for a ventilator, or severe mental retardation[3], impact the emotional and mental health of thousands of families in the United States each year.

Preceding GBS neonatal disease is successful colonization of the maternal vaginal tract, and at the beginning this dissertation project in 2011, very little was known about GBS factors promoting vaginal colonization, and concurrent host factors responding to GBS presence. There was one study conducted by former lab members identifying GBS surface proteins Srr-1 and PilA as playing roles in GBS vaginal colonization using a preliminary mouse model[4]. Only one other group had utilized a mouse model of GBS to study the therapeutic potential for a phage lytic enzyme to reduce GBS vaginal colonization[5]. Since the commencement of this dissertation research, several other groups have begun to developed working animal models of GBS vaginal colonization[6,7], and GBS accession into the uterus during pregnancy[8], which highlights the interest and necessity of these types of models within the field of GBS pathogenesis research. This

final chapter of the dissertation will summarize the key discoveries within this work, and suggest future work to continue to elucidate the cellular and molecular mechanisms governing GBS vaginal colonization.

SUMMARY OF RESULTS

Host immune responses to GBS within the vaginal tract

Prior to the start of this dissertation, to our knowledge the human host response to GBS vaginal presence was entirely undescribed. One of the key experiments in this work was the global transcriptional profiling performed on human vaginal epithelial cells (Chapter 2, Fig. 2.1), which were infected with wild type GBS, several GBS mutants, or a non-pathogenic *Lactobacillus* species. This microarray data revealed that even within a robust keratinocyte-type epithelial cell, GBS elicits inflammatory pathways involved in both innate and adaptive immune responses, as well as cellular stress responses including apoptosis. Additionally, this experiment demonstrated the importance of the two component regulatory system CovRS and GBS toxin production in repressing and stimulating the host immune response respectively.

This preliminary microarray led us to investigate the role of GBS CovR and the β-H/C toxin in our mouse model. We determined that CovR is vital in down-regulating GBS virulence factors to promote colonization, and in the absence of CovR regulation, GBS induces a robust innate immune response, in part through the CXCL2 receptor, resulting in enhanced neutrophil activation and subsequent clearance of GBS from the murine vaginal tract. Furthermore, we found that many of the human cytokines stimulated by GBS were reliant on presence of the β-H/C production-linked gene *cylE*. GBS deficient in *cylE* were significantly less inflammatory in human reproductive tract

epithelial cells, and were also less able to colonize mice by 2 days post-inoculation compared to the wild type parental strain. Although we were the first to publish on the effect of *cylE* on vaginal colonization, others have also since noted that *cylE*-deficient strains are less able to colonize the vaginal tract [6,8].

Chapter 3 was also initiated from observations within the human vaginal epithelial cell microarray. Further examination of the microarray data revealed that factors such as IL-1β, IL-6, IL-23, and IL-36γ were stimulated, which have previously been implicated in the T_H17 response[9,10]. This observation led us to describe IL-17 production in response to GBS. We found that not only does GBS stimulate IL-17 production within the vaginal tract after several weeks of colonization, but furthermore, this is a beneficial host response which correlates with GBS clearance. This is an important discovery and may aid in future strategies of mucosal vaccination against GBS. While our manuscript was in production, another group also discovered that neonatal mononuclear cells produced significantly less IL-17 in response to GBS compared to adult cells, which may be one explanation for the increase susceptibility of newborns to GBS infection[11]. Although this dissertation has described both innate and adaptive immune stimulation, there are likely many additional nuances of host immune responses to GBS in the reproductive tract, including other T cell responses, resident dendritic cell and macrophage stimulation, and local prostaglandin production.

Previous work has identified increased Prostaglandin E_2 (PGE_2) production in monocytes exposed to GBS[12] and has demonstrated inhibition of PGE_2 is protective in a GBS sepsis model[13]. However, heat-killed GBS did not induce increased PGE_2 in human amnion cells *in vitro*[14]. In our human vaginal epithelial cell microarray, we observed a

moderate increase in Prostaglandin-endoperoxide synthase 2 (COX-2) upon exposure to A909, however, expression levels in hyper-hemolytic (pigmented) strain NCTC 10/84 and A909Δ*covR* were increased by 10-fold or higher. Of note, the β-hemolysin deficient mutant Δ*cylE* and *L. crispatus* did not alter COX-2 expression above the media control. Additionally, PGE_2 synthase and EP4 expression were somewhat elevated in NCTC 10/84 and Δ*covR* groups, and EP2 expression was slightly depressed in HVEC exposed to GBS strains as compared to *L. crispatus* and media controls. Prostaglandin E_2 (PGE_2) is a lipid autacoid derived from arachidonic acid that mediates blood pressure, fertility, and inflammatory responses to pathogenesis[15]. PGE_2 production is initiated by cyclooxygenase (COX)-2, which converts arachidonic acid into PGH_2, in response to inflammatory stimuli or growth factors[16]. PGE_2 signaling is achieved through activation of four rhodopsin-like seven transmembrane G protein-coupled receptors named E prostanoid receptor 1 (EP1), EP2, EP3, and EP4[15]. EP2 and EP4 receptors both activate adenylyl cyclase through G protein signaling which increases intracellular cAMP, however EP4 is more abundant in most tissues and has higher affinity for PGE_2 than EP2[15]. Although well-known for pro-inflammatory signaling and vasodilation, PGE_2 also has immunosuppressive activity including inhibiting macrophage phagocytosis[16] and neutrophil adhesion[17] so the specific role of PGE_2 is context dependent. Recently, PGE_2 was also demonstrated to synergize with IL-23 and IL-1β to expand T_H17 cells, and specifically, EP4-mediated cAMP amplified IL-17A production in the T cell line HuT-102[18]. Future work should be done to confirm our microarray data through both *in vitro* and *in vivo* experiments comparing gene expression and protein production of COX-2, receptors EP4 and EP2, and other cytokines, including IL-17A, between A909, Δ*covR*,

Δ*cylE*, and NCTC 10/84. The contribution of PGE_2 to GBS vaginal colonization and host immune response can be further evaluated through inhibiting COX-2 by administering NSAIDs[19] or by comparing WT and mPGES-$1^{-/-}$ mice[16]. At this point, PGE_2 synthesis in response to GBS in the female reproductive tract has not been explored, and future work will provided valuable information regarding the immunomodulatory effects of PGE_2 during GBS vaginal colonization.

GBS factors that promote vaginal colonization

Prior to the start of this dissertation, there were only two GBS factors that had been studied in the context of vaginal colonization: Srr-1 and PilA[4]. During the development of the mouse model, we noted differences in the ability of several GBS strains to colonize, and these strains represented a variety of serotypes; however, aside from mentioning this observation in Chapter 3, we did not uncover the molecular basis for these strain differences. One of the main goals of this dissertation was to examine the contributions of GBS two component regulatory systems to allowing for persistence in the vaginal tract. As mentioned in Chapter 2, CovR of the CovRS system, down regulates the expression of 27 genes and activates the expression of 3 genes in all GBS strains examined [20-22]. CovR an important regulator of virulence factors such as repression of fibrinogen-binding proteins A and B (FbsA, FbsB), genes involved in iron uptake, and in particular, repression of *cylE*, the gene encoding GBS β-H/C[23]. In Chapter 2, CovR was demonstrated to promote GBS vaginal colonization and to dampen the host immune response. In Chapter 4, we established that the regulator CiaR, part of the CiaRH system which regulates at least 5 GBS genes, was also critical for vaginal persistence of GBS. Although not appearing in this dissertation, the author of this dissertation also discovered

that a novel two component system, that we named FspSR, contributes to colonization through regulation of fructose-6-phosphate metabolism[24]. Since all of the two component systems tested in this work demonstrated importance in promoting vaginal persistence, it is highly likely that the other GBS regulatory systems have yet unrecognized contributions to allowing for GBS colonization, and for subsequent transition to pathogenesis in vulnerable individuals.

Aside from global regulators of gene expression, we also examined several distinct GBS factors to vaginal persistence and host responses. In Chapter 2, we compared the ability of a β-hemolysin-deficient strain with wild type, and noted a decreased recovery of GBS by 2 days post-inoculation of the Δ*cylE* mutant. Additionally, we described a novel peptidoglycan hydrolase in Chapter 4, which we named zoocin A, and determined that this GBS factor promoted vaginal colonization as well, probably through providing GBS a competitive edge with other vaginal microbes. Another factor studied during this dissertation by the author, but not appearing in this printed version, was the latch domain of the fibrinogen-binding surface protein Srr-1. Using the mouse model developed in this dissertation, we determined that the latch domain effectively stabilizes GBS-fibrinogen binding, and promotes vaginal colonization[25]. All these studies combined barely begin to describe all of the constituents which GBS uses to survive in the dynamic, mucosal environment of the vagina, yet they provide a promising strategy to continue to discover important GBS factors and therapeutic targets to control host colonization.

Potential therapeutic strategies to control GBS vaginal colonization

The most direct applications of this dissertation lie in the two therapeutic-based research projects in Chapter 5 and Chapter 6. We described the serendipitous discovery that a immunostimulatory peptide, named EP67, which was originally designed as a vaccine adjuvant[26], also possessed a bacteriocidal activity specifically towards *Streptococcus* species. This peptide was remarkably effective in reducing GBS from the murine vaginal or the peritoneal cavity in two models, demonstrating potential in controlling GBS colonization in a matter of days, or GBS sepsis in a matter of hours, respectively. Native antimicrobial peptides that directly target bacterial membranes, such as LL-37 or its derivatives, can permeabilize Gram-positive and Gram-negative membranes within a matter of minutes[27]. In contrast, because EP67 initiates a microbicidal effect that leads to GBS cell death and membrane damage over a longer time period, we suggest the possibility of its interaction with an internal target(s) to exert its antimicrobial effect. Moreover, we could not completely rule out EP67 immune signaling through the C5a receptor as a potential explanation for GBS vaginal reduction. Although the mechanism needs more investigation, EP67 represents an exciting new strategy to thwart GBS colonization and disease, which merits further considerations for immunostimulatory drugs as a GBS therapy.

Another potential therapeutic strategy was addressed in Chapter 6 of this dissertation. Here, we examined the ability of a probiotic microbe, *Streptococcus salivarius* K12 (K12), to reduce GBS from the vaginal tract. Although *S. salivarius* is primarily considered an oral microbe, it has been recovered from the vaginal tract[28], yet an inverse correlation between *S. salivarius* and GBS has not been observed at this point. In Chapter 6, we demonstrated that K12 had bacteriostatic activity towards GBS in a

plasmid-dependent manner. Furthermore, we showed that K12 was capable of both colonizing the mouse vaginal tract, and concurrently reducing GBS bacterial loads. In the current times where natural remedies are highly favored by the certain populations in developed countries, a probiotic therapy, like K12, may serve as a desirable strategy to limit GBS vaginal colonization in pregnant women.

FUTURE STUDIES

Follow-up studies on discoveries made in this dissertation

There are several key discoveries made in this dissertation that warrant further study. The novel therapeutic strategies, *S. salivarius* K12 and EP67, showed promising results in our animal models. However, a next step in therapeutic development would be the conduction of preliminary clinical trials, particularly in the instance of K12. EP67 toxicity has not been examined thoroughly, and would be a necessary step prior to beginning human studies. Since K12 has already been demonstrated to be safe for oral treatment in human trials[29], approval for K12 clinical trials to combat GBS should be approved fairly readily. Additionally, one very small clinical trial (20 individuals)[30] of a probiotic treatment, called Florajen3, to control GBS vaginal colonization has already been conducted, so there is definite interest in the field of probiotic therapy.

Another general observation made in this dissertation is the variability in vaginal persistence of clinical isolates of GBS as seen in Chapter 3 and Appendix B. We observed that each GBS strain tested had a different length in persistence from several days (strain NCTC 10/84) to several months (CJB111) in the CD1 mouse. Since all of the strains we have tested so far have been fully sequenced[31,32], genomic differences may reveal potential mechanisms controlling GBS vaginal persistence. For example, CJB111

possesses a set of 20 unique genes compared to other fully sequenced GBS strains[31]. Moreover, CJB111 is adept at forming biofilms *in vitro*[33]. Alternatively, gene expression may also explain strain variability, so transcriptional profiling may also be required to determine GBS factors contributing to vaginal colonization.

Screening studies and subsequent experimental verification

Many of the discoveries made in this dissertation stemmed from our initial human vaginal epithelial cell microarray data in Chapter 2, and more human microarray data could be collected from endocervical and ectocervical cell lines to better understand human reproductive tract responses to GBS. Recently, some additional microarray studies have been done to evaluate GBS global gene expression changes in a variety of host tissue models such as decidual and epithelial cells[34], or amniotic fluid[35]. Thus far, these have been limited to fairly rudimentary *in vitro* and *ex vivo* human models, but as models are improved, the quality of obtained data will allow for even more realistic analysis of GBS gene expression in different host tissues. However, due to the descriptive nature of microarray data, follow up experimentation including molecular cloning techniques may be necessary to verify microarray results.

Development of new tools to study GBS vaginal colonization

To further the advancement of our understanding of GBS interactions with the both the host, and other microbes within the context of the host, our animal models must be improved. A molecular tool that would broadly enhance our understanding of the changes that occur both in the host and the microbial community during GBS establishment of colonization is RNA sequencing (RNA-Seq). RNA-Seq has been performed on total RNA recovered from the human vaginal tract via swabbing and the

microbial RNAs compared between healthy women and those with bacterial vaginosis[36], but to our knowledge, transcriptional profiles of GBS in the vaginal tract have not been examined. Others have performed microarray analysis on whole murine vaginas to study mouse transcriptome profiles[37]. However, we would need to develop a protocol to recover enough microbial mRNA from a murine vaginal swab to allow for RNA-Seq of the same mouse over a time course. This would allow us to observe the transcriptional changes that occur in GBS over the course of vaginal transfer, colonization establishment, and prolonged persistence within the host vaginal tract.

Another tool that would widen our understanding of GBS interaction in the vaginal mucosa would be a stable immunofluorescent or bioluminescent GBS strain. We have tested a plasmid GFP-expressing GBS strain in the mouse, and have been able to detect GBS both adhering to murine epithelium (Fig. 7.1A) and in close proximity to other vaginal flora (Fig. 7.1B). However, to observe later time points, we would need a chromosomal GFP gene under a ubiquitous promoter. This tool would allow us to visualize co-localization of GBS with host surface proteins and perhaps even GBS invasion into host tissues or intracellular niches.

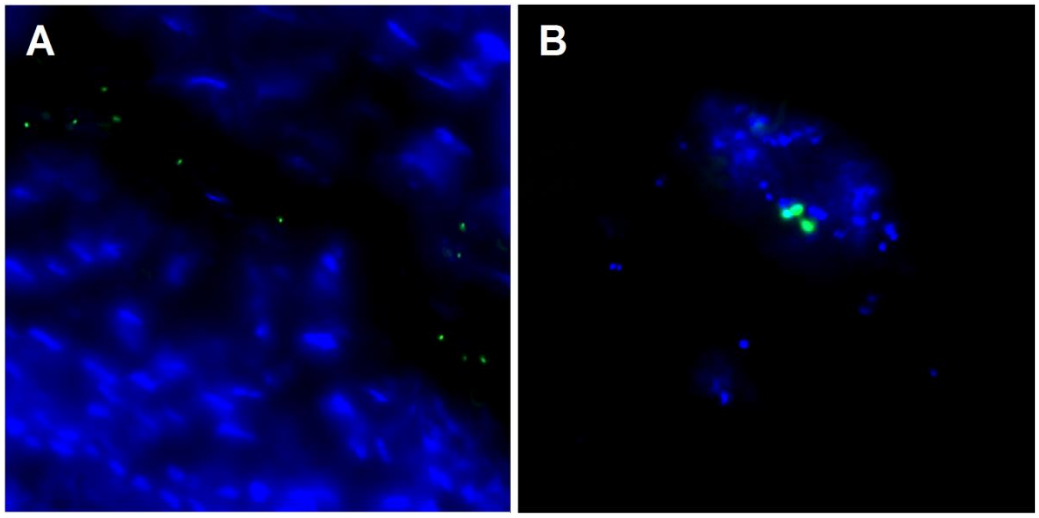

Figure 7.1 Fluorescent imaging of GBS within the mouse vaginal tract. Visualization of GFP-GBS (green) along the vaginal epithelium at magnification = 630X (A), and in close proximity to normal flora at magnification = 1000X (B). Blue stain = DAPI.

Continued development of mouse models

Lastly, we should continue to improve on our mouse model to better mimic GBS interactions with human hosts and microbes native to the human vagina. To better portray GBS interactions with native vaginal flora, gnotobiotic mice could be colonized with vaginal flora collected from healthy women, with or without GBS colonization. To better understand GBS induction of host immune responses, we could use humanized mice which imitate human neonatal host responses and which have already been used to characterize immune profiles during GBS sepsis[38]. Finally, all of the work performed in this dissertation has been done in a post-pubertal, non-pregnant mouse model. One group has begun to investigate the ascension of GBS from the vaginal tract to the uterus during pregnancy[8]. In this study, they inoculated mice on day E13 and examined uterine, placental, and fetal damage on day E17. However, they used a hyperhemolytic GBS strain (NCTC 10/84) and the inbred mouse line C57Bl/6 which are not necessarily

representative examples of invasive GBS and outbred human populations respectively. A better pregnancy model would consist of mice colonized with GBS prior to pregnancy in a robust, outbred mouse line such as CD1. Moreover, pregnancies should be allowed to go full term, or to whelping in the case of pre-term births, to better understand GBS transmission to the fetus during pregnancy or during birth.

In summary, this dissertation sought to examine host and bacterial factors that govern GBS vaginal colonization. Primarily, the robust, innovative animal model of GBS vaginal colonization developed in this work was used to describe complex host-bacterial interactions in an *in vivo* vaginal environment. The information obtained from this dissertation has greatly increased our knowledge of host immune components and specific GBS genes that control GBS vaginal persistence. Moreover, these results have raised additional questions and will be useful for further development of novel therapeutics to limit maternal GBS vaginal colonization and subsequent transmission to the newborn.

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APPENDIX A

GBS VAGINAL COLONIZATION MOUSE MODEL PROTOCOLS

Intraperitoneal injection of β-estradiol

Intraperitoneal injection of β-estradiol

Purpose: Synchronize the estrus cycle of mice and promote bacterial persistence prior to inoculation with GBS.

Materials:

- Sesame oil (Sigma Aldrich, Cat: S3547-250ML)
- β-Estradiol (Sigma Aldrich, Cat: E8875-1G)
- weigh paper
- 15mL conical tube
- 0.45μm syringe filter
- 10mL syringe
- 1mL tuberculin syringe
- 18G 1" needle
- 26G ½" needle

1. Measure out β-estradiol on weigh paper while wearing appropriate PPE. B-estradiol can be absorbed through the skin and mucosal surfaces. Each mouse will receive 0.5mg of β-estradiol (10 mice = 5mg).
2. Transfer β-estradiol to 15mL conical tube and vortex until lumps are removed and β-estradiol is a fine powder.
3. Draw up sesame oil in a 10mL syringe. Each mouse will receive 100μL (10 mice = 1mL). The final concentration of β-estradiol will be 5mg/mL.
4. Syringe filter sesame oil into 15mL conical tube containing β-estradiol using a 0.45μm filter.
5. Vortex conical tube until β-estradiol is a homogenous suspension in the sesame oil.
6. Draw up β-estradiol/sesame oil suspension in a new 10mL syringe.
7. With an 18G needle, aliquot 100μL of mixture into each 1mL tuberculin syringe (one syringe per mouse).
8. Place a fresh 26G needle on each tuberculin syringe.
9. Inject each mouse with the in the peritoneal cavity just to the right or left of the midline.

Vaginal inoculation with GBS

Purpose: Colonize the murine vaginal tract with 1×10^7 CFU GBS.

Materials:

- Overnight culture of GBS strain of interest
- Todd Hewitt broth (THB)
- 5mL culture tubes
- 15mL conical tubes
- 1X PBS
- 200µL gel loading pipette tips (USA Scientific, Cat: 1252-0610)

1. Grow subculture of overnight in THB in 5mL culture tube to OD_{600} = 0.4-0.5.
2. Transfer subculture to 15mL conical and pellet bacteria at 4000rpm for 5 minutes.
3. Resuspend pellet in 200µL of PBS.
4. Bring pellet to exactly OD_{600} = 0.4 in PBS in a new culture tube. This will be a concentration of ~1×10^8CFU/mL.
5. Transfer to a new 15mL conical and repellet bacteria at 4000rpm for 5 minutes.
6. Resuspend pellet in PBS to 1/10 the original volume. For example, if 3mL of OD_{600} = 0.4 was pelleted, then resuspend in 300µL of PBS. This will be a concentration of ~1×10^9CFU/mL.
7. Serially dilute and plate 10^{-4} thru 10^{-6} dilutions on THB agar plate to determine exact inoculum.
8. Inoculate each mouse with 10uL of final bacterial suspension so that each mouse will receive 1×10^7CFU.
9. To inoculate a mouse, restrain via scruffing and inoculate using the 200µL gel loading tip by inserting ¼ inch into the vaginal lumen and dispensing the full 10µL of GBS.
10. Immediately following inoculation, release the scuff and elevate the mouse's hind end by lifting the mouse by the tail and walking the front paws on a hard surface for about one minute. Monitor vaginal opening for any backflow.

Swabbing the vaginal lumen to quantify GBS load

Purpose: Remove GBS from the mouse vaginal lumen to determine bacterial load over time.

Materials:

- Urethro-genital, sterile, calcium alginate swabs (Fisher Scientific, Cat: 22-029-501)
- 1X PBS
- 1.5mL microfuge tubes
- CHROMagar StrepB plates (DRG International, Cat: SB282)

1. Prepare one 1.5mL microfuge per mouse with 100µL of PBS.
2. Prior to swabbing, pre-wet swab in PBS.
3. Restrain mouse via scruffing, and insert the swab ¼ inch into the vaginal lumen.
4. Gently rotate swab 4 times clockwise and 4 times counter clockwise applying slight pressure to the vaginal wall.

5. Transfer the swab to the 1.5mL microfuge tube with 100µL of PBS.
6. Vortex microfuge tube for ~15 seconds to release bacteria from swab.
7. Serially dilute each sample and plate 20µL of 10^{-1} thru 10^{-4} dilutions on CHROMagar plates.
8. GBS colonies will appear either light pink or mauve in color.

Lavaging the vaginal lumen

Purpose: Collect vaginal lavage for use in staging estrous cycle, quantifying bacterial loads, or quantifying secreted cytokines.

Materials:

- 200µL gel loading pipette tips (USA Scientific, Cat: 1252-0610)
- 1X PBS
- 0.7mL microfuge tubes (for cytokines or CFU quantification)
- Glass slides (for staging estrus)

1. Using a 200µL gel loading pipette tip, pipet 20µL of PBS into the vaginally lumen, gentle pipet the entire volume up and down 4 times, and then withdraw the entire volume in the same pipette tip.
2. If the lavage fluid is thick with mucous, a standard 200µL pipette tip can be used to collect the final lavage fluid.
3. If saving lavage fluid for cytokines or CFU quantification, dispense into 0.7mL microfuge tube.
4. If staging estrus, dispense at least 5µL on to a glass slide and observe cells under a 10X objective on a light microscope. For examples of estrous stages, see Appendix B.

Tissue dissection and homogenization

Purpose: Collect and process reproductive tract tissues for bacterial and/or cytokine quantification.

Materials:

- Razor blades
- Petri dishes
- Dissection trays, forceps and scissors
- 1X PBS
- 2mL screw cap tubes
- 1mm zirconia beads
- Mini beadbeater (BioSpec Products)

1. For each mouse, prepare three 2mL screw cap tubes (one for each tissue: vagina, cervix, and uterus). Each tube should contain 500mL of PBS and enough 1.0mm zirconia beads to cover the conical-shaped bottom of the tube.
2. Autoclave the prepared tubes prior to collecting tissue, especially if quantifying bacterial load.
3. Weigh each tube after autoclaving and record for future reference to help determine tissue weight.
4. Sacrifice mouse using approved methods such as CO_2 asphyxiation and/or cervical dislocation.
5. Open abdominal pelvic cavity and with sterile scissors (can be done with 70% EtOH cleaning) cut both uterine horns mid-length between uterine body and ovaries.
6. Separate fat, membranes, and urinary bladder away from reproductive tract moving caudally.
7. With freshly cleaned scissors, transversely cut vagina as close to the vulva as possible.
8. Remove intact reproductive tract and place in a sterile petri dish.
9. Using a new razor blade, separate the uterus from the cervix in one cut. There will most likely be a small amount of uterine tissue next to the cervix.
10. Wipe the razor blade with 70% EtOH, and then separate the cervix from the vagina in one cut. There will most likely be a small amount of vaginal tissue next to the cervix.
11. Using clean forceps transfer each of the tissues into their respective 2mL tubes containing PBS and beads. Clean the forceps in between handling each tissue.
12. Reweigh screwcap tubes and subtract original weight of tube to determine tissue weight. Tissue weights typically vary between 20-100mg.
13. Tightly seal screwcap tubes, and homogenize tissues for 1 minute at maximum speed.
14. For quantifying bacterial load, serially dilute 25μL of tissue homogenate and plate dilutions 10^{-1} thru 10^{-4} on appropriate agar plates.
15. For quantifying cytokines, store tissue homogenates at -20°C until performing ELISA.

Tissue digestion for flow cytometry

Purpose: Digest murine reproductive tract tissues into single cell suspensions to identify cell populations using flow cytometry.

Materials:

- RPMI 1640
- Fetal Bovine Serum (FBS)
- PBS
- Collagenase (Sigma-Aldrich, Cat:)
- Brefeldin A (for intracellular staining, BD Biosciences, Cat:)
- 1.7mL microfuge tubes

1. Dissect murine reproductive tract tissues as described in the above protocol "Tissue dissection and homogenization" Steps 4-10.
2. Finely dice separate tissues into as small of pieces as possible using a razor blade. Wipe blade with 70% EtOH in between dicing each tissue as to not cross-contaminate.
3. Transfer tissues to separate microfuge tubes. Each microfuge tube should contain 400μL of RPMI 1640 with 10% FBS, 1:1000 Brefeldin A (0.4μL, only for intracellular staining), and 50μL of collagenase (60mg/mL in PBS).
4. Incubate tubes at 37°C for a total 2.5 hours. Every 30 minutes, pipette samples up and down with progressively smaller pipet tips to break down tissues.
5. If doing surface staining only, proceed with surface blocking and staining as desired. Pass samples through a 40μm filter prior to running on a flow cytometer.
6. If doing intracellular staining, spin down samples for 10 minutes at 1200 rpm, remove supernatant containing collagenase, and replace with 450μL of fresh RPMI 1640 with 10% FBS and 1:1000 Brefeldin A.
7. Incubate samples for an additional 3.5 hours and then proceed with surface/intracellular permeablization and staining protocols as desired.
8. Make sure to pass samples through a 40μm filter prior to running on a flow cytometer.

APPENDIX B

FACTORS AFFECTING GBS VAGINAL PERSISTENCE AND OBSERVATIONS

The following observations were made while establishing a murine model for Group B Streptococcus (GBS) vaginal colonization, and subsequent experiments are the work of the dissertation author. The vast majority of this work is unpublished, but has been referenced in the work and papers of this dissertation project.

Staging estrous cycle from vaginal lavage fluid

It is well-known that the reproductive stage of mice can be determined from unstained vaginal lavage fluid, and this process has been described previously[1]. There are four main stages of estrus in the female mouse: proestrus, estrus, metestrus, and diestrus. The stages can be classified from vaginal lavage fluid depending on the relative abundance of 3 different types of murine cells shed into the vaginal lumen: nucleated squamous epithelial cells, cornified squamous epithelial cells, and leukocytes. All three of these cell types can be distinguished, unstained, under a low power light microscope. The stages are characterized as follows: proestrus, pre-ovulatory and where estrogen increases, consists of predominantly nucleated squamous epithelial cells; estrus, where estrogen remains elevated, consists predominantly of cornified epithelial cells; metestrus, where estrogen decreases, consists of all 3 cell types; and diestrus, where estrogen begins to increase again, is characterized by low cell numbers with a predominance of leukocytes[1].

For our GBS vaginal colonization mouse model, we used the protocol "Lavaging the vaginal lumen" in Appendix A to determine estrous stages via vaginal lavage. Lavage fluid was imaged under a light microscope, and the stage of estrus determined and recorded. Figure B1 contains representative images of the four stages of the estrous cycle.

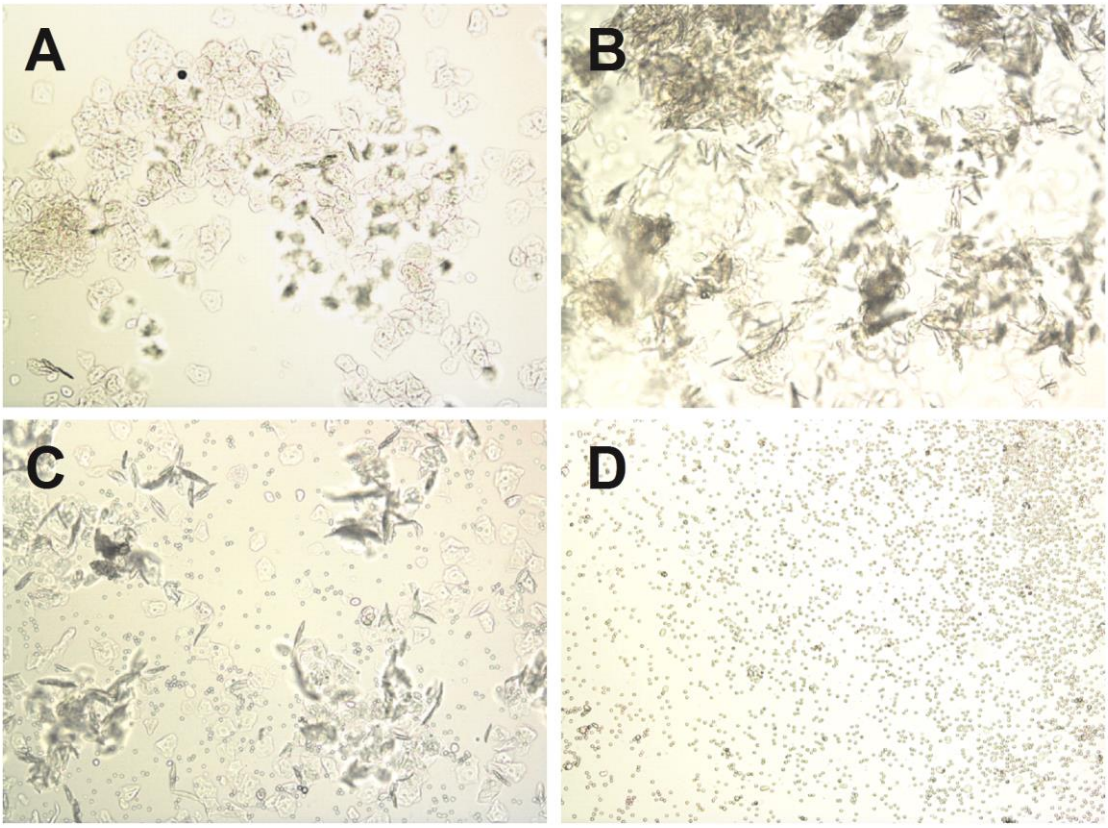

Figure B1. Identifying stage of estrus from unstained murine vaginal lavage fluid. (A) Proestrus. (B) Estrus. (C) Metestrus. (D) Diestrus. Magnification = 100X.

Staging estrous cycle from vaginal histology

An alternative route to staging the estrous cycle is to examine vaginal histology from mice at the experimental endpoint. This is not as desirable typically as using vaginal lavage fluid because of the increased length of time needed to process the samples, as well as this type of sample cannot be collected on a daily basis, but only at the experimental end point. However, if taking vaginal tissue for another purpose, this method is useful to obtain additional information on the estrous cycle stage. The changes that occur in the vaginal epithelium have been well-documented in rodents[2], and they mimic what is seen in vaginal lavage fluid. The stages are characterized as follows: proestrus: mucous layer present along with stratum granulosum; estrus: thick, cornified layer with abundant cell debris shed into the lumen; metestrus: continued cell debris present in the lumen accompanied with leukocyte infiltration; and diestrus: thin epithelium with variable leukocyte infiltration[2].

For our GBS vaginal colonization mouse model, we used the protocol "Tissue dissection and homogenization" in Appendix A Steps 4-6 to collect the mouse reproductive tract. Whole reproductive tracts were paraformaldehyde-fixed, paraffin-embedded, sectioned, and stained with H&E per standard lab protocols. Mounted tissues

were imaged under a light microscope, and the stage of estrus determined and recorded. Figure B2 contains representative images of the four stages of the estrous cycle.

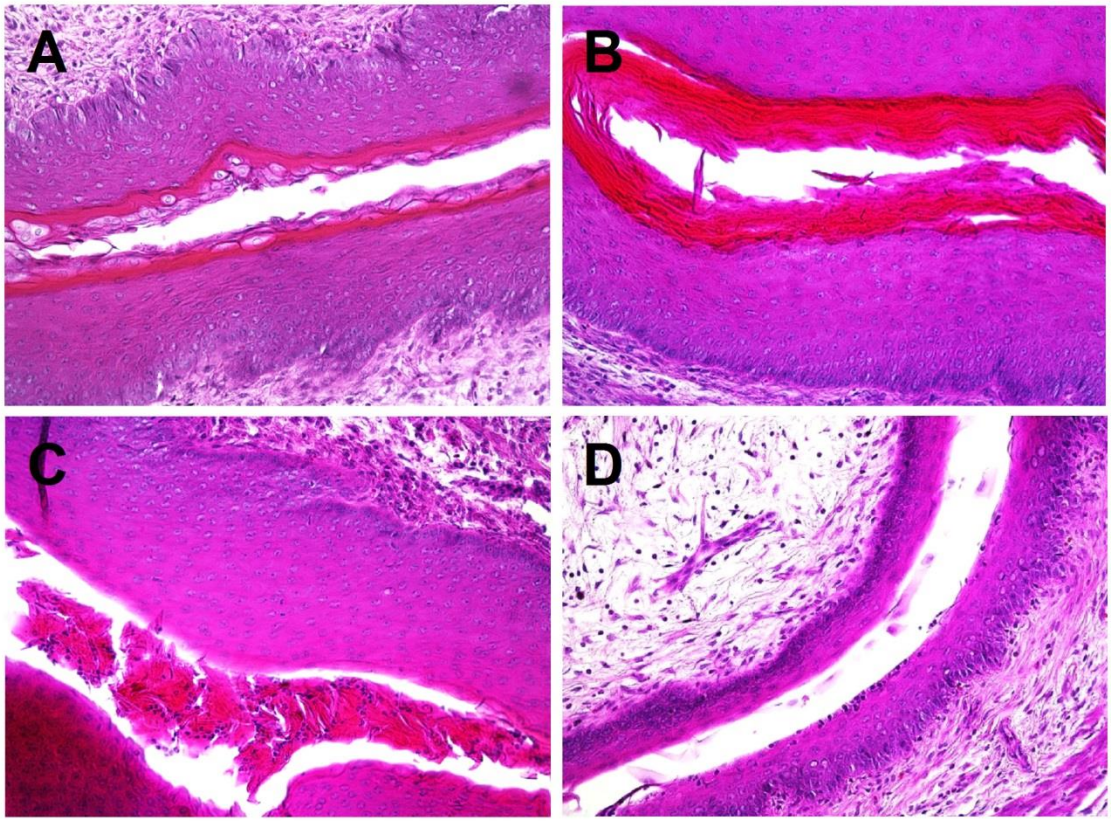

Figure B2. Changes to the murine vaginal epithelium throughout the estrous cycle. Hematoxylin and eosin stain of paraformaldehyde-fixed, paraffin-embedded 5μm tissue sections. (A) Proestrus. (B) Estrus. (C) Metestrus. (D) Diestrus. Magnification = 200X.

Estrous stage impacts GBS vaginal persistence

Previous work has noted that the total rodent vaginal flora increases during estrus, when cornified cells are abundant, and decreases during diestrus, when leukocytes are abundant[3]. To observe whether this effect also occurred in mice in our model system, total aerobic bacteria was collected via vaginal swab and grown on TSA media. Mice were grouped via estrous stage as categorized by vaginal lavage. Mice in estrus possessed significantly higher aerobic CFU compared to mice in the diestrus stage (Supplemental Figure 2.4.A).

To determine if GBS bacterial persistence was impacted by the stage of estrus at the time of GBS inoculation, mice were staged on the day of inoculation via vaginal lavage, divided into groups based on this initial stage, and GBS persistence was monitored over time via vaginal swabbing. As shown in Figure B3, mice inoculated at the proestrus stage were colonized with GBS longer than any other group of mice, particularly those in diestrus at the time of inoculation. Based on these results, in our

model we adopted to treat mice with β-estradiol one day prior to GBS inoculation to synchronize them into the proestrus stage.

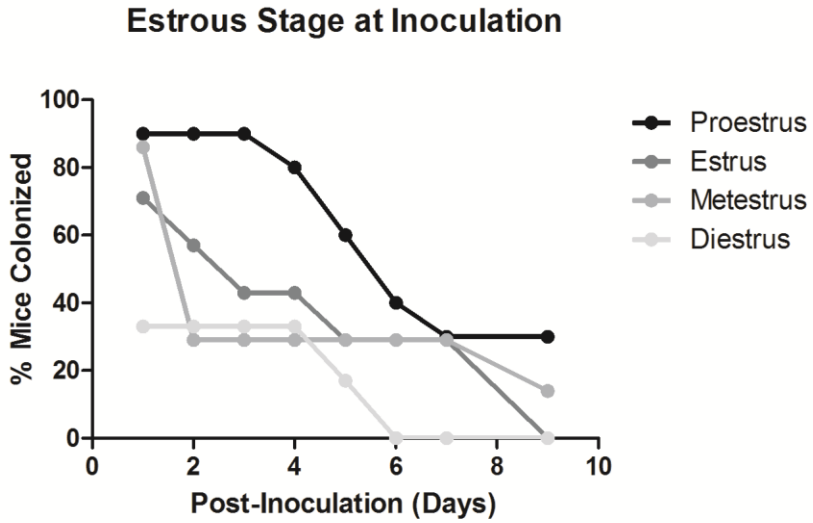

Figure B3. Estrous stage impacts persistence of GBS. Percent of mice colonized with 1×10^7 CFU GBS original inoculum over time. Mice were grouped based on estrous stage at the time of GBS inoculation as determined by vaginal lavage.

GBS persistence with continued β-estradiol treatment

Other murine models of reproductive tract infections have noted an increased ability of the pathogenic organism to persist in the mouse when the mouse was treated with exogenous estradiol[4]. To see if this phenomenon also occurred during vaginal colonization with GBS, I monitored GBS persistence during repeated estradiol treatment. Mice were injected with β-estradiol one day prior to GBS inoculation. On days 1, 3, 5, 21and 23 days post-inoculation, mice received additional β-estradiol injections. Early treatment with β-estradiol promoted GBS A909 persistence in 90% of CD1 mice up to two weeks (Figure B4A). Most experiments with only one dose of β-estradiol result in about 50% of mice being cleared by one week (see Figure B5). Later treatment with β-estradiol at 3 weeks with 20-30% of mice colonized could not rescue GBS load from mice that had cleared the bacteria (Figure B4A). The mean CFU recovered from these mice mimics the percentage of colonization (Figure B4B). Our conclusion is that keeping mice in continuous estrus promotes GBS vaginal colonization in the majority of mice.

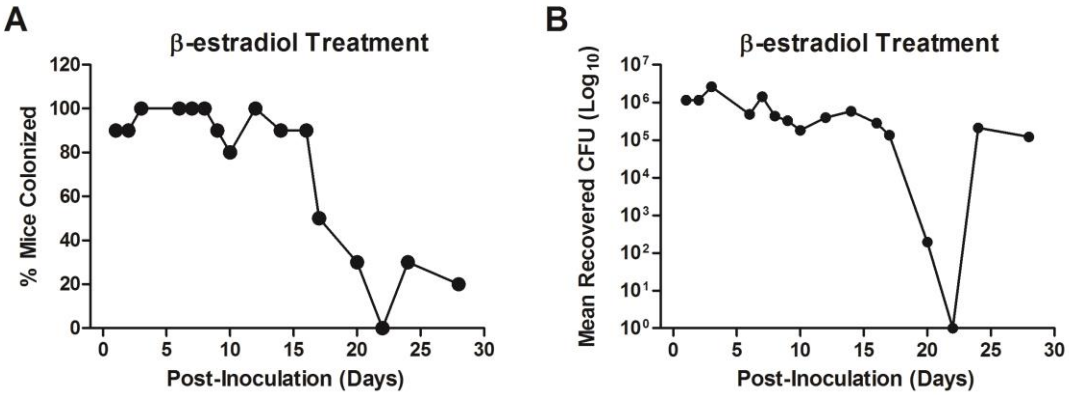

Figure B4. Continued treatment with β-estradiol promotes GBS vaginal persistence. Percent colonized (A) or mean recovered CFU (B) of mice inoculated with 1×10^7 CFU GBS and maintained on β-estradiol treatment as described in the above text.

GBS strain differences in vaginal persistence

The majority of mouse experiments in this dissertation project were performed using outbred CD1 mice. While conducting various experiments using different GBS strains, we observed that different strains varied in their ability to colonize the CD1 mouse ranging from several days to beyond a month. Strain NCTC 10/84 has the shortest duration, whereas strain CJB111 persists in the majority of mice for more than 4 weeks (Figure B5). Both of these strains are serotype V, so at this time, we have observed no correlation of serotype and ability to persist in the mouse vaginal tract. Strains A909 (serotype Ia) and COH1 (serotype III) showed intermediate persistence lengths (Figure B5). Other strains of GBS should also be tested to better characterize persistence phenotypes.

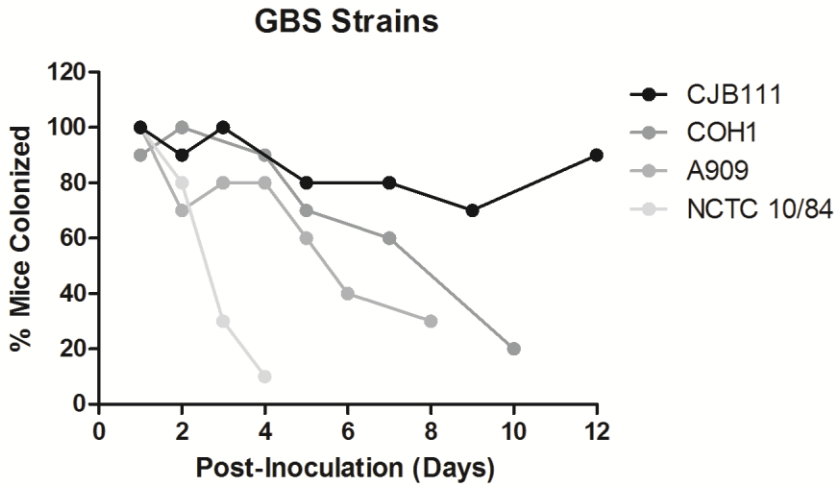

Figure B5. GBS strains differ in their ability to persist in the vaginal tract. Outbred CD1 mice were inoculated with 1×10^7 CFU of given GBS strains. Experiments with each strain were carried out as independent experiments.

Mouse strain differences in vaginal persistence

Although the majority of experiments in this dissertation were carried out in outbred CD1 mice, due to the use of knockout mice or other opportunities, we were also able to test GBS vaginal persistence in several inbred strains. We observed with GBS strain A909 that GBS persisted in the vaginal tract for approximately one week in outbred CD1 mice and inbred FVB mice (Figure B6). Alternatively, well-characterized inbred BALB/c and C57BL/6 mice were colonized for longer, up to a month or beyond (Figure B6). Others have also tested other GBS strains 874391 and NCTC 10/84 in C57BL/6 backgrounds and have observed longer persistence times as well[5,6]. These differences may be explained by a variety of factors including immune responses and normal vaginal flora. Although each mouse background comes with their own advantages and disadvantages, more work should be done in outbred strains that mimic the variability and robust immune responses of the human population.

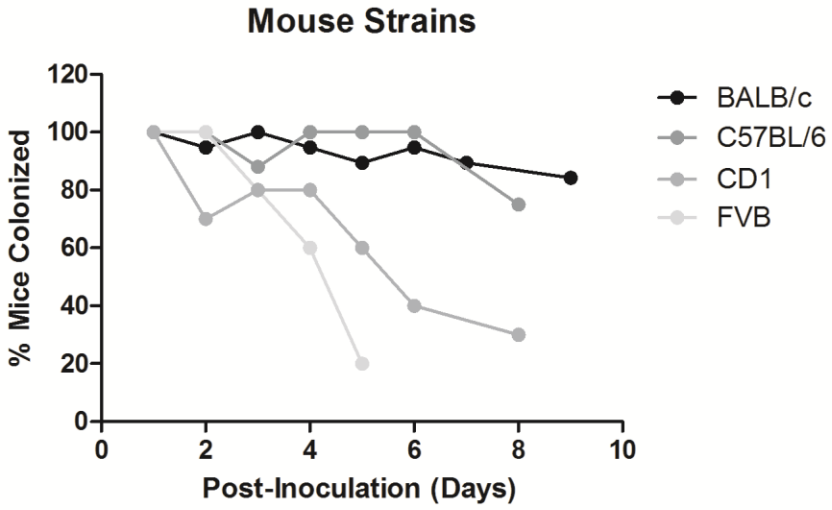

Figure B6. Mouse strains differ in their ability to be colonized with GBS in the vaginal tract. Outbred CD1 mice were inoculated with 1×10^7 CFU of GBS A909. Experiments with each mouse strain were carried out as independent experiments.

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