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

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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 vs. other GBS strains. We also demonstrate that the persistent strain more readily invades cervical cells compared with vaginal cells, suggesting that GBS may potentially use the cervix as a reservoir to establish long-term colonization. Furthermore, we have identified interleukin-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 ~2,000 live births per year in the United States alone.[3] Maternal vaginal colonization rates appear similar in developed and developing countries across all six inhabited continents, ranging from 8 to 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-2a.[10–12] These, and other GBS surface proteins, also facilitate adherence to extracellular matrix 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 extracellular matrix proteins,[18] which may aid in tissue invasion or niche establishment. Within the vaginal environment, GBS invokes innate immune responses

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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 three 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 nonpregnant mice. We further establish key host immune responses including a previously unidentified GBS stimulation of the interleukin-17 (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 also likely 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, whereas GBS strain, CJB111 (serotype V), persisted beyond several weeks in $>50\%$ of mice (**Figure 1a**). Furthermore, although these three 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 (**Figure 1b**). At 1 month after inoculation, CJB111 was readily isolated from the vagina, cervix, and uterus, whereas A909 and COH1 were not detected (**Figure 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 three 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 h, and nonadherent bacteria washed away before 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 (**Figure 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.[23] Additionally, CJB111 exhibited increased ability to invade certain cell lines compared with COH1 and A909 (**Figure 2d–f**). To access invasive capability, we recovered and quantified viable intracellular bacteria from cell lysates after a 2-h infection and a 2-h antibiotic treatment to kill all extracellular bacteria. CJB111 showed significantly increased invasion over COH1 and A909 in both vaginal and cervical cell lines (**Figure 2d–f**). Similarly, for intracellular survival, cell monolayers were infected for 2 h, 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 with COH1 and A909, but no striking differences were observed in vaginal cells (**Figure 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* (**Figure 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 cervicovaginal 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 with other GBS strains. Our previous studies with human vaginal epithelial cells using microarray, reverse transcription-PCR, and protein analysis revealed increased transcription and production of multiple proinflammatory 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 helper type 17 (T_H17) cell response, which contributes to neutrophil homeostasis and sustained inflammatory signaling.[24,25]

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 upregulated in all three cell lines compared with media controls (**Figure 3a–c**). Interestingly, COH1 and A909 invoked more significant upregulation of IL-36γ in all

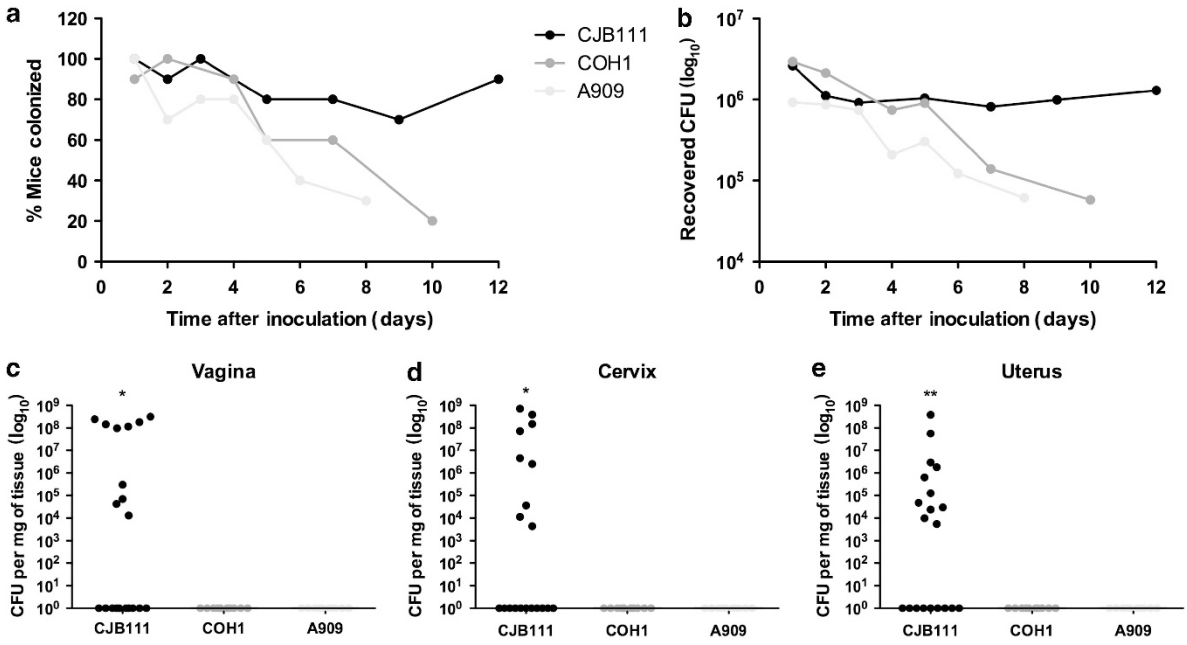

Figure 1 *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) strain persistence in the murine vaginal tract. CD1 mice ($n = 10$ per group) were colonized with 1×10^7 CFU (colony-forming unit) of GBS clinical isolates CJB111, COH1, or A909 in the vaginal lumen. GBS persistence was determined by 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 after inoculation with GBS strains. Bacterial load was quantified by plating serially diluted tissue homogenates. *In vivo* experiments were conducted independently at least two times and data from one representative experiment is shown. Data were analyzed using Kruskal–Wallis with Dunn's multiple comparisons post-test. $^*P<0.05$; $^{**}P<0.01$.

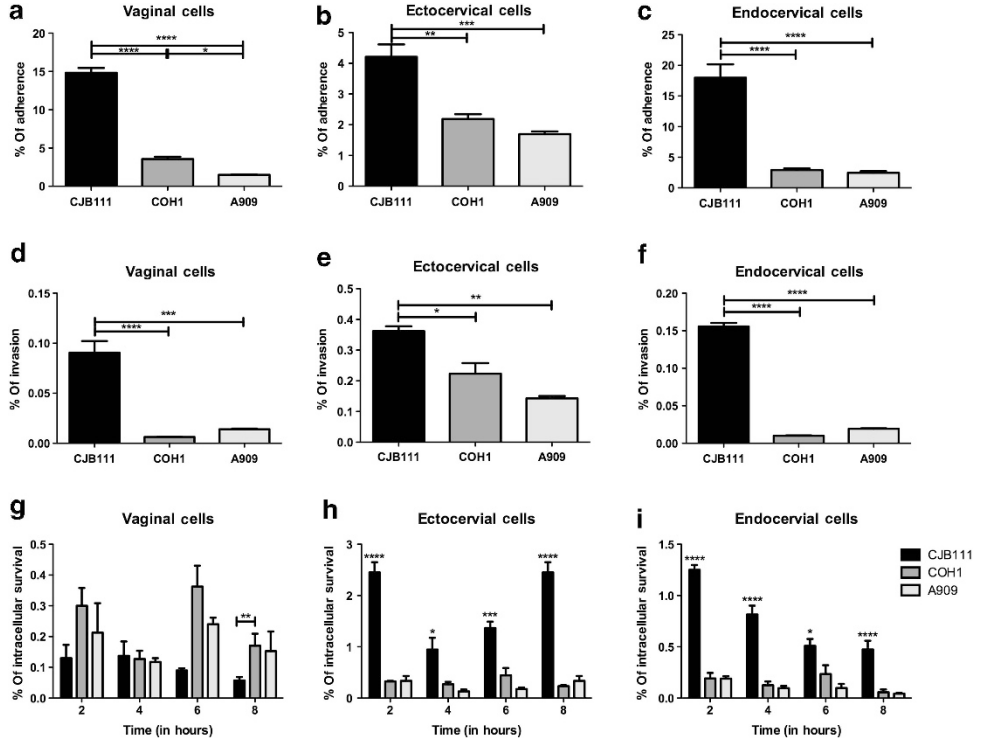

Figure 2 Interaction of *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) strains with human female reproductive epithelial cells. (**a**–**c**) Adherence, (**d**–**f**) invasion, or (**g**–**i**) intracellular survival of GBS CJB111, COH1, or A909 with indicated epithelial cells, multiplicity of infection (MOI) = 10 for **a**–**f** and MOI = 1 for **g**–**i**. For **h**–**i**, significance given is CJB111 in comparison with both COH1 and A909. Experiments were repeated at least two times with four replicates and data from a representative experiment is shown. Data were analyzed by one-way analysis of variance (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**. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

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 with 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 h after infection compared with media controls; however, CJB111 did not illicit this same response (**Figure 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 h, and in endocervical cells treated with A909 at 6 h (**Figure 3h,i**). Although we did note increased IL-36γ production in vaginal cells in response to CJB111 at 6 h (**Figure 3g**), we also observed lower levels of intracellular CJB111 in the vaginal cell assays (**Figure 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 nonpregnant 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 the presence of interferon-γ, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p70, keratinocyte-derived chemokine/growth-related oncogene alpha (KC/GROα), and tumor necrosis factor-α in the vagina, cervix, and uterus 2 days after inoculation with GBS. We observed stimulation of IL-1β, IL-6, and KC, whereas the other cytokines on the panel were

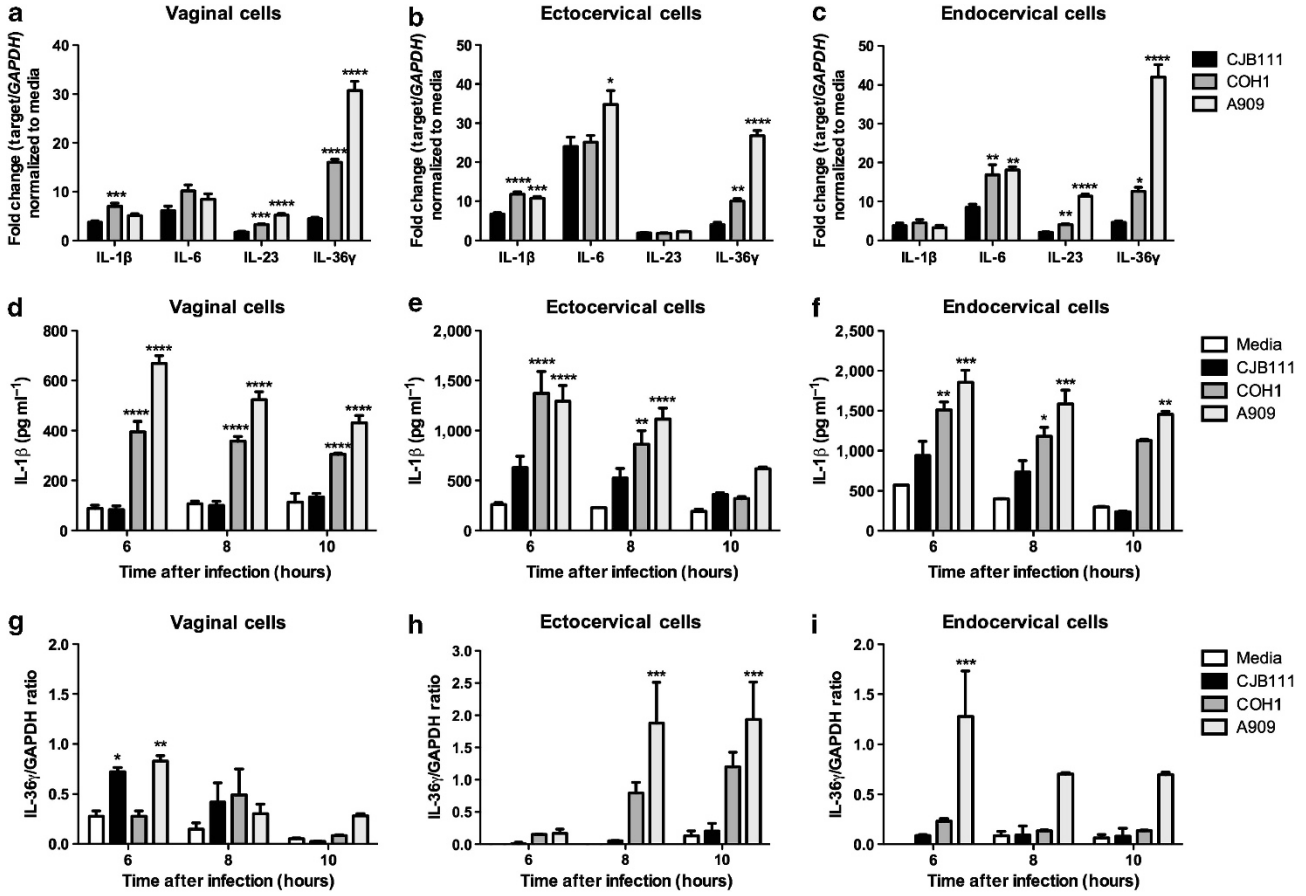

Figure 3 *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) cytokine induction in human female reproductive epithelial cells. (**a**–**c**) Transcript abundance of interleukin-1β (IL-1β), IL-6, IL-23, and IL-36γ in human epithelial cells was determined using quantitative reverse transcription-PCR following infection with CJB111, COH1, or A909 (multiplicity of infection (MOI) = 10). Fold change was calculated using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and then normalized to media controls as described in Methods section. 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 Methods section 4 h after infection with CJB111, COH1, or A909 at an MOI of 10. Statistical values are in reference to media controls. Data are one representative experiment of at least two independent experiments performed in four replicates at minimum. Data were analyzed by one-way analysis of variance (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**). $^{*}P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; $^{****}P<0.0001$.

detected at much lower levels (data not shown). To confirm this preliminary screen, mice were colonized with CJB111, COH1, and A909, and 2 days after 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 macrophage inflammatory protein-2 (MIP-2), but no increased production of IL-23 (**Figure 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 colony-forming units (CFUs) were recovered than the other strains (**Figure 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 4 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 with phosphate-buffered saline (PBS) controls, but not in mice colonized with COH1 or A909 (**Figure 4f**). Additionally, we analyzed IL-17A levels at days 2, 5, and 10 after 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*[26] and *Neisseria gonorrhoeae*[27] 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.

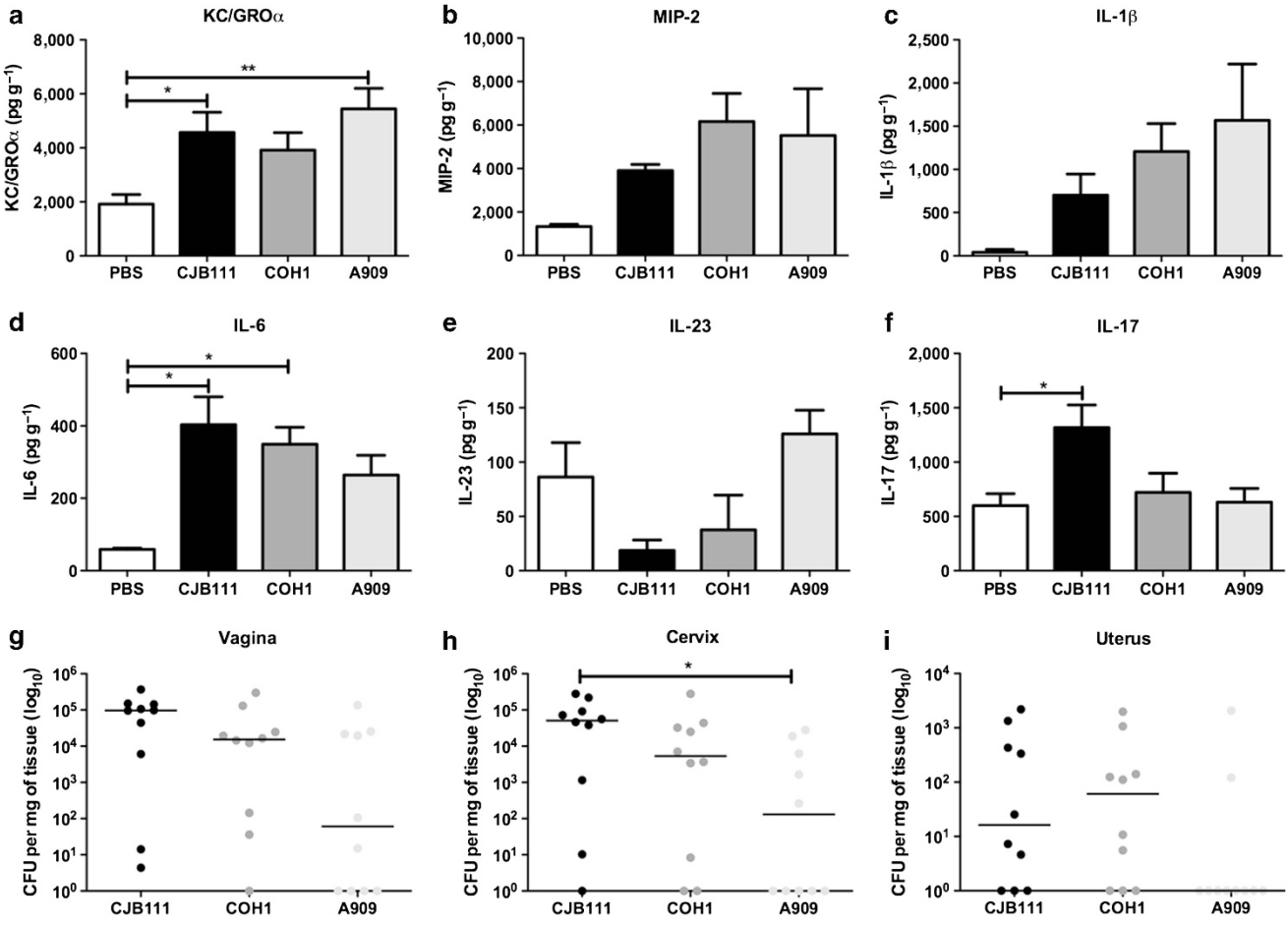

Figure 4 *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) colonization and cytokine production in the murine reproductive tract. (**a**) Keratinocyte-derived chemokine/growth-related oncogene alpha (KC/GROα), (**b**) macrophage inflammatory protein-2 (MIP-2), (**c**) interleukin-1β (IL-1β), (**d**) IL-6, and (**e**) IL-23 levels in vaginal homogenates from mice 2 days after inoculation, or (**f**) IL-17A levels at 4 weeks after inoculation, were quantified by enzyme-linked immunosorbent assay (ELISA). Data are 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 after inoculation ($n = 10$ per group). Lines represent median values of each group. Data were analyzed by Kruskal–Wallis with Dunn's multiple comparisons post-test. $^*P < 0.05$; $^{**}P < 0.01$.

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 (**Figure 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 and 4 weeks after 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 killing, and mice that had cleared CJB111 to below our limit of detection. By 2 weeks after 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 ($P = 0.0979$) (**Figure 5a**). The average basal levels of total IL-17A+ cells in uninfected mice were 0.8% 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 (**Figure 5b–d**). At 4 weeks after 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 vs. those that remained colonized (**Figure 5g**). This effect was not seen in the vagina or uterus, both of which contained much lower quantities of total IL-17A+ cells (**Figure 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 h, and then began daily vaginal administration of recombinant IL-17 (rIL-17) as described in the Methods section. We found that after 2 days of rIL-17 treatment, treated mice had significantly lower bacterial load than nontreated controls (**Figure 5k**). Taken together, these

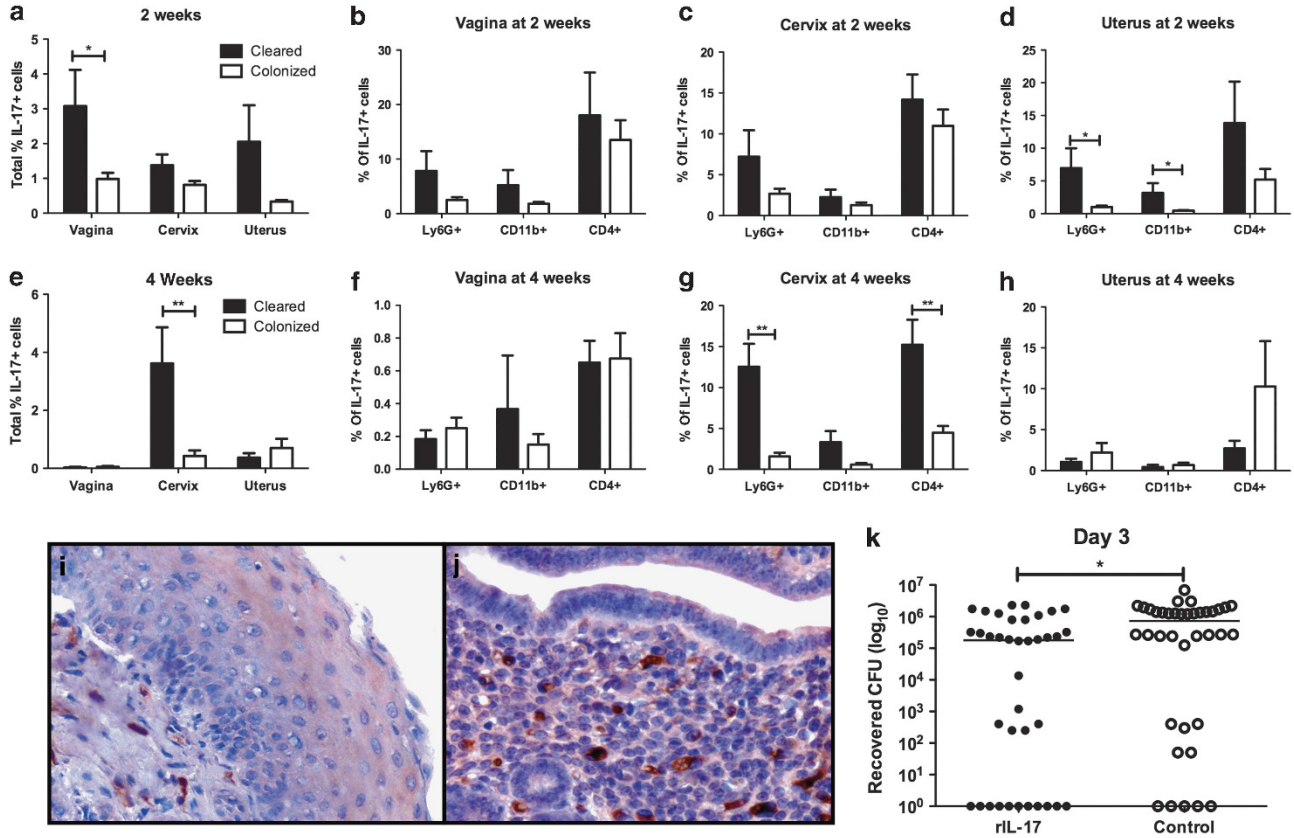

Figure 5 Presence of interleukin-17 (IL-17) within the reproductive tract is associated with reduced clearance of *Streptococcus agalactiae* (Group B *Streptococcus*, GBS) CJB111. (**a** and **e**) Total IL-17A+ cells present in reproductive tract tissues collected from mice ($n = 4$–6 per group) at indicated time points after inoculation with CJB111 were quantified by flow cytometry as described in Methods section. Cleared mice (black bars) were separated from colonized mice (white bars) if GBS counts were below the limit of detection at the time of killing (50 CFU per tissue). (**b**–**d** and **f**–**h**) Surface markers Ly6G, CD11b, and CD4 present on populations identified within total IL-17A+ cells from **a** and **e**. Representative data from one of two independent experiments is shown. Immunohistochemistry of the (**i**) vagina and (**j**) uterus of GBS-colonized mice with IL-17+ cells visualized with diaminobenzidine (brown) and counterstained with hematoxylin (blue). Original magnification, ×200. (**k**) Mice were colonized with CJB111, and 1 day later, received daily treatment of recombinant IL-17 (rIL-17) within the vaginal lumen. Data shown are from 3 days after inoculation with CJB111, and is the result of three independent experiments combined (total $n = 38$ per group). Lines represent median values of each group. Data were analyzed by Mann–Whitney. $^*P < 0.05$; $^{**}P < 0.01$.

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 the chemical structure (Ia, Ib, II, III, IV, V, VI, VII, VIII, and IX).[28] Epidemiologic studies have revealed that serotype III strains are significantly more prevalent in neonatal meningitis,[29] 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 three of these strains have been fully or partially sequenced,[30] the molecular machinery determining the 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 dialog with the host immune system. CJB111 possesses a set of 20 unique genes compared with other fully sequenced GBS strains,[30] 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 with 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 nonpregnant women,[33] whereas 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 hyperhemolytic GBS,[10] and another group has noted increased reproductive tract levels of tumor necrosis factor-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 used 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 carried out with this exact vaginal cell line was also unable to detect IL-23 production either spontaneously or with lactic acid stimulation;[39] thus, 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 three 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 that germ-free mice possess significantly 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 vascular endothelial growth factor.[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.[26,46] Additionally, the timing of IL-17A production at the mucosal surface varies across pathogenic agents and host tissues, with responses occurring within 2–3 days or up to 6 weeks in the lung,[46,47] or ~2 weeks in the vaginal tract.[26] 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 (**Figure 5**). Interestingly, enzyme-linked immunosorbent assay (ELISA) analysis identified increased IL-17A in the vaginal tract at 4 weeks (**Figure 4**), even though flow cytometry displayed very low populations of IL-17A+ cells at this time point (**Figure 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 after inoculation, although this difference was not significant.[19] Even though this work was carried out 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 with 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 with 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 (**Figure 1c**), and thus we do not believe that the diluent impacted these results. This information combined with further studies may be useful for developing pharmacologic 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, American Type Culture Collection, Manassas, VA) were grown aerobically in Todd–Hewitt broth (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 (Life Technologies, Carlsbad, CA) with 0.5 ng ml^{-1} human recombinant epidermal growth factor and 0.05 mg ml^{-1} 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 before 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 h of incubation, cells were washed six times with PBS. Cells were lifted from plates by adding trypsin-EDTA and then lysed with 0.025% Triton X-100. Lysates were serially diluted and plated on Todd–Hewitt broth agar plates to quantify adherent CFUs. 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 h, monolayers washed three times with PBS, treated with media containing antibiotics, and incubated for an additional 2 h for invasion assays, or as given for survival assays. Cells were washed three times with PBS, lysed as described above, and viable intracellular GBS determined by serial dilution plating as quantified above.

Reverse transcription (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 before 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 h. Cells were lysed, then total RNA was extracted, and reverse transcription (Macherey-Nagel, Düren, Germany) and qPCR (quantitative PCR) were performed (Quanta Biosciences, Gaithersburg, MD). Human primer sequences used are as follows: GAPDH (glyceraldehyde 3-phosphate dehydrogenase)—forward sequence, 5′-GAAGGTGAAGGTCGGAGTGAA-3′ and reverse sequence, 5-TCCTGGAAGATGGTGATGGGA-3′, IL-1β—forward sequence 5′-GCCCTAAACAGATGAAGTGCTC-3′ and reverse sequence, 5′-GAACCAGCATCTTCCTCAG-3′; IL-6—forward

sequence, 5′-GGAGACTTGCCTGGTGAAAA-3′ and reverse sequence, 5′-CAGGGGTGGTTATTGCATCT-3′; IL-23 (α-subunit)—forward sequence, 5′-GCTTCAAAATCCTTCGCAG-3′ and reverse sequence, 5′-TATCTGAGTGCCATCCTTGAG-3′; and IL-36γ—forward sequence, 5′-GAAACCCTTCCTTTTCTACCGTG-3′ and reverse sequence, 5′-GCTGGTCTCTCTTGGAGGAG-3′.

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 h, washed, and cell lysates were analyzed for cytokine production using human IL-1β ELISA Kit (R&D Systems, Minneapolis, MN) according to the 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. The 8- to 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 10 µl 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 killed 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 1.0 mm 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) as per the 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 the 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 2 h at 37 °C in RPMI-1640 containing 10% fetal bovine serum, 0.4 mg ml^{-1} collagenase, and 1:1,000 brefeldin A (BD Biosciences, San Jose, CA). During this incubation, tissues were vigorously pipetted through 1,000 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 1:1,000 brefeldin A and incubated for an additional 4 h 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 (BD Biosciences) per manufacturer's instructions, and stained with IL-17-AlexaFluor647 (BD Biosciences). Before 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 the percent of fluorescent staining and staining brightness using Accuri analysis software (BD Biosciences).

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, Cambridge, MA) at 5 µg ml^{-1} overnight, then incubated with goat anti-rabbit IgG-HRP, and developed with diaminobenzidine chromogen (Sigma-Aldrich). Tissues were counter-stained with hematoxylin and visualized on a Zeiss upright microscope (Zeiss, Thornwood, NY) with attached Axiocam Icc3 camera (Zeiss) at $\times 200$ 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 analysis of variance (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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DISCLOSURE

The authors declare no conflict of interest.

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