Cervicovaginal Bacteria Are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract

Highlights

- Most African women studied had genital communities with low *Lactobacillus* abundance

- Unlike the gut, high-diversity cervicovaginal communities are pro-inflammatory

- Specific bacteria induce cytokine production from genital APCs and epithelial cells

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In Brief

Vaginal colonization by a single bacterium, *Lactobacillus*, is considered healthy. Kwon and colleagues show that in a cohort of asymptomatic African women, the majority have high-diversity genital bacterial communities that are closely associated with local inflammation. This work suggests specific genital bacteria might increase HIV acquisition risk in women.

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Cervicovaginal Bacteria Are a Major Modulator of Host Inflammatory Responses in the Female Genital Tract

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SUMMARY

Colonization by *Lactobacillus* in the female genital tract is thought to be critical for maintaining genital health. However, little is known about how genital microbiota influence host immune function and modulate disease susceptibility. We studied a cohort of asymptomatic young South African women and found that the majority of participants had genital communities with low *Lactobacillus* abundance and high ecological diversity. High-diversity communities strongly correlated with genital pro-inflammatory cytokine concentrations in both cross-sectional and longitudinal analyses. Transcriptional profiling suggested that genital antigen-presenting cells sense gram-negative bacterial products in situ via Toll-like receptor 4 signaling, contributing to genital inflammation through activation of the NF-κB signaling pathway and recruitment of lymphocytes by chemokine production. Our study proposes a mechanism by which cervicovaginal microbiota impact genital inflammation and thereby might affect a woman's reproductive health, including her risk of acquiring HIV.

INTRODUCTION

The female genital tract (FGT) maintains a finely-tuned immune response that balances reproductive tolerance with protection against genital infections. While inflammatory responses are beneficial and required to effectively eliminate several sexually transmitted infections (STIs), the presence of elevated genital inflammation in women prior to HIV exposure paradoxically increases the risk of disease acquisition (Lajoie et al., 2012; Masson et al., 2015; Morrison et al., 2014). Studies of the microbial causes of this inflammation have primarily focused on established pathogens such as *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, HSV-2, and *Trichomonas vaginalis*. The potential role of commensal cervicovaginal bacteria, which number ~10^8 per gram of vaginal fluid (Delaney and Onderdonk, 2001), in modulating immune responses in the FGT is largely unknown.

The bacterial microbiome of the healthy FGT is thought to be exceedingly simple, predominated by a single *Lactobacillus* species in the majority of white premenopausal women (Chaban et al., 2014; Drell et al., 2013; Huttenhower, 2012; Ravel et al., 2011; Zhou et al., 2007). These lactobacilli benefit the host by inhibiting the growth of pathogenic bacteria and fungi through the production of bacteriocins, lactic acid, and hydrogen peroxide (Aroutcheva et al., 2001; Ghartey et al., 2014). Bacterial vaginosis (BV) is an alteration of microbial communities in this ecological niche in which lactobacilli are replaced by *Gardnerella* and *Mobiluncus* species. BV is associated with a 1.5- to 2-fold increased risk of acquiring *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and HIV (Atashili et al., 2008; Brotman et al., 2010), which raises the question of whether specific genital microbial communities in asymptomatic women might more broadly modulate disease susceptibility.

To determine the contribution of cervicovaginal microbiota to genital inflammation, we evaluated a cohort of asymptomatic HIV-negative young South African women. Only a minority of
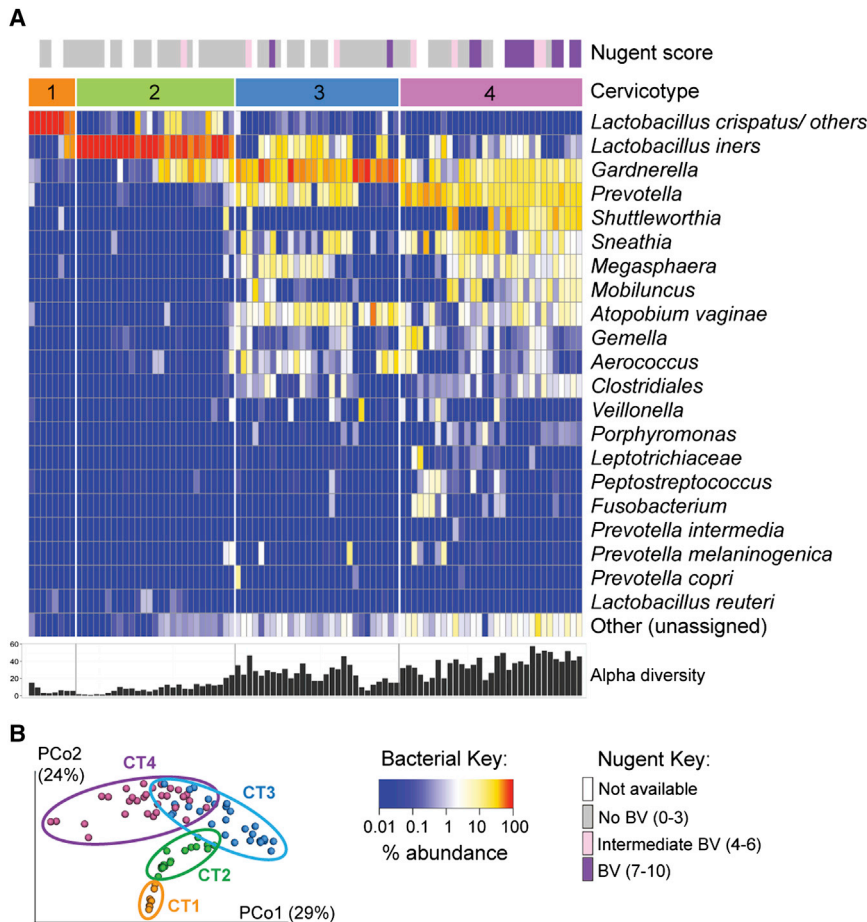
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(A) Heatmap of bacterial taxa identified by 16S rRNA V4 sequencing of cervical swabs collected from 94 women. Cervicotypes (CTs) were determined based on the dominant species: *Lactobacillus crispatus* (CT1), *L. iners* (CT2), *Gardnerella* (CT3), and mixed microbial community containing *Prevotella* (CT4). Nugent scores and bacterial alpha diversity are also shown.

(B) Principal coordinates analysis using the weighted UniFrac distance metric on the same 94 samples, colored by CT.

See also Figure S1 and Table S1.

observed bacterial communities into four distinct community types based on the dominant bacterial species, herein referred to as "cervicotypes" (CTs). CT1 was primarily composed of non-*iners* *Lactobacillus* (i.e., higher percentage of sequencing reads from non-*iners* *Lactobacillus* than *L. iners*, *Gardnerella*, or *Prevotella*); CT2 was *Lactobacillus iners* dominant; CT3 had *Gardnerella* dominance; and CT4 lacked a consistent dominant species but communities all included *Prevotella* (Figures 1A and S1). Visualization of the same samples using a principal coordinates plot, which represents the phylogenetic distance between samples, supported the dominance-based clustering, though CT3 and CT4 are a continuum (Figure 1B).

We found that only 37% of participants had *Lactobacillus* dominant cervicovaginal communities. This is in contrast to published reports of white and black women in developed countries in which 90% and 62% of women respectively demonstrated *Lactobacillus* dominance (Ravel et al., 2011; Zhou et al., 2007). Of those women with *Lactobacillus* dominance in our study, 77% primarily had *Lactobacillus iners* (CT2). *L. iners* is biologically distinct from other lactobacilli because of its unique adaptation to survive with diverse community members (Macklaim et al., 2013) and greater pathogenic potential (Doerflinger et al., 2014; Rampersaud et al., 2011).

Of the 63% of women in our cohort who did not have *Lactobacillus* dominance, 45% had *Gardnerella*-dominant communities (CT3). The remaining 55% of women did not have a consistent predominant bacterial taxon, though each community was found to have at least 10% *Prevotella* abundance (CT4). Additionally, only half of the women in CT4 had BV, as measured by the Nugent criteria, which assesses a Gram-stained vaginal wet prep based on bacterial morphology; none of these women reported symptoms. Overall, the bacterial community structures seen in these asymptomatic young South African women were characterized by a predominance of communities with high ecological diversity and low abundance of non-*iners* *Lactobacillus*.

study participants had *Lactobacillus*-dominant cervicovaginal communities, despite a lack of clinical symptoms, redefining what is considered to be normal in this region. We found a strong in vivo relationship between high-diversity bacterial communities lacking *Lactobacillus* dominance and genital pro-inflammatory cytokine levels. We identified specific bacterial species within the high-diversity communities that elicit pro-inflammatory cytokines and provide evidence that endocervical antigen-presenting cells (APCs) sense microbial lipopolysaccharide (LPS) and produce myriad pro-inflammatory cytokines and T cell chemoattractants. Our data provide important insight into the mechanism by which bacterial microbiota impact host immunity and suggest potential interventions to reduce disease susceptibility in women in sub-Saharan Africa.
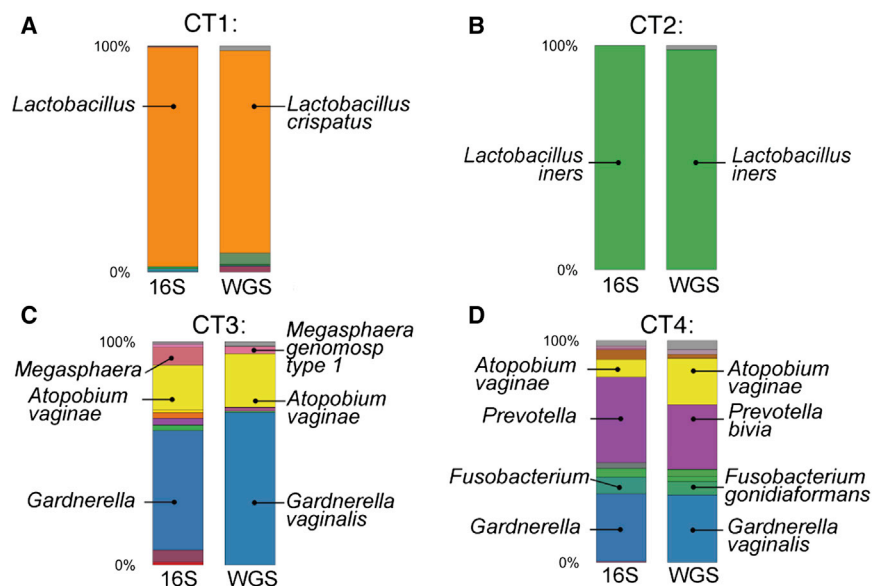
RESULTS

A Minority of South African Women in FRESH Have *Lactobacillus* Dominant Genital Communities

We began by assessing the baseline bacterial microbiome in participants from the FRESH (Females Rising through Education, Support and Health) study, a cohort enrolling HIV-negative, 18- to 23-year-old, black South African women. Following isolation of nucleic acid from cervical swabs, we sequenced variable region 4 (V4) of the bacterial 16S rRNA gene to assess bacterial abundances (Caporaso et al., 2012) (Figure 1A). We clustered the

We next confirmed the 16S rRNA gene sequencing results and resolved specific community members at a species level by performing whole-genome shotgun sequencing (WGS) on cervical bacterial DNA from a subset of 12 women representing all four CTs (Figure S2A). WGS has proven useful for species-level taxonomic resolution when different bacterial species share nearly identical 16S rRNA variable regions (Jaspers and Overmann, 2004; Segata et al., 2012). The taxonomic classification by WGS had good agreement with 16S-based classification and STI PCR results (Figures 2 and S2B). The additional species-level resolution provided by WGS also revealed that both of the women with CT1 analyzed by WGS had *Lactobacillus crispatus* dominant microbiota (Figures 2A and S2B). We extended the analysis to every woman within CT1 by using taxonomic identification by oligotyping, a method that detects patterns in subtle nucleotide variations within the 16S rRNA gene (Eren et al., 2011) and found that nearly all women had identical sequences that aligned to *L. crispatus* (Figure S2C), further validating that CT1 was mostly composed of *L. crispatus*. We therefore demonstrated close agreement between taxonomic classifications obtained by 16S rRNA sequencing and WGS and further used WGS to provide species level resolution of specific bacterial community members.

Cervicovaginal Bacterial Communities Are Not Associated with Sexually Transmitted Infections, Hormonal Contraceptive Usage, or Sexual Behavior

Women with BV have been shown to have a higher incidence and prevalence of sexually transmitted infections (STIs) (Brotman et al., 2010; Wiesenfeld et al., 2003), to be less likely to use hormonal contraceptives (Bradshaw et al., 2013a), and to be more likely to engage in high-risk sexual behavior (Bradshaw et al., 2013b). However, we did not find a difference in the prevalence of an active STI or STI symptoms between cervicotypes, though the association with *N. gonorrhoeae* was difficult to ascertain due to its low prevalence in this group (Table S1). Additionally, we saw no difference in hormonal contraceptive method, condom use, or reported sexual activity (Table S1).

Thus, there was no evidence that the high ecological diversity in CT4 was due to behavioral, infectious, or exogenous hormonal etiologies.

### Large Variation in Baseline Genital Immune Activation Is Not Explained by STIs

Genital immune activation has been described as an important risk factor for disease of the FGT, such as obstetric complications (Inglis et al., 1994; Lockwood et al., 1994) and HIV acquisition (Lajoie et al., 2012; Masson et al. 2015; Morrison et al., 2014). We therefore assessed the cohort's baseline genital immune activation by measuring the concentrations of 17 soluble cytokines in cervicovaginal lavage (CVL) fluid from all participants who had completed at least one mucosal sampling. The cytokine panel included canonical pro-inflammatory cytokines (interleukin-1α [IL-1α], IL-1β, tumor necrosis factor alpha [TNF-α]), interferons (IFN-γ, IFN-α2), chemokines (MIP-1α, MIP-1β, RANTES, IP-10, IL-8), regulators of inflammation (IL-1 receptor antagonist [IL-1RA], IL-10), differentiation markers (IL-4, IL-12p70), and activation and proliferation markers (soluble CD40L, FLT-3L, IL-2). There was a wide range in CVL pro-inflammatory cytokine levels, with over a 1,000-fold difference in some cytokines (e.g., IL-8 and IL-1α) among participants. We performed unsupervised clustering to visualize inflammatory patterns (Figure 3A) and found strong positive correlations (Spearman's p > 0.4) between 45% of cytokine pairs (Figure S3A).

To reduce the dimensionality of the cytokine dataset to a smaller number of covarying components, we performed principal component analysis (PCA) on the normalized cytokine concentrations. We found that the first principal component (PC1) explained 41% of the variation in cytokines; PC1 represented the general presence of inflammatory cytokines, with high and nearly equal loading by all cytokines except for IL-1RA and IP-10, which had a negligible contribution (Figure S3B).

We next asked whether women with the highest levels of cytokine inflammation had active STIs. *Chlamydia trachomatis* was the most prevalent STI in this cohort, with 13% of women testing positive at the first visit. We found that women with *C. trachomatis* had higher inflammation than women without an STI (p = 0.0226, Mann-Whitney test) but saw no trend with *N. gonorrhoeae* (Figures S3C–S3E), consistent with previous studies (Hedges et al., 1998; Rasmussen et al., 1997). Of the 35 women in the top quartile of inflammation, 75% of them did not have a detectable STI (Figure 3B). Cytokine levels also did
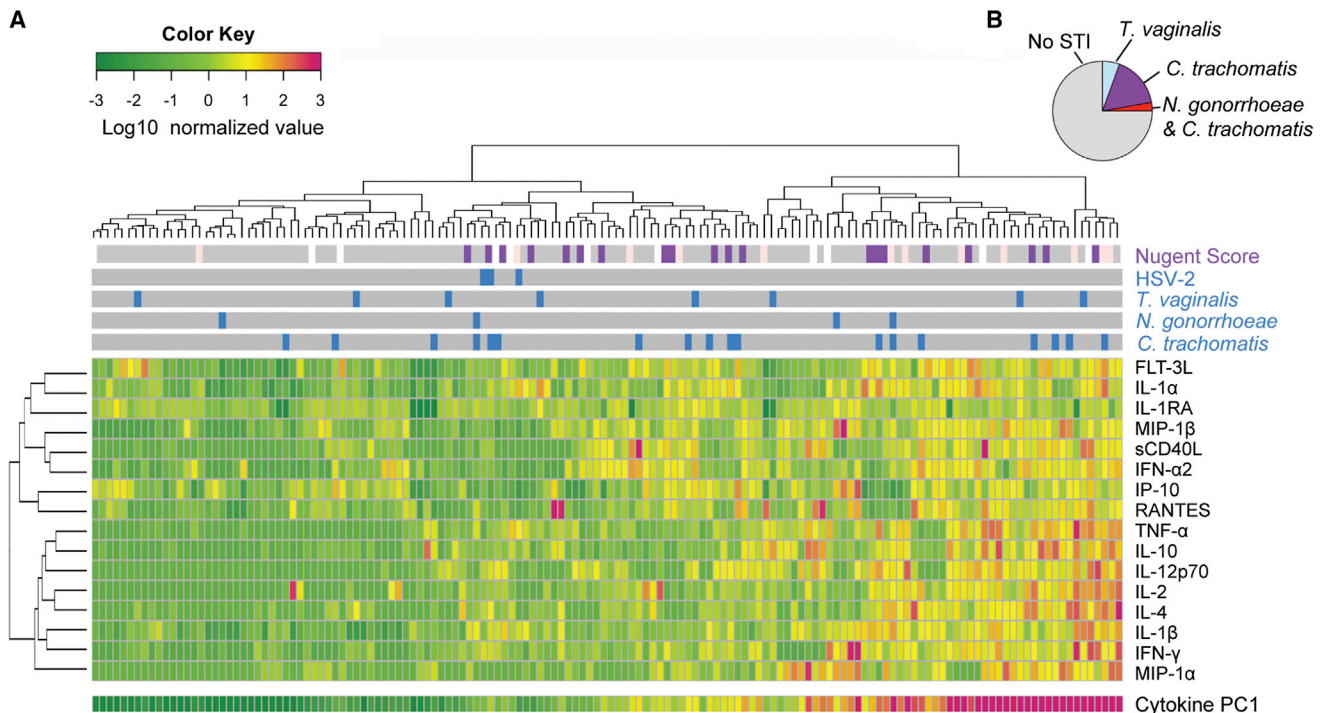
Figure 3. Asymptomatic Women Display a Broad Range of Baseline Genital Inflammation that Is Not Explained by STIs

(A) Heatmap of cervicovaginal lavage cytokines from 146 women, each represented by a column. Nugent scores and active STIs (blue: *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and/or HSV-2 positive; gray: negative for that STI) are also displayed. Principal component (PC) analysis was performed on the normalized cytokine concentrations and the first PC (PC1) explained 41% of variation.

(B) Pie chart of the STI prevalence in women in the highest quartile of inflammation (n = 35).

See also Figure S3 and Table S2.

not correlate with sexual frequency, condom use, hormonal contraception use, or self-reported STI symptoms (Fisher's exact test with p = 0.05 significance level, Table S2). Thus, the cause of elevated genital inflammation in the majority of women remained unexplained.

High-Diversity Bacterial Communities Are Strongly Associated with Pro-inflammatory Genital Cytokines

To assess whether microbial communities might account for non-STI associated genital inflammation, we compared genital pro-inflammatory cytokine levels among CTs, with the hypothesis that *Lactobacillus crispatus* dominant CT1 would have the lowest inflammation due to the beneficial effects attributed to *L. crispatus* (Ghartey et al., 2014). To determine aggregate differences in cytokine concentrations, we first examined the value of cytokine PC1 in each CT. There was a highly significant difference across the cervicotypes (p < 0.0001); between CT1 and CT4 (p < 0.01); CT2 and CT4 (p < 0.001), and between CT3 and CT4 (p < 0.05) (Kruskal-Wallis with post-test, Figure 4A), even after excluding women with any STIs (Figure S4A).

To determine how strongly the presence of CT4, STIs, or BV associated with inflammation (defined by highest quartile of cytokine PC1), we calculated the odds ratios for each and found that only CT4 was significantly associated with cytokine PC1 (OR 4.33, 95% CI: 1.575 –11.92; p = 0.0046), though there

was a trend for PCR-positive chlamydia (OR 2.380, 95% CI: 0.8497 – 6.666; p = 0.1329) (Figure 4B). This indicated that women with CT4 were over four times more likely to have elevated genital pro-inflammatory cytokines than those with CT1–CT3, and that CT4 was a better predictor of inflammation than STIs or BV.

We next determined which of the cytokines that comprise PC1 were different between CTs. We observed significant increases in IL-1α, IL-1β, TNF-α, IFN-γ (p < 0.001, Figures 4C–4F), IL-10 (p < 0.01, Figure 4G), and IL-8 (p < 0.05, Figure 4H), as well as IL-12p70, IL-4, and FLT-3L (p < 0.01, Figures S4B–S4D) in CT4 relative to CT1. Even after excluding women with any STIs, individuals with CT4 still had significantly higher levels of each of these cytokines relative to those with CT1 (Figures S4E–S4M). We observed a less pronounced increase in cytokines in CT3, with significantly higher IFN-γ than CT1, and a trend toward higher TNF-α, IL-8, and IL-10. Thus, the highly diverse bacterial communities in CT4, and to a lesser extent CT3, were strongly associated with the presence of multiple pro-inflammatory cytokines, suggesting that specific genital bacteria could induce a robust local immune response.

Longitudinal Changes in Genital Microbial Communities Correlate with Pro-inflammatory Cytokines

Because of the close association between genital bacterial communities and inflammation in the cross-sectional analysis,
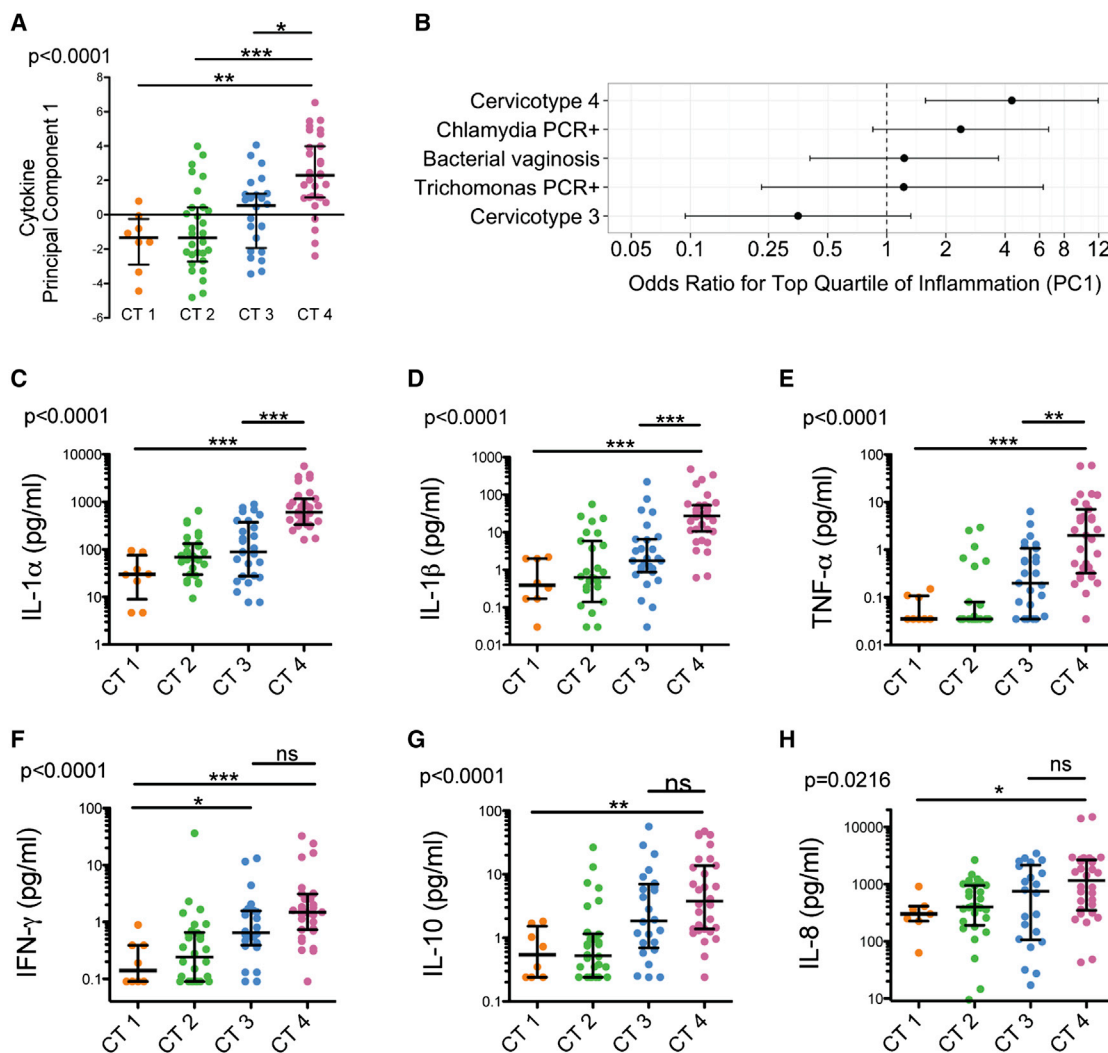
Figure 4. Genital Pro-inflammatory Cytokine Levels Vary Significantly with Microbial Community Structure and Most Strongly Correlate with CT4 Bacterial Communities

(A) Cytokine PC1 values from women with bacterial communities CT1–CT4.

(B) Odds ratios and 95% confidence intervals representing the likelihood that a woman with cervicotype 4, an active chlamydia infection, bacterial vaginosis (Nugent score > 7), an active Trichomonas infection, or cervicotype 3 is within the top quartile of pro-inflammatory cytokine levels (as determined by cytokine PC1) versus below the 75th percentile.

(C–H) Cervicovaginal lavage IL-1α, IL-1β, TNF-α, IFN-γ, IL-10, and IL-8 concentrations in women with bacterial communities CT1-4. Median and IQR are shown. Significance levels were determined by a Kruskal-Wallis test, and asterisks denote post-test significance level (*p < 0.05; **p < 0.01; ***p < 0.001; n = 94). See also Figure S4.

we assessed the stability of these factors over time. We performed 16S rRNA sequencing and measured soluble cytokines on matched longitudinal cervical swabs and CVLs from all women who had been followed for 6–12 months at the time of analysis. In 58% of sequential time points, women remained in the same CT (Figure S5A). Of those who had changes in microbial communities, there was a close association between CTs and IL-1α, IL-1β, and TNF-α, which we focused on because they had the strongest associations with CTs in the cross-sectional analysis. Two representative individuals who experienced CT changes are shown in Figure 5A (also see Figure S5, and associated metadata in Table

S3). When we assessed sequential time points for which there was not a CT change, we did not find a significant difference in the canonical pro-inflammatory cytokines (p > 0.05, one-tailed Wilcoxon matched pairs test; Figure 5B). However, there were significant increases in IL-1α (p = 0.033), IL-1β (p = 0.042), and TNF-α (p = 0.030) in adjacent time points with transitions between a lower and higher CT (one-tailed Wilcoxon matched pairs test; Figure 5C). Thus, intra-individual changes in genital bacterial communities were tightly linked to inflammatory changes and further support the reciprocal relationship between bacterial communities and genital inflammation.
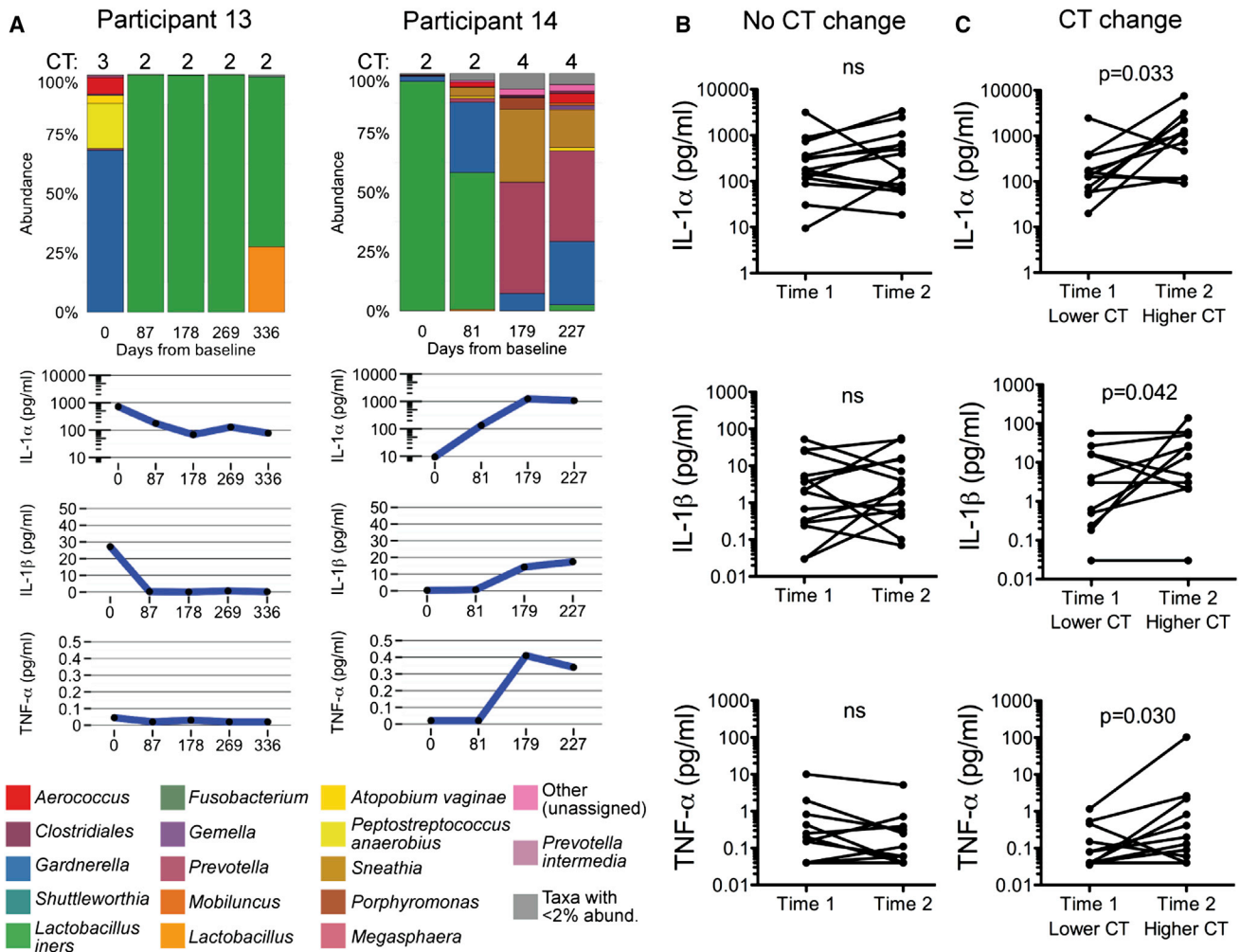
Figure 5. Intraindividual Longitudinal Genital Microbiome Changes Correlate with Pro-inflammatory Cytokine Levels

(A) Cervicovaginal IL-1α, IL-1β, and TNF-α concentrations with bacterial taxa identified by 16S rRNA sequencing from matched longitudinal cervical swabs and CVLs collected from two representative participants, #13 and #14.
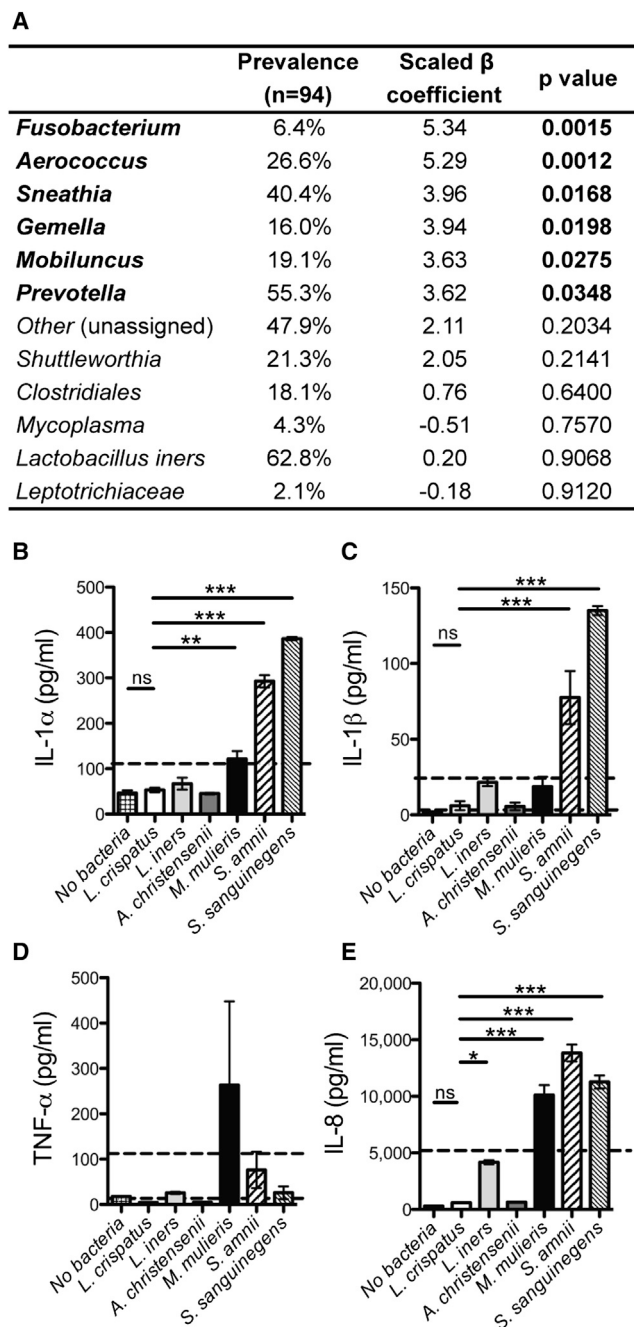
(B and C) Cervicovaginal IL-1α, IL-1β, and TNF-α concentrations from serial time points with either no change in CT (B) or a change (C) (one-tailed Wilcoxon matched pairs test). The order of time points was standardized such that the lower cervicotype was entered as the first time point and the higher cervicotype was entered as the second time point, regardless of the actual chronological order. Any transition, e.g., from CT1 to CT2 and from CT3 to CT4, was considered to be a CT change.

See also Figure S5 and Table S3.

Identification of Bacterial Species that Cause Pro-inflammatory Cytokine Secretion

We next asked which bacteria in CT4 were most highly correlated with inflammation. To select bacterial taxa to use in a multivariate model, we performed univariate Spearman correlations between cytokine PC1 and each taxon found with over 0.1% abundance (Figure S6A). The 11 taxa with highly significant correlations (p ≤ 0.005) were included as predictor variables in the multivariate analysis. Because many taxa were highly correlated (Figure S6B), the ridge method was used to correct for multicollinearity and revealed six genera significantly correlated with cytokine PC1: *Fusobacterium*, *Aerococcus*, *Sneathia*, *Gemella*, *Mobiluncus*, and *Prevotella* (Figure 6A). Species-level identification from whole-genome sequencing (Figure S2A) provided a set of candidate bacteria to test in vitro.

We used these candidates to test the causative relationship between individual bacterial species and genital inflammation with an in vitro epithelial cell co-culture assay, because epithelial cells play critical roles as anatomic barriers and in the innate immune response to bacteria in the FGT (Fichorova et al., 2011; Wira et al., 2005). We co-cultured immortalized human vaginal epithelial cells with bacterial isolates and measured secreted pro-inflammatory cytokines in the supernatant. Consistent with our in vivo findings, *Prevotella amnii*, *Mobiluncus mulieris*, *Sneathia amnii*, and *Sneathia sanguinegens* induced higher levels of IL-1α, IL-1β, and IL-8 than *Lactobacillus crispatus* (Figures 6B–6E, Table S4). *Prevotella*, the genus consistently present in CT4, was able to induce significant cytokine secretion even at an input colony-forming unit (CFU) as low as 2 log₁₀ (Table S4). Additionally, *L. iners* induced moderate IL-8 secretion,

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	Prevalence (n=94)	Scaled β coefficient	p value
Fusobacterium	6.4%	5.34	**0.0015**
Aerococcus	26.6%	5.29	**0.0012**
Sneathia	40.4%	3.96	**0.0168**
Gemella	16.0%	3.94	**0.0198**
Mobiluncus	19.1%	3.63	**0.0275**
Prevotella	55.3%	3.62	**0.0348**
Other (unassigned)	47.9%	2.11	0.2034
Shuttleworthia	21.3%	2.05	0.2141
Clostridiales	18.1%	0.76	0.6400
Mycoplasma	4.3%	-0.51	0.7570
Lactobacillus iners	62.8%	0.20	0.9068
Leptotrichiaceae	2.1%	-0.18	0.9120

Figure 6. Bacterial Species Highly Correlated with Pro-inflammatory Cytokines In Vivo Also Stimulate Vaginal Epithelial Cells to Produce the Same Cytokines In Vitro

(A) Ridge regression analysis with cytokine PC1 as the response variable and bacterial abundances as the predictor variables. The bacterial prevalence among the 94 subjects is also shown; taxa must have an abundance of at least 1% within a sample to be considered present.

(B–E) IL-1α, IL-1β, TNF-α, and IL-8 secretion by vaginal epithelial cells after in vitro application of 15 $\log_{10}$ CFU/cm^2 of each bacterial species. MALP-2 (a TLR 2/6 agonist; 25 nM) treatment was used as a positive control (value denoted by dashed line). p values were determined by a Dunnett test, compared to *Lactobacillus crispatus*. Data are shown as mean and SEM, with two biological replicates.

See also Figure S6 and Table S4.

consistent with reports that it can have moderate pro-inflammatory activity, whereas *L. crispatus* and *Aerococcus christensenii* did not elicit any cytokine secretion relative to the absence of bacteria. Low levels of TNF-α were detected in response to these bacteria. These data suggest that epithelial cells sense cervicovaginal bacteria in a species-specific manner and contribute to the observed pro-inflammatory cytokines found in women in CT4.

Genital APCs from Women with High-Diversity Genital Communities Are Activated by Microbial Products In Vivo

APCs in the cervix are closely associated with the epithelium (Figures 7A and 7B) and sense microbes in conjunction with epithelial cells (Hickey et al., 2011). Because the context in which APCs are stimulated can have important effects on their phenotype and ability to prime adaptive immune cells (Littman and Pamer, 2011; Ochiel et al., 2010), we performed phenotypic and transcriptional analyses on sorted APCs obtained by cervical cytobrush to understand bacterial sensing in situ (Figure S7A). We compared APCs from women in CT1 or CT2, who had gram-positive *Lactobacillus* dominance, to those in CT4, additionally excluding anyone with an active STI. Using flow cytometry, we saw no significant difference in the frequency of cervical APC subsets (CD11c$^+$ dendritic cells or CD14$^+$ monocytes or macrophages) (Figure S7B). However, there were pronounced transcriptional differences among the cervical APCs; 35 genes were significantly upregulated and two were downregulated in women with *Prevotella*-dominant communities relative to those with *Lactobacillus* dominance (false discovery rate [FDR] < 0.05; Figures 7C and S7C). The genes upregulated in CT4 were enriched in NF-κB, Toll-like receptor (TLR), NOD-like receptor, and TNF-α signaling pathways. Gene set enrichment analysis revealed that 20 of the 35 genes upregulated in CT4 APCs were shared with monocytes treated with a low dose of LPS (a TLR4 agonist; q = 6.16e-36) and 19 genes were shared with dendritic cells treated with LPS (q = 1.11e-33). Ingenuity Pathway Analysis also identified LPS as the most likely upstream regulator to be activated in CT4 APCs (p = 9.47e-26). These results are consistent with our bacterial pathway analysis using the microbial DNA shotgun sequences, which demonstrated that LPS biosynthesis was the second most enriched pathway in the high inflammation samples compared to those with low inflammation (Table S5).

In addition to LPS, Ingenuity Pathway Analysis also predicted IFN-γ (p = 6.31e-24), IL-1β (p = 1.51e-23), and CSF2 (colony stimulating factor 2, p = 6.74e-22) to be upstream regulators of the CT4 APCs, which was consistent with higher levels of IFN-γ and IL-1β in the CVLs of women with CT4. Also consistent with the CVL cytokine measurements, CT4 APCs had significantly higher expression of many pro-inflammatory cytokine genes, such as *IL1A* (5.8-fold), *IL1B* (4.3-fold), *TNF* (6.5-fold), *IL10* (10.6-fold), *IFNB1* (23.4-fold), *IL23A* (7.3-fold), and *IL6* (8.9-fold), compared to CT1 and CT2 APCs (Figure 7C). Of relevance to cellular recruitment, CT4 APCs also upregulated the chemokines *CCL4* (MIP-1β, a CCR5 ligand, 5.7-fold), *CXCL9* (MIG, a CXCR3 ligand, 9-fold), and *CCL8* (MCP-2, a CCR1 and CCR5 ligand, 6.3-fold). These transcriptional differences were not observed when comparing APCs from peripheral
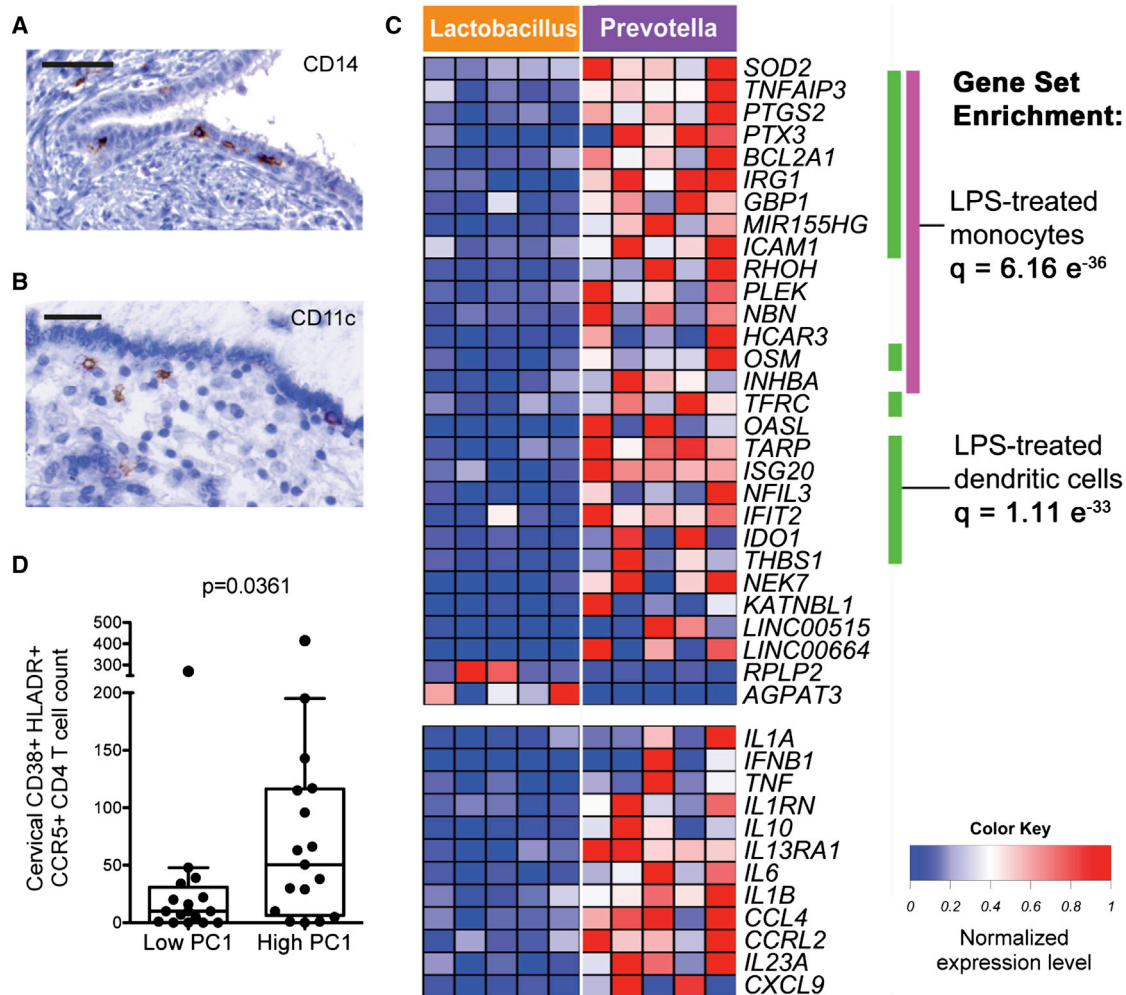
Figure 7. Cervical APC Sensing of Genital Bacteria

Transcriptional profiling suggests that CT4 bacterial products are sensed by cervical APCs, which contribute to genital inflammation by producing a myriad of pro-inflammatory cytokines and critical T cell chemoattractants.

(A and B) CD14 (A) and CD11c (B) staining of endocervical tissue. Scale bar represents 50 μm.

(C) Heatmap of genes differentially expressed in cervical APCs from women with *Lactobacillus*-dominant communities (> 95% abundance; from left to right: 2 in CT1, 3 in CT2) versus those with high *Prevotella* abundance (> 25%; n = 5 in CT4). Women with STIs were excluded. Only genes with a false discovery rate < 0.05 are shown. GSEA was used to determine statistically significant similarities with annotated gene sets.

(D) Number of live endocervical CD38+ HLADR+ CCR5+ CD4+ T cells from women in the highest and lowest quintile of cytokine PC1 (Mann-Whitney, n = 16 per group). Data shown as Tukey box plots.

See also Figure S7 and Table S5.

blood between CTs (Figure S7C). Thus, the transcriptional profile of cervical APCs indicated that they were responding to a CT4 bacterial product such as LPS and subsequently initiating acute inflammation and lymphocyte recruitment and that these differences were not a reflection of systemic inflammatory activation.

Women with Elevated Pro-inflammatory Genital Cytokines Have Increased Activated Cervical HIV Target Cells

Given the close linkage between CT4 bacteria and genital inflammation, we sought to determine whether pro-inflammatory cytokines would correlate with the presence of activated

HIV target cells in the genital tract. Activated CCR5+ CD4+ T cells at the cervical mucosal surface are likely the first susceptible cells to encounter HIV upon sex with an infected partner (Haase, 2010) and support greater HIV replication upon infection than resting cells (Meditz et al., 2011). We found a significantly higher number of activated (CD38+ HLA-DR+) CCR5+ CD4+ T cells in the endocervical canal of women in the top quintile of cytokine PC1 relative to those in the bottom quintile (p = 0.0361, Figure 7D), but no significant difference in activated CCR5+ CD4+ T cells in the peripheral blood (Figure S7D). Thus, the observed increase in genital HIV target cells supports the link between high-diversity microbial communities and HIV acquisition risk.

DISCUSSION

By integrating metagenomic, immunologic, transcriptional, and behavioral datasets from a population of young South African women, we demonstrated a strong link between high-diversity cervicovaginal bacterial community structures and genital inflammation in vivo in both cross-sectional and longitudinal analyses. Women with CT4 were over four times more likely to have high pro-inflammatory genital cytokine levels than those with other CTs and, on average, had higher levels of genital inflammation than women with BV or STIs. We provide evidence that these high-diversity communities in the FGT are sensed by both epithelial cells and APCs. APCs specifically appear to respond to LPS via Toll-like receptor 4 signaling, contributing to genital inflammation through NF-κB activation and recruitment of lymphocytes by chemokine production. This was consistent with the bacterial pathway analysis that demonstrated that LPS biosynthesis was highly enriched in bacterial communities from women with high genital inflammation. This genital inflammation was accompanied by increased numbers of activated HIV infectable CD4$^+$ T cells in the cervix, providing a potential cellular link to increased HIV acquisition risk. Our study therefore identified a mechanism by which the predominant cervicovaginal communities found in our cohort of South African women impact genital inflammation and thereby likely modulate HIV acquisition risk.

Only 37% of participants had CT1 or CT2 bacterial communities, clearly indicating that *Lactobacillus* dominance was not typical in this population. This is in contrast to studies in the U.S., in which 90% of white women were found to have *Lactobacillus* dominance (Ravel et al., 2011), but consistent with previous studies that use Gram stain analyses to demonstrate a high prevalence of BV in sub-Saharan African women (Jespers et al., 2014; Myer et al., 2005). We reported a common occurrence of low *Lactobacillus*, high-diversity communities even in the absence of BV and provided a comprehensive characterization of bacterial taxa by using high-throughput sequencing.

The etiology of the low *Lactobacillus* abundance in our cohort remains unexplained. It was not associated with sexual behavior, contraceptive usage, or demographic characteristics, and we speculate that host genetic factors might play a role. Previous studies demonstrate a lower frequency of *L. crispatus* and *L. jensenii* and a higher frequency of *Prevotella* in black and Hispanic women compared to white and Asian women in the U.S. (Ravel et al., 2011; Zhou et al., 2007). The high-diversity vaginal communities seen in black American women show general similarities to those seen in black South African women, specifically with high levels of *Prevotella*, but the prevalence of these high-diversity communities was approximately half of that observed in South Africans. Studies of African cohorts have also demonstrated a higher intestinal *Prevotella* abundance than in American and European cohorts (De Filippo et al., 2010; Yatsunenko et al., 2012) suggesting that vaginal seeding from the bacteria-rich rectum (El Aila et al., 2009) might also influence the observed differences. Overall, our findings support the growing understanding that *Lactobacillus* dominance is not tantamount to a "healthy" female genital tract microbiome in all women (Ravel et al., 2011), and they highlight the value of bacterial characterization in broader geographic regions.

Within CT4, we found that *Sneathia sanguinigens*, *Sneathia amnii*, *Mobiluncus mulieris*, and *Prevotella amnii* were capable of inducing the secretion of IL-1α, IL-1β, and IL-8 from human vaginal epithelial cells in vitro. *Aerococcus* was highly associated with inflammation in vivo but did not induce cytokine secretion in vitro, possibly due to the particular bacterial isolate that we tested or because it is merely well-adapted to surviving in inflamed tissue, but not a primary driver of inflammation. We did not test *Fusobacterium*, but *Fusobacterium nucleatum* has been shown to be pro-inflammatory and tumorigenic in the gut (Kostic et al., 2013). Overall, several CT4 bacteria were capable of directly inducing pro-inflammatory cytokine production from epithelial cells in vitro. The strong correlation between bacterial communities and genital inflammation that we demonstrated might help explain the increased genital immune activation seen in other sub-Saharan cohorts compared to those in the U.S. (Cohen et al., 2010). While the proportion of American women with high-diversity communities might be lower, preliminary results demonstrated that they were also associated with elevated pro-inflammatory genital cytokines (data not shown).

To begin to understand how CT4 bacteria are sensed by the host immune system, we performed transcriptional analyses on APCs isolated from cervical cytobrushes. APCs from women with high *Prevotella* abundance demonstrated a marked response to LPS, as well as to IFN-γ and IL-1β. This most likely reflects both the stimulation by gram-negative bacteria and effects of the cytokine milieu, because women with CT4 had higher levels of both IFN-γ and IL-1β in the CVL. The CT4 APCs also were more activated and mature, with higher expression of CD80, ICAM-1, and MHC II and were likely contributing to both T cell priming and effector function control (Iijima et al., 2011). Additionally, miR-155 was strongly upregulated in CT4 APCs and has been shown previously to be critical for APCs to effectively activate T cells (Rodriguez et al., 2007).

Based upon transcriptional data and in vitro assays, we propose that cervicovaginal bacteria are sensed through epithelial cells and APCs, activating TLR and NF-κB pathways that cause secretion of chemokines that recruit activated CCR5$^+$ CD4$^+$ T cell targets to the genital tract. Additionally, cytokines such as TNF-α and IFN-γ might directly damage the columnar epithelial barrier of the endocervix by disrupting tight junctions, which enables both bacterial and viral translocation (Madara and Stafford, 1989; McGee et al., 1992). Thus, bacteria-induced genital inflammation might increase HIV acquisition through multiple local mechanisms in the FGT. This is supported by prior observations that pro-inflammatory cytokines in CVLs are associated with an over 3-fold increased risk of HIV acquisition in women (Masson et al., 2015) and that HIV-1 exposed women who remain seronegative have lower levels of genital IFN-γ, IL-1α, and IP-10 (Lajoie et al., 2012).

Our work supports the role of the bacterial microbiome in modulating HIV risk and suggests that interventions targeting genital microbiota, such as narrow-spectrum antibiotics or probiotics, might reduce HIV acquisition in women. In addition, our results indicate that the genital microbiome can significantly alter local host inflammation and might more broadly impact efforts to create effective microbicides, induce mucosal vaccine responses, curtail cervical cancer progression, and improve reproductive health in women.

EXPERIMENTAL PROCEDURES

Study Cohort

Participants were recruited through the Females Rising through Education, Support, and Health (FRESH) study, a prospective, 12-month observational study of women 18–23 years old conducted outside of Durban, South Africa. At the time of data analysis, 146 participants had completed at least one mucosal sampling. The study protocol was approved by the Biomedical Research Ethics Committee of the University of KwaZulu-Natal and the Massachusetts General Hospital Institutional Review Board (2012P001812/MGH). Informed consent was obtained after the nature and possible consequences of the study were explained.

Sample Collection

Twice weekly volunteers had a finger-prick blood draw for HIV RNA testing and every 3 months had a pelvic exam, peripheral blood draw, and completed an HIV risk questionnaire administered by a counselor. The mucosal sample collection involved the collection of ectocervical and midvaginal swabs (Catch-All, Epicenter), a cervicovaginal lavage, and an endocervical cytobrush, as described in the Supplemental Experiment Procedures.

BV and Sexually Transmitted Infection Detection

Vaginal smear Gram staining, Nugent scoring for BV, and STI testing were performed by Global Labs.

Bacterial 16S rRNA Gene Sequencing

Nucleic acid was extracted from cervical swabs by bead beating in phenol:chloroform:isoamyl alcohol (25:24:1, pH 7.9, Ambion), followed by an isopropanol precipitation, as described in the Supplemental Experimental Procedures. Extracted DNA was amplified with the 16S rRNA gene V4 primer constructs (Caporaso et al., 2012) 515F and Golay-barcoded 806R for PCR amplification and Illumina sequencing. The cleaned amplicon pool was sequenced on a single 1×300 bp run on an Illumina MiSeq machine. 16S rRNA sequencing reads were processed with the QIIME (Caporaso et al., 2010) version 1.8.0 analysis pipeline. Samples from the full dataset were then assigned to the four cervicotypes based on a simple assessment of dominance (i.e., abundance). Details can be found in Supplemental Experimental Procedures.

Metagenomic Shotgun DNA Sequencing and Taxonomic Classification

Shotgun DNA libraries were made from total nucleic acid isolated from 12 cervical swab samples; the same total nucleic acid was used for 16S rRNA amplification. DNA was isolated from the total nucleic acid with an AllPrep DNA/RNA Micro Kit (QIAGEN) and sheared (S2 sonicator, Covaris). DNA libraries were prepared with the NEBNext DNA Library Construction kit (New England Biolabs) and sequenced on a single paired-end 250 bp Illumina MiSeq run. Bacterial species were identified with the MetaPhlAn2 package v.2.0.0.b (Segata et al., 2012).

Bacterial Functional Pathways Analysis

Reads aligning to the human genome or PhiX were removed from quality controlled and adaptor trimmed shotgun DNA sequences. The remaining reads were assigned to KEGG genes via a translated BLAST search, and HUMAnN was used to identify the differentially abundant microbial pathways (Abubucker et al., 2012).

Cytokine Measurements in CVLs

Cytokines were measured by a human cytokine/chemokine multiplexed bead assay (Millipore). CVLs were analyzed within 6 months of collection and underwent a single freeze-thaw cycle. Additional information can be found in the Supplemental Experimental Procedures.

Bacterial Strains and Colonization Assays

Isolates were obtained from the Culture Collection University of Göteborg, the American Type Culture Collection (ATCC), and collaborators. See Supplemental Experimental Procedures for further information.

Immunophenotyping of Cervical Cytobrush Samples

Cervical and peripheral blood cells were stained with a violet viability dye (Life Technologies) and labeled with a cocktail of antibodies, described in detail in the Supplemental Experimental Procedures. Cells were sorted on a FACS Aria III (BD Biosciences) directly into TRIzol (Life Technologies), vortexed vigorously, and stored at -80°C. Data were processed with FlowJo software (Tree Star). See Figure S7A for gating strategy.

Transcriptional Analysis of Sorted Cells

RNA was extracted from cells sorted into TRIzol by a chloroform-based method. RNA quality and quantity were assessed with the Bioanalyzer (Agilent) and RINs were consistently above 9. The RNA-seq libraries were prepared according to the Single Cell RNA Barcoding and Sequencing method originally developed for single cell RNA-seq (Soumillon et al., 2014) and adapted to extracted total RNA. Digital gene-expression values were analyzed with the edgeR package (Robinson et al., 2010).

Statistics

Mann-Whitney Wilcoxon test was used to compare continuous data between two groups. For comparisons between more than two groups, Kruskal-Wallis test with post hoc analyses was used. Wilcoxon signed rank was used to compare continuous data between two time points. Fisher's exact test was used for comparing categorical data among two or more groups. Spearman's correlation coefficients were used to examine associations between variables; only correlations with a p value lower than the specified cutoff are displayed. Principal component and cluster analyses were used to obtain summary measures for cytokine/bacteria variables and to identify study subjects with similar cytokine/bacteria profiles. Multivariate analyses using ridge regression (Cule and De Iorio, 2013) were used to identify the correlates of higher cytokine values. p values are two-sided and are not adjusted for multiple testing. The analyses were performed with R Studio and Prism (GraphPad).

ACCESSION NUMBERS

The accession number for the gene expression data reported in this paper is GSE68452.

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures, five tables, and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.04.019.

AUTHOR CONTRIBUTIONS

M.N.A., E.H.B., and B.A.B. performed the nucleic acid extraction and bacterial sequencing; K.E.D., N.P., M.N.A., and D.S.K. performed flow cytometry; M.N.A. performed the CVL cytokine measurements and data analyses; H.S.Y. and R.N.F. designed and performed the in vitro bacterial-epithelial cell co-culture experiments and cytokine measurements; R.N.F. and M.E.S. provided expertise in vaginal mucosal immunity and genital sampling; K.E.D. and A.M. collected behavioral data; N.I. processed and managed clinical samples; M.S.G. was involved with statistical analyses; M.S. and C.N. performed the RNA sequencing and data processing; C.H. and H.W.V. were involved with experimental design and data analysis; T.N., K.L.D., B.D.W., and D.S.K. designed and managed the clinical study; M.N.A. and D.S.K. prepared the manuscript. All authors discussed the results and commented on the manuscripts.

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