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Deciphering the Role of Mucosal Immune Responses and the Cervicovaginal Microbiome in Resistance to HIV Infection in HIV-Exposed Seronegative (HESN) Women

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ABSTRACT The female genital tract (FGT) is an important site of human immunodeficiency virus (HIV) infection. Discerning the nature of HIV-specific local immune responses is crucial for identifying correlates of protection in HIV-exposed seronegative (HESN) individuals. The present study involved a comprehensive analysis of soluble immune mediators, secretory immunoglobulins (sIg), natural killer (NK) cells, CXCR5⁺ CD8⁺ T cells, T follicular helper (Tfh) cells, and T regulatory cells (Tregs) in the vaginal mucosa as well as the nature and composition of the cervicovaginal microbiome in HESN women. We found significantly elevated antiviral cytokines, soluble immunoglobulins, and increased frequencies of activated NK cells, CXCR5⁺ CD8⁺ T cells, and Tfh cells in HESN females compared to HIV-unexposed healthy (UH) women. Analysis of the genital microbiome of HESN women revealed a greater bacterial diversity and increased abundance of *Gardnerella* spp. in the mucosa. The findings suggest that the female genital tract of HESN females represents a microenvironment equipped with innate immune factors, antiviral mediators, and critical T cell subsets that protect against HIV infection.

IMPORTANCE The vast majority of human immunodeficiency virus (HIV) infections across the world occur via the sexual route. The genital tract mucosa is thus the primary site of HIV replication, and discerning the nature of HIV-specific immune responses in this compartment is crucial. The role of the innate immune system at the mucosal level in exposed seronegative individuals and other HIV controllers remains largely unexplored. This understanding can provide valuable insights to improve vaccine design. We investigated mucosal T follicular helper (Tfh) cells, CXCR5⁺ CD8⁺ T cells, natural killer (NK) cells subsets, soluble immune markers, and microbiome diversity in HIV-exposed seronegative (HESN) women. We found a significantly higher level of mucosal CXCR5⁺ CD8⁺ T cells, CD4⁺ Tfh cells, activated NK cell subsets, and antiviral immune cell mediators in HESN women. We also found a higher abundance of *Gardnerella* spp., microbiome dysbiosis, and decreased levels of inflammatory markers to be associated with reduced susceptibility to HIV infection. Our findings indicate that increased distribution of mucosal NK cells, CXCR5⁺ CD8⁺ T cells, Tfh cells, and soluble markers in HIV controllers with a highly diverse cervicovaginal microbiome could contribute effectively to protection against HIV infection. Overall,

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our findings imply that future vaccine design should emphasize inducing these highly functional cell types at the mucosal sites.

KEYWORDS cytobrush, cervicovaginal lavage, HESN, NK cells, TSCM cells, Tfh cells, CXCR5⁺ CD8⁺ cells, B cells, CBA, cervicovaginal microbiota, memory B cells

Mucosal surfaces are primarily protected by innate immune mechanisms, but adaptive immune mechanisms also operate (1). The natural barriers present in the female genital tract (FGT) are insufficient to protect against all infections, although the FGT is a site of immunological balance (2). Innate immune cells play a vital role in the nonspecific destruction of foreign organisms. Natural killer (NK) cells recognize infected host cells via surface receptors and use various mechanisms to kill them (3). Increased NK cell activity is reflected by the higher cytotoxic capacity of NK cells and increased production of NK cell-specific cytokines and chemokines associated with control of infection (4–6). Perturbations in the NK cell receptor repertoire, including cells expressing natural cytotoxicity receptors (NCRs) and killer inhibitory receptors (KIRs), have been reported in human immunodeficiency virus (HIV) and other viral infections (7). Elevated NK cell activity has been correlated with protection against infection in several high-risk cohorts of HIV-exposed seronegative (HESN) subjects, including intravenous drug users, HIV-1-discordant couples, and perinatally exposed infants (8). Studying the role of KIR- and NCR-expressing NK cell subsets may help us understand NK cell-mediated control of early HIV infection.

Neutralization has long been viewed as the primary effector function of humoral immunity and is considered to be the primary correlate of antibody-mediated protection in HIV infection (9). However, the role of mucosal antibodies in HIV infection continues to be controversial (10). Soderlund et al. (2007) detected HIV-1-specific neutralizing IgA antibodies in some infected persons, but these antibodies were unable to block the transfer of the virus from dendritic cells (DC) to susceptible target cells (11). However, Nag et al. found that HIV-1 gp120-specific IgGs in the cervical fluid could mediate antibody-dependent cellular cytotoxicity (ADCC) activity and that their levels correlated inversely with genital viral load (12).

Antibody responses are dependent mainly on CD4⁺ T cell help, and the significant subset that provides this help is the T follicular helper (Tfh) cell. These cells help in B cell affinity maturation, isotype switching, and the development of long-lived plasma cells (13). A high frequency of peripheral blood Tfh cells has been reported to correlate with high titers of broadly neutralizing antibodies (bNAb) and reduced viral load in HIV-infected individuals (14). Similarly, infiltration of CD8⁺ T cells into the B cell follicle area has been reported in HIV-infected individuals. More recently, Shen et al. demonstrated that CXCR5⁺ CD8⁺ T cells residing in the germinal center follicular area play an essential role in neutralizing virus-infected target cells (15). However, the role of CXCR5⁺ CD8⁺ T cells or Tfh cells in disease control in highly exposed uninfected individuals and the correlation of these cell types with HIV-specific antibody titers is yet to be assessed.

The cervicovaginal microbiome is a complex ecosystem with a preponderance of *Lactobacillus* spp. Vaginal lactobacilli prevent the colonization of the female genital tract by pathogenic microbes by maintaining an acidic pH. In addition, the cervicovaginal mucus functions as a physical barrier for the ascent of exogenous pathogens in women with a healthy vaginal microbiome (16). Depletion of vaginal lactobacilli and cervicovaginal mucus is commonly observed among women with conditions such as bacterial vaginosis (BV), aerobic vaginitis, genital chlamydia, and human papilloma virus (HPV) infection (17). Many studies have reported cervicovaginal dysbiosis as an essential risk factor for the acquisition of HIV-1 infection (16, 18, 19). Cervicovaginal colonization by bacterial species, such as *Prevotella bivia*, *Sneathia* spp., and *Mycoplasma hominis*, has been reported to be associated with increased concentrations of proinflammatory cytokines and chemokines in the FGT and enhanced susceptibility to HIV-1 acquisition (20–22).

The present study attempted to characterize in detail the role of mucosal NK cells, CXCR5⁺ CD8⁺ T cells, Tfh cells, soluble immune mediators, antiviral cytokines and chemokines, as well

TABLE 1 Demographic and clinical characteristics of HESN and HIV-unexposed healthy women

Parameters ^a	HESN women ^a (N = 37)	Unexposed women (N = 35)
Age, yrs		
Median	36	33
Range	27–42	22–42
Vaginal douching	37 (100%)	35 (100%)
Cervical cancer (HPV)-positive	0	0
STD (BV, CT, NG)-positive	0	0
Avg no. of sexual contacts per mo		
With condom (median)	2	2
Without condom (median)	1	1
Unknown (median)	1	0
HIV-infected partner on ART		
Yes	37 (100%)	0
NA	0	35 (100%)

^aHESN, HIV-1-exposed seronegative; HPV, human papilloma virus; STD, sexually transmitted disease; BV, bacterial vaginosis; CT, *Chlamydia trachomatis*; NG, *Neisseria gonorrhoeae*; ART, antiretroviral therapy, NA, not applicable.

as the composition of the cervicovaginal microbiome in the early control of infection in HIV-exposed seronegative women.

RESULTS

Sociodemographic features of the study population. There was no significant difference between the two groups for age, practice of vaginal douching, presence of bacterial vaginosis, *Chlamydia trachomatis* (CT), or *Neisseria gonorrhoeae* (NG) infection. All study participants were screened for cervical cancer by testing for HPV infection using the Pap smear test. The HESN group reported sex an average of 2 times a month with their infected partner with or without condom use. The mucosal specimens collected for this study were obtained at least a week after the last sexual act and precisely 2 weeks after the start of the last menstrual period in the volunteers (Table 1).

Presence of increased frequency of mucosal CD8⁺ T cells expressing follicular homing receptor in HESN individuals. Studies have shown that the frequency of CD8⁺ T cells expressing the follicular homing receptor, CXCR5 correlates inversely with viral load in both human and animal models (15, 23). A recent study reported that elite controllers had increased numbers of CXCR5⁺ CD8⁺ T cells exhibiting increased effector function and suggested that these cells have a potential role in the natural control of HIV infection (24). Similarly, it is well known that follicular T helper cells play an important role in class switching and antibody production. However, the distribution of Tfh cells in the mucosal microenvironment has not been fully elucidated. Hence, we analyzed the frequencies of CXCR5⁺ CD8⁺ T cells, Tfh cells, and T regulatory cells (Tregs) in cytobrush specimens obtained from HESN women and compared these values with those detected in unexposed healthy (UH) women.

To examine the frequency and distribution of follicular homing/CXCR5⁺ CD8⁺ T cells, Tfh cells, and Tregs, we stained cervical cells obtained by cytobrush sampling of the cervix of HESN and UH women with fluorochrome-tagged monoclonal antibodies and performed multicolor flow cytometry (Table S1; <http://www.niir.res.in/pdf/HIV/Supplemental%20File.pdf>). As previously described, CD4⁺ Tfh cells were defined as CD4⁺ CD45RO⁺ CCR7⁺ PD1⁺ CXCR5⁺ CXCR3⁻ cells (14), and follicular homing CD8⁺ T cells were defined as CD8⁺ CD45RO⁺ CCR7⁺ PD1⁺ CXCR5⁺ CXCR3⁻ cells (23). Tregs were defined as CD3⁺ CD4⁺ CD127⁻ CD25⁺ cells (Fig. 1a).

We found that the HESN group had significantly increased numbers of mucosal follicular homing CXCR5⁺ CD8⁺ T cells ($P = 0.017$) (Fig. 1b) compared to the UH group. We further analyzed the expression of CXCR5 on CD4⁺ Tfh cells and found that the frequency of CXCR5-expressing CD4⁺ Tfh cells was significantly higher in the HESN group than in the UH group

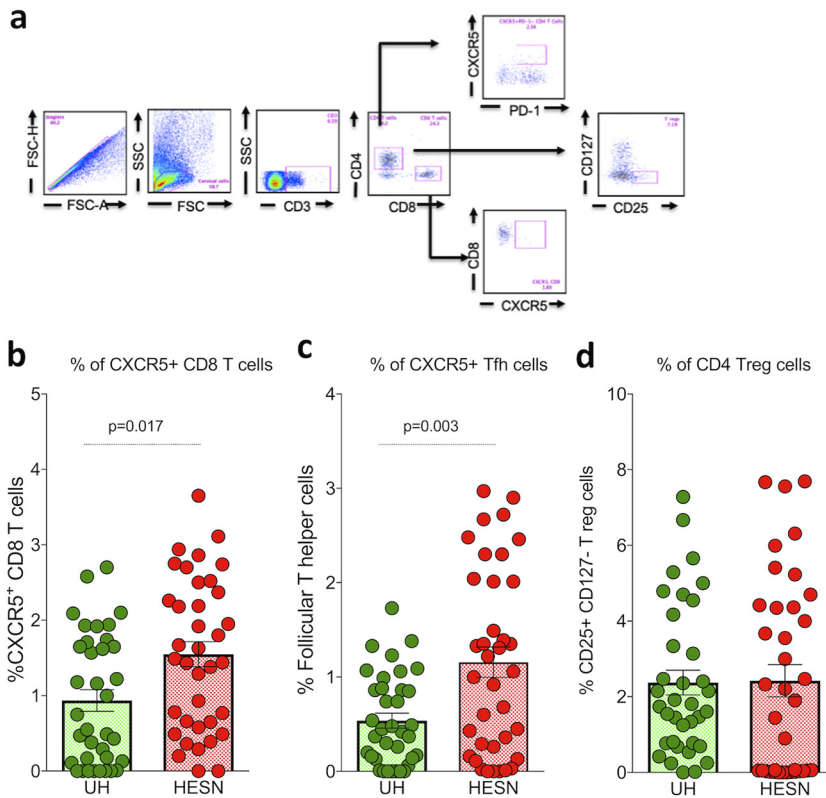


FIG 1 Mucosal CXCR5⁺ CD8⁺ T cells, T follicular helper cells and Tregs. (a) Representative flow plots showing the percent frequency of CXCR5⁺ T cells and Tregs in the study groups (HESN, HIV-exposed seronegative [*N* = 37]; UH, HIV-unexposed seronegative [*N* = 35]). (b) Figure showing the cumulative frequency of CXCR5⁺ CD8⁺ T cells in the HESN and UH groups. (c) Figure showing the cumulative frequency of T follicular helper cells in the HESN and UH groups. (d) Figure showing the cumulative frequency of CD127⁻ CD25⁺ T regs in the HESN and UH groups. The scatter dot plots summarize the percent frequency of total CD8⁺ CXCR5⁺ T cells, Tfh cells, and Tregs (median, 1st, and 3rd quartiles). *P* values were determined using the Mann-Whitney test.

(*P* = 0.003) (Fig. 1c). The median frequency of mucosal CXCR5⁺ CD8⁺ T cells was 1.49% (range, 0.65 to 2.37%) compared to 0.65% (range, 0.11 to 1.69%) in the UH group, and the median frequency of mucosal Tfh cells among the CD4⁺ T cell subset was 1.06% (range, 0.26 to 2.01%) in the HESN group compared to 0.44% (range, 0.14 to 0.87%) in the UH group. However, there was no difference in the distribution of mucosal T regulatory cells between the HESN and UH groups (Fig. 1d).

Abundance of natural cytotoxicity receptor-expressing NK cells in the cervical mucosa of HESN individuals. NK cell-mediated antiviral effector function and ADCC mediated by NK cells and antiviral antibodies have been implicated in the control of HIV infection (25, 26). This evidence suggests a role for NK cells in the control of HIV and opens up a new avenue for research in the development of HIV vaccines (27). However, NK cell function and distribution in the mucosal tissue are not fully understood. We analyzed the distribution of NK cells expressing NCRs and KIRs in the genital mucosa of HESN women and UH women using multicolor flow cytometry on cells present in the cervical cytobrush specimens. NK cells expressing activating natural cytotoxicity receptors (Table S2; <http://www.nirt.res.in/pdf/HIV/Supplemental%20File.pdf>) were defined as CD3⁻ CD16⁺ CD56⁺ CD27⁺ NKG2D⁺ Nkp30⁺/Nkp44⁺/Nkp46⁺ cells, and NK cells expressing inhibitory killer cell immunoglobulin-like receptors were defined as CD3⁻ CD16⁺ CD56⁺ CD27⁺ KLRG1⁺ CD158a⁺/CD158b⁺/CD158e1⁺ cells as described by Kulkarni et al. (5) (Fig. S1 and S2; <http://www.nirt.res.in/pdf/HIV/Supplemental%20File.pdf>).

We found significantly higher frequencies of NK cells expressing NCRs, such as Nkp30, Nkp44, and Nkp46, in the genital mucosa of HESN women than in UH women. We performed

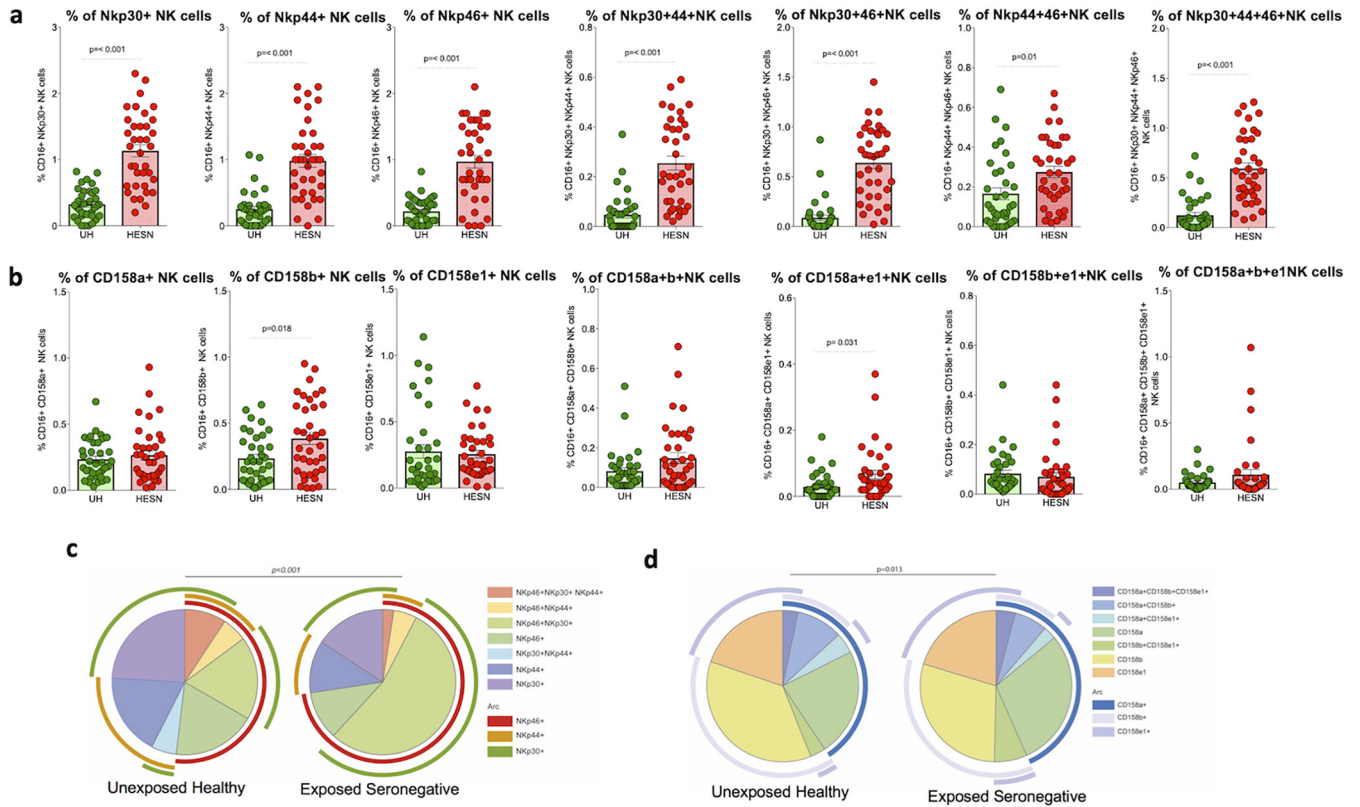


FIG 2 Distribution of natural cytotoxicity receptor (NCR)- and killer cell immunoglobulin-like inhibitory receptor (KIR)-coexpressing mucosal NK cells. (a) Proportion of NK cells expressing each activating receptor (Nkp30, Nkp44, Nkp46). (b) Proportion of NK cells expressing each inhibitory receptor (CD158a, CD158b, CD158e1). The scatter dot plots summarize the percent frequency of total NCR- and KIR-expressing NK cells from cytobrush specimens (HESN, HIV-exposed seronegative [$N = 37$]; UH, HIV-unexposed seronegative [$N = 35$]). The graphical plots show the median, 25th, and 75th percentiles and interquartile range (IQR). P values were determined using the Mann-Whitney test. (c) SPICE analysis of different combinations of NCR-expressing NK cells indicated statistically significant differences in the permutation of different combinations. (d) SPICE analysis of the different combination of KIR-expressing NK cells indicated statistically significant differences in the permutation of different combinations.

Boolean analysis of NCR-expressing cells to identify multiple receptor-expressing cells and found that dual NCR-expressing NK cells (Nkp30⁺ Nkp44⁺, Nkp44⁺ Nkp46⁺, and Nkp30⁺ Nkp46⁺) were also significantly elevated in the HESN group (Fig. 2a). In contrast, the frequency of NK cells expressing inhibitory receptors was similar in the HESN and UH groups. However, CD158b⁺ and CD158a⁺ CD158e1⁺ dual receptor-expressing NK cells alone were found to be significantly higher in the genital mucosa of HESN women than in UH women ($P = 0.018$ and $P = 0.031$, respectively) (Fig. 2b).

We also found that NK cells expressing all 3 NCRs (Nkp30⁺ Nkp44⁺ Nkp46⁺) were significantly more abundant in the HESN group. However, there was no difference in the frequency of NK cells expressing multiple KIRs (CD158a⁺ CD158b⁺ CD158e1⁺ cells) between the HESN and UH groups ($P > 0.950$). These data taken together demonstrate that NK cells expressing NCRs are more abundant in the mucosa of HESN women than in UH women (Fig. 2c and d).

Increased levels of total secretory IgG and IgA in the mucosal microenvironment of HESN individuals. Mucosal immunoglobulin (IgG, IgA, and IgM) levels were determined in the study participants' cervicovaginal lavage using a BD cytometric bead array. We found increased levels of total IgG and IgA in HESN women compared to in UH women. The median level of mucosal IgG was 10,000 ng/ml (2,874 to 55,454 ng/ml) in the HESN group and 454 ng/ml (101 to 3,516 ng/ml) in the UH group. The median level of mucosal IgA was 6,447 ng/ml (1,984 to 11,442 ng/ml) in the HESN group and 1,955 ng/ml (487 to 7,351 ng/ml) in the UH group. Thus, it is evident that the HESN participants had significantly higher levels of total IgG and IgA in their cervicovaginal secretions than UH women (IgG, $P < 0.001$; IgA, $P = 0.024$); however, there was no significant difference in IgM levels between the two groups (Fig. 3).

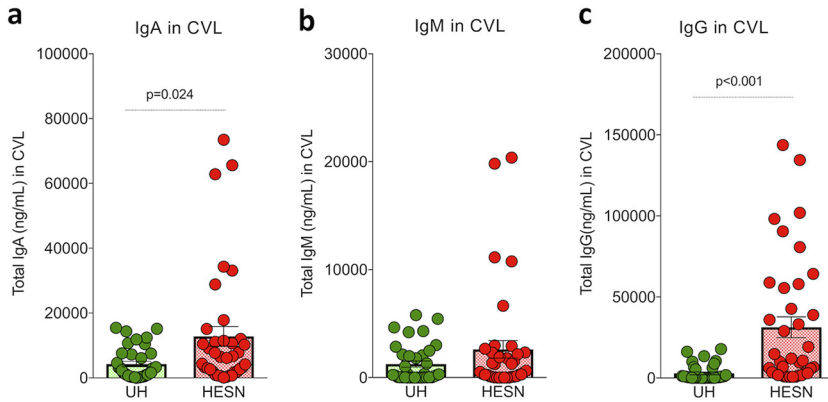


FIG 3 Levels of mucosal immunoglobulins (IgG, IgA, and IgM) in cervical vaginal lavage. Mucosal immunoglobulin (IgG, IgA, and IgM) levels in CVL specimens measured by cytometric bead array and reported in ng/ml. (a) Total IgA level in cervicovaginal lavage. (b) Total IgM level in cervicovaginal lavage. (c) Total IgG level in cervicovaginal lavage. The box-and-whisker plots represent median, 25th, and 75th percentiles and range (IQR). *P* values were determined using the Mann-Whitney test. (HESN, HIV-exposed seronegative [*N* = 37]; UH, HIV-unexposed seronegative [*N* = 35]).

Differences in dynamics of proinflammatory molecules, antiviral cytokines, and chemokines in the cervicovaginal secretions of HESN individuals. Earlier studies that reported elevated antiviral responses against HIV in HIV controllers, slow progressors, and HIV-exposed seronegative individuals (27, 28) have demonstrated a significant role for cytokines and chemokines in HIV disease progression (29). As part of our analysis, we measured antiviral cytokines and chemokines in the cervicovaginal lavage of HESN and UH individuals using a cytometric bead assay (LEGENDplex).

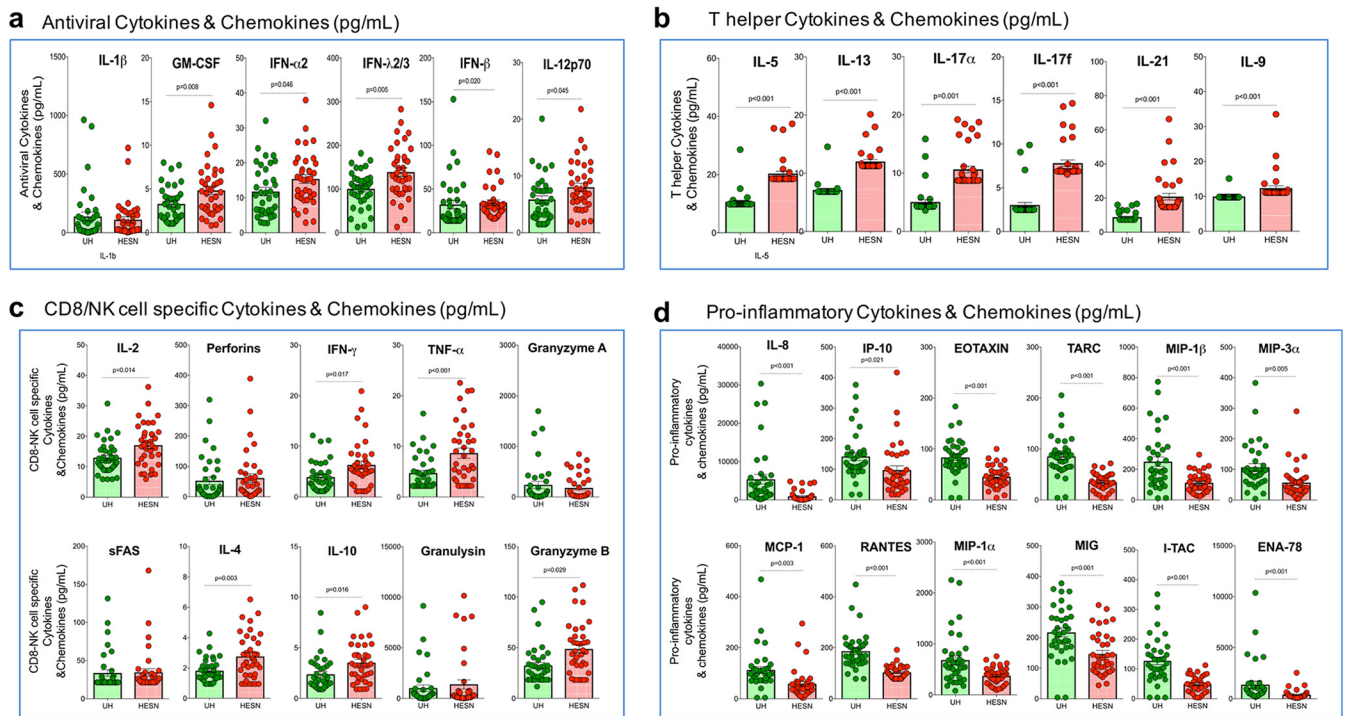


FIG 4 Levels of antiviral mediators, T helper cell-specific and CD8⁺ T cell/NK cell-specific effector molecules, cytokines and chemokines in the cervicovaginal lavage. Levels of (a) antiviral molecules (IL-1, GM-CSF, IFN-2, IFN-λ2/3, IFN-γ, IL-12p70), (b) T helper cell cytokines (IL-5, IL-13, IL-17α, IL-17f, IL-21, IL-9), (c) CD8/NK cell specific cytokines (IL-2, perforin, IFN-γ, TNF-α, granzyme A, sFAS, IL-4, IL-10, granulysin, granzyme B) (d) Pro-inflammatory cytokines and chemokines (IL-8, IP-10, EOTAXIN, TARC, MIP-1β, MIP-3α, MCP-1, RANTES, MIP-1α, MIG, I-TAC, ENA-78) in the CVL of HESN (*N* = 37) and UH (*N* = 35) groups. The data shown is the median level of cytokines and chemokines (pg/ml). The scatter dot plots show the median, 25th, and 75th percentiles and range (IQR). *P* values were determined using the Mann-Whitney test. HESN, HIV exposed seronegative; UH, HIV unexposed seronegative.

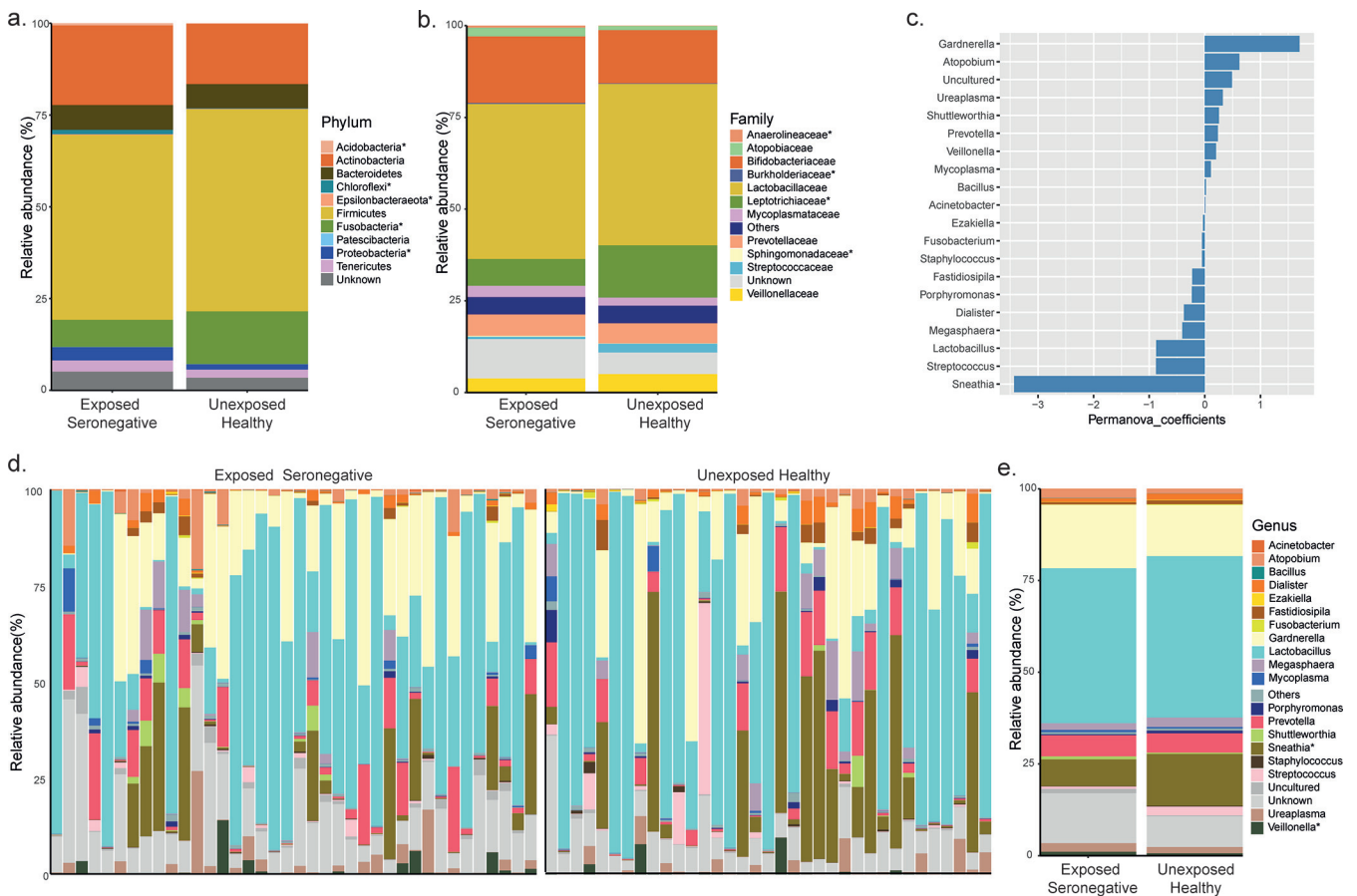


FIG 5 Composition of the cervicovaginal microbiome in the study population. (a) Mean relative abundance in the phylum. (b) Mean relative abundance in family. (c) Top 20 taxa at the genus level that have a significant effect on separating the groups as per PERMANOVA analysis. (d) Visualization of sample-wise relative abundance (%) in the two experimental groups. (e) Mean relative abundance in genus. The asterisk (*) denotes significance in the Mann-Whitney test.

Significantly elevated levels of several T cell/NK cell cytokines and soluble factors, such as interleukin-2 (IL-2), interferon- γ (IFN- γ), tumor necrosis factor- α (TNF- α), IL-17 α , IL-17f, IL-21, IL-4, IL-5, IL-9, IL-10, IL-13, and granzyme B, were detected in the HESN group (Fig. 4b and 4c). The HESN cohort also had significantly higher levels of antiviral cytokines, such as IFN- α 2, IFN- β , IFN- λ 2/3, IL-12p70, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 4a) (Table S4; <http://www.nirt.res.in/pdf/HIV/Supplemental%20File.pdf>). In contrast, levels of all the proinflammatory chemokines measured were found to be significantly lower in the HESN group. These included interleukin-8 (IL-8), interferon- γ -induced protein 10 kDa IFN-gamma-inducible protein 10 (IP-10), eotaxin, thymus- and activation-regulated chemokine (TARC), monocyte chemoattractant protein 1 (MCP-1), RANTES, macrophage inflammatory protein-1 α (MIP-1 α), MIP-1 β , monokine induced by gamma (MIG), epithelial-neutrophil activating peptide (ENA-78), MIP-3 α , human growth regulated oncogene alpha (GRO α), and interferon-inducible T cell alpha chemoattractant (I-TAC) (Fig. 4d; Table S5; <http://www.nirt.res.in/pdf/HIV/Supplemental%20File.pdf>). These findings suggest that the mucosal microenvironment in HESN individuals is more quiescent and has a good supply of antiviral effector cytokines and chemokines, which makes it unsuitable for the establishment of HIV infection.

Composition of the cervicovaginal microbiome differs between the HESN and UH groups. Microbiome analysis revealed that bacteria belonging to the phyla *Proteobacteria*, *Epsilonbacteraeota*, *Acidobacteria*, and *Chloroflexi* were relatively more abundant ($P < 0.001$) in the HESN group than in the UH group (Fig. 5a). The family-level relative abundance of the microflora in the two groups is presented in Fig. 5b. Group-wise comparison of the

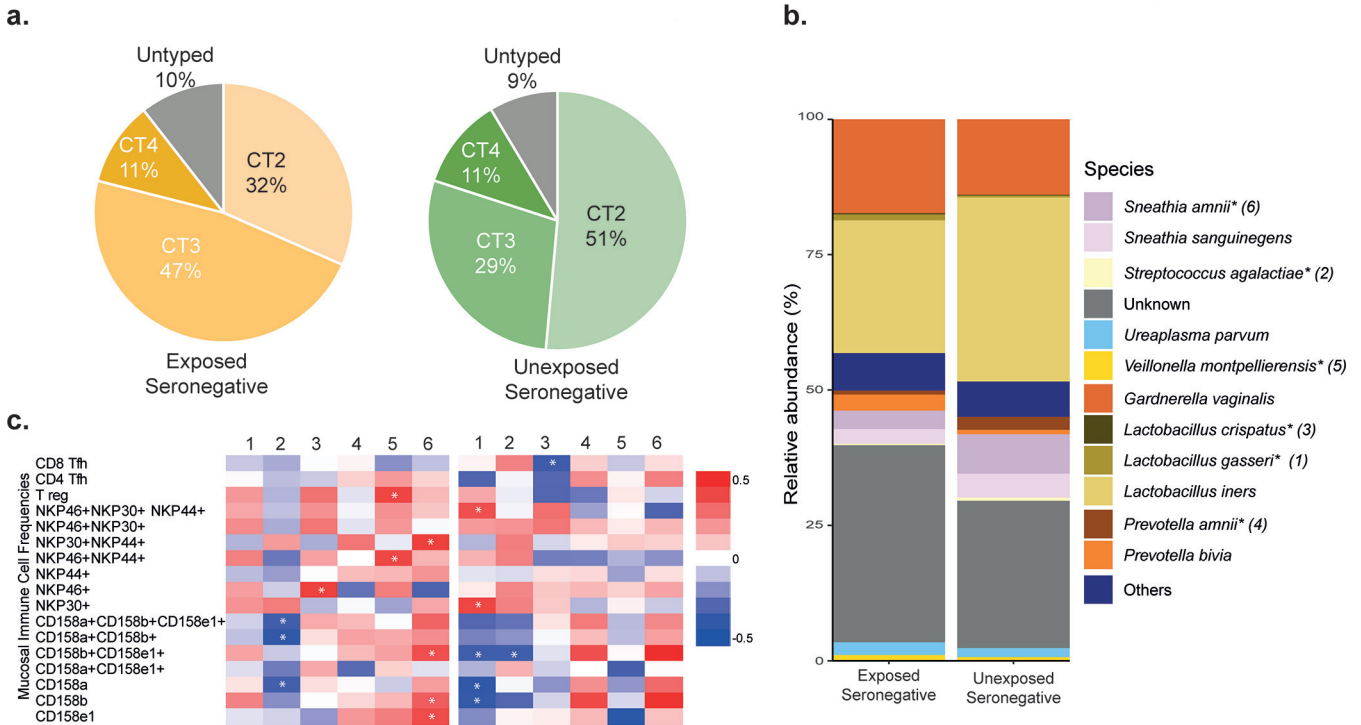


FIG 6 Distribution of bacterial species and their association with inflammation. (a) Distribution of CT2, CT3, and CT4 bacteria in HESN and UH women. (b) Bar graph showing average relative abundance (%) of various species. The asterisk (*) denotes significance in the Mann-Whitney test. (c) Heat map showing Spearman's correlation values between significant species (from Mann-Whitney test) and mucosal immune cell frequencies. The asterisk (*) denotes a significant correlation.

microbiome composition at the genus level was performed using a permutational multivariate analysis of variance (PERMANOVA), which indicated that *Gardnerella* had the highest PERMANOVA coefficient (Fig. 5c). Our findings are in agreement with previous observations of higher abundance of *Gardnerella* and intermediate abundance of *Lactobacillus* spp. in highly exposed commercial sex workers from the Nairobi cohort (30). Microbiome distribution at the genus level is shown in Fig. 5d and 5e. At the species level, we found that a majority (51%) of the UH women had a preponderance of *L. iners* (cervicotype-2 [CT-2] flora), followed by *Gardnerella vaginalis* (CT-3 flora) in 29% and mixed anaerobic bacteria (CT-4 flora) in 11% (Fig. 6a). Among the HESN group, CT-2, CT-3, and CT-4 flora were observed in 32, 47, and 11% of the women, respectively. The comprehensive cervicotype classification system was adapted from Anahtar et al. (21). Taken together, our findings reveal significantly higher microbial diversity (abnormal flora belonging to CT-3 and CT-4) in the HESN cohort (58%) than in the control group (40%) (Fig. 6a). Statistically significant differences were detected between the study groups in the relative abundance of *Veillonella montpellierensis*, *L. gasseri*, *L. crispatus*, *Prevotella amnii*, *Sneathia amnii*, and *Streptococcus agalactiae* (Fig. 6b). While *Veillonella montpellierensis*, *L. gasseri*, and *L. crispatus* were more abundant in the HESN group, *Prevotella amnii*, *Sneathia amnii*, and *Streptococcus agalactiae* were more common in the UH group (Fig. 6b). Higher abundance of ungrouped bacteria was also observed in the HESN group than in the UH group in our cohort.

Interestingly, we observed a positive correlation between the frequency of mucosal NCR- and KIR-expressing NK cell subsets and *L. gasseri*, *L. crispatus*, *Veillonella montpellierensis*, *Prevotella amnii*, and *Sneathia amnii* in the HESN population. A significant correlation was observed between Tregs, Nkp44⁺ Nkp46⁺, and *Veillonella montpellierensis*. Similarly, Nkp30⁺ Nkp44⁺, CD158b⁺ CD158e1⁺, CD158e1⁺, and CD158b⁺ significantly correlated with *Sneathia amnii*. On the other hand, in UH individuals, NK cells expressing Nkp30⁺, Nkp46⁺, Nkp44⁺, and Nkp30⁺ were significantly associated with *L. gasseri*. The correlation observed between bacterial species and immunological markers in the study groups is shown in Fig. 6c. The positive association with immune cell frequencies and

higher microbial diversity in the HESN group suggests that the increased colonization of the genital mucosa with diverse microbial species might lead to increased recruitment of critical components of the innate immune system that coordinate the early control of HIV infection. The intricate role of mucosal immune cell activation in determining the pattern and diversity of the local microbiome remains incompletely understood. Understanding the interplay between the microbiome and the innate and adaptive immune system may shed light on some of the critical events that orchestrate protection against HIV infection.

DISCUSSION

Mucosal immune responses present at the surfaces that serve as points of contact with the pathogen are believed to play a critical role in controlling/preventing HIV infection (31). Innate immune responses are the first line of defense against microbes (32). Earlier studies have documented that host factors present in the FGT, including the natural microbiota, play a role in both protection and susceptibility to infection (1, 33). Protective mucosal responses against HIV are best characterized in individuals who remain relatively resistant to HIV infection despite repeated exposure (34). This phenomenon has been documented in many groups, including commercial sex workers (35), serodiscordant couples (36), infants born to HIV-infected mothers, and others. Long-term nonprogressors (LTNPs) constitute another group of individuals who are HIV infected but relatively resistant to disease progression. These individuals sometimes share common immunological features with HIV controllers and have been used to understand the correlates of resistance/susceptibility to HIV infection and disease progression (1, 36, 37).

Several correlates of protection against HIV infection have been previously described in both systemic and genital compartments. HIV-specific CD8⁺ T cell responses (38–40) and the presence of anti-HIV IgA (41) have been implicated as markers of protection or indicators of exposure (42). Increased levels of the molecules RANTES, serpin B, alpha-2 macroglobulin-like molecule, and cystatin have been correlated with resistance to infection (43). Although multiple correlates of resistance to infection and/disease progression have been described, the mechanisms through which the different factors operate have not been deciphered. Many future investigations are required to understand the mechanisms involved in the protection against HIV-1 infection.

Characterizing the subset of innate immune cells like the NK cells present in the FGT may help us to understand the nature of the innate immune mechanisms operating in the mucosal microenvironment and provide a clue to the development of an adaptive immune process. An earlier study reported elevated levels of NK cell activity and higher secretion of NK cell-specific cytokines in highly exposed uninfected intravenous drug users compared to controls (44). In addition to the evidence supporting the role of NK cells and elevated levels of IFN- γ in mediating control of HIV infection, changes in expression of different cell surface receptors on NK cells have also been implicated in HIV infection and control (45).

Stimulation of NK cell cytotoxicity involves interaction between target cell antigens and stimulatory surface receptors on NK cells (5, 46–48). Wang et al. reported that elevated expression of NCR correlated with low viremia and that NKp30 plays a significant role in NK cell activation, degranulation, and cytotoxicity (4). Another study reported that increased expression of activating receptors and potency of cytotoxic NK cells correlated with reduced viral load in recently infected individuals and long-term nonprogressors (5). Our study also found significantly higher levels of NK cells expressing NKp46, NKp44, and NKp30 in the HESN cohort than in the UH population. We further analyzed frequencies of dual receptor- and triple receptor-expressing NK cells using Boolean gating and found that the proportion of activated NK cells expressing multiple NCRs was also significantly higher in the HESN cohort. This observation supports the hypothesis that the activating receptor-expressing NK cell subset might have a role to play in the early control of HIV infection. We also examined the expression of inhibitory receptors (CD158a, CD158b, CD158e1, and NKG2A) on NK cells and found that the proportion of cells expressing these receptors was correspondingly lower in the HESN group than in the UH group. Alter et al. also suggested that activating KIRs, or

weakly inhibitory KIRs, may have a significant role to play in viral infections (7). Zwolinska et al. found that the frequency of NK cells expressing KIRs was elevated during HIV disease progression and suggested a role for these cells in regulating the exaggerated innate and adaptive antiviral response (49). Studies done in African female sex workers (FSWs) have shown decreased genetic pairing of inhibitory KIRs with HLA ligand, leading to a decrease in inhibitory signaling. These observations support a role for NK cells in protecting against virus transmission and demonstrate that improved NK activation or reduced NK inhibition is linked with resistance to HIV infection (50, 51).

High levels of inflammatory cytokines and tissue damage in the FGT have been suggested to increase susceptibility to HIV infection (52). Earlier studies have documented increased inflammation in the FGT of healthy donors compared to the periphery. These observations suggest that decreased levels of proinflammatory mediators in the FGT would lower inflammation of the mucosa and reduce the risk of HIV acquisition. The findings of our study provide evidence for decreased levels of proinflammatory mediators in the mucosa of HESN women compared to UH women as well as higher levels of antiviral molecules, such as type I and III interferons and cytotoxic effector molecules, suggesting that mechanisms are in place to control inflammation of the FGT in HESN women while responding to low-level viral replication. Lajoie et al. showed that the mucosal immune system of FSWs is less activated than that of low-risk HIV-negative women; they also found that this group of individuals had much lower mucosal levels of proinflammatory cytokines/chemokines than sexually active non-FSWs (53). These findings indicate that low levels of mucosal immune activation trigger the local innate response without increasing the number of potential target cells (53). Our study also found lower levels of proinflammatory cytokines in the mucosal compartment of HESN women than in UH individuals. Lower levels of proinflammatory molecules in the mucosa possibly contribute to the integrity of the genital mucosa by controlling inflammation and maintaining tight junctions, thus favoring the control of HIV infection. It is, therefore, crucial to consider the genital tract as a separate entity when studying HIV-1 transmission and evaluating HIV resistance.

Few studies in HESN individuals have shown that these individuals have neutralizing IgA antibodies in the genital mucosa, which could protect against infection (54). Some reports have suggested that the presence of anti-HIV IgA antibodies in genital secretions correlates more strongly with exposure than resistance to HIV infection (42). In the present study, we measured total IgG, IgA, and IgM in the cervicovaginal lavage (CVL) specimens of HESN and UH cohorts and observed elevated levels of IgA and IgG in the HESN group compared to the UH group. However, there was no difference in the level of IgM between the two groups. Although the study did not measure HIV-specific antibodies, we hypothesize that the abundance of antibodies at the mucosal surface of HESN women may contribute to protection against HIV infection, perhaps by the indirect killing of HIV-infected cells through antibody-dependent cell-mediated cytotoxicity coordinated by NK cells (55).

Antibody responses typically require CD4⁺ T cell help, and the major helper subset that provides this help is the T follicular helper (T_{fh}) cell (56) that primes B cells to produce antibodies. In HIV-infected individuals, a high frequency of circulating T_{fh} cells has been found to correlate with high titers of broadly neutralizing antibodies (bNAb) and reduced viral load (14, 57). Sivasankaran et al. reported that a subtype C-specific HIV vaccine induced circulating memory-like T_{fh} cells that correlated positively with variable region-specific antibody production (58). More recent studies have shown that CXCR5⁺ CD8⁺ T cells traffic into the B cell follicle area of simian immunodeficiency virus (SIV)-infected rhesus macaques and suppress residual viral replication (59). In the present study, we evaluated the frequencies of mucosal CD4⁺ T_{fh} and CXCR5⁺ CD8⁺ T cells in cytobrush specimens and found elevated levels of these T cell subsets in HESN women compared to UH women.

However, in a parallel study, we also analyzed the systemic immune response in HESN individuals and compared it with that seen in HIV-infected women on antiretroviral therapy (ART) as well as those who were naive to ART from another cohort along with UH women. We found significantly elevated frequencies of CXCR5⁺ CD8 T cells and polyfunctional T cells, with stem cell likeness in HESN individuals. In addition, we also demonstrated

a potent *in vitro* viral inhibition activity in the HESN population compared to HIV-infected and HIV-unexposed counterparts (60). To our knowledge, this is the first study reporting higher frequencies of Tfh and CXCR5⁺ CD8⁺ T cells in HESN cohorts from India. These results suggest that the induction of mucosal CD4⁺ Tfh cells and CXCR5⁺ CD8⁺ T cells might contribute to the early control of infection, possibly through induction of a significant antibody response or some other unknown mechanism to eradicate infected cells at the mucosal level.

HIV infection results in the production of proinflammatory cytokines, and their levels increase with the progression of HIV disease (61). Many soluble mediators can suppress HIV replication *in vitro* (62). The number of cytokines produced and cytokine receptor density on the host cell surface are believed to be key determinants for the control of HIV infection (61). Many innate soluble markers possess an inhibitory effect on HIV, including α , β , and θ defensins, IL-6, IL-10, IL-16, TNF- α , interleukin-1 β , and type I interferons α and β (63). In the present study, we analyzed several antiviral cytokines and chemokines in the CVL of HESN and UH women using a cytometric bead array and observed significantly elevated levels of IL-12p70, IFN- α 2, IFN- λ 2/3, GM-CSF, IFN- β , and IL-10 in the HESN group. We also analyzed cytokines produced by CD8 and NK cells among others and found that IL-2, IL-4, IL-10, TNF- α , IFN- γ , and granzyme B were present at significantly increased levels in HESN women. Additionally, soluble Fas (sFas), soluble Fas ligand (sFasL), and perforin levels were also higher in the mucosal compartment of HESN women, although the difference between groups was not significant. Furthermore, we analyzed the levels of Th cytokines and found that IL-5, IL-13, IL-9, IL-17 α , IL-17f, IL-21, and IL-22 were very high in HESN women. The presence of high levels of immune cell-specific cytokines and antiviral cytokines and chemokines at the mucosal surface is thought to be instrumental in instructing the development of different types of immune mechanisms that contribute to the early control of HIV infection.

The cervicovaginal microbiome is a dynamic ecosystem, and the diversity of its composition can vary due to multiple factors, including ethnicity, socioeconomic status, level of education, sexual behavior, and lifestyle factors (18). An interesting finding from the present study concerning the cervicovaginal microbiome was a higher relative abundance of *L. iners* in both groups than of *L. crispatus*, unlike in other studies (21, 22, 64). Reasons for the low abundance of *L. crispatus* in our cohort could not be assessed due to the lack of epidemiological data (socioeconomic status, level of education, and lifestyle factors) regarding vaginal health status (by Gram-stained smear examination) of the study participants. Low et al. reported that intravaginal practices, as well as the presence of bacterial vaginosis (BV), can increase the risk of HIV acquisition. However, intravaginal cleaning with soap, causes disruption of vaginal flora, and usage of vaginal microbicides can reduce the likelihood of HIV acquisition (19). In a recent study on South African women, it was reported that women with a predominance of *L. crispatus* were more resistant to the acquisition of HIV than those with a predominance of *L. iners* and other anaerobic bacterial flora. The investigators also reported an increase in proinflammatory cytokines among women colonized with bacteria such as *Vellionella montpellierensis*, *Prevotella bivia*, and *Sneathia sanguinegens* (22). However, in the present study, we observed a predominance of *L. iners* and a high level of diversity in the cervicovaginal microbiome of HESN individuals. Studies report that a *Lactobacillus*-dominant vaginal microbiota generally reflects vaginal health and is associated with a decreased risk of HIV acquisition (16, 22). We observed higher abundance of *L. crispatus* in the HESN group and a low level of proinflammatory mediators that may help maintain mucosal surface integrity and reduce the risk of HIV acquisition in these individuals. Further, we observed a correlation between mucosal NCR-expressing, activated NK cells and mucosal microbiota in HESN women, suggesting that immune cell trafficking and activation of immune cells in the cervicovaginal surfaces may reduce the risk of HIV infection in association with vaginal dysbiosis.

To summarize, the majority of HIV infections occur via the vaginal or rectal route, and, therefore, many researchers believe that a strong preexisting immune response in the mucosa-associated lymphoid tissue (MALT) may be able to protect against HIV infection. In addition to antigen-specific adaptive immune responses, which were not evaluated in this study, there is mounting evidence that factors associated with

inflammation, or alternatively immune quiescence, may contribute to HIV susceptibility at the mucosal surface (28, 38). The present study explored some of the immune mechanisms operating in the mucosal microenvironment of HESN women and identified mucosal natural cytotoxicity receptor-expressing NK cells, CXCR5⁺ CD8⁺ T cells, follicular T helper cells, and soluble markers as well as a highly diverse cervicovaginal microbiome as potential correlates of protection against HIV-1 infection. As an additional control group, we originally planned to recruit HIV-positive women not on antiretroviral therapy. However, due to implementation of the Treat All policy as required by the new National Technical Guidelines for Antiretroviral Therapy (2017), we were unable to recruit any ART-naïve women. However, further investigation is necessary to determine the functional capabilities of these factors in abrogating HIV infection. The multifactorial nature of HIV resistance emphasizes the importance of the interplay between immune cells and the composition of the vaginal microbiome at the mucosal level. Further understanding in this line would help us to devise strategies to enhance useful innate and adaptive immune responses to prevent HIV infection. This knowledge would also prove to be useful for the identification of novel candidate vaccines.

MATERIALS AND METHODS

Ethics statement. The study protocol was approved by the Scientific Advisory Committee of the Indian Council of Medical Research (ICMR)-National Institute for Research in Tuberculosis (NIRT), Chennai, India. The study was conducted under good clinical laboratory practice (GCLP) guidelines. The study protocol was reviewed and approved by the Institutional Ethics Committee of ICMR-NIRT (IEC ID-2015015) and the Institutional Review Board of Y. R. Gaitonde Centre for AIDS Research and Education (YRG CARE; YRG-302), Chennai, India.

Study participants. The study participants consisted of two groups of individuals: seronegative female spouses of HIV-1-seropositive men (HIV-discordant couples) (HESN, $n = 37$) and HIV-unexposed seronegative women (UH, $n = 35$). Enrollment into the study required willingness to provide written informed consent for specimen collection and storage. HIV-exposed seronegative women aged between 20 and 35 years with a history of multiple unprotected sex events with an HIV-infected partner during the past 1 year were recruited into the HESN group, and HIV-negative healthy women aged between 20 and 35 years with no history of HIV exposure were recruited into the control group. Women outside the reproductive age group, women on menstruation, those with sexually transmitted infections (STIs) or other major illnesses, moribund individuals, and pregnant and lactating women were excluded from the study.

Sample collection. *Collection and processing of cervical cytobrush specimens.* Mucosal specimens were collected by a trained physician. At least two cytobrush specimens were obtained from each study participant. The cytobrush was inserted just within the cervical os and rotated to one 360° turn. Samples contaminated visibly with blood were discarded. Immediately after sampling, the cytobrush was placed in a 15-ml tube containing 3 ml of RPMI 1640 medium containing 100 U/ml penicillin, 100 µg/ml streptomycin, and 2.5 µg/ml amphotericin B and placed on ice. The specimens were transported on ice to the laboratory and processed within 3 h of collection. The cytobrush was gently rotated several times in the transport medium to release all the cells into the medium and then discarded. The cell suspension was centrifuged at $330 \times g$ for 10 min, and the pellet was resuspended in complete RPMI medium containing 100 U/ml penicillin and 100 µg/ml streptomycin and used for flow cytometric analyses.

Collection and processing of cervicovaginal lavage. CVL samples were collected by gently washing the cervicovaginal area with 10 ml of sterile normal saline (pH 7.2) and withdrawing the fluid using a 5-ml syringe. Following CVL collection, samples were frozen as quickly as possible at -80°C . At the time of analysis, samples were thawed at room temperature and centrifuged at $10,000 \times g$ for 5 min, and supernatants were analyzed for soluble antiviral and innate immune factors.

Quantification of total IgG, IgA, and IgM antibodies. Total IgG, IgA, and IgM antibodies were quantified in the CVL specimens using the human immunoglobulin cytokine bead array (CBA) flex set assay (BD Biosciences, San Jose, CA, USA). The Ig CBA assay was performed as per the manufacturer's instructions. Briefly, 50 µl of sample, 1:2 diluted standards (1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, and 1:256) and negative control (plain medium) were added to the respective tubes. About 50 µl of capture bead was added to each tube and incubated for 1 h at room temperature. The tubes were washed with 1 ml of wash buffer at $200 \times g$ for 5 min, and the supernatant was removed. Phycoerythrin (PE) detection reagent (50 µl) was added to the pelleted beads and incubated for 2 h at room temperature. The washing procedure was repeated, and the pellet was resuspended in 300 µl of wash buffer and mixed well. Samples were acquired on a FACS ARIA III SORP flow cytometer (BD Biosciences), and the fluorescence correlation spectroscopy (FCS) files were analyzed using the FCAP array v3 software (BD Biosciences).

Measurement of soluble markers in CVL specimens using cytometric bead array. Proinflammatory chemokines, including MCP-1 (CCL2), RANTES (CCL5), IP-10 (CXCL10), eotaxin (CCL11), TARC (CCL17), MIP-1α (CCL3), MIP-1β (CCL4), MIG (CXCL9), MIP-3α (CCL20), ENA-78 (CXCL5), GROα (CXCL1), I-TAC (CXCL11), and IL-8 (CXCL8), CD8⁺ T cell/NK cell cytokines and cytolytic molecules, including IL-2, IL-4, IL-10, IL-6, IL-17, TNF-α, sFAS, sFASL, IFN-γ, granzyme A, granzyme B, perforin, and granulysin, antiviral cytokines, including IL-1β, IL-6, IL-8, IL-10, IL-12p70, IFN-α, IFN-β, IFN-λ1, IL-29, IFN-λ2/3, IL-28, IFN-γ, TNF-α,

IP-10, and GM-CSF, and Th cell cytokines, including IL-5, IL-13, IL-2, IL-6, IL-9, IL-10, IFN- γ , TNF- α , IL-17A, IL-17F, IL-4, IL-21, and IL-22 were measured in CVL using the BioLegend LEGENDplex human multianalyte kit-based cytometric bead array (CBA) (BioLegend, CA, USA) following the manufacturer's recommendations. Briefly, beads coated with 13 specific capture antibodies were mixed. Subsequently, 50 μ l of the mixed capture beads, 50 μ l of CVL diluted 1:2 or 1:20, and 50 μ l of detector antibody were added and incubated for 2 h on a plate shaker (250 rpm) at room temperature in the dark. Fifty microliters of streptavidin-phycoerythrin (SA-PE) detection reagent was then added to each assay tube and incubated for 30 min on a plate shaker (approximately 250 rpm) at room temperature in the dark. The samples were washed with 1 ml of wash buffer (at $200 \times g$) for 5 min. The bead pellet was resuspended in 100 μ l of wash buffer after discarding the supernatant. Samples were analyzed on a BD FACS ARIA III SORP flow cytometer and analyzed using the LEGENDplex data analysis software, v8.0 (BioLegend, CA, USA). Individual cytokine concentrations were indicated by their fluorescence intensities. Cytokine standards were serially diluted to facilitate the construction of calibration curves, which were necessary for determining the protein concentration of the test samples (65). The lower limit of detection of each molecule is provided in Table S3; <http://www.nirt.res.in/pdf/HIV/Supplemental%20File.pdf>.

Characterization of immune cell types in cervical cytobrush specimens. Mucosal cytobrush-derived cells were washed twice, and viability was evaluated by using the trypan blue dye exclusion method. The average percentage of viability was in the range of 80%. The cells were stained with the following cocktails of monoclonal antibodies for enumeration of the different immune cell types (Table S1 and S2; <http://www.nirt.res.in/pdf/HIV/Supplemental%20File.pdf>).

T follicular helper and Treg panel: CD3-allophycocyanin (APC) H7 (SK7, BD Bioscience), CD4-peridinin chlorophyll protein (PerCP) Cy5.5 (RPA-T4, BD Bioscience), CD8-Brilliant UltraViolet 737 (BUV737) (RPA-T8, BD Bioscience), CD45RO-BUV395 (UCHL-1, BD Bioscience), CCR7-PE-cyanine 7 (PEcy7) (G043H7, BD Bioscience), CXCR3-APC R 700 (IC6, BD Bioscience), CXCR5-Brilliant blue 515 (BB515) (RF8B2, BD Bioscience), PD-1-PE (EH12.1, BD Bioscience), CD25-APC (M-A251, BD Bioscience), and CD127-PE-CF594 (HL7R.M21, BD Bioscience).

NCR-expressing NK cells: CD3-APC H7 (SK7, BD Bioscience), CD16-BUV737 (3G8, BD Bioscience), CD56-APC Alexa 700 (NCAM, BioLegend), CD27-BB515 (MT271, BD Bioscience), NKG2D-PE-CF594 (ID11, BD Bioscience), NKP44-PE (p44-8, BD Bioscience), NKP46-PEcy7 (9E2, BD Bioscience), and NKP30-APC (p30-15, BD Bioscience).

KIR-expressing NK cells: CD3-APC H7 (SK7, BD Bioscience), CD16-BUV737 (3G8, BD Bioscience), CD56-BUV396 (NCAM 16.2, BD Bioscience), CD27-PEcy7 (MT271, Bio Legend), KLRG1-PE-CF94 (231A2, BioLegend), CD158a-PE (CH-L, BD Bioscience), CD158b-BB515 (CH-L, BD Bioscience), and CD158e1-APC (DX9, BD Bioscience).

The cells were stained for 20 min at 4°C. About 0.5 million cells were stained for each panel. After staining, the cells were washed, fixed with BD Cytotfix (2% paraformaldehyde), and analyzed on a FACS ARIA III SORP flow cytometer (Becton Dickinson) (58). A minimum of 300,000 total events were acquired for each panel, and data were analyzed using FlowJo software, version 10.5.4 (Tree Star Inc., Ashland, OR, USA).

Analysis of the vaginal microbiome by next-generation sequencing. The V3-V4 region of 16S rRNA was sequenced using an Illumina HiSeq2500 with 2×250 cycle chemistry. The raw reads were checked for base quality, base composition, and GC content using the FastQC tool (version 0.11.8). After the quality check, preprocessing steps were used on the data. First, forward V3 and reverse V4 primer sequences were trimmed using an in-house Perl script. Properly paired paired-end reads with a Phred quality score of >20 were considered for V3-V4 consensus generation. The preprocessed reads were then analyzed using QIIME (66) software (version 1.9.1) to pick up operational taxonomic units (OTUs) with 97% similarity cutoff and taxonomy classification with the SILVA database as a reference (67). Various R packages were used for all downstream statistical analyses and figure generation. Mann-Whitney test was performed at the phylum, family, genus, and species levels between the two groups using the R package stats since the data followed a nonnormal distribution. PERMANOVA significance test was performed at the genus level using the R package vegan (68). Correlation analysis was performed between relative abundance at the species level, immune cell frequencies, and soluble markers by Spearman correlation using the R package psych (69).

Statistical analysis and graphical representation of the soluble factors and immunological markers. The statistical analysis and graphical representation of immunological markers were performed using the data analysis program SPICE (version 6.0) (70) and ggplot2 version 3.2.0 (<https://ggplot2.tidyverse.org/>). Statistical analyses of mucosal immune cells and soluble markers were performed using GraphPad Prism, version 7.05 (GraphPad Software, Inc., CA). Values are presented as median, interquartile range, and percentage. Mann-Whitney test was used to examine the difference in frequency (%) of different immune cell subsets and levels of soluble markers between the two groups. Correlation analysis was performed to determine the relationship between the frequency of different immune cell types and levels of mucosal soluble markers. For all analyses, differences were considered significant if the *P* value was less than 0.05.

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We declare no conflicts of interest.

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