Infection and Cellular Defense Dynamics in a Novel 17β-Estradiol Murine Model of Chronic Human Group B Streptococcus Genital Tract Colonization Reveal a Role for Hemolysin in Persistence and Neutrophil Accumulation

Alison J. Carey, Chee Keong Tan, Shaper Mirza, Helen Irving-Rodgers, Richard I. Webb, Alfred Lam and Glen C. Ulett

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Genital tract carriage of group B streptococcus (GBS) is prevalent among adult women; however, the dynamics of chronic GBS genital tract carriage, including how GBS persists in this immunologically active host niche long term, are not well defined. To our knowledge, in this study, we report the first animal model of chronic GBS genital tract colonization using female mice synchronized into estrus by delivery of 17β-estradiol prior to intravaginal challenge with wild-type GBS 874391. Cervicovaginal swabs, which were used to measure bacterial persistence, showed that GBS colonized the vaginal mucosa of mice at high numbers (10^6–10^7 CFU/swab) for at least 90 d. Cellular and histological analyses showed that chronic GBS colonization of the murine genital tract caused significant lymphocyte and PMN cell infiltrates, which were localized to the vaginal mucosal surface. Long-term colonization was independent of regular hormone cycling. Immunological analyses of 23 soluble proteins related to chemotaxis and inflammation showed that the host response to GBS in the genital tract comprised markers of innate immune activation including cytokines such as GM-CSF and TNF-α. A nonhemolytic isogenic mutant of GBS 874391, Δcycle9, was impaired for colonization and was associated with amplified local PMN responses. Induction of DNA neutrophil extracellular traps, which was observed in GBS-infected human PMNs in vitro in a hemolysin-dependent manner, appeared to be part of this response. Overall, this study defines key infection dynamics in a novel murine model of chronic GBS genital tract colonization and establishes previously unknown cellular and soluble defense responses to GBS in the female genital tract. The Journal of Immunology, 2014, 192: 1718–1731.

**Infection and Cellular Defense Dynamics in a Novel 17β-Estradiol Murine Model of Chronic Human Group B Streptococcus Genital Tract Colonization Reveal a Role for Hemolysin in Persistence and Neutrophil Accumulation**

Alison J. Carey, * Chee Keong Tan, * Shaper Mirza, † Helen Irving-Rodgers, ‡, ¹ Richard I. Webb, § Alfred Lam, § and Glen C. Ulett*
hormone cycling confirmed that GBS has robust fitness for chronic surveillance systems. Unexpectedly, inflammatory activity triggered a unique host–pathogen interaction for GBS compared with other organism. We examined bacterial colonization dynamics and show valuable to gain insight into the mechanisms of microbe persistence for up to 77 d (32). Steroid hormones such as estradiol are typically bacteria disseminate to the upper reproductive tract and persist there limiting but is associated with reproductive tract pathology and our understanding of this organism’s fitness for this unique host genital tract and will be essential for studies aimed at improving in this study defines new factors affecting GBS survival in the colonization in individual carriers or the dynamics of GBS survival in this niche. Mothers with high genital tract GBS colonization loads generally have low levels of protective Abs because of the poor immunogenicity of the GBS capsule (25). The contribution of maternal Abs to the protection of newborns against GBS disease is discussed elsewhere in relation to pathogenesis (26). GBS β-hemolysin contributes to pathogenesis (27, 28), but the role of this virulence factor in the female genital tract is essentially unknown. Moreover, the cellular and immunological basis of long-term genital tract carriage of GBS, including how the bacteria persist in this immunologically active host niche (29), remains largely undefined. A constraint to this understanding is the lack of a model of chronic human GBS genital tract colonization. Murine models of genital tract infection have been reported for other pathogens such as Neisseria gonorrhoea, for example, in which the bacteria’s ability to avoid host responses and persist for 10 d can lead to ascending infection and pelvic inflammatory disease (30). In Chlamydia infection, disease is self-limiting but is associated with reproductive tract pathology and infertility (31). In a model of Mycoplasma genitalium infection, the bacteria disseminate to the upper reproductive tract and persist there for up to 77 d (32). Steroid hormones such as estradiol are typically used in such murine models to synchronize the estrous cycle into a state of estrus for consistency of infection. Such models are invaluable to gain insight into the mechanisms of microbe persistence and local immune defenses in the female genital tract, as reviewed elsewhere (33, 34).

In this study, we report the development and characterization of the first murine model of chronic human GBS genital tract colonization. We examined bacterial colonization dynamics and show a unique host–pathogen interaction for GBS compared with other bacteria so far examined in the female genital tract in other similar models. We show that, for GBS, this interaction involves a distinct cellular infiltrate comprising neutrophils and lymphocytes at the vaginal mucosal surface as well as various soluble markers of inflammation that reflect activation of genital tract immune surveillance systems. Unexpectedly, inflammatory activity triggered by GBS was unable to clear the bacteria. Analysis of the effects of hormone cycling confirmed that GBS has robust fitness for chronic persistence in the female genital tract regardless of estrous phase. Finally, high-level persistence of GBS in the genital tract relied on the organisms’ β-hemolysin, which impacted local neutrophil responses, and activated DNA extracellular trap release in human PMNs in vitro leading to host cell destruction. The model reported in this study defines new factors affecting GBS survival in the genital tract and will be essential for studies aimed at improving our understanding of this organism’s fitness for this unique niche.

Materials and Methods

Mice and steroid hormone treatment

Female C57BL/6 mice (8–10 wk) were purchased from the Animal Resources Centre (WA, Australia) and received food and water ad libitum. All procedures were approved by and conducted within the guidelines of the Griffith University Animal Research Ethics Committee (approval: MSC/14/08/AEC). Mice received 0.1 mg 17β-estradiol (Sigma–Aldrich, Castle Hill, NSW, Australia) in castor oil s.c. 1 d prior to infection to synchronize mice into a state of estrus (35) for consistency of infection, unless otherwise indicated. For some experiments, subsequent injections of 17β-estradiol were administered weekly to maintain mice in a consistent stage of estrus. Individual mice in all groups were staged using vaginal swab smears (collection described below) for cytological assessment of the genital tract cellular conditions prior to challenge. Staging of the hormone cycle in mice was also conducted throughout the infection assays to monitor the genital tract conditions during the course of colonization.

Bacteria

Wild-type (WT) GBS 874391, a commonly used virulent serotype III strain (36), was grown on Todd–Hewitt agar and in Todd–Hewitt broth at 37°C. For infections, GBS was grown shaking overnight in Todd–Hewitt broth (200 rpm), centrifuged (8000 × g, 10 min), washed three times in PBS, and resuspended at ∼107 CFU/ml in sterile PBS. Bacterial challenges were performed using an AxioImager.M2 microscope (Carl Zeiss MicroImaging, Jena, Germany), fitted with Plan-Apochromat × 63/1.40 and × 200/0.8 magnifications, and AxiosCam MRm Rev.3 and MRC 5 cameras. Other groups of

Vaginal vault infection and measurement of GBS load

One hour prior to vaginal infection, GBS were cultured in 40 ml 0.2% Triton X-100 in 0.9% saline, followed by 40 ml 0.9% saline. Mice were then infected with 107 CFU GBS in 10 μl PBS using a micropipette; sham-infected mice received 10 μl PBS only. Cervicovaginal swabs (Copan, Murrieta, CA) were collected every 3 d for 90 d to monitor bacterial load and the dynamics of GBS colonization in the lower genital tract. Each experiment was terminated at 90 d. For collection of samples, swabs were inserted into the vaginal vault, rotated 10 times to collect shedding cells and bacteria, and placed into tubes with 300 μl PBS. Tubes were vortexed (30 s) to remove bacteria from swabs, and suspensions were serially diluted for colony counts. The following media were used for total and differential counts: tryptic soy agar (TSA) 5% horse blood (Oxoid, Adelaide, SA, Australia), Columbia agar 5% horse blood containing 15 μg/ml nalidixic acid and 10 μg/ml colistin (CoNAC) (Sigma–Aldrich) (38), and Granada agar (BioMérieux, Marcy l’Étoile, France), which is highly selective for GBS (39–41). All media were used for each sample to determine total bacterial load (TSA 5% horse blood), Gram-positive load (CoNAC), and specific GBS load (Granada) in the genital tract of each mouse. Groups of 12 mice were used for each treatment, and data are expressed as mean CFU/swab ± SEM. Individual experiments were repeated at least three times.

Colony blots for GBS

To confirm the identity of GBS recovered on selective media, individual colonies grown on Granada agar were patched onto TSA 5% horse blood and analyzed by colony blotting using a GBS-specific Ab. Prewet nitrocellulose was used to blot bacteria from the agar surface (5 min), which were fixed over steam for 5 min, and then placed in 1% Triton X-100/PBS for 20 min. Membranes were washed with PBS for 20 min and blocked in 2% BSA for 1 h. Membranes were incubated for 1 h in primary Ab (1/1000 dilution), specific for GBS surface binding protein 1 (42), washed, and incubated for 1 h in secondary Ab (goat anti-rat IgG, alkaline phosphatase conjugated; Santa Cruz Biotechnology, Santa Cruz, CA). Membranes were washed and developed with NBT chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt (Sigma–Aldrich). A positive blot reaction from a colony cultured from Granada agar and TSA 5% horse blood was considered confirmatory for GBS.

Cellular assessment: vaginal smears and histopathology

Cervicovaginal swabs, which were collected at 3-d intervals, were used to prepare smears for analysis of local cellular shedding and inflammatory infiltrate in the vaginal mucosal epithelium. Smears of glass slides were air-dried and fixed in methanol (30 s) and then stained with Diff-Quick (Lab Aids, Narrabeen, NSW, Australia), according to the manufacturer’s instructions. Slides were scanned using a × 40 magnification on an Aperio Scanscope (Vista, CA), and the images were used to enumerate lymphocytes, PMNs, and monocytes. Smears also were used to stage individual mice according to the presence of keratinized epithelial cells and other cell types, as described previously (35, 43). Enumeration of inflammatory cells was performed only for those mice identified to be in estrus, so as to enable comparisons between animals in different groups. The mice were euthanized at 90 d, and the reproductive tracts were collected, fixed in 4% buffered formalin, and prepared for histological assessment. Tissues were processed according to standard techniques for H&E, Giemsa, and Gram staining. Additional mice were sacrificed at days 3, 15, and 30 postinfection. Bright-field and fluorescence imaging (below) were performed using an AxioImager.M2 microscope (Carl Zeiss MicroImaging, Jena, Germany), fitted with Plan-Apochromat × 63/1.40 and × 200/0.8 magnifications, and AxiosCam MRm Rev.3 and MRC 5 cameras. Other groups of
mice were used for flow cytometry analysis of cellular infiltrates in the genital tract tissue as below.

Flow cytometry

17β-Estradiol–primed mice were infected with either WT or Δcyl63 GBS or sham-infected (PBS), and tissue samples were collected at days 15 and 30 postinfection. The reproductive tracts were removed and dissected to separate cervix/vaginal tissue and uterine tissue. Samples were cut into small (~1 × 1 mm) fragments and digested in RPMI 1640 medium supplemented with 20 mM HEPES (Invitrogen, North Ryde, NSW, Australia), 10% FBS (Moregate Biotech, Bulimba, QLD, Australia), 2 mg/ml collagenase A (Roche, Castle Hill, NSW, Australia), and 0.057 KU/ml DNase I (Sigma-Aldrich). Digestion was performed in a volume of 2 ml in 15 ml Eppendorf tubes incubated at 37°C, shaking (750 rpm) for 2 h (Eppendorf Thermostat). The samples were repeatedly passed through a 19-gauge needle attached to a 1-ml syringe to enhance digestion at 45 min into the incubation. Single-cell suspensions were obtained by passing the digested fragments through a 45-μm cell strainer (BD Biosciences, Franklin Lakes, New Jersey) into a well of a 6-well plate. Samples were collected and centrifuged at 500 × g for 5 min. Cells were washed with wash buffer (PBS containing 1% FBS) and centrifuged again. Cells were then blocked with wash buffer containing 2% BSA for 1 h at room temperature, and FcRs were blocked using 1% mouse serum (collected from healthy mice) in wash buffer for 1 h at room temperature. Cells were centrifuged and resuspended in 200 μl wash buffer; cells were counted and resuspended in flow cytometry tubes at 106 cells/tube. Cells were stained with a primary Ab for neutrophils (rat anti-mouse Ly-6G [Gr-1]; eBioscience, San Diego, CA) for 30 min, followed by secondary Ab (anti-rat IgG-FITC conjugated; eBioscience), and anti-mouse F4/80 (APC-conjugated; eBioscience) for 30 min in the dark. Cells were washed (three times), and the final resuspension was 4% PFA. Cells were analyzed using a BD Biosciences LSRII Fortessa, and data were analyzed using the FACSDiva version 6 software package.

Electron microscopy

Reproductive tracts were fixed in 4% paraformaldehyde in PBS and stored at 4°C. For immunolabeling, the tissue was divided into two and labeling was performed in a Pelco Biowave Microwave Oven (Ted Pella, Redding, CA) at 150 W with the samples sitting on a ColdSpot recirculating water system, which was set to 22°C. After blocking (20 mM glycine, 0.2% fish skin gelatin, and 0.2% BSA), samples were incubated with antimouse GBS Ab (Acris, San Diego, CA), dilution 1:50 in blocking buffer for 6 min, and then incubated in Protein A/gold (10 nm) (University Medical Center, Utrecht, the Netherlands) diluted 1:60 in blocking buffer for 6 min, then washed again. Samples were then dehydrated through a graded ethanol series and dried in a Tousimis Samdri 815a critical point dryer. After carbon coating, they were observed in a JEOL 7001F scanning electron microscope operated at 10 kV. Backscatter and secondary electron images were obtained. For morphological studies, samples were fixed in 2.5% glutaraldehyde and postfixed in 1% osmium tetroxide before being dehydrated and dried as above. These samples were sputter coated with platinum and observed using a JEOL 7001F SEM operated at 10 kV.

Analysis of GBS-induced inflammation in the genital tract

On the basis of our initial findings of a cellular inflammatory infiltrate triggered by GBS, we measured the levels of 23 cytokines and other soluble factors in the genital tract of mice following infection. This panel included several markers related to chemotaxis and function of PMNs and lymphocytes, which were shown to make up the inflammatory infiltrate at the vaginal mucosal surface according to swab-smear and histopathology analyses. For measurement of these soluble factors, the entire genital tracts were collected and homogenized in PBS with 150 μl protease inhibitor (Roche, Castle Hill, NSW, Australia). The genital tract homogenates were clarified (8000 × g, 10 min), and the supernatants were stored at −80°C until assay. Fifty-microliter samples were analyzed using biological replicates of n = 12/group in 23-target multiplex protein assays (Bio-Rad, Gladesville, NSW, Australia). The results are presented as means ± SEM pg/ml for whole tissue for each protein.

In vitro GBS–PMN interaction assays

A PMN infiltrate that was detected at the vaginal mucosal surface in the genital tracts of GBS-colonized mice prompted us to investigate GBS–PMN interactions further. For this, we performed in vitro assays to determine whether PMNs respond to GBS by neutrophil extracellular trap (NET) formation as a form of defense, as occurs for other streptococci (44). In vitro experiments were performed using primary human PMNs isolated by density gradient centrifugation of whole blood collected in Vacutainer citrate tubes (BD Biosciences) using Polymorphprep (Axis-Shield, Rodelloka, Norway), according to the manufacturer’s instructions. PMNs were washed with ice-cold PBS supplemented with 2% human serum albumin, erythrocytes were removed by washing in lysis buffer, and cells were resuspended in culture media (RPMI 1640 medium with 2% human serum albumin and 10 mM HEPES; Life Technologies). Twenty thousand cells in 500 μl were seeded into multiwell culture slides (354118; BD Biosciences), incubated for 30 min at 37°C with 5% CO2, and challenged with GBS at a multiplicity of infection (MOI) of 100. At selected times, cells were fixed in 3.7% paraformaldehyde for 45 min and stained with 0.3 μg/ml propidium iodide (PI) in 2× SSC buffer (5 min, room temperature) for the detection of DNA, or were prepared for immunohistochemistry (IHC), described below.

We used IHC for the detection of neutrophil elastase and histones, which are PMN NET markers. For this, the PMNs were fixed, permeabilized with 0.5% Triton X-100 (5 min, room temperature), blocked with 2% BSA in PBS (15 min, room temperature), and stained with primary and secondary Abs (60 min, room temperature). Abs were as follows: 1/100 rabbit IgG to PMN elastase (481001; Calbiochem, Massachusetts), followed by 1/500 goat anti-rabbit IgG (4050-02-FITC; Southern Biotechnology Associates, Birmingham, AL), and 1/100 chicken IgY to histone [1H] (ab114028), followed by 1/500 rabbit anti-chicken IgY (ab6751-texas red, Abcam, Cambridge, MA USA). In some assays, GBS were stained with FITC (0.25 mg/ml, 60 min, 37°C) prior to infection to colorolate bacteria and PMN NETs. Cells were counter stained with the nuclear dye Hoechst 33258 (4 min, 20 μg/ml, B-2833, Sigma-Aldrich), mounted in 0.25% n-propyl gallate in 90% glycerol: 10% PBS, and viewed with a Leica Microsystems SP2 confocal laser-scanning microscope.

For dose escalation studies, PMNs were prepared as above using multilayer culture slides (154941; Nunc Lab-Tek) seeded with 5 × 105 cells in 500 μl. WT and Δcyl63 GBS were stained with FITC prior to infection. Cells were challenged at MOIs of 5, 50, or 500 and were incubated for 10 min treatment with GBS (Thermoblock, Monash University, VIC, Australia) for 120 min was used as a positive control for NET induction. Cells were then fixed and stained using PI. Images were taken using an AxioImager.M2 microscope. Quantification of DNA NETs was performed using mean fluorescent intensity (MFI) calculations. First, we used Photoshop CS3.1 (Adobe) to remove whole PMNs from the image, leaving behind only DNA NETs. The images were imported back into the AxiosVision software (Carl Zeiss MicroImaging), and MFI was calculated. This was performed using 8–10 images for each sample. The assays were repeated independently in three separate experiments. We also assessed the cellular integrity of PMNs during the infection and the process of NET formation by measuring lactate dehydrogenase (LDH) release as a marker of cell lysis to determine whether DNA release from PMNs was merely a result of cell death. LDH assays were performed using supernatants from PMNs in LDH in vitro toxicity kits (Sigma-Aldrich), according to the manufacturer’s instructions.

Statistics

Bacterial loads over time were compared between groups using an area under the curve analyses, followed by a Mann–Whitney U test, with significance set at a p value < 0.05. Leukocyte cell numbers for inflammatory infiltrate analyses derived from vaginal swab smears and flow cytometry data were compared using a Mann–Whitney U test, as were cytokine expression levels and MFI, with significance set at a p value < 0.05. Bacterial load comparisons were reproduced in three independent experiments, and infiltrate counts were repeated twice. GraphPad Prism software package 5.0 and SPSS version 19 were used for all statistical comparisons.

Results

GBS chronically colonizes the female genital tract of mice during continual estrus

We initially measured GBS loads in the genital tract of C57BL/6 mice for 90 d following intravaginal challenge of 17β-estradiol–treated animals, given the variable fitness of other bacteria for colonization of this niche in the first 30 d postinfection. Initial assays revealed extraordinarily long-term, persistent GBS colonization in the reproductive tract of mice (Fig 1A). Weekly injections of 17β-estradiol were used in these assays to maintain mice in estrus, and this was associated with recovery of high numbers of GBS at all time points from the majority of mice in each group. Typical recovery
of GBS on Granada agar in these assays ranged between $10^6$ and $10^7$ CFU/swab for most of the time points assessed, with the identity of GBS confirmed by colony blots using specific anti-GBS Ab (data not shown). The early phase of colonization observed in mice was dynamic and generally followed a pattern of an initial decline in GBS load to $\sim 3 \times 10^5$ CFU/swab during the first 3 wk postinfection. This decline preceded an increase in GBS loads, which were subsequently maintained at or above $10^6$ CFU/swab for at least 90 d (Fig. 1A). To determine whether persistent GBS colonization had any major effect on the overall numbers of resident genital tract commensal flora, we measured total bacterial loads using TSA 5% horse blood and total Gram-positive bacteria using ColNAC. Overall, there were no significant differences in the numbers of these commensal bacteria in the genital tract of GBS-colonized mice compared with control mice (Fig. 1B, 1C). We did observe however, that the early, reproducible decline phase in GBS numbers was consistently preceded by an immediate sharp rise in the total number of Gram-positive bacteria (also noted in sham infected; Fig. 1C), which subsequently stabilized during the second week until day 90.

**Chronic GBS colonization triggers an inflammatory infiltrate in the genital tract**

Numbers of PMNs, lymphocytes, and monocytes within the superficial layers of the genital tract mucosa were measured in infected and control mice over time using vaginal swab smears every 3 d to investigate the local inflammatory infiltrate at the mucosal surface during GBS colonization. Differential cell counts, performed only on mice in estrus, encompassed almost all mice (>90%) at each time point and showed a stable low level of lymphocytes (less than five cells per swab) in control mice over time (Fig. 2A). In comparison, mice colonized with GBS exhibited a significant increase in the numbers of lymphocytes over time (day 30: $p = 0.015$; day 60: $p = 0.036$; Fig. 2A), and by 90 d postinfection, colonized mice had an average of 15 lymphocytes/swab, which was significantly higher than controls ($p = 0.042$).
Simultaneous enumeration of PMNs in vaginal swab smears also revealed an infiltrate of these cells due to GBS infection (Fig 2B). The number of PMNs peaked at day 30 postinfection in control mice (illustrating a response to the challenge procedure itself), with an average of eight PMNs per smear; these cells then decreased until the end of the experiment. Similar to the dynamics of lymphocyte infiltration observed in GBS-colonized mice, numbers of PMNs gradually increased until day 60 in colonized mice and were significantly higher compared with controls ($p = 0.041$) (Fig. 2B). Fewer PMNs were detected at day 90 in GBS-colonized mice, although numbers of cells remained significantly elevated compared with controls ($p = 0.038$). A representative image of this genital tract cell infiltrate is shown in Fig. 2C, which illustrates colonized cornified vaginal epithelial cells (D), PMNs (E), and lymphocytes (F). Slides were scanned using an Aperio Scanscope and counted using Imagescope version 10. Data are mean ± SEM of 12 mice and are representative of two separate experiments, each with 12 mice/group. Scale bars: 500 μm (C), 10 μm (D–F). Mann–Whitney $U$ tests were performed. # $p < 0.05$.

Differential cell counts revealed few monocytes in swab smears over the course of each assay (data not shown). Flow cytometry analysis of vaginal tissue, used to investigate the cell infiltrate in tissue submucosa rather than the superficial mucosal layers as is provided by swab smears, showed that there was no increase in Gr-1$^+$ cells in the submucosa of infected or control mice at day 15 and 30 (absolute numbers: $8 \pm 4$ and $4 \pm 1$ Gr-1$^+$ cells/mg in each group on days 15 and 30, respectively; Fig. 3A). Differences in numbers of F4/80$^+$ cells detected in colonized mice and controls at day 30 did not reach statistical significance ($p = 0.072$; Fig. 3B). We observed a significantly higher increase in the number of cells positive for the F4/80 marker in mice colonized with a GBS hemolysin–deficient mutant, Δcyt9, at 30 d postinfection compared with both WT-colonized and PBS control animals ($p = 0.0038$ and $p < 0.0001$, respectively; absolute numbers: $6 \pm 1, 6.3 \pm 1,$ and $8.1 \pm 1.8$ F4/80$^+$ cells/mg on day 15, and $6.1 \pm 0.3, 7.2 \pm 0.3$, and $8.1 \pm 0.2$ F4/80$^+$ cells/mg on days 30 [PBS, WT, and Δcyt9 groups, respectively]; Fig. 3B). Thus, chronic GBS genital tract colonization in mice induces responses at the vaginal mucosal surface made up of increased numbers of lymphocytes and PMNs and increased numbers of F4/80$^+$ cells in tissue submucosa that are amplified in the absence of GBS hemolysin.

Genital tract histopathology analysis of cellular infiltrate in GBS-colonized mice

The gross pathology of entire genital tracts was similar between GBS-colonized and control mice after 90 d in groups that were...
maintained in continual estrus for the duration of the experiment (Supplemental Fig. 1). The use of 17β-estradiol did, however, cause enlargement of the genital tract in some mice, with notable solid masses in the upper reproductive tract and edema, caused by weekly administration of the steroid hormone (Supplemental Fig. 1A, 1B, solid arrows). Serial sections of the genital tract stained with H&E revealed a cellular infiltrate in the superficial luminal regions of the vaginal mucosal surface in GBS-colonized mice, which was most notable at days 15 (Fig. 4A, 4B, solid arrows) and 30 (Fig. 4C) postinfection, which was consistent with findings of lymphocyte and PMN infiltrates noted in vaginal swab smears after day 15 (Fig. 2). An inflammatory infiltrate was not detected in the luminal regions of genital tracts in sham-infected animals (Fig. 4D–F) in the sections examined. Gram staining revealed many Gram-positive bacteria in both GBS-colonized and control mice, which were bound to cornified vaginal epithelial cells (Fig. 4G, 4H) as well as to PMNs (Fig. 4I). These stains also demonstrated an increase in the cell inflammatory infiltrate in the superficial luminal regions of the vaginal mucosa in GBS-colonized mice over the course of infection (Fig. 4G–I) compared with sham-infected mice (Fig. 4J–L). These inflammatory infiltrates were consistent with cytology swab-smear data that showed infiltrates in the superficial vaginal mucosal layers (Fig. 2). We also noted a marked increase in the number of Gram-positive bacteria in the vaginal submucosa in GBS-colonized mice at day 30 postinfection (Fig. 5A), compared with sham-infected controls (Fig. 5B). Parallel Giemsa staining of serial sections supported these findings (data not shown).

We next analyzed genital tracts of GBS-colonized mice using high-resolution SEM to complement colonization data on numbers of resident genital tract commensal flora and investigate the distribution of bacteria including GBS on the vaginal mucosa. SEM of genital tracts at day 1 postinfection revealed a diverse collection of morphological cell types of normal flora bound to the surface of the vaginal epithelium (Fig. 6A–F). This flora represents a mixed community with which GBS might compete or synergize for adherence in the genital tract. To assess the distribution of GBS within these tissues, we used specific immune labeling using anti-capsular polysaccharide Ab conjugated to gold, which showed GBS bound among a mixed microbial flora to the vaginal epithelium (Fig. 6G, 6H). This labeling was highly specific and none of the mixed flora of any other cell morphological types reacted with this Ab (Fig. 6I, 6J). Other labeling controls were tissues from mice administered PBS, Ab isotype controls, and no secondary conjugate, and these also exhibited no labeling (data not shown), confirming the specificity of this SEM immune labeling approach for GBS in vivo.

Regular hormone cycling does not restrict chronic GBS genital tract colonization

We next sought to determine whether the frequency of normal hormone cycling in mice might affect the progression of carriage of GBS in the genital tract, and if long-term, stable carriage is possible in the absence of continual estrus. For this, mice received only a single dose of 17β-estradiol prior to challenge to synchronize their state of estrus and achieve consistent infection. Development of GBS infection under these conditions still provided long-term, high-level colonization for at least 90 d, as shown in Supplemental Fig. 2A. Staging of these mice using vaginal swab smears collected at daily intervals showed reversion to normal hormone cycling at day 10 (± 1 d). GBS loads in normal hormone cycling mice were more dynamic compared with mice in continual estrus; however, the pattern of an early decline phase with fewer GBS in the first month was consistent with prior assays. This phase was followed by an increase in GBS numbers to >10⁶ CFU/swab; changes in GBS loads in these mice did not correlate with the stage of the estrous cycle, as was determined using vaginal swab smears. For example, at several time points (e.g., days 42 and 63; Supplemental Fig. 2A), GBS loads decreased during different phases of the cycle. Details of cycle stage and GBS loads that showed no correlation between estrous cycle stage and GBS load are summarized in Supplemental Table I. Statistical analyses comparing GBS loads over 90 d in mice in continual estrus versus those given a single dose of 17β-estradiol confirmed that these did not differ significantly. No control mice in our study exhibited
positive GBS cultures from the genital tract at any time point. Cellular infiltrates in normal hormone cycling mice were not assessed because of effects of cycle stage on leukocyte presence. Finally, neither total nor Gram-positive bacterial loads differed significantly between GBS-colonized and control mice in normal hormone cycling (Supplemental Fig. 2B, 2C). Taken together, these data show that GBS readily establishes chronic colonization in the female genital tract of C57BL/6 mice even in conditions of normal hormone cycling, and stable carriage can be generated in the absence of continual estrus.

**GBS β-hemolysin aids chronic genital tract colonization and inhibits PMN accumulation**

We next examined whether GBS hemolysin might contribute to chronic colonization in the genital tract in mice. Direct comparison of bacterial loads in groups of challenged mice revealed that the nonhemolytic Δcyle9 mutant was unable to colonize the genital tract as efficiently compared with the WT and was recovered in significantly fewer numbers compared with the parent strain (Fig. 7A). Colony blots were used to confirm GBS colonies on Granada agar in these assays. Repeat assays confirmed that colonization loads for Δcyle9 were significantly lower compared with the WT strain over 90 d with averages between 10^3 and 10^5 CFU/swab versus 10^6 CFU/swab (p < 0.001). Recovery of the nonhemolytic Δcyle9 mutant was below the limit of detection of the assay (10^5 CFU/swab) at days 33 and 48, which preceded an increase in numbers during days 60–90 (Fig. 7A). The local cell infiltrate in Δcyle9-colonized mice was also strikingly different compared with mice colonized with the WT strain. Analysis of vaginal swab smears showed high numbers of lymphocytes in vaginal mucosa of mice that were colonized with Δcyle9, whereas these differences were not significantly different compared with WT GBS (Fig. 7B). There was a massive neutrophilia noted in the vaginal mucosa of Δcyle9-colonized mice, which comprised significantly higher numbers of PMNs compared with mice infected with WT GBS (Fig. 7C). The cell infiltrate induced by the mutant was more pronounced than that observed in H&E sections of genital tracts from mice colonized with WT GBS (Fig. 7D compare Fig. 4A–C). The cell infiltrate in Δcyle9-colonized mice was also most notable at the vaginal mucosal surface, where we noted Gram-positive bacteria (Fig. 7E). These data are consistent with cytology, histopathology, and flow cytometry findings of an infiltrate localized to the vaginal mucosal surface (and lumen) at these time points rather than the submucosa and a greater inflammatory infiltrate in Δcyle9-colonized mice (compared with WT and PBS groups). Thus, GBS hemolysin is necessary for efficient, high-level chronic colonization of the female genital tract and inhibits local accumulation of PMNs at the vaginal mucosal surface and accumulation of F4/80+ cells in the vaginal submucosa.

**GBS induces multiple soluble inflammatory markers during genital tract colonization**

GBS-induced cell infiltrates observed in the genital tract prompted us to examine soluble inflammatory markers to gain further insight into the local host response to GBS colonization. For this, we examined 23 proteins related to chemotaxis and inflammation including keratinocyte-derived chemokine (KC/CXCL1), IL-1β, TNF-α, IL-1α, MCP-1, GM-CSF, RANTES, and IL-17 (45–48). Measurement of these markers revealed distinct patterns of production in the genital tracts of GBS-colonized mice. There were no significant differences in levels of KC and IL-1β between GBS-colonized mice and controls (Fig. 8); however, levels of other cytokines including GM-CSF, TNF-α, IL-1α, and MCP-1 were significantly elevated in colonized mice between days 15 and 30 postinfection (p < 0.001; Fig. 8). Levels of RANTES and IL-17 were divergent between the groups, but these differences were not statistically significant (RANTES: day 30, p = 0.069 IL-17; day 30, p = 0.141; Fig. 8). Other immune-regulatory markers including IL-12 (both p40 and p70 subunits), IL-3, IL-4, IL-5, IL-9, IL-10, and eotaxin were significantly elevated at one or more time points during chronic GBS genital tract colonization compared with controls. Others including IFN-γ, IL-13, G-CSF, IL-2, and IL-6 were unchanged as a result of infection (data not shown). Thus, GBS stimulates a local soluble inflammatory pattern of multiple cytokines and chemokines in the genital tract of mice consisting of GM-CSF, TNF-α, IL-1α, MCP-1, and several other cytokines.

**GBS infection of PMNs induces NET formation and cell death, dependent on hemolysin**

Our findings of a PMN infiltrate and local inflammation in the genital tract in response to GBS in mice prompted us to further investigate the interactions between GBS and PMNs in vitro. During our examination of H&E sections prepared from GBS-colonized mice at day 60 (peak PMN responses according to vaginal swab smears; Fig. 2B), we noted strands of darkly stained DNA in regions of infected mucosa, which were colocalized with bacteria and intact PMNs (Fig. 9A, 9B). Patterns of DNA stranding were sporadic but were not observed in control mice, suggesting the presence of DNA NETs in GBS-colonized genital tracts, as recently described in other in situ responses to infection (49). We therefore chose to examine PMN NET formation in response to GBS in vitro. Infection assays using human PMNs challenged with WT GBS 874391 at a MOI of 100 revealed rapid NET formation following infection; immunofluorescence of PI-stained DNA stranding showed release of NETs within 5 min of infection (Fig. 9C). Colocalization using FITC-stained GBS and confocal microscopy illustrated distinct GBS binding to PMNs (Fig. 9C) and to NETs (Fig. 9C, inset). IHC for detection of PMN elastase, a NET marker (50), confirmed elastase along the length of NET strands (Fig. 9D, 5 min), which extended between and around some PMNs (Fig. 9D, inset; 30 min). IHC for histones also showed presence along these NET strands (Fig. 9E, 5 min) and in regions to which GBS had bound (Fig. 9F, 30 min).

GBS β-hemolysin activates and subverts PMN killing mechanisms (28, 51, 52), and our findings on NET induction prompted us to investigate whether this response might be influenced by β-hemolysin. For this, we compared NET induction in human PMNs following infection with either WT GBS or Δcyle9. In

![FIGURE 5.](http://www.jimmunol.org/) Gram-positive bacteria in the vaginal submucosa of C57BL/6 mice during chronic GBS colonization. Reproductive tracts were sectioned and Gram stained at various time points postinfection, which revealed an increase in clusters of Gram-positive bacteria (solid arrows) within the vaginal submucosal tissue at day 30 postinfection in mice that were infected with GBS (A), compared with sham (PBS)-infected controls (B). Scale bars: 50 μm (A, B), 10 μm (insets).
contrast to rapid NET induction by WT GBS (Fig. 10A), the Δcyle9 strain failed to induce NET release at the same MOI, despite binding to the PMNs (Fig. 10B). Quantitative dose response analyses showed that increasing MOIs caused more NET formation compared with lower MOIs, and WT GBS induced 4-fold more NETs than did the Δcyle9 mutant at an MOI of 500 (Fig. 10C).

FIGURE 6. GBS adhere among diverse normal flora during chronic colonization of the murine genital tract. Mice were infected and genital tracts were processed for SEM at day 1. A diverse collection of microbial morphological cell types, including widely distributed bacilli (A–C), coccobacilli (D), and clusters and chains of cocci (E, F) are shown. Immune labeling of GBS capsular polysaccharide, shown in SEM and backscatter images (G, H), illustrates specific labeling (arrows) and attachment to the epithelium. Inset in (H) shows higher magnification of specific conjugated gold particles bound to GBS. Most other mixed genital tract normal flora did not label (I, J). Scale bars: 10 μm (A), 1 μm (B, F, I, and J) and 100 μm (C–E, G, and H).
In comparison, PMA treatment induced major NET induction at 120 min (Fig. 10C). We also undertook LDH assays to measure the viability of PMNs in vitro to examine cell viability following infection and determine whether the DNA release simply reflected rapid hemolysin-induced cell lysis. These assays showed that PMN cultures expelling NETs in response to GBS did not exhibit elevated LDH levels compared with control cultures; LDH release at the time of, and immediately following, GBS-induced NET release was equivalent to noninfected controls (Fig. 10D). These data demonstrate that the PMNs in GBS-infected cultures exhibiting NET formation were not compromised in terms of cell viability and were not lysed. Several hours postinfection, however, LDH levels increased as viability of PMNs decreased (data not shown). Collectively, these data show that human PMNs secrete DNA NETs in response to GBS infection and this response depends on β-hemolysin, which ultimately results in killing of the PMNs.

Discussion
Colonization of the female genital tract with potential pathogens is complex and influenced by the local cellular inflammatory response and the pathogenic potential of the microbe. GBS is unique in its ability to cause acute disease but also colonize the genital tract of adult women asymptptomatically, long term. These lifestyle traits of GBS are not well understood, and the roles of GBS virulence factors and host responses are unknown. Precisely how GBS persists within the female genital tract long term is not understood.
genital tract irrespective of regular shedding of the vaginal mucosal epithelium during repeated shifts in hormonal cycle. Temporal analysis of bacterial, histological, and immune parameters showed that establishment of consistent, long-term colonization required at least one pretreatment with estradiol to synchronize the mice in the estrus phase of the reproductive cycle. Within 24 h of hormone treatment, all mice entered estrus, which is the optimal phase of the cycle for experimental infection because of the
GBS maintains high-level infection despite regular sloughing and regeneration of superficial genital tract epithelial cell layers is unknown. GBS is able to maintain high-level colonization in this model despite the activation of local inflammatory mechanisms, including a major influx of PMNs and lymphocytes to the superficial vaginal mucosa of the genital tract.

In relation to the adherence of GBS to the genital tract mucosa, in vitro binding studies using human vaginal and cervical epithelial cells have provided insight into this process. GBS has a binding affinity for these cells at pH similar to vaginal physiological pH (<4.5), a trait that underscores the organisms’ adaptation for colonization of the genital tract (62). GBS also is able to translocate epithelial barriers (63, 64), which may aid invasion into the genital tract submucosa. Interestingly, cellular translocation of GBS in vitro causes minimal destruction to epithelial monolayers (64).

In our model, there was no obvious tissue damage in the genital tract of mice chronically colonized with GBS according to histological analysis. However, there was an increase in the number of Gram-positive bacterial aggregates in the submucosal region of vaginal epithelia. This finding is notable because the lack of tissue damage observed in our model of chronic GBS colonization contrasts with severe pathology in the genital tract during infection with other pathogens that colonize this niche such as Chlamydia (31).

The significant influx of PMNs triggered by GBS throughout estrous challenges the notion that GBS is entirely benign in the female genital tract, and this has implications for understanding how the bacteria avoid immune clearance in this niche. To our knowledge, no studies to date have reported an inflammatory infiltrate in the female genital tract in response to GBS, nor have any soluble inflammatory markers been associated with colonization. Neutrophil infiltration is considered a primary line of host defense against GBS infection in general (51), and our findings establish that these cells are active in defense against GBS in the genital tract. Numbers of PMNs steadily increased in the surface mucosa of the genital tract in response to GBS over time, according to swab-smear analysis and histopathology, and peaked between days 30 and 60. This influx of PMNs parallels an infiltrate of PMNs observed in GBS neonatal disease (26). Precisely when this influx wanes in the genital tract during chronic GBS colonization is unclear because we concluded our assays at 90 d. The small increase in PMN responses at day 30 in control mice observed in our model likely relates to the invasive nature of the challenge procedure and the trauma of inoculation into the vaginal vault. In other models of genital tract infection, rapid PMN infiltrates are effective at killing the infecting microbes (30, 59), which contrasts with the long-term persistence of GBS reported in this paper. This suggests that the PMN infiltrate in this model is not effective at eliminating GBS from the female genital tract. Concomitant lymphocyte infiltrates also support a chronic inflammatory nature of the host response to GBS in this model. For other genital tract pathogens, such a response can have consequences for reproductive function. Persistent inflammation in the genital tract and ascending infection to the upper reproductive tract, for example, can cause tubal blockage and infertility (65). Aside from some tissue enlargement and edema, no mice in our study showed signs of hydrosalpinx, an indicator of tubal blockage and infertility (66), and GBS is not associated with infertility in women. This indicates that the inflammatory mechanisms activated in the genital tract during chronic GBS colonization in this model are distinct from those triggered by other pathogens such as C. trachomatis and N. gonorrhoeae.

Several of the soluble inflammatory makers of inflammation examined in this study have been implicated in GBS disease in prior studies. GM-CSF was particularly significant in the genital

FIGURE 9. GBS-induced DNA stranding in the murine genital tract and NET formation in human PMNs. H&E sections of vaginal submucosa from a GBS-colonized mouse at ×20 magnification (A) and ×63 magnification (B) illustrating DNA stranding (open arrow), localized with PMNs (closed arrow), and bacteria (arrowhead). Primary human PMNs, infected with WT GBS (MOI 100), and stained with PI showing DNA release within 5 min (C). IHC for elastase in (D) (FITC) at 30 min showing distribution along DNA NETs. IHC for histones in (E) and (F) (Texas red) showing distribution along NETs and GBS (FITC) colocalization. Scale bars: 10 μm (A), 5 μm (B), 20 μm (C, inset 8 μm), and 25 μm (D–F, insets 15–20 μm). Counterstain in (D–F) is Hoechst.

presence of keratinized and cornified epithelial cells, which promote adherence of colonizing bacteria (55). Estrus is also characterized by sparse leukocytes in the vaginal submucosa in contrast to other stages of the cycle in which leukocytes are abundant (35). Synchronization of mice in estrus would allow bacteria to avoid early interactions with leukocytes and permit binding to epithelial cells to establish infection. GBS readily adheres to human vaginal epithelial cells (56), which increases progressively as women reach day 14 of their hormonal cycle (55). Our results are consistent with these findings and show the utility of using estradiol for modeling human GBS genital tract colonization in mice as for other models of genital tract infection (32). The finding of GBS colonization for at least 12 d in a short-term infection model in CD1 mice (56).

A remarkable finding of this study was that GBS maintained stable infection of the vaginal mucosa after the effects of estradiol subsided at day 10. This represents a striking fitness trait of GBS compared with other genital tract pathogens characterized in mice to date, which require repeated hormone treatments to enable long-term infection (57, 58). In murine models of genital tract Chlamydia trachomatis and Neisseria gonorrhoeae infection, for example, the bacteria are cleared within 1 mo, even with continual progesterone (59, 60), or 17β-estradiol (61) therapy. Thus, how
FIGURE 10. GBS-triggered PMN NET formation requires β-hemolysin expression. WT GBS 874391 (FITC) was incubated with human PMNs and stained with PI to detect extracellular DNA NETs [(inset), original magnification ×1000] (A). Isogenic mutant Δcyle9 GBS, which lacks β-hemolysin, was incubated with PMNs at the same MOI and stained with PI but did not induce NETs (B). Quantitative dose-response analyses using increasing MOIs showed the most NET formation at MOI 50 (10 min shown), compared with positive control treatment using 25 nM PMA for 120 min (C). Lactate dehydrogenase assay (D) was performed on supernatants from control PMNs (no treatment), Triton X-100–lysed PMNs (positive control), PMNs infected with WT GBS, and PMNs infected with Δcyle9 GBS to assess cell viability during NET release. Dose-response data are mean ± SEM of MFI and is representative of three separate experiments, each with eight images (×63 magnification) per sample. Mann–Whitney U tests were performed with significance set at a p value < 0.05. Scale bars, 50 μm. *p = 0.021, #p = 0.015.

The tract response to GBS and occurred alongside TNF-α, IL-1α, and MCP-1. Several of these cytokines have previously been associated with streptococcal infection (28, 67–69). The absence of a response for other markers may relate to the time points examined, or GBS might suppress production of KC, IL-1β, and other pro-inflammatory mediators, as it has been shown to do in another model of streptococcal infection (69). Our finding of a GM-CSF peak at day 30 postinfection correlates with in vivo and in vitro studies that have shown production of GM-CSF during GBS infection (70–72). In studies that have used macrophages, GM-CSF enhanced the killing of GBS (70, 71), possibly through the production of superoxide and hydrogen peroxide (70). GBS can survive for long periods inside macrophages and can induce macrophage apoptosis by β-hemolysin–dependent (73) and independent mechanisms (37, 74, 75). A novel function of secreted GBS GAPDH as an inducer of macrophage apoptosis also has been shown recently (76). Induction of apoptosis represents a possible mechanism of immune suppression by GBS, as occurs for other Gram-positive pathogens (77). Although the role of GM-CSF in macrophage responses to GBS appears to be beneficial for bacterial killing, studies are now required to define its role in macrophage anti-microbial responses in the GBS-colonized genital tract (e.g., the F4/80+ cell response). Our flow cytometry analysis indicated minor and major infiltration of F4/80+ cells into the genital tract submucosa at day 30 in mice infected with WT GBS and non-hemolytic Δcyle9, respectively. In this study, it is also important to highlight the potential limitations of the flow cytometry approach used for measuring surface localized cell infiltrate in our model. The vast majority of infiltrating leukocytes were observed at the vaginal surface mucosa and luminal region, rather than within the submucosa, according to quantitative vaginal swab smears and histology. This finding was somewhat surprising because any infiltrate at the vaginal surface mucosa, as observed, would normally also be expected to occur concurrently in submucosal areas. This may reflect the specific time points chosen for assay, however, also may be affected by technical limitations of the approaches used. For example, in our flow cytometry approach, mucous-bound leukocytes at the vaginal mucosal surface may be lost during the digestion and cell isolation process because mucin, present in the female genital tract in abundance, is reportedly difficult to break down (78–80). Standard tissue digestion protocols exclude mucin(s), and cell aggregates are unlikely to pass through the cell sieve to single-cell suspensions. Another limitation in our study was use of commercial Gr-1 Abs that have been reported to cross-react with monocytic cells and macrophages (81). We reduced the potential effect of this through placement of gates; however, cross-reactivity still may have masked group differences. Future studies will assess the inflammatory infiltrate within the vaginal submucosa of colonized mice in this model in more detail. Finally, we noted a small, statistically insignificant increase in IL-17 in GBS-colonized mice. IL-17 promotes production of some cytokines and helps to recruit and activate PMNs and macrophages (82). These data highlight the need to better understand these responses and their effects on GBS persistence and local immune responses in this model.

GBS β-hemolysin is a key virulence factor for the organism, and this study shows that this factor contributes to GBS survival in the female genital tract. A recent study of 4-d infection showed that GBS pili and serine-rich repeat proteins also contribute to early colonization of the murine genital epithelium (56). A single dose of a phage lysin, PlyGBS, in a 1-d vaginal GBS infection model showed a significant reduction in bacterial load within just a few hours (83). In the future, it will be interesting to assess the role of these factors in the chronic infection model described in our study. In terms of the interactions between PMNs and GBS, other streptococci have been shown to induce DNA NETs, which is believed to aid bacterial clearance. Our in vitro findings, based on primary human PMNs, show not only does GBS induce DNA NETs but also that this response depends on the expression of the bacteria’s β-hemolysin. Because β-hemolysin expression was also associated with increased GBS survival in the genital tract and a lower local PMN response (in WT GBS–colonized mice), overall, this suggests an effect of the hemolysin on either PMN survival or recruitment, or both, in the genital tract. We addressed the former...
The authors have no financial conflicts of interest.

Disclosures

References


