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

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Summary

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

Keywords

Streptococcus agalactiae; transcription; regulation; two component system; vaginal tract

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Introduction

Streptococcus agalactiae (Group B *Streptococcus*, GBS) is an encapsulated Gram-positive bacterium that commensally colonizes the lower gastro-intestinal tract, and in females, the lower reproductive tract, of 20–30% of healthy adults (Doran et al., 2004). However, in immune compromised individuals, such as neonates, pregnant women, and the elderly, GBS may transition to an invasive pathogen, resulting in pneumonia, sepsis, urinary tract infections, and meningitis (Maisey et al., 2008). Despite currently recommended intrapartum antibiotic prophylaxis for GBS-positive mothers, GBS remains the leading cause of early-onset neonatal sepsis (Verani et al., 2010). Whether *in utero* or during labor, neonatal exposure to GBS requires survival, transversal through a number of host environments and barriers, immune evasion, and in the case of meningitis, crossing of the highly regulated blood-brain barrier (Maisey et al., 2008). Upstream of these virulent interactions within the neonate, is the initial commensal colonization of the maternal vaginal tract.

Vaginal colonization by GBS is believed to be somewhat transient, and likely dependent on vaginal pH, normal flora, pregnancy, and estrous cycle, among many other constituents. Factors that favor the persistence of GBS in this complex biological niche are not well understood. GBS biofilm formation has been demonstrated in simulated vaginal fluids, with bacterial survival and growth improving as pH approaches neutral (Borges et al., 2012, Ho et al., 2012). However, optimal pH for GBS biofilm formation is unclear, as one group reported biofilm production increased as pH rose from 4.2 to 6.5 (Borges et al., 2012), and another group, that GBS biofilm formation is enhanced at pH 4.5 compared to pH 7.0 (Ho et al., 2012). Although so much is still unknown, a few GBS determinants have been shown to contribute to vaginal cell adherence and colonization, including surface Serine Rich Repeat (Srr) proteins, Srr-1 and Srr-2, and pili protein, PilA of GBS Pilus Island (PI)-2a (Sheen et al., 2011). Interestingly, it has been recently observed that GBS PI-1 does not mediate attachment to vaginal cells *in vitro* (Jiang et al., 2012). Others have also begun exploring bacteriocin-like inhibitory substances produced by native vaginal species that negatively impact GBS growth (Ruiz et al., 2012). Nevertheless, the molecular mechanisms governing GBS vaginal persistence and competition with normal microbiota remain to be elucidated.

GBS has several regulatory systems in place that may control the transition of the organism from a commensal niche (e.g. vaginal tract) to invasive niches (e.g. blood, lung, brain, and other organs). Bacteria respond to changes in environmental stimuli using two-component systems (TCS) to alter gene expression. TCS typically consist of a membrane-associated histidine kinase and a cytoplasmic transcriptional regulator (Beier et al., 2006). In GBS, a TCS consisting of a sensor histidine kinase CovS (Cov, control of virulence), also known as CsrS (Csr, capsule synthesis regulator), and a response regulator CovR (CsrR) down regulates the expression of 27 genes and activates the expression of 3 genes in all GBS strains examined (Lamy et al., 2004, Jiang et al., 2008, Lembo et al., 2010). The conserved regulatory functions of CovR in GBS include repression of fibrinogen-binding proteins A and B (FbsA, FbsB), genes involved in iron uptake, and in particular, repression of *cylE*, the gene encoding GBS toxin β-hemolysin/cytolysin (β-H/C) (Pritzlaff et al., 2001), as CovR-deficient strains exhibit increased hemolytic activity (Lamy et al., 2004, Rajagopal et al., 2006, Jiang et al., 2008, Lembo et al., 2010). The β-H/C toxin is a well-established virulence factor known to promote GBS cellular interactions, and to provoke host innate immune responses in human epithelial and endothelial cell models by activating transcription of host genes encoding the chemokines IL-8, CXCL1, and CXCL2 for neutrophil recruitment (Doran et al., 2002, Doran et al., 2003). *In vivo* studies have further indicated that the β-H/C contributes to the development and severity of meningitis (Doran et al., 2003), pneumonia (Hensler et al., 2005), arthritis (Puliti et al., 2000), and sepsis (Ring et al., 2002). The cytotoxic and proinflammatory properties of the β-H/C toxin work to the detriment of the

host. The CovR regulatory system itself has also been shown to contribute to disease progression (Lembo et al., 2010). GBS CovR/S mutation has been observed in clinical isolates (Sendi et al., 2009) and CovR/S mutations are frequently observed in *Streptococcus pyogenes* (Group A *Streptococcus*) (Engleberg et al., 2001, Sumby et al., 2006).

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

Experimental Procedures

Bacterial strains and growth conditions

Streptococcus agalactiae (GBS) strains were grown in Todd-Hewitt broth (THB) (Hardy Diagnostics) at 37°C. The wild-type (WT) clinical isolates used in this study are A909 (serotype Ia) (Madoff et al., 1991) and COH1 (serotype III) (Wessels *et al.*, 1991). Both A909 and COH1 Δ*cylE* mutants were constructed previously (Pritzlaff *et al.*, 2001), as were the A909Δ*covR* and Δ*covR*/Δ*cylE* mutants (Rajagopal et al., 2006, Lembo et al., 2010). The COH1Δ*covR* was derived using methods described (Rajagopal et al., 2006). The A909Δ*fbsB*/Δ0956 strain was constructed using methods described (Shelver et al., 2003), where the gene encoding kanamycin (Ω*km*-2) was used for allelic replacement. The complemented *covR* strain (p*covR*) was generated using methods as described previously (Rajagopal et al., 2003). Briefly, the gene encoding CovR was amplified using primers 5'GCGCGGAGCTCTTGTTAAGTAAAGAATAAG 3' and 5'GCGCGAGGATCCTTTATTTTTCACGAATCAC 3' and the PCR products were digested and ligated into the complementation vector pDC123 (Chaffin et al., 1998) downstream to the tetracycline promoter, P_{tet} followed by electroporation into the GBS A909Δ*covR* mutant. When necessary, mutants were maintained and grown in antibiotics at the following concentrations: spectinomycin (300 μg/mL), chloramphenicol (2 μg/mL), and kanamycin (500 μg/mL). *Lactobacillus crispatus* (LC) (Strain # 33820, ATCC) was grown in Lactobacilli MRS broth (BD Biosciences) at 37°C.

Cell lines

Immortalized human vaginal epithelial cell (HVEC) line, VK2/E6E7, and ectocervical epithelial cell line, Ect1/E6E7, were obtained from the American Type Culture Collection (ATCC CRL-2616 and ATCC CRL-2614 respectively) (Fichorova et al., 1997). Passages 5–20 were used for all cell assays. Cells were maintained at 37°C in a 5% CO_2 atmosphere in keratinocyte serum-free medium (KSFM) (Invitrogen) with 67.419 pg/mL human

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recombinant epidermal growth factor and 65 μg/mL bovine pituitary extract as described previously (Sheen et al., 2011).

Vaginal cell infection and microarray analysis

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

In vitro cell assays

GBS adherence and invasion assays of HVEC were conducted as described previously (Sheen et al., 2011) with minor modifications. Concisely, cells were grown to confluency in 24 well tissue culture treated plates and washed prior to bacterial addition. Bacteria were grown to mid-log phase and added at an MOI of 1 for adherence assays. After 30 minutes of incubation, cells were washed 6 times with PBS and lysed by adding trypsin-EDTA and Triton X-100. Lysate was serially diluted and plated on THB agar plates to enumerate bacterial cfu. Total adherent cfu was calculated as (total cfu recovered/total cfu of original inoculum)×100%. To quantify intracellular bacteria, cells were incubated at MOI of 10 for 2 hours, monolayers washed, treated with antibiotics, and incubated for an additional 2 hours (Sheen et al., 2011). Cells were washed 3 times with PBS, lysed as described above, and intracellular GBS determined by serial dilution plating and total intracellular bacteria quantified as above.

RT-qPCR and ELISA

For analysis of gene expression induction, HVEC were grown to confluency in 24 well tissue culture-treated plates and washed prior to bacterial exposure. Bacteria were grown to mid-log phase and added at an MOI of 10 and incubated for 4 hours. Cells were then lysed, total RNA extracted (Macherey-Nagel), and qPCR performed (Quanta Biosciences). Primers and primer efficiencies for IL-8, CXCL1, CXCL2, CCL20 and GAPDH were utilized as previously described (van Sorge et al., 2008). For ELISA assays, HVEC were infected as described above with several modifications. Bacteria were added at an MOI of 10–50 as listed in figure legends, and cells were incubated with bacteria for 1.5–2 hours as indicated. After initial incubation, cells were washed with once with PBS, and fresh KSFM added containing 5 μg/mL penicillin and 50 μg/mL gentamicin. Cells were incubated an additional 3.5–4 hours in the presence of antibiotics and cell supernatants were analyzed for chemokine secretion using human IL-8 (R&D Systems), CXCL1 (R&D Systems), and CXCL2 (Antigenix America) ELISA kits according to manufacturer’s instructions.

In vitro HVEC viability assay

To determine viability of HVEC infected with GBS *in vitro*, adherence and invasion assays were performed as described above. Afterwards, supernatant was aspirated and placed into microfuge tubes. To dislodge adherent cells, 100 μL of 0.25% trypsin-EDTA (Gibco) was added to each well and incubated at 37°C for 5 minutes. Trypsin activity was halted by adding 200 μL of DMEM F12 (Cellgro) with 10% fetal bovine serum (Invitrogen). Cells were gently removed from wells by pipetting up and down several times and suspended cells were added to supernatant. To distinguish live cells from dead cells (with permeable

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membranes), 0.4% Trypan Blue (Life Technologies) was added at 1:10, and live cells (unstained) and dead cells (stained) were quantified using a hemocytometer. Percent of live cells per sample was calculated as (number of live cells/total cells counted)×100%. Two grids per sample were counted and results averaged.

In vivo mouse model of GBS vaginal colonization

All mouse work was approved by the Office of Lab Animal Care at San Diego State University and conducted under accepted veterinary standards. Female CD1 and BALB/c mice (8–16 weeks old) were obtained from Charles River Laboratories and used for colonization assays adapted from previous work (Sheen et al., 2011). Breeding pairs of CXCr2 (CXCL2 receptor) knock out (KO) mice (formerly IL8r KO mice), were originally purchased (C.129S2(B6)-Cxcr2^{tm1Mwm}/J, Jackson Laboratories). The mutation was crossed onto a BALB/c background prior to being deposited at Jackson Laboratories. We established a homozygous×homozygous breeding colony at the UCSD VA Hospital using mice that were maintained on water containing co-trimoxazole (200 μg/mL sulfamethoxazole and 40 μg/mL trimethoprim). For the 17 week old females used in this study, antimicrobial treatment was terminated 48 hours prior to inoculation with GBS. To synchronize estrus and promote bacterial colonization (Furr et al., 1989, Cheng et al., 2005), mice were injected intraperitoneally with 0.5mg 17β-estradiol suspended in sesame oil (Sigma) 24 hours prior to inoculation. Mice were inoculated with ~1×10^7 cfu (in 10 μL PBS) GBS in the vaginal lumen. Immediately prior to inoculation, vaginal lavage was performed by pipetting the lumen with 20 μL of PBS several times to collect cells and cytokines as described elsewhere (Sonoda et al., 1998, Caligioni, 2009). On successive days, the vaginal lumen of each mouse was first lavaged for cytokine analysis and then swabbed with ultrafine calcium alginate-tipped swabs. Bacterial load was determined by serial dilution plating of swab samples. WT or mutant GBS strains were identified as mauve or light pink-pigmented colonies on CHROMagar Strep B agar (DRG International Inc.) (Poisson et al., 2011). For tissue collection, mice were sacrificed using CO_2 asphyxiation and reproductive tracts excised from mid-uterine horn to just proximal of the vulva. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. ELISA assays were performed on vaginal lavages for KC (R&D Systems), MIP-2 (R&D Systems) and IL-1β (eBiosciences) as described by manufacturer.

Myeloperoxidase Assay

Neutrophil recruitment and activation was determined by quantifying the neutrophil enzyme, myeloperoxidase (MPO), as described previously (Banerjee et al., 2011), but modified for murine vaginal samples. Cells were collected from swabs as described above and suspended in 100 μL PBS. 50 μL of the swab sample was added to 100 μL of 0.05% hexadecyltrimethylammonium bromide (HTAB) buffer and MPO was released from neutrophils by bead-beating with 1.0 mm diameter zirconia beads (BioSpec) for 1 minute using a Mini BeadBeater (BioSpec). Samples were centrifuged at 13,000 rpm for 15 minutes at 4°C, and 10 μL of supernatant was added to 190 μL phosphate buffer containing 0.167 mg/mL o-dianisidine dihydrochloride and 0.0005% hydrogen peroxide. Absorbance was measured at 450 nm in 96-well flat bottom plates and MPO activity calculated as milliunits per volume of homogenate supernatant (mU/mL of supernatant).

Microscopy

Vaginal cell monolayers were propagated on glass cover slips within 24 well plates. Following a standard adherence assay (MOI = 100), monolayers were washed 6 times with DPBS and the cover slips were then removed from the trays. Cover slips were air-dried and heat fixed then subjected to a standard Gram stain protocol as described previously (Sheen et al., 2011). Paraffin embedded reproductive tracts were sectioned on a Leica RM 2125

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microtome at 5 μm and stained with a standard H&E staining protocol. All images were taken on a Zeiss upright microscope with attached Axiocam Icc3 camera at indicated magnification.

Statistical Analysis

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

Results

Differential gene expression profile of vaginal epithelium induced by GBS

To determine the response of vaginal epithelium to GBS colonization, we performed global transcriptional profiling on human vaginal epithelial cells (HVEC) during infection with WT GBS (strain A909, serotype Ia) or isogeneic Δ*covR* and Δ*cylE* mutant strains. As a control, we exposed HVEC to a nonpathogenic vaginal organism, *L. crispatus* (Wira et al., 2011). As can be expected, exposure to the native flora species, *L. crispatus*, did not induce an inflammatory response compared to media alone (Fig. 1A and Suppl. Table 1). However, when HVEC were infected with WT GBS, numerous immune pathways were activated including proinflammatory cytokines IL1-α and IL1-β, and chemokines involved in leukocyte recruitment and activation such as CCL20, IL8, CXCL1, and CXCL2 (Fig. 1A and Suppl. Table 1). A similar global pattern of gene induction was observed during infection with another WT (serotype V) GBS strain (data not shown). Of particular interest, infection with the Δ*covR* mutant induced higher levels of expression of several of these genes, including IL-8 (2-fold) and CXCL2 (5-fold) when compared to WT. GBS β-H/C has previously been shown to activate neutrophil recruitment in other models of infection (Doran et al., 2003), and here we show that infection with the Δ*cylE* mutant resulted in lower induction of genes required for leukocyte recruitment and activation as a whole, including 4-fold lower transcription of IL-8 and CXCL2, indicating that β-H/C mediates similar responses in the vagina. Moreover, infection with the Δ*covR* mutant resulted in the upregulation of 708 genes (>2-fold) in vaginal epithelial cells as compared to 310 or 125 genes induced by WT or Δ*cylE* infection respectively (Fig. 1B). These results illustrate the impact of β-H/C alone in the vaginal tract by comparing effects of WT and Δ*cylE*, and demonstrate a tremendous shift in vaginal epithelium response to GBS when CovR regulation is removed.

To verify the microarray data *in vitro*, we examined differences in transcript and protein levels of highly induced genes involved in neutrophil recruitment. Additionally, we examined the effect of infection with a Δ*covR*/Δ*cylE* double mutant to determine the proportion of increased inflammatory response contributed by increased β-H/C production in Δ*covR*. We observed that infection with the Δ*covR* mutant resulted in increased transcription of IL-8 and CXCL2 compared to WT infection, while transcription of CXCL1 and CCL20 was not significantly different. When comparing the response to WT and Δ*cylE* infection, loss of β-H/C reduced chemokine transcription of IL-8, CXCL1, CXCL2, and CCL20. Interestingly, transcription of all chemokines was also down regulated in the Δ*covR*/Δ*cylE* mutant compared to WT, highlighting the inflammatory role of β-H/C in human vaginal cells.

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

CovR moderates adherence and invasion in vaginal epithelial cells

Several GBS WT strains, including A909, have already been shown to readily attach to HVEC *in vitro* (Sheen et al., 2011, Jiang et al., 2012). To determine the effect of CovR regulation on GBS interaction with vaginal epithelium, we characterized attachment and invasion of WT, Δ*covR*, Δ*cylE*, and Δ*covR*/Δ*cylE* strains in HVEC using standard methods as previously described (Doran et al., 2005, Sheen et al., 2011). After 30 minutes of incubation, and multiple washes to remove nonspecific interactions, adherent GBS was quantified from monolayer lysates. We observed that both Δ*covR* ($p = 0.0068$) and Δ*covR*/Δ*cylE* ($p = 0.0136$) mutants adhered significantly more than WT, with Δ*covR* approaching 100% adherence of original inoculum (Fig. 3A). Adherence of the Δ*cylE* mutant did not significantly differ from that observed for WT GBS. Adherent GBS in association with vaginal epithelium were visualized by microscopy following multiple wash steps and Gram staining (Fig. 3C). Additionally, we quantified viable intracellular bacteria from cell lysates following a 2 hour incubation period and a 2 hour antibiotic treatment to kill any extracellular bacteria. Our data show that, when compared to WT, Δ*covR* and Δ*covR*/Δ*cylE* mutants exhibited reduced recovery of intracellular GBS (Fig. 3B). We recovered a relatively low level of intracellular bacteria (0.5% of the initial inoculum of WT GBS), but we were able to recover only 0.002% of the Δ*covR* mutant. Thus approximately 0.021% and 0.000016% of the adherent WT and Δ*covR* GBS, respectively, had invaded the intracellular compartment. Similar results were observed when CovR was deleted from serotype III GBS strain, COH1 (Suppl. Fig. 2A,B). Interestingly, the A909Δ*cylE* mutant had significantly more intracellular colony forming units than WT, indicating expression of β-H/C may decrease intracellular survival in vaginal epithelial cells, however, this effect was not seen in Δ*covR*/Δ*cylE*, suggesting that additional genes regulated by CovR impact GBS intracellular survival.

To verify that the Δ*covR* mutant phenotype could be complemented, we cloned the A909 *covR* sequence into a GBS expression vector and transformed this plasmid (p*covR*) into the Δ*covR* mutant as described in Experimental Procedures. GBS strains expressing more β-H/C typically produce more red pigment (Liu et al., 2004), such as our Δ*covR* mutants, and we observed a loss of pigmentation when the Δ*covR* mutant was complemented with p*covR* (Suppl. Fig. 3C). Additionally the complemented strain exhibited WT levels of β-H/C activity (data not shown). In both adherence and invasion assays, the complemented p*covR* strain differed significantly from the Δ*covR* strain, exhibiting significantly lower adherence

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

Role of CovR in vaginal persistence *in vivo*

Our *in vitro* data indicates that CovR regulation alters both vaginal epithelial interactions (Fig. 3) as well as host immune response (Fig. 1, 2). To confirm these results *in vivo*, we used a mouse model of GBS vaginal colonization adapted from previous work by our lab (Sheen et al., 2011) and others (Cox et al., 1993, Cheng et al., 2005). In rodents, normal flora load and novel bacterial colonization ability appear to peak at estrus (Koiter et al., 1977, Larsen et al., 1977, Furr et al., 1989). We found this to be true in our model of GBS colonization (Suppl. Fig. 4A,B). Consequently, we treated 8-week old CD1 mice with 17β-estradiol one day prior to bacterial inoculation. We inserted ~1×10^7 cfu GBS into the vagina, and on successive days, the vaginal lumen was swabbed and recovered bacteria quantified on agar plates to determine changes in bacterial load over time. The Δ*covR* mutant exhibited decreased persistence in the vaginal tract by day 2 post-inoculation, as significantly more mice treated with Δ*covR* had cleared GBS when compared to WT (p = 0.0108) (Fig. 4A) in the combined results of 4 independent experiments. Similarly, a significant reduction was observed when analyzing the isogenic Δ*covR* mutant in the COH1 background (Suppl. Fig. 2C). We also examined the colonization of other GBS mutants deficient in genes highly regulated by CovR in the A909 strain: Δ*cylE*, Δ*covR*/Δ*cylE,* and a double mutant in *fbsB* (SAK_0955) and adjacent gene (SAK_0956) encoding a hypothetical protein. Although in all instances, the mean number of mutant bacterial cfu recovered from mice decreased during colonization compared to the WT strain over the course of 5 days, the differences were not significant largely due to the high level of variation observed in this model (Suppl. Fig 4C). However, at day 5 there was an 8.0-fold decrease in mean bacterial load recovered from mice inoculated with Δ*covR* compared to only a 1.6-fold reduction in WT bacteria recovered.

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

Since we observed an increase in numerous innate immune factors in vaginal cells exposed to Δ*covR in vitro* (Fig. 1, 2), as well as a decreased vaginal persistence (Fig. 4A), we sought to characterize host immune response *in vivo*. Vaginal lavage from CD1 mice was collected for cytokine analysis by gently pipetting PBS in the vaginal lumen multiple times as described (Sonoda et al., 1998) prior to GBS inoculation and then throughout GBS colonization. Cytokine levels of murine chemokines KC and MIP-2, as well as IL-1β, were measured by ELISA. We observed large variation in cytokine concentrations between individual mice, which has been seen previously (Sonoda et al., 1998). Even so, by day 2 post-inoculation, significantly more mice inoculated with Δ*covR* secreted MIP-2 (11/12 vs. 4/12, p = 0.0094) and IL-1β (8/12 vs. 2/12, p = 0.0361) compared to WT (Fig. 4B). Additionally, more Δ*covR* treated mice secreted chemokine KC than WT treated mice (5/12 vs. 1/12), but this difference was not significant.

Since MIP-2 and KC are both key chemoattractants involved in the recruitment of neutrophils to the vaginal epithelium (Sonoda et al., 1998, Hickey et al., 2012), we assessed neutrophil activation and influx into the vaginal lumen by measuring neutrophil enzyme myeloperoxidase (MPO) during GBS colonization. MPO serves as an effective indication of

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neutrophil infiltration (Bradley et al., 1982). We observed that mice inoculated with Δ*covR* expressed significantly higher fold levels of MPO than mice treated with WT (Fig. 4C). To visualize neutrophil infiltration, we collected vaginal tissues 2 days post GBS inoculation for histopathologic analysis. Consistent with our previous results there were fewer mice colonized with Δ*covR* at day 2 post inoculation compared to WT (data not shown). At this time point, neutrophils were present in most of the mice, however, mice treated with Δ*covR* showed greater inflammation, epithelial rearrangement and hemorrhaging than WT and Δ*cylE* treated mice, with two representative images shown (Fig. 4D–I). Mice inoculated with Δ*cylE* exhibited more variation within the epithelium than either of the other groups (Fig. 4H–I), in combination with significantly fewer cfu recovered compared to WT (Fig. 4A), which may be explained by individual immune system differences between mice in response to a less virulent form of GBS.

The CXCL2 receptor contributes to host immune response *in vivo*

Thus far, we have demonstrated that infection with the Δ*covR* mutant results in a heightened inflammatory response *in vitro* and *in vivo*. Moreover, we have observed a decreased persistence of the Δ*covR* mutant in the vaginal environment compared to WT, and we hypothesized that enhanced immune response during infection with Δ*covR* is responsible for this difference. Because neutrophil chemokines were differentially induced by the Δ*covR* mutant, both *in vitro* and *in vivo*, we used mice that lack a receptor for these chemokines. We inoculated CXCr2 KO mice, formerly known as IL-8r KO mice, and control BALB/c mice with either WT GBS or the Δ*covR* mutant as described in Experimental Procedures. Mice were swabbed and lavage fluid collected daily to enumerate bacterial load and cytokine production respectively. GBS persisted longer in BALB/c mice than in CD1 mice (data not shown), but by day 5, mean cfu values, although not significant, were distinctly different with fewer Δ*covR* (4.1×10^5) recovered compared to WT (1.6×10^6) (Fig. 5A). However, we did not observe a difference in recovered bacteria between the WT and Δ*covR* over time in CXCr2 KO mice (Fig. 5A). Our results revealed that, like CD1 mice, WT BALB/c exhibited significantly increased MIP-2 ($p = 0.0372$) and KC ($p = 0.0328$) levels in mice receiving Δ*covR* as compared to WT by day 2 post-inoculation (Fig. 5B,C). At no time point examined did we observe significant differences in KC and MIP-2 levels in CXCr2 KO mice in either group (Fig. 5B,C). Furthermore, we did not observe neutrophils in vaginal lavage fluid of CXCr2 KO mice at any time point examined (Suppl. Fig. 5). In combination, this data suggests that increased host inflammatory response to Δ*covR* occurs at least in part by signaling through the CXCL2 receptor.

Discussion

GBS poses a severe threat to newborn infants worldwide. GBS propagates through vertical transmission as 50–70% of infants born to colonized mothers become colonized (Baker *et al*., 2001). A better understanding of mechanisms important for GBS colonization of the vaginal tract, identification of host and bacterial factors contributing to colonization, and genetic and environmental stimuli that promote GBS colonization and subsequent transmission is essential. Through combining our *in vitro* and *in vivo* models, we have continued to identify host and microbe features that impact GBS persistence in the vaginal environment. Herein, we have demonstrated that the CovR/S regulatory system is necessary for limiting the expression of virulence factors during vaginal colonization, thereby reducing the host innate immune response to promote colonization.

GBS CovR/S has been shown to positively and negatively regulate a variety of genes, of which, *cylE* (β-H/C) is one of the most highly repressed (Lamy et al., 2004, Jiang et al., 2008, Lembo et al., 2010). Host global transcriptional profiling during infection with WT, Δ*covR*, or Δ*cylE* GBS revealed the β-H/C toxin as a key mediator in provoking an acute

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inflammatory response in the vaginal epithelium, which was further confirmed by analysis of specific gene transcripts during infection with a Δ*covR*/Δ*cylE* mutant. Most dramatic was the shift in gene induction profiles when CovR regulation was absent, including chemokines IL-8 and CXCL2, which are involved in neutrophil activation and recruitment (Baggiolini et al., 1994). Additionally, vaginal epithelial cells responded to GBS infection with increased production of proinflammatory factors such as IL-1β, which promotes neutrophil recruitment and bacterial clearance (Miller et al., 2007), GM-CSF (CSF2), which controls the production, differentiation, and function of granulocytes, macrophages and other leukocytes (Zhan et al., 2012), and oxidized low density lipoprotein (lectin-like) receptor 1 (OLR1), a leukocyte cell adhesion molecule (Honjo et al., 2003). Furthermore, GBS infection stimulated an increased production of extracellular matrix modifiers, including those associated with inflammation and epithelial disruption such as VEGF, MMP1, MMP3, MMP9, plasminogen activators and HAS3 (Mannelqvist et al., 2011, Yang et al., 2011, Noel et al., 2012, Rilla et al., 2012, Rodriguez-Flores et al., 2012). While these proteins are associated with inflammation and neutrophil migration, their expression may also allow GBS access to underlying tissues and bloodstream by disruption of the epithelial barrier.

CovR regulates many secreted or cell wall and envelope-associated GBS factors (Lamy et al., 2004, Lembo et al., 2010); thus logically, CovR regulation may impact host cell interactions. We have shown previously that the A909 CovR deficient strain exhibits increased adherence and decreased invasion in brain endothelium and lung epithelium (Lembo et al., 2010). Although the exact mechanism is not known, we reveal that loss of CovR in two different GBS serotypes, Ia and III, promotes adherence to and limits invasion of vaginal epithelial cells independent of *cylE* expression. Additionally, we observed that WT GBS adherence and invasion levels can be restored by complementing the CovR deficient strain. However, the invasion phenotype was not completely rescued, which may suggest a more complex regulation of this function. Consistent with our results, a recent study demonstrated that a CsrR/S (CovR/S) deficient strain exhibited increased adherence to epithelial and abiotic surfaces (Park et al., 2012). Environmental signals such as vaginal pH may alter CovR regulation as neutral, and not acidic, pH enhanced GBS binding to vaginal epithelial cells, and this was partially dependent on CsrR/S (CovR/S) (Park et al., 2012). Furthermore, CovR/S down regulates virulence factors such as *cylE* and *scpB* (a C5a peptidase) in acidic pH (Santi et al., 2009). The enhanced adherence observed in the CovR-deficient strain may prove detrimental to colonization, as increased host-microbe interaction may provoke the increased immune activation and cytokine production that we observed. Additionally, host intracellular responses may also explain the decreased invasion seen in Δ*covR* and Δ*covR*/Δ*cylE* mutants (Fig. 3B), because it has been demonstrated that, although a CovR deficient mutant was phagocytosed more efficiently by macrophages, it exhibited decreased ability to survive intracellularly (Cumley et al., 2012). Therefore, we cannot exclude the possibility that CovR regulation effects both bacterial invasion and intracellular survival, which consequently may impact colonization.

Other CovR-regulated factors, FbsA and FbsB, which are fibrinogen binding proteins, have been shown to contribute to bacterial attachment and invasion of other host cells (Gutekunst et al., 2004, Schubert et al., 2004, Tenenbaum et al., 2005). In the A909Δ*covR* mutant, these genes are highly expressed; transcription of FbsA (SAK_1142) was increased 25-fold, and FbsB (SAK_0955) and an adjacent gene (SAK_0956) encoding a hypothetical protein was increased 151-fold and 157-fold respectively (Lembo et al., 2010). We constructed a mutant lacking FbsB and the adjacent gene (Δ*fbsB*/Δ*0956*), and subsequent analysis revealed that it exhibited similar levels of vaginal cell adherence and invasion to the WT strain (data not shown), and further, it was not cleared from the mouse vagina as readily as the Δ*covR* mutant (Suppl. Fig. 4C). This suggests that these factors are not responsible for the altered

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adherence and invasion phenotype of the CovR deficient strain. However, fewer Δ*fbsB*/Δ*0956* bacteria were recovered from the mouse vaginal tract compared to WT (Suppl. Fig. 4C) indicating fibrinogen binding may contribute to GBS colonization *in vivo*.

Murine chemokines KC (CXCL1) and MIP-2 (CXCL2) are both functional homologs of human CXCL8 (IL-8) and bind to CXCr2 (IL-8 receptor homolog) on neutrophils, resulting in their migration to the site of chemokine production (Olson et al., 2002). To determine the functional role of chemokine signaling during GBS colonization, we utilized CXCr2 KO mice in our murine vaginal model and observed no difference in cytokine levels between GBS WT or Δ*covR* groups, starkly contrasting our results in CD1 and BALB/c mice. Cytokine levels in CXCr2 KO mice steadily increased in vaginal lavage over the time course examined when compared to BALB/c mice, indicating that chemokine production was not directly affected by the lack of the receptor. Steady increase of MIP-2 levels in CXCr2 KO mice has been observed in other models of pathogenic infection and is believed to occur because of lack of effective down-regulation and/or continued stimuli (Hang et al., 2000); KC was not examined in this particular study. Of note, CXCr2 KO mice did not have neutrophils visible in vaginal lavage fluid either prior to or during the experimental period when viewed under the microscope, whereas neutrophils were present in the control mice, both prior to inoculation as part of the estrous cycle, and following GBS inoculation (Suppl. Fig. 5). This supports previous work showing MIP-2 expression is necessary to recruit neutrophils to the vaginal lumen during the normal estrous cycle (Sonoda et al., 1998). This data also suggests that the increased cytokine levels observed in BALB/c mice treated with Δ*covR* may be explained by paracrine signaling and secretion by neutrophils that have infiltrated the vaginal epithelium.

Within the vaginal tract, mucosal epithelial immunity is tightly controlled by the estrous cycle, with sex steroids affecting all aspects of innate and adaptive immunity, whether directly or indirectly (Hickey et al., 2011). In human vaginal fluid samples, IL-8 and IL-1β production positively correlate with pattern-recognition proteins, and these cytokines levels vary with stage of estrous cycle (Macneill et al., 2012). In the mouse, neutrophils are recruited after the estrogen peak in each estrous cycle with MIP-2 (CXCL2) controlling the majority of neutrophil migration (Sonoda et al., 1998). KC (CXCL1) and IL-1β are also implicated in this process (Hickey et al., 2012). In our mouse model of vaginal colonization, we have observed GBS persist for several weeks (Sheen et al., 2011), or up to several months (Patras, Doran, unpublished data), depending on the stage of estrus, estradiol treatment regime, or specific mouse strain used. We have observed nearly 100% colonization of mice within the first 24 hours post-inoculation, and subsequently, GBS bacterial strain, immune response, and normal flora determine the course of persistence (Patras, Doran, unpublished data). Furthermore, we have also observed that mice were either chronically or intermittently colonized, mimicking human GBS persistence (Yow et al., 1980). We recognize that there are many differences between humans and our murine model counterparts including vaginal pH, normal flora, length of estrous cycle, and immune repertoire. However, given the success of other vaginal disease mouse models (Furr et al., 1989, Jerse, 1999, Escario et al., 2010), and our findings thus far, we believe this model has useful applications in studying host-GBS interactions in the vaginal environment. Herein, we show CovR deficiency hinders GBS persistence *in vivo*, coinciding with increased innate immune markers known to recruit neutrophils, which have been described as first responders in other GBS infection models (Doran et al., 2003). To our knowledge, this is the first time vaginal immune response to GBS has been characterized *in vivo*.

In summary, we have shown that CovR deficiency provokes increased inflammatory response both *in vitro* and *in vivo,* as well as increased adherence to a vaginal epithelial cell line. This heightened response may contribute to the decreased persistence observed in our

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mouse model of colonization. Finally, a functional chemokine receptor, CXCr2, may contribute to the differential increase in host immune signaling pathways in response to loss of CovR regulation *in vivo*. Collectively, our work indicates that GBS virulence regulation by the CovR/S two-component system is critical for niche establishment and maintaining a commensal state in the female vaginal tract.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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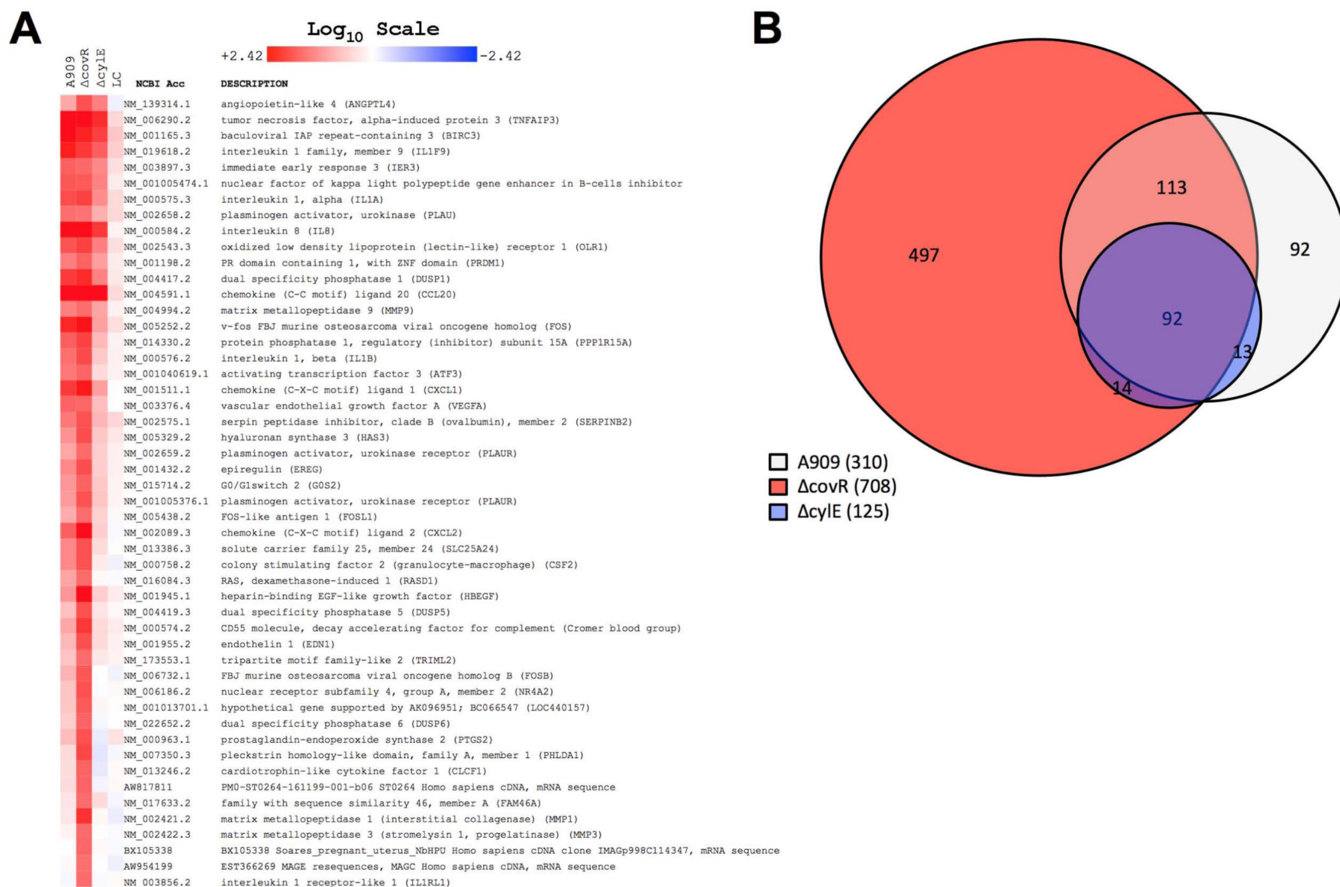

Figure 1. Microarray of gene expression levels in HVEC upon infection with A909, Δ*covR*, Δ*cylE* or *Lactobacillus crispatus* (LC)

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

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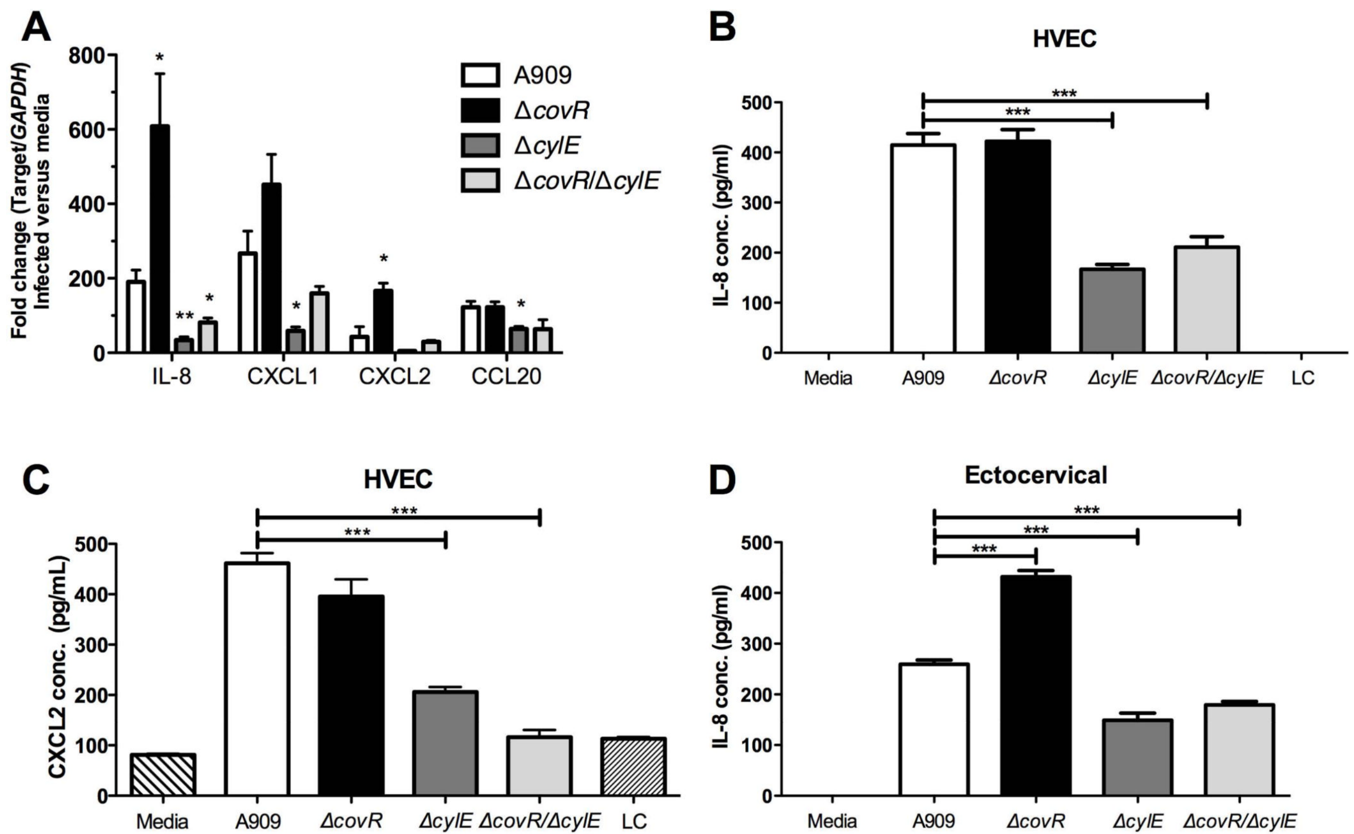

Figure 2. mRNA expression of IL-8, CCL20, CXCL1 and CXCL2 and protein production of IL-8 in HVEC and ectocervical cells upon infection with WT and mutant strains

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

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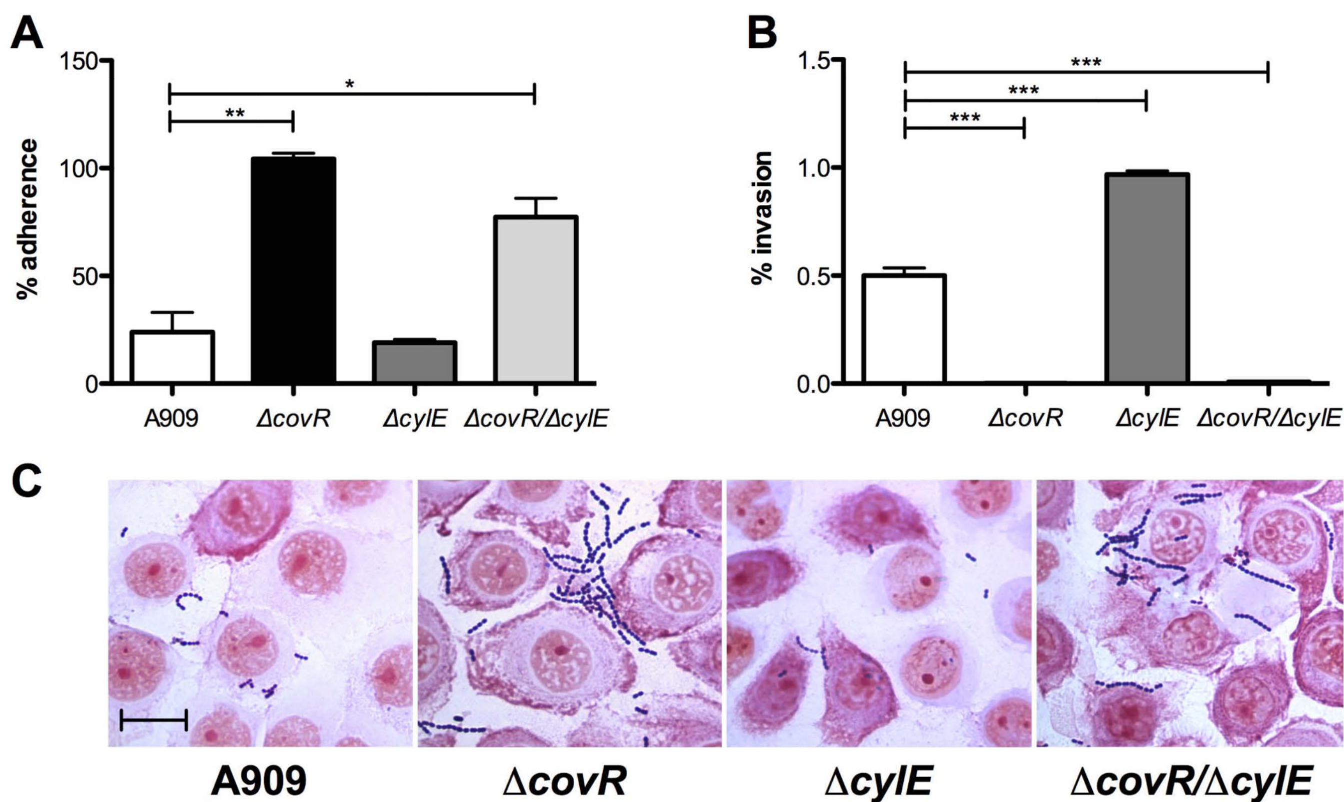

Figure 3. GBS interaction with vaginal epithelium *in vitro*

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

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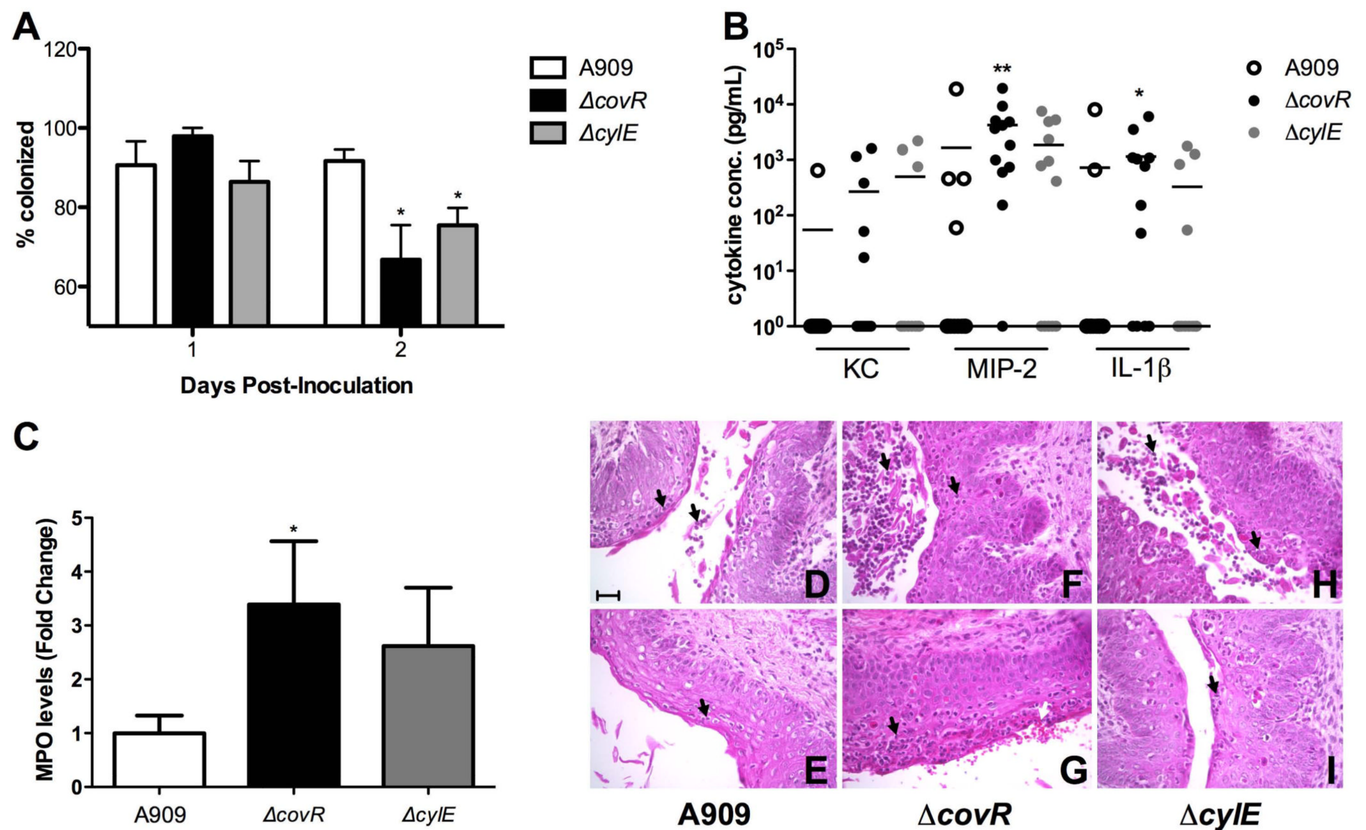

Figure 4. CovR regulation affects host immune response *in vivo* soon after inoculation

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

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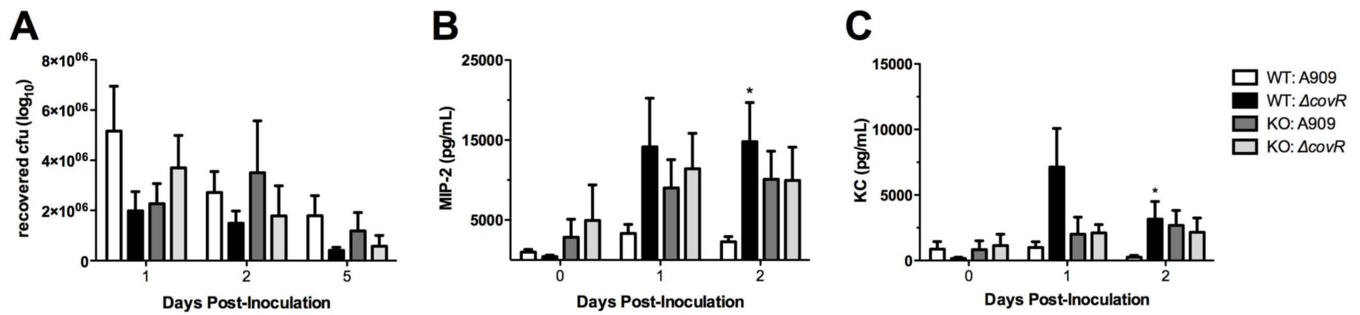

Figure 5. Role of the CXCL2 receptor during GBS infection *in vivo*

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