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

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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.

Keywords

Streptococcus agalactiae; GBS; vaginal colonization; innate immunity; IL-17

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],

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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.

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. 1C–E).

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.

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.

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).

Presence of IL-17 within the reproductive tract is associated with reduced 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.

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.

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°C.

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 possibly 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°C 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°C. 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 200× 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 $P<0.05$.

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References

1. Ohlsson A, Shah VS. Intrapartum antibiotics for known maternal Group B streptococcal colonization. Cochrane Database Syst Rev. 2014; 6:CD007467.10.1002/14651858.CD007467.pub4 [PubMed: 24915629]
2. Le Doare K, Kampmann B. Breast milk and Group B streptococcal infection: vector of transmission or vehicle for protection? Vaccine. 2014; 32:3128–3132.10.1016/j.vaccine.2014.04.020 [PubMed: 24736004]
3. Prevention, C. f. D. C. a. Active Bacterial Core Surveillance Report, Emerging Infections Program Network, Group B Streptococcus, 2012. 2013.

4. Stoll BJ, Schuchat A. Maternal carriage of group B streptococci in developing countries. Pediatr Infect Dis J. 1998; 17:499–503. [PubMed: 9655542]
5. Regan JA, Klebanoff MA, Nugent RP. The epidemiology of group B streptococcal colonization in pregnancy. Vaginal Infections and Prematurity Study Group. Obstet Gynecol. 1991; 77:604–610. [PubMed: 2002986]
6. Verani JR, McGee L, Schrag SJ. Prevention of perinatal group B streptococcal disease--revised guidelines from CDC, 2010. MMWR Recommendations and reports : Morbidity and mortality weekly report Recommendations and reports / Centers for Disease Control. 2010; 59:1–36. [PubMed: 21088663]
7. Phares CR, et al. Epidemiology of invasive group B streptococcal disease in the United States, 1999–2005. Jama. 2008; 299:2056–2065. [PubMed: 18460666]
8. Falagas ME, Rosmarakis ES, Avramopoulos I, Vakalis N. Streptococcus agalactiae infections in non-pregnant adults: single center experience of a growing clinical problem. Med Sci Monit. 2006; 12:CR447–451. [PubMed: 17072267]
9. Park SE, Jiang S, Wessels MR. CsrRS and environmental pH regulate group B streptococcus adherence to human epithelial cells and extracellular matrix. Infect Immun. 2012; 80:3975–3984. [PubMed: 22949550]
10. Patras KA, et al. Group B Streptococcus CovR regulation modulates host immune signalling pathways to promote vaginal colonization. Cell Microbiol. 2013; 15:1154–1167. [PubMed: 23298320]
11. Wang NY, et al. Group B streptococcal serine-rich repeat proteins promote interaction with fibrinogen and vaginal colonization. J Infect Dis. 2014; 210:982–991. [PubMed: 24620021]
12. Sheen TR, et al. Serine-rich repeat proteins and pili promote Streptococcus agalactiae colonization of the vaginal tract. J Bacteriol. 2011; 193:6834–6842. [PubMed: 21984789]
13. Banerjee A, et al. Bacterial Pili exploit integrin machinery to promote immune activation and efficient blood-brain barrier penetration. Nat Commun. 2011; 2:462.10.1038/ncomms1474 [PubMed: 21897373]
14. Schubert A, et al. A fibrinogen receptor from group B Streptococcus interacts with fibrinogen by repetitive units with novel ligand binding sites. Mol Microbiol. 2002; 46:557–569. [PubMed: 12406229]
15. Tamura GS, Rubens CE. Group B streptococci adhere to a variant of fibronectin attached to a solid phase. Mol Microbiol. 1995; 15:581–589. [PubMed: 7783628]
16. Franken C, et al. Horizontal gene transfer and host specificity of beta-haemolytic streptococci: the role of a putative composite transposon containing scpB and lmb. Mol Microbiol. 2001; 41:925–935. [PubMed: 11532154]
17. Zegels G, Van Raemdonck GA, Coen EP, Tjalma WA, Van Ostade XW. Comprehensive proteomic analysis of human cervical-vaginal fluid using colposcopy samples. Proteome Sci. 2009; 7:17.10.1186/1477-5956-7-17 [PubMed: 19374746]
18. Soares GC, et al. Metallopeptidases produced by group B Streptococcus: influence of proteolytic inhibitors on growth and on interaction with human cell lineages. Int J Mol Med. 2008; 22:119–125. [PubMed: 18575784]
19. Carey AJ, et al. Infection and cellular defense dynamics in a novel 17beta-estradiol murine model of chronic human group B streptococcus genital tract colonization reveal a role for hemolysin in persistence and neutrophil accumulation. J Immunol. 2014; 192:1718–1731. [PubMed: 24453257]
20. Randis TM, et al. Group B Streptococcus beta-hemolysin/cytolysin breaches maternal-fetal barriers to cause preterm birth and intrauterine fetal demise in vivo. J Infect Dis. 2014; 210:265–273. [PubMed: 24474814]
21. Faralla C, et al. Analysis of two-component systems in group B Streptococcus shows that RgfAC and the novel FspSR modulate virulence and bacterial fitness. mBio. 2014; 5:e00870–00814.10.1128/mBio.00870-14 [PubMed: 24846378]
22. Yow MD, et al. The natural history of group B streptococcal colonization in the pregnant woman and her offspring. I Colonization studies. Am J Obstet Gynecol. 1980; 137:34–38. [PubMed: 6989247]

23. Tettelin H, et al. Genome analysis of multiple pathogenic isolates of Streptococcus agalactiae: implications for the microbial “pan-genome”. Proc Natl Acad Sci U S A. 2005; 102:13950–13955. [PubMed: 16172379]
24. Bodaszewska-Lubas M, et al. Adherence of group B streptococci to human rectal and vaginal epithelial cell lines in relation to capsular polysaccharides as well as alpha-like protein genes - pilot study. Pol J Microbiol. 2013; 62:85–90. [PubMed: 23829083]
25. Mei J, et al. Cxcr2 and Cxcl5 regulate the IL-17/G-CSF axis and neutrophil homeostasis in mice. J Clin Invest. 2012; 122:974–986. [PubMed: 22326959]
26. Iwakura Y, Ishigame H. The IL-23/IL-17 axis in inflammation. J Clin Invest. 2006; 116:1218–1222. [PubMed: 16670765]
27. Pietrella D, et al. Th17 cells and IL-17 in protective immunity to vaginal candidiasis. PloS One. 2011; 6:e22770.10.1371/journal.pone.0022770 [PubMed: 21818387]
28. Feinen B, Jerse AE, Gaffen SL, Russell MW. Critical role of Th17 responses in a murine model of Neisseria gonorrhoeae genital infection. Mucosal Immunol. 2010; 3:312–321. [PubMed: 20107432]
29. Berti F, et al. Structure of the Type IX Group B Streptococcus Capsular Polysaccharide and Its Evolutionary Relationship with Types V and VII. J Biol Chem. 2014; 289:23437–23448. [PubMed: 24990951]
30. Baker CJ, Barrett FF. Group B streptococcal infections in infants. The importance of the various serotypes. JAMA. 1974; 230:1158–1160. [PubMed: 4608888]
31. Rinaudo CD, et al. Specific involvement of pilus type 2a in biofilm formation in group B Streptococcus. PloS One. 2010; 5:e9216.10.1371/journal.pone.0009216 [PubMed: 20169161]
32. Baker CJ, et al. Immune response of healthy women to 2 different group B streptococcal type V capsular polysaccharide-protein conjugate vaccines. J Infect Dis. 2004; 189:1103–1112. [PubMed: 14999615]
33. Nikolaitchouk N, Andersch B, Falsen E, Strombeck L, Mattsby-Baltzer I. The lower genital tract microbiota in relation to cytokine-, SLPI- and endotoxin levels: application of checkerboard DNA-DNA hybridization (CDH). APMIS. 2008; 116:263–277. [PubMed: 18397461]
34. Mitchell K, et al. Group B Streptococcus colonization and higher maternal IL-1beta concentrations are associated with early term births. J Matern Fetal Neonatal Med. 2013; 26:56–61. [PubMed: 22946471]
35. van de Veerdonk FL, et al. Th17 responses and host defense against microorganisms: an overview. BMB Rep. 2009; 42:776–787. [PubMed: 20044948]
36. Buonocore S, et al. Innate lymphoid cells drive interleukin-23-dependent innate intestinal pathology. Nature. 2010; 464:1371–1375.10.1038/nature08949 [PubMed: 20393462]
37. Vigne S, et al. IL-36R ligands are potent regulators of dendritic and T cells. Blood. 2011; 118:5813–5823.10.1182/blood-2011-05-356873 [PubMed: 21860022]
38. Fichorova RN, Anderson DJ. Differential expression of immunobiological mediators by immortalized human cervical and vaginal epithelial cells. Biol Reprod. 1999; 60:508–514. [PubMed: 9916021]
39. Mossop H, Linhares IM, Bongiovanni AM, Ledger WJ, Witkin SS. Influence of lactic acid on endogenous and viral RNA-induced immune mediator production by vaginal epithelial cells. Obstet Gynecol. 2011; 118:840–846. [PubMed: 21934447]
40. Fanibunda SE, Modi DN, Bandivdekar AH. HIV gp120 induced gene expression signatures in vaginal epithelial cells. Microbes Infect. 2013; 15:806–815. [PubMed: 23867815]
41. Ye P, et al. Requirement of interleukin 17 receptor signaling for lung CXC chemokine and granulocyte colony-stimulating factor expression, neutrophil recruitment, and host defense. J Exp Med. 2001; 194:519–527. [PubMed: 11514607]
42. Edwards LA, et al. Delineation of the innate and adaptive T-cell immune outcome in the human host in response to Campylobacter jejuni infection. PloS One. 2010; 5:e15398.10.1371/journal.pone.0015398 [PubMed: 21085698]
43. Ivanov II, et al. Specific microbiota direct the differentiation of IL-17-producing T-helper cells in the mucosa of the small intestine. Cell Host Microbe. 2008; 4:337–349. [PubMed: 18854238]

44. Kirjavainen PV, et al. Abnormal immunological profile and vaginal microbiota in women prone to urinary tract infections. Clin Vaccine Immunol. 2009; 16:29–36. [PubMed: 19020112]
45. Kubota T, Nojima M, Itoh S. Vaginal bacterial flora of pregnant women colonized with group B streptococcus. J Infect Chemother. 2002; 8:326–330. [PubMed: 12525892]
46. Ferretti S, Bonneau O, Dubois GR, Jones CE, Trifilieff A. IL-17, produced by lymphocytes and neutrophils, is necessary for lipopolysaccharide-induced airway neutrophilia: IL-15 as a possible trigger. J Immunol. 2003; 170:2106–2112. [PubMed: 12574382]
47. Zhang Z, Clarke TB, Weiser JN. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. J Clin Invest. 2009; 119:1899–1909. [PubMed: 19509469]
48. Lieberman JA, Moscicki AB, Sumerel JL, Ma Y, Scott ME. Determination of cytokine protein levels in cervical mucus samples from young women by a multiplex immunoassay method and assessment of correlates. Clin Vaccine Immunol. 2008; 15:49–54. [PubMed: 17978011]
49. Madoff LC, Michel JL, Kasper DL. A monoclonal antibody identifies a protective C-protein alpha-antigen epitope in group B streptococci. Infect Immun. 1991; 59:204–210. [PubMed: 1702759]
50. Wilson CB, Weaver WM. Comparative susceptibility of group B streptococci and Staphylococcus aureus to killing by oxygen metabolites. J Infect Dis. 1985; 152:323–329. [PubMed: 2993435]

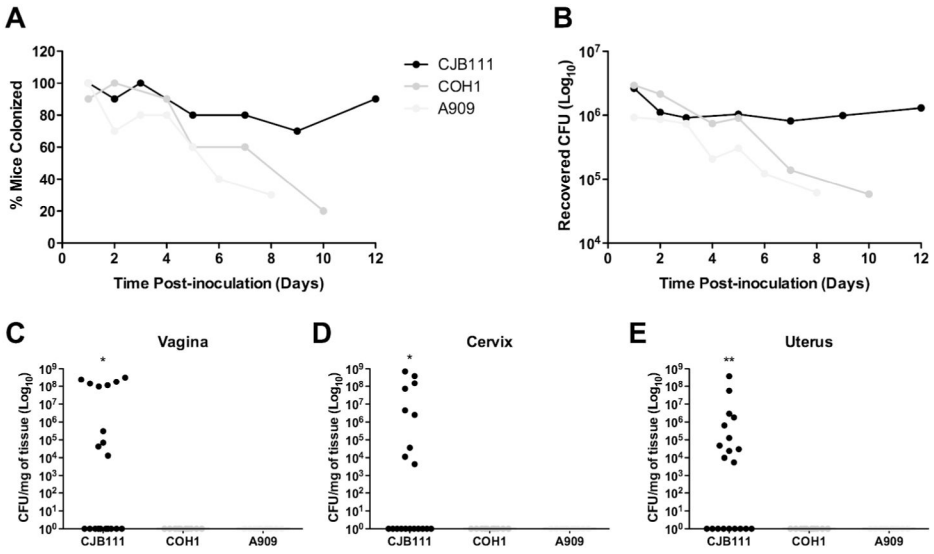

Figure 1. GBS strain persistence in the murine vaginal tract

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. *, $P<0.05$; **, $P<0.01$.

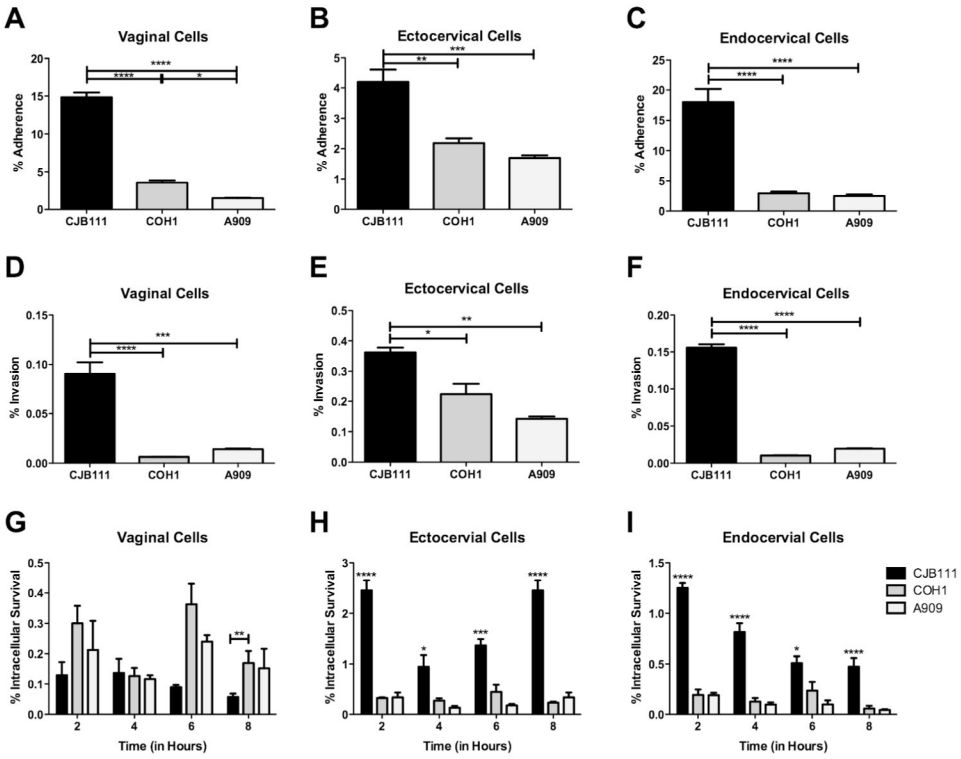

Figure 2. Interaction of GBS strains with human female reproductive epithelial cells

(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 MOI = 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.

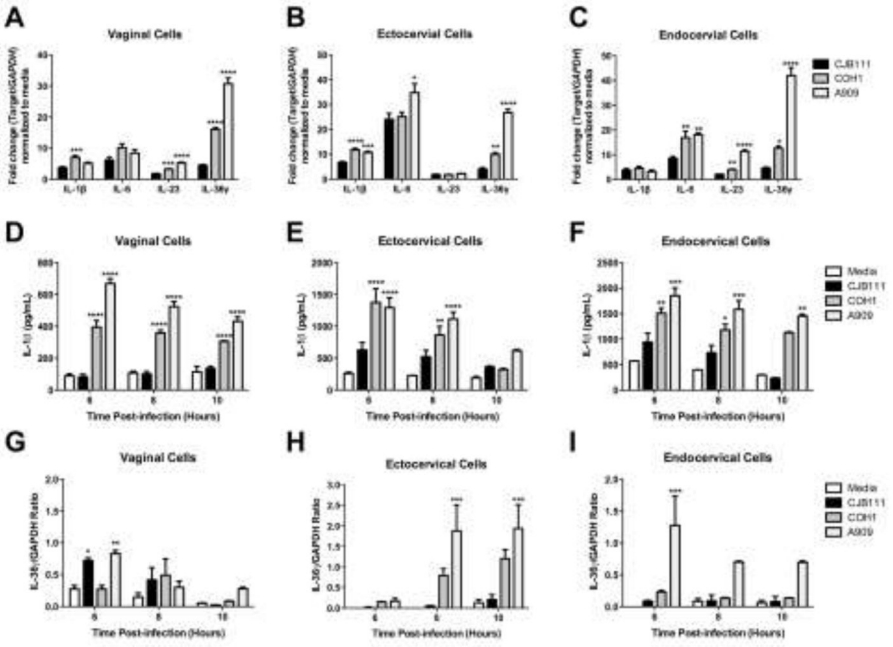

Figure 3. GBS cytokine induction in human female reproductive epithelial cells

(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.

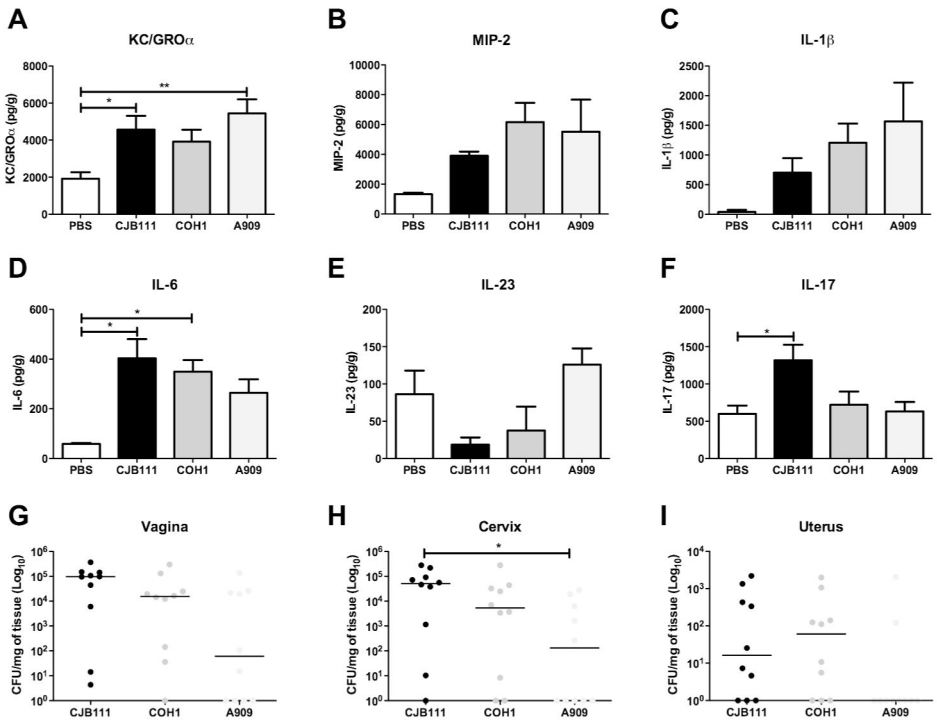

Figure 4. GBS colonization and cytokine production in the murine reproductive tract

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.

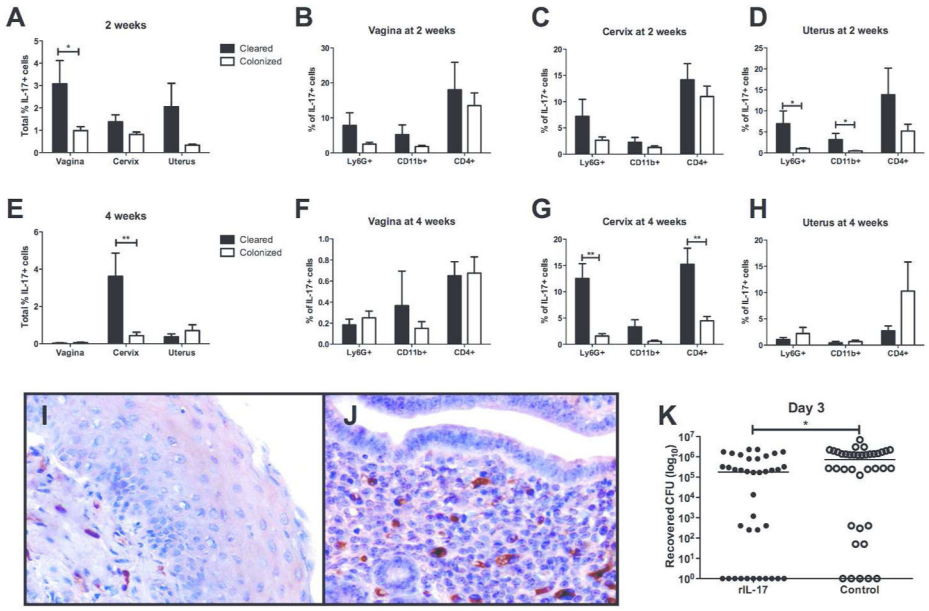

Figure 5. Presence of IL-17 within the reproductive tract is associated with reduced clearance of GBS CJB111

(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 = 200×. (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.