RESEARCH ARTICLE

Enhanced Trapping of HIV-1 by Human Cervicovaginal Mucus Is Associated with *Lactobacillus crispatus*-Dominant Microbiota

Kenetta L. Nunn,[a] Ying-Ying Wang,[b] Dimple Harit,[c] Michael S. Humphrys,[d] Bing Ma,[d,e] Richard Cone,[b] Jacques Ravel,[d,e] Samuel K. Lai[a,c,f]

UNC/NCSU Joint Department of Biomedical Engineering, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA[a]; Department of Biophysics, Johns Hopkins University, Baltimore, Maryland, USA[b]; Division of Molecular Pharmaceutics, Eshelman School of Pharmacy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA[c]; Institute for Genome Sciences, University of Maryland School of Medicine, Baltimore, Maryland, USA[d]; Department of Microbiology and Immunology, University of Maryland School of Medicine, Baltimore, Maryland, USA[e]; Department of Microbiology and Immunology, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina, USA[f]

ABSTRACT **Cervicovaginal mucus (CVM) can provide a barrier that precludes HIV and other sexually transmitted virions from reaching target cells in the vaginal epithelium, thereby preventing or reducing infections. However, the barrier properties of CVM differ from woman to woman, and the causes of these variations are not yet well understood. Using high-resolution particle tracking of fluorescent HIV-1 pseudoviruses, we found that neither pH nor Nugent scores nor total lactic acid levels correlated significantly with virus trapping in unmodified CVM from diverse donors. Surprisingly, HIV-1 was generally trapped in CVM with relatively high concentrations of D-lactic acid and a *Lactobacillus crispatus*-dominant microbiota. In contrast, a substantial fraction of HIV-1 virions diffused rapidly through CVM with low concentrations of D-lactic acid that had a *Lactobacillus iners*-dominant microbiota or significant amounts of *Gardnerella vaginalis*, a bacterium associated with bacterial vaginosis. Our results demonstrate that the vaginal microbiota, including specific species of *Lactobacillus*, can alter the diffusional barrier properties of CVM against HIV and likely other sexually transmitted viruses and that these microbiota-associated changes may account in part for the elevated risks of HIV acquisition linked to bacterial vaginosis or intermediate vaginal microbiota.**

IMPORTANCE **Variations in the vaginal microbiota, especially shifts away from *Lactobacillus*-dominant microbiota, are associated with differential risks of acquiring HIV or other sexually transmitted infections. However, emerging evidence suggests that *Lactobacillus iners* frequently colonizes women with recurring bacterial vaginosis, raising the possibility that *L. iners* may not be as protective as other *Lactobacillus* species. Our study was designed to improve understanding of how the cervicovaginal mucus barrier against HIV may vary between women along with the vaginal microbiota and led to the finding that the vaginal microbiota, including specific species of *Lactobacillus*, can directly alter the diffusional barrier properties of cervicovaginal mucus. This work advances our understanding of the complex barrier properties of mucus and highlights the differential protective ability of different species of *Lactobacillus*, with *Lactobacillus crispatus* and possibly other species playing a key role in protection against HIV and other sexually transmitted infections. These findings could lead to the development of novel strategies to protect women against HIV.**

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Address correspondence to Samuel K. Lai, lai@unc.edu.

The vaginal epithelium produces little to no secreted mucins; instead, mucus coating the female reproductive tract is primarily derived from mucin-secreting glands in the cervix (1). Cervical mucus flows by gravitational and/or abdominal pressure through the cervical os into the vaginal canal (2, 3), where it is modified by fluid and ion exchange (4, 5), shed epithelial cells, and the vaginal microbiota (6, 7). Thus, mucus secretions coating the vaginal epithelium are frequently referred to as cervicovaginal mucus (CVM) to emphasize both its origin and the unique physicochemical properties that differentiate CVM from cervical mucus. CVM is critical to reproductive health not only as a lubricant that minimizes physical trauma to the underlying epithelium during coitus but also by serving as the first line of defense against transmission of infectious virions.

Vaginal microbes (8) can modify CVM biochemically to an extent not yet fully understood (6). Until recently, the primary method for characterizing the vaginal microbiota was Nugent scoring, a morphology-based evaluation of the abundance of rod-shaped, Gram-positive *Lactobacillus* spp. (the most prevalent bacteria in the vagina) and Gram-variable polymicrobial communities (7). Advances in high-throughput-sequencing technology based on analysis of the 16S rRNA gene now afford high-resolution culture-independent molecular methods that reveal the full diversity of *Lactobacillus* spp. and other commensal or

pathogenic microbes present in the vaginal microbiota, as well as the dynamic nature of shifts between different microbial communities over short temporal scales (8, 9). Among the four most common *Lactobacillus* species (*L. crispatus*, *L. iners*, *L. jensenii*, and *L. gasseri*), an *L. crispatus*-dominant microbiota is found in about 14% of African-American women and over 45% of Caucasian women, while an *L. iners*-dominant microbiota and a microbiota comprising *Gardnerella vaginalis* and other bacteria associated with bacterial vaginosis (BV) (10) are found in 36% and 40%, respectively, of African-American women and in 27% and 10%, respectively, of Caucasian women (8).

For vaginal transmission to occur, HIV must penetrate CVM to reach target cells in the vaginal epithelium (or penile epithelium, in the case of female-to-male transmission). CVM that either retards or immobilizes HIV virions can directly reduce the effective viral load that arrives at target cells and can potentially prevent initial infections altogether. In prior work, we measured the mobility of HIV in fresh human CVM and found that native CVM from a limited number of college students, the majority with vaginal microbiota likely dominated by *L. crispatus*, effectively trapped HIV-1 virions (11). A follow-up study on a different population of women by a coauthor of that initial work reported that CVM generally failed to trap HIV-1 virions (12). Since there are substantial variations in the vaginal microbiota between women and within the same woman over time (8, 9), differences in the vaginal microbiota between the subject populations may well account for the reported differences between the two studies. Thus, we sought to explore whether the vaginal microbiota, including different strains of *Lactobacillus* spp., can alter the diffusional barrier properties of CVM against HIV.

RESULTS

CVM with high D-lactic acid (D-LA) concentrations consistently traps HIV-1. To reconcile the contrasting observations of HIV mobility in CVM reported in the two previous publications, we screened a larger subject pool than was included in our original study and observed significant variation in the mobility of HIV-1 virus-like particles (pseudotyped with a YU2 envelope) in fresh, minimally modified CVM that could be broadly divided into 2 categories (see, e.g., Movies S1 and S2 in the supplemental material): CVM that traps the vast majority of HIV-1 virions ($n = 17$ of 31 women) and CVM with a substantial population of rapidly diffusing HIV-1 virions ($n = 14$ of 31). On average, the mobile fraction of HIV-1 was only 1.3% ± 0.6% in CVM samples that trapped HIV-1 compared to 45% ± 8% in CVM samples with a substantial fast-moving population (see Table S1), and the average effective diffusion coefficient (D_{eff}) of HIV-1 in CVM with substantial fast-moving viral populations was ~20-fold greater than in CVM that effectively trapped HIV-1. In CVM samples that trapped HIV-1, virions were trapped immediately after addition into mucus and remained trapped for over more than 1 h of incubation; similarly, in samples that failed to trap HIV-1, virions remained diffusive even after extended incubation.

To begin to understand what might contribute to these variations in the diffusional barrier properties of CVM samples against HIV, we first attempted to correlate the observed HIV-1 mobility with three traditionally measured properties of CVM: pH, Nugent score, and lactic acid (LA) content (Fig. 1). Although CVM generally did not trap HIV-1 at high vaginal pH, lower vaginal pH (pH ≤ 4.2) was not predictive of HIV-1 trapping (Fig. 1B and E; note that a pH level of 4.2 is the upper limit of CVM pH measured under physiologically hypoxic conditions for women with what is thought to be a clinically "normal" microbiota [13], while the clinical criterion of a pH level of >4.5 for BV is based on measurements in ambient air). Over 25% of the CVM specimens with a pH level of ≤4.2 failed to trap HIV-1, and the average pH of CVM samples that trapped HIV-1 (pH 3.99 ± 0.04) was not statistically different from that of samples that failed to trap HIV-1 (pH 4.03 ± 0.03) (see Fig. S1A and Table S1 in the supplemental material). HIV-1 mobility also tended to be greater with higher Nugent scores (see Fig. S1B), which is not surprising given the correlation between BV and greater risk for HIV transmission (14, 15). However, nearly a third of the CVM samples that exhibited low Nugent scores in the "normal" range (0 to 3 [7]) failed to trap HIV-1 (Fig. 1C and F), and the difference between low-Nugent-score samples that trapped HIV-1 and those that did not trap HIV-1 was not significant (average Nugent score of 1.07 ± 0.30 versus 1.67 ± 0.49; see Fig. S1B). Likewise, high levels of total LA were not predictive of HIV-1 trapping in CVM (Fig. 1D and G), with average LA levels of 0.96 ± 0.09% (wt/vol) versus 0.85 ± 0.07% (wt/vol) for samples that trapped or did not trap HIV-1 among those with "normal" LA levels (at least 0.7% [wt/vol] LA [13]) (see Fig. S1C). We thus decided to seek other biomarkers that could more accurately predict the barrier properties of CVM against HIV.

Humans can secrete only L-LA, which does not contribute substantially to the total LA found in the vagina (6). In contrast, *Lactobacillus* spp. can produce both D-LA and L-LA, and different species of *Lactobacillus* may differ in D-LA versus L-LA production (6, 16, 17). Therefore, we tested whether D-LA or L-LA content might help reveal differences in the barrier properties of CVM. We found that CVM samples that trapped HIV-1 at native pH levels generally possessed substantial quantities of D-LA (Fig. 2A and D); in contrast, CVM that failed to trap HIV-1 generally possessed significantly lower levels of D-LA. Among samples with relatively high levels of D-LA (>0.3% [wt/vol]), 10 of 11 samples trapped HIV-1, with an average D-LA level of 0.52% ± 0.07% (see Fig. S1D in the supplemental material). While the lone sample that did not trap HIV-1 had a D-LA level of 0.39%, this sample also had a Nugent score of 6 (in the "intermediate" range bordering on bacterial vaginosis [7]). HIV-1 trapping in CVM did not correlate with either the L-LA level or the D:L ratio (Fig. 2B and E or C and F, respectively), and average differences in the L-LA or D:L ratio between samples that trapped versus those that did not trap HIV-1 were not statistically significant (see Fig. S1E and F).

Given that the presence of high levels of D-LA appears to be an effective predictor of HIV-1 trapping in CVM, we sought to evaluate whether D-LA directly mediates HIV trapping by adding D-LA to CVM specimens with low endogenous D-LA levels. The addition of D-LA to reach a final concentration in excess of 1% (wt/vol) did not increase HIV-1 trapping in CVM (data not shown), suggesting that D-LA is likely simply a surrogate indicator of the diffusional barrier properties of CVM against HIV rather than directly participating in interactions between HIV and mucins.

The specific composition of the vaginal microbiota determines CVM barrier properties against HIV. Unlike humans, a variety of bacteria can produce D-LA, with the majority of vaginal D-LA presumably produced by commensal *Lactobacillus* spp., as other bacteria such as *Atopobium* spp. produce relatively little lac-

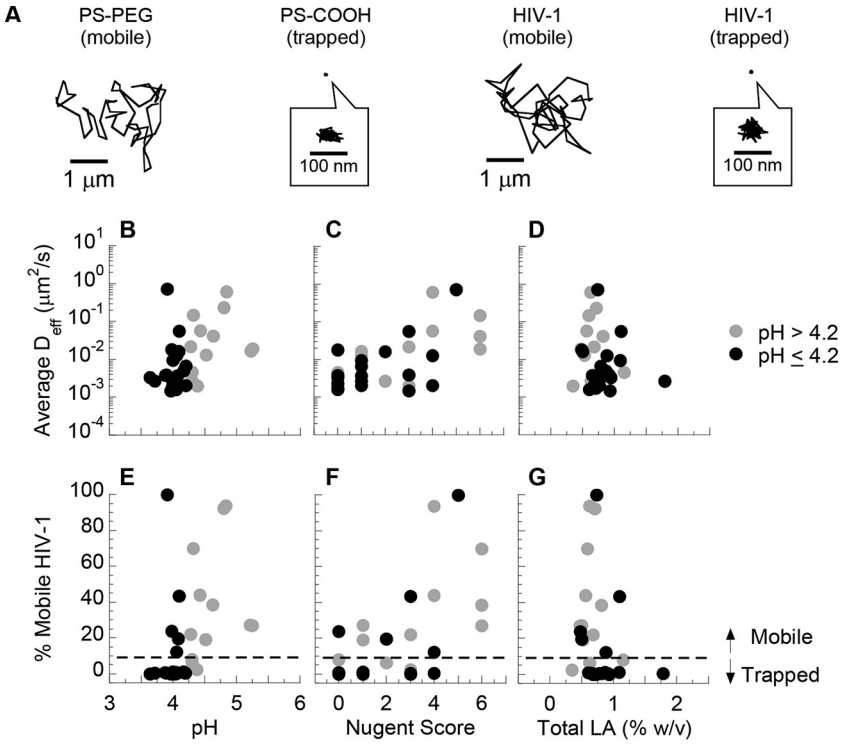

FIG 1 (A) Representative traces of mobile versus trapped HIV-1 virions compared to PS-PEG nanoparticles that are mucoinert and readily diffuse through CVM and compared to PS-COOH nanoparticles that are mucoadhesive and trapped in CVM. (B to G) HIV-1 mobility measured by average effective diffusivity (D_{eff}) (B to D) values or fraction of mobile virions (E to G) versus pH (B and E), Nugent score (C and F), or total LA (D and G) for $n = 31$ CVM samples with a pH level of >4.2 ($n = 12$) or ≤4.2 ($n = 19$). Dashed lines indicate the cutoff between samples with a significant mobile HIV-1 population (≥10% mobile) and those in which HIV-1 is largely trapped (<10% mobile). w/v, weight/volume.

tic acid (6, 9). Thus, we speculated that differences in D-LA levels between CVM specimens likely reflect the presence of different species of *Lactobacillus*. RNA sequencing (RNAseq) analysis has shown that *L. crispatus* generally expresses high levels of both D- and L-lactate dehydrogenase leading to high levels of both D-LA and L-LA, whereas many strains of *L. iners* exhibit reduced expression of D-lactate dehydrogenase relative to L-lactate dehydrogenase, resulting in low levels of D-LA but not of L-LA (17). We thus hypothesized that the correlation between HIV mobility and the level of D-LA in CVM likely reflects distinct microbial communities present in CVM. Using 16S rRNA gene sequencing, we characterized the microbial composition and structure of a subset of CVM specimens from our studies described above (Fig. 3A), and we indeed found that CVM samples with high D-LA levels that trapped HIV generally possessed an *L. crispatus*-dominant microbiota (group 1), whereas the majority of CVM that failed to trap HIV possessed either an *L. iners*-dominant microbiota (group 2) or significant quantities of *G. vaginalis* (group 3) (Fig. 3B). Group 1 CVM samples also had significantly lower pH and Nugent scores and higher total and D-LA levels than other samples (Fig. 3C).

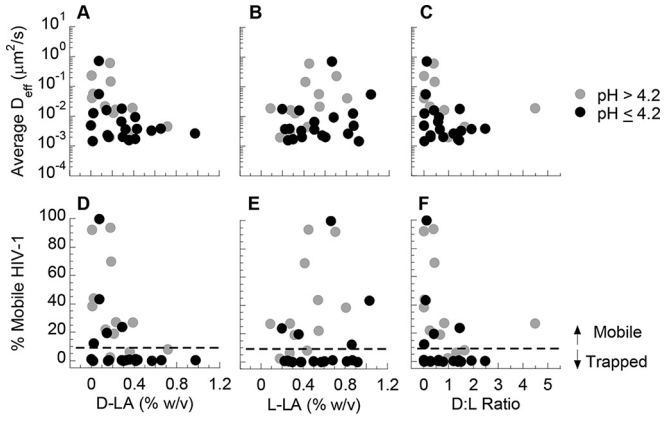

FIG 2 HIV-1 mobility measured by average effective diffusivity (D_{eff}) (A to C) values or fraction of mobile virions (D to F) versus D-LA (A and D), L-LA (B and E), or D:L ratio (C and F) for $n = 31$ CVM samples with a pH level of >4.2 ($n = 12$) or ≤4.2 ($n = 19$). Dashed lines indicate the cutoff between samples with a significant mobile HIV-1 population (≥10% mobile) and those in which HIV-1 is largely trapped (<10% mobile).

HIV-mucin interactions may be mediated by carboxyl groups present on the surface of HIV virions. To begin to understand mechanistically how HIV is trapped in CVM, we first investigated whether HIV-1 virions were trapped due to steric obstruction from the dense mucin mesh or due to adhesive interactions with mucus constituents. We had previously measured the average mesh spacing in CVM to be ~340 nm, with over 80% of spacings larger than 200 nm (18). This suggests that the majority of HIV-1 virions (diameter, ~100 nm) should not be slowed appreciably by steric obstruction, although it was unclear whether the pore size of CVM could be substantially reduced by specific vaginal microbes. To test this, we prepared polymeric nanoparticles (diameter, ~200 nm) coated with polyethylene glycol (PEG) to produce a mucoinert surface that prevents adhesive interactions with mucins, whereby the diffusion of the beads is limited only by

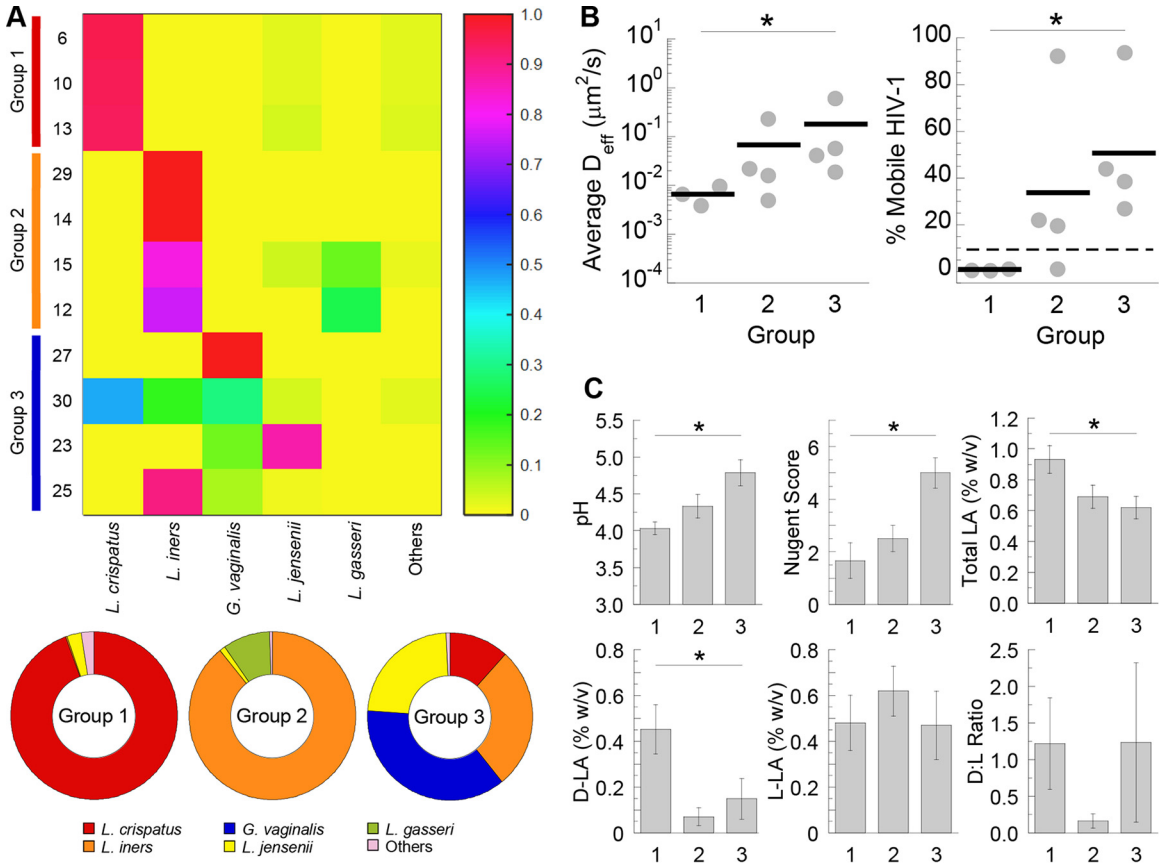

FIG 3 (A) 16S rRNA sequencing and RNAseq analysis of CVM specimens reveals groups with distinct vaginal microbiotas: *L. crispatus*-dominant microbiota (group 1), *L. iners*-dominant microbiota (group 2), and microbiota containing a significant fraction of *G. vaginalis* (group 3). The color bar indicates the abundance of different bacterial species as a proportion of all species in the sample. Annulus charts depict the average distributions of the bacterial population across species of lactobacilli, *Gardnerella vaginalis*, and other species within each group. Donor identification numbers (IDs) are indicated to allow comparisons to the biochemical characterization data and bacterial abundance values in Tables S1 and S2 in the supplemental material. (B) HIV-1 mobility measured by average effective diffusivity (D_{eff}) values or fraction of mobile virions in CVM samples within each group. The dashed line indicates the cutoff between samples with a significant mobile HIV-1 population (≥10% mobile) and those in which HIV-1 is largely trapped (<10% mobile). (C) Average pH, Nugent score, total LA, D-LA, L-LA, and D:L ratio for CVM samples within each group. *, $P < 0.05$ (statistically significant difference between group 1 and groups 2 and 3).

physical obstruction from the mucus mesh. In this study, all CVM specimens that trapped HIV-1 failed to trap the larger PEG-coated beads (Fig. 4A; see also S2A and E in the supplemental material). This confirms that HIV-1 was trapped by adhesive interactions with mucus constituents.

We next sought to determine whether the adhesive interactions that facilitate HIV trapping are specific to HIV-1 by investigating whether the interactions involve trimeric gp120/gp41 Env spikes. To do so, we prepared ΔEnv mCherry-Gag-tagged pseudoviruses (i.e., without the HIV-specific envelope glycoprotein) and compared their mobility in the same CVM specimens to that of virions with intact envelope glycoprotein. We found that the ΔEnv pseudoviruses exhibited comparable trapping in CVM with high D-LA content as well as similar mobile fractions in CVM with low D-LA content (Fig. 4B; see also Fig. S2B and F in the supplemental material). This indicates that the adhesive interactions responsible for trapping HIV-1 viruses in CVM do not involve HIV-specific viral proteins and may instead be based on interactions between mucins and components of the lipid membrane of the viruses.

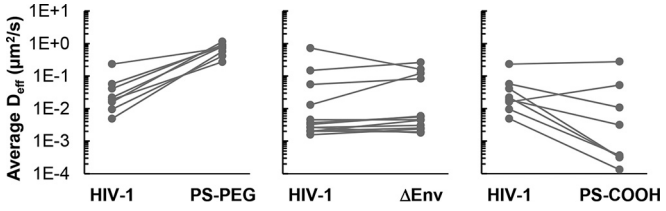

FIG 4 Comparison of average effective diffusivity (D_{eff}) values for mucoinert synthetic beads (PS-PEG) (A), HIV-1 ΔEnv pseudovirus (B), or PS-COOH (C) to that of HIV-1 in native CVM. Data represent $n = 8$, 13, and 8 distinct CVM samples for PS-PEG, HIV-1 ΔEnv, and PS-COOH, respectively. Lines connect pairs of data points for the same sample.

Mucins, due to their dense glycosylation, contain many carboxyl groups that are negatively charged at neutral pH (i.e., COO^-) but that become increasingly neutral as pH decreases and COO^- groups are protonated (i.e., COOH). Similarly, the surfaces of HIV and other enveloped viruses also contain a high density of carboxyl groups derived from either glycans on viral proteins or, more generally, human glycoproteins and glycolipids naturally incorporated into the viral membrane. Protonation of carboxyl groups both on the virus surface and on mucins may

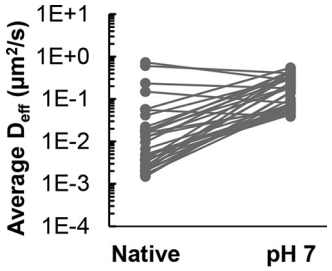

FIG 5 Comparison of average effective diffusivity values for HIV-1 in native versus neutralized (pH 7) CVM. Data represent $n = 31$ distinct CVM samples. Lines connect pairs of data points for the same sample.

supply the protons necessary to form hydrogen bonds, and the extent of such hydrogen bonding increases as mucus pH decreases to approach the pK_a of the carboxyl groups (~3 [19]). Thus, we speculated that virions in native, acidic CVM samples that trap HIV-1 may be immobilized via multiple hydrogen bonds between surface carboxyl groups and mucins and that protonation of even a small fraction of carboxyl groups may be adequate to create sufficient hydrogen bonds to stop the diffusion of viruses through mucus. To test this hypothesis, we measured the mobility of carboxyl-modified latex beads (polystyrene [PS]-COOH) in a subset of CVM specimens. As we expected, PS-COOH was mostly trapped in CVM specimens in which the HIV-1 virions were largely trapped and displayed substantial mobility in CVM specimens that failed to trap HIV-1 (Fig. 4C; see also Fig. S2C and G in the supplemental material). We further found that neutralizing CVM, which eliminates essentially all protonated carboxyl groups, reduced the adhesive interactions between HIV-1 and mucus constituents (Fig. 5; see also Fig. S2D and H). Since ΔEnv pseudoviruses that lack viral glycoproteins on the viral surface also exhibited diffusion kinetics similar to those of pseudoviruses with Env, it is likely that the bulk of the interactions between the viral surface and mucins are via carboxyls on host-derived envelope glycolipids or glycoproteins rather than via carboxyls on viral glycoproteins.

DISCUSSION

Although CVM is densely populated by vaginal microbes, the relationship between the vaginal microbiota and the barrier properties of CVM remains poorly understood. The primary association between mucus and HIV transmission is typically in the elevated risks for women with episodes of BV of acquiring HIV and various other sexually transmitted infections (STI). BV is commonly diagnosed in research settings by Nugent scoring or in clinical settings by Amsel's criteria (20), which include an elevated pH (>4.5) and increased watery vaginal discharge. It is thought that watery vaginal fluid allows HIV to more easily reach target cells in the vaginal epithelium (21), resulting in a greater risk of transmission. Consistent with this notion, we found that CVM with substantial (i.e., >5%) quantities of *G. vaginalis*, a bacterium frequently associated with BV, failed to trap HIV-1. Surprisingly, we discovered that even differences in specific *Lactobacillus* spp. can directly impact the barrier properties of CVM: CVM from women with an *L. crispatus*-dominant microbiota consistently trapped HIV-1, whereas CVM from women with an *L. iners*-dominant microbiota mostly failed to do so. Importantly, CVM that trapped HIV-1 virions also trapped ΔEnv pseudoviruses and carboxyl-modified latex beads, suggesting that CVM with *L. crispatus*-dominant microbiota may broadly preclude the penetration of HIV across different clades/strains as well as that of other enveloped viruses transmitted sexually.

Previously, *Lactobacillus* spp. were thought to be associated with only a moderate reduction in the risks of acquiring HIV and other STIs. Indeed, epidemiological studies suggest that the risk for women with intermediate microbiota or BV of acquiring HIV or other STIs is only ~2-fold to 4-fold higher than the risk for women with *Lactobacillus*-dominant microbiota. Unfortunately, the common method of categorizing the vaginal microbiota in these studies, Nugent scoring (7), does not differentiate between different species of *Lactobacillus* or account for the potentially rapidly fluctuating nature of the vaginal microbiota (Nugent scoring is typically performed only at the onset of a clinical trial rather than throughout the trial, while episodes of BV may last only a few days at a time [22, 23]). Recent evidence has shown that *L. iners* is commonly found in women who have had episodes of BV or intermediate microbiota (23). It is thus very likely that a significant population of women with an *L. iners*-dominant microbiota that frequently shifts to an intermediate microbiota or BV may have been included in the "healthy" protective vaginal microbiota group in prior epidemiological studies. This, in turn, suggests that the risks of acquiring STIs when a woman possesses an *L. crispatus*-dominant microbiota (and, potentially, other *Lactobacillus* spp. besides *L. iners*) are likely markedly lower than previously estimated. If true, this implies that methods ensuring an *L. crispatus*-dominant microbiota may be among the most effective means of reducing vaginal HIV transmission.

We posit that *L. crispatus* may mediate vaginal protection against HIV by at least three mechanisms: (i) enhanced trapping of viruses via hydrogen bonding to mucins at low pH, (ii) direct inactivation of cell-free or cell-associated HIV through secreted lactic acid (24), and (iii) inhibition of other bacteria that could compromise CVM barrier properties and thereby elevate risks for HIV acquisition. First, lactic acid continuously secreted by *Lactobacillus* spp. creates an acidic environment in the range of pH 3.5 to 4 under anaerobic conditions (13, 25). Numerous studies have shown that *L. crispatus* produces not only more D-LA but also more total LA than *L. iners*, with correspondingly lower pH (Fig. 3C) (8, 17). Lower pH, in turn, promotes hydrogen bonding among carboxylic acid groups, which our studies suggest likely play a role in interactions between the virus surface and mucins. Greater LA secretion may also lead to more-rapid restoration of the native acidic environment upon exposure to semen. Second, in addition to serving as a marker of the diffusional barrier properties of CVM, there is emerging evidence that LA may facilitate protection by other mechanisms. For example, LA acidification can rapidly inactivate sperm as well as prevent vaginal colonization by nonindigenous organisms (16, 25–28). Recent evidence suggests that LA possesses increased antimicrobial activity beyond acidity alone (11, 24, 25), and Aldunate et al. showed that vaginal concentrations of LA can potently inactivate HIV (24). Furthermore, low (<5.8) pH quickly immobilizes and kills human leukocytes (29), which, combined with the CVM diffusional barrier surrounding the cells, is likely to effectively inhibit any cell-associated transmission of HIV via immune cells present in the mucus layer. Finally, cultivation-based studies have shown that women with vaginal microbiota colonized by *L. crispatus* have a lower risk of BV acquisition than women colonized by other spe-

cies of lactobacilli (30). Lactic acid at low (<4.5) pH is a potent bactericide against BV-associated bacteria (25), and *L. crispatus* may also produce bacteriocins that ward off other bacteria, including *L. iners* and *G. vaginalis* (31). *G. vaginalis* can secrete high levels of sialidases and other mucin-degrading enzymes (21, 32), and it is possible that some strains of *L. iners* may also secrete enzymes that reduce HIV trapping in CVM, either by destroying the mucin network that holds adhered viruses in place or by cleaving binding groups on mucins that HIV virions bind to in *L. crispatus*-dominant CVM. The various extents to which HIV and carboxyl-modified latex beads were trapped in native CVM may thus reflect various concentrations of bacterial enzymes in CVM, which in turn would impact the extent to which mucins are altered biochemically. Once mucins are degraded, their ability to trap HIV is likely irreversibly compromised. Thus, addition of exogenous D-LA did not enhance trapping, and we speculate that addition of *L. crispatus* to *ex vivo* CVM also would not facilitate trapping. In contrast to *L. crispatus*, *L. iners* does not appear to produce bacteriocins (33), which may contribute to a higher frequency of shifts to intermediate or BV microbiota. In general, the presence of *L. iners* has been found to correlate with vaginal metabolic profiles that are intermediate with respect to those correlated with BV-associated bacteria and *L. crispatus* (34), although such studies have yet to reveal additional metabolite differences specifically attributable to *L. iners* versus *L. crispatus*. (Note that while *L. crispatus* is thought to be a hydrogen peroxide producer and *L. iners* a nonproducer [35], this difference is unlikely to be relevant *in vivo*, since hydrogen peroxide production requires aerobic conditions and the vagina is typically hypoxic [13].) The combination of the factors discussed above may explain why no single CVM characteristic measured in this study—pH, Nugent scores, total lactic acid, or even D-LA— correlated as strongly with HIV mobility as did the vaginal microbiota.

CVM can exhibit markedly different macroscopic characteristics (e.g., color and consistency) over time as well as between women. Nevertheless, it is important to note that the physical properties of the local environment (i.e., at the length scale of HIV virions) that enable or block HIV diffusional mobility in CVM are distinct from the macroscopic physical properties of the mucus gel (36, 37). For example, in our previous study (11), as well as the current work, the bulk viscoelasticities of CVM did not appear to differ between the CVM specimens that trapped HIV and those that failed to trap HIV, including those with substantial quantities of *G. vaginalis*. Likewise, variations in the macroscopic properties of CVM do not necessarily compromise its ability to serve as an effective and consistent diffusional barrier against HIV and other STIs. Consequently, the key to harnessing CVM as a protective barrier is to better understand the complex molecular and biophysical interactions that occur within CVM, and our current effort to link vaginal microbial communities to HIV mobility in CVM provides important first clues as to how the innate diffusional barrier properties of CVM may be reinforced against STIs.

Trapping or even slowing viruses in mucus is likely an effective mechanism of mucosal protection that operates not only by reducing the viral load arriving at target cells and purging trapped viruses by natural mucus clearance mechanisms but also by increasing the likelihood of inactivation via other innate protective mechanisms (e.g., defensins, thermal inactivation, etc. [38, 39]) while virus penetration of mucus is delayed. The potential effectiveness of trapping in mucus is perhaps best exemplified by studies in reproductive biology: sperm trapped in mucus by antisperm antibodies are excluded from contacting target cells (i.e., eggs), and the inability of sperm to penetrate mucus correlates strongly with infertility (40). Recently, we showed that mucosal IgG against herpes simplex virus (HSV-1) can trap HSV-1 in CVM at subneutralizing concentrations and that even a nonneutralizing IgG can block vaginal Herpes transmission in mice by trapping viruses in mucus (41). (Note that while it is also possible for HIV to be trapped by HIV-binding IgG, this mechanism is unlikely to have played a role in the current study, since samples were obtained from individuals who self-reported as HIV negative.) Our current finding that *L. crispatus* can enhance the diffusional barrier properties of native CVM adds to emerging insights into the mechanisms of both innate and adaptive vaginal mucosal immune protection based on trapping viruses in mucus.

It is important to note that our discoveries have been made possible only by investigation of pathogen mobility in fresh, minimally modified CVM collected from a relatively large number of women. Few prior studies of mucosal protection have been performed with human mucus secretions *ex vivo*, since fresh mucus gel is more difficult to obtain and handle than diluted fluids from lavages or swabs or from mucins isolated from cell cultures. We anticipate that investigating the effect of exposure to semen will also be critical to fully understanding CVM barrier functions *in vivo*. Semen contains degradative enzymes that may alter mucins and/or the HIV surface (42). In addition, HIV transmitted from ejaculate to CVM may be coated with proteins or other semen components that affect HIV diffusion in CVM. Perhaps most importantly, semen transiently dilutes and neutralizes CVM (the entire vagina becomes acidic again within minutes to hours, depending on the rate of postcoital discharge of semen [43]), which can interfere with its ability to block HIV. Nevertheless, semen is unlikely to fully mix with mucus, and there is likely to be a pH gradient within the mucus layer that is more acidic closer to the epithelium, where lactobacilli are most abundant. Further studies using fresh *ex vivo* semen and mucus gels from women with diverse microbiota, and in greater numbers of samples, will afford a better understanding of the barrier properties of physiological mucus secretions and will likely lead to the development of novel approaches to reinforce the mucosal barrier against pathogens.

MATERIALS AND METHODS

Preparation of fluorescent HIV-1 and ΔEnv pseudoviruses. Replication-defective HIV-1, internally labeled with an mCherry-Gag construct to avoid alteration of the viral surface, was prepared by transfection of 293T cells with plasmids encoding NL4-3Luc$^+$Vpr$^-$Env$^-$, Gag-mCherry, and YU2 Env in a 4:1:1 ratio. The cell supernatant was collected 48 h later, and fluorescently tagged virions from the cell supernatant were purified by centrifugation through 25% sucrose at 160,000 × *g* for 2.5 h. The virions were then washed, resuspended in phosphate-buffered saline (PBS), divided into aliquots, and stored at −80°C. ΔEnv pseudoviruses were similarly prepared without incorporating the Env plasmid.

Nanoparticle preparation and characterization. Fluorescent, carboxyl-modified latex beads (PS-COOH) (200 nm in diameter) were purchased from Molecular Probes (Eugene, OR). These particles feature a high surface density of COOH groups (~7 to 8 COOH/nm^2) and a negatively charged surface at neutral pH (−49.3 ± 2.1 mV). Mucoinert nanoparticles (PS-PEG) were prepared by conjugating 2 kDa of amine-modified polyethylene glycol (PEG; Rapp Polymere, Tuebingen, Germany) to PS-COOH particles via a carboxyl-amine reaction, as published previously (44). PEG conjugation was confirmed by a near-neutral ξ-potential (−3.23 ± 0.32 mV), measured in 10 mM NaCl solution

(pH 7) using a Zetasizer Nano ZS90 system (Malvern Instruments, Southborough, MA).

CVM collection and characterization. Cervicovaginal mucus (CVM) collection was performed as published previously (11, 18, 44). Briefly, undiluted CVM secretions, averaging 0.3 g per sample, were obtained from 31 women (one sample per participant) of reproductive age ranging from 19 to 33 years of age (mean ± standard error of the mean [SEM], 24.5 ± 0.7 years) by using a self-sampling menstrual collection device (Instead Softcup) following a protocol approved by the Institutional Review Board of the University of North Carolina at Chapel Hill. Informed consent of participants was obtained after the nature and possible consequences of the study were explained. Participants inserted the device into the vagina for at least 30 s, removed it, and placed it into a 50-ml centrifuge tube. Samples were centrifuged at 230 × *g* for 2 min to collect the secretions. Samples were collected at random times with respect to the menstrual cycle, and cycle phase was estimated based on the last menstrual period date normalized to a 28-day cycle. While a woman's vaginal microbiota may vary throughout the menstrual cycle (9, 23), to avoid potential biases due to menstrual cycle effects, no participant was sampled more than once in this study. No samples were ovulatory as judged on the basis of visual observation (none exhibited "spinnbarkeit" [spinnability]). Samples that were nonuniform in color or consistency were discarded. Donors stated they had not used vaginal products or participated in unprotected intercourse within 3 days prior to donating and indicated whether they had participated in protected intercourse within 24 h prior to donating. Donors also reported whether they had been using a hormonal contraceptive in the 3 weeks prior to donating. No donors reported having had STIs or other vaginal conditions within 7 days prior to donating. HIV-1 mobility in CVM did not correlate with donor age, race, contraceptive use, or menstrual cycle phase (see Table S1).

The pH of CVM samples was measured immediately after sample collection using a pH microelectrode (Microelectrodes, Inc., Bedford, NH) calibrated to pH 4, 7, and 10 buffers. Vaginal smear slides for Gram staining and aliquots of CVM for lactic acid measurements (diluted 1:5 with 1× PBS and stored at −80°C) were also prepared immediately, and the remainder of the sample was stored at 4°C until microscopy, typically within a few hours. Gram-stained slides were viewed under a 100× objective, and Nugent scores were calculated following scoring criteria described previously (7). For lactic acid measurements, CVM aliquots were thawed and centrifuged for 2 min at 21,130 × *g* to obtain cell-free supernatant containing lactic acid, which was measured using a D/L-lactic acid kit (R-Biopharm, Darmstadt, Germany) following the manufacturer protocol adapted to a 96-well format.

Multiple-particle tracking of HIV-1 and nanoparticles in CVM. Fluorescent virions or beads (approximately 10^8 to 10^9 particles/ml) were added at 5% (vol/vol) to 20 μl of CVM placed in a custom-made glass chamber and incubated for 1 h at 37°C prior to microscopy. In a subset of samples, an aliquot of CVM was first titrated to pH 6.8 to 7.1 using small volumes (~3% [vol/vol]) of 3 N NaOH to mimic neutralization of CVM by alkaline seminal fluid. The translational motions of the particles were recorded using an electron multiplying charge-coupled-device (EMCCD) camera (Evolve 512; Photometrics, Tucson, AZ) mounted on an inverted epifluorescence microscope (AxioObserver D1; Zeiss, Thornwood, NY) equipped with an Alpha Plan-Apo 100×/1.46 numerical-aperture (NA) objective, an environmental (temperature and CO_2)-control chamber, and a light-emitting-diode (LED) light source (Lumencor Light Engine DAPI/GFP/543/623/690). Videos (512 pixels by 512 pixels, 16-bit image depth) were captured with MetaMorph imaging software (Molecular Devices, Sunnyvale, CA) at a temporal resolution of 66.7 ms and a spatial resolution of 10 nm (nominal pixel resolution, 0.156 μm/pixel) for 20 s. The tracking resolution was determined by tracking the displacements of particles immobilized with a strong adhesive, following a previously described method (45). Particle trajectories (*xy* locations) were obtained using MatLab software, following methods originally developed in IDL by Crocker and Hoffman (46). Subpixel tracking resolution was achieved by determining the precise location of particle centroids by light-intensity-weighed averaging of neighboring pixels. Trajectories were analyzed using a frame-by-frame approach (Y.-Y. Wang, K. L. Nunn, D. Harit, S. A. McKinley, and S. K. Lai, submitted for publication) in which mean squared displacements (MSD) and effective diffusivities (D_{eff}) are first calculated for individual particle traces, as previously described (11, 18, 44), and averages and distributions are then calculated at each frame based on only the particles present in that frame before averaging across all frames in the movie. This approach minimizes bias toward faster-moving particle subpopulations due to the particles potentially moving into and out of the focal plane multiple times throughout the course of a movie (this results in multiple traces, whereas only a single trace is typically recorded for a trapped particle). Trajectories of ≥40 particles per frame on average were analyzed for each experiment, which typically corresponds to >100 individual particle traces throughout the video and is consistent with the extent of sampling in our previous work (Wang et al., submitted). Trapped particles were defined by a D_{eff} value of $<10^{-1.5}$ $\mu m^2/s$ at a τ value of 0.2667 s (time scale corresponding to a minimum trajectory length of 5 frames); for particles 200 nm in diameter and larger, this cutoff translates to particles moving a distance that is much less than their diameter within 0.2667 s.

16S rRNA sequencing and analysis. DNA extractions for microbiome analysis were performed using previously described protocols (23) on aliquots of the same CVM samples used for microscopy studies and other characterizations described above. The detailed method of Fadrosh et al. (47) was used to analyze the vaginal microbiota composition and structure and relied on amplification and sequencing on an Illumina MiSeq instrument (300-bp paired-end reads) of the V3-to-V4 region of the 16S rRNA gene. Sequence analyses and taxonomic assignments were performed using a custom pipeline, freely available on GitHub (https://github.com/cwzkevin/MiSeq16S). The resulting taxonomic assignments are shown in Table S2 in the supplemental material. To assess whether the vaginal microbiota affects the barrier properties of CVM, we first grouped any CVM samples with an appreciable (>5%) abundance of *G. vaginalis* (group 3), since *G. vaginalis* is thought to potentially increase the risks of HIV transmission by secreting sialidases and other enzymes that degrade mucins (21, 32). Thus, any CVM specimens with appreciable quantities of *G. vaginalis*, even at relatively low levels, are likely to have had their barrier properties compromised to some extent. We next grouped the remaining CVM specimens based on whether they were *L. crispatus*-dominant (group 1) or *L. iners*-dominant (group 2) to determine whether *L. crispatus* and *L. iners* have differential effects on CVM barrier properties.

Statistical analysis. Statistical comparisons were limited to two groups (CVM samples that trapped HIV-1 versus those that did not trap HIV-1 and CVM samples with microbiota dominated by *L. crispatus* versus those dominated by *L. iners* or containing substantial *G. vaginalis*). A one-tailed Student's *t* test, assuming unequal variances, was used for all comparisons. Differences were deemed significant at an alpha level of 0.05. All values are reported as means ± SEM unless otherwise indicated.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01084-15/-/DCSupplemental.

Movie S1, AVI file, 1.2 MB.
Movie S2, AVI file, 1.2 MB.
Figure S1, PDF file, 0.3 MB.
Figure S2, PDF file, 0.3 MB.
Table S1, PDF file, 0.3 MB.
Table S2, PDF file, 0.5 MB.

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