MAJOR ARTICLE

Elevated T Cell Counts and RANTES Expression in the Genital Mucosa of HIV-1–Resistant Kenyan Commercial Sex Workers

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The initial site of exposure to human immunodeficiency virus (HIV)–1 during heterosexual transmission occurs in the genital tract. Although the majority of immunological studies have focused on the immune response to HIV-1 at the systemic level, our understanding of tissue-specific immunity is deficient. The goal of the present study was to characterize T cell populations found in the cervix of women shown to be resistant to infection by HIV-1. Levels of both systemic and cervical mucosal lymphocytes were compared between HIV-1–resistant, HIV-1–uninfected, and HIV-1–infected commercial sex workers (CSWs) as well as HIV-1–uninfected non-CSW control subjects at low risk for exposure. The HIV-1–resistant CSWs had increased cervical $CD4^+$ and $CD8^+$ T cell counts, compared with the HIV-1–uninfected CSWs; importantly, these increases were not reflected in the systemic lymphocyte compartment. There was a 2-fold increase in $CD4^+$ T cell counts in the HIV-1–resistant CSWs, compared with both the HIV-1–infected and the HIV-1–uninfected CSWs. Expression of the HIV-1 coreceptors CCR5 and CXCR4 was also determined, and cytokine and β chemokine levels in the genital mucosa were assessed. The HIV-1–resistant CSWs had a 10-fold increase in RANTES expression, compared with the HIV-1–uninfected CSWs. This is the first study to show elevated levels of β chemokines and $CD4^+$ T cells in the genital tracts of women who are exposed to HIV-1 and yet are uninfected.

The mechanisms of HIV-1 transmission at the initial site of infection has become an important question in recent years [1, 2]. Although much of the literature on HIV-1 infection and AIDS is based on studies performed in the systemic immune system, this does not reflect initial viral-host interactions. Initial exposure to HIV-1 during sexual transmission occurs in the genital tract; however, little is known about HIV-1–specific immune responses at this site [3], in part due to difficulties in acquiring adequate samples for immunological studies of the genital tract [4]. Recently, researchers have begun to appreciate the complexity of the immunoregulatory environment at this site and the role it plays during the initial phases of HIV-1 transmission [3]. Understanding the immune response to HIV-1 at this site and its subsequent dissemination throughout the body will inform the design of a vaccine based on mucosal immunity.

Studies of the initial interactions between a susceptible host and HIV-1 at the mucosal level have primarily been conducted in animal models [3] and have demonstrated that HIV-1 elicits active immune responses in the genital tract. Vaccination and/or simian immunodeficiency virus (SIV) challenge at the mucosal level results in the production of SIV-specific cytotoxic T lymphocyte (CTL) responses and neutralizing IgA [5–7]. A study in which 3 immunized macaques were pro-

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Table 1. Summary of clinical information from all commercial sex workers (CSWs) enrolled in the present study.

Group	Age, years	Days from last menses	Duration of sex work, years	Contraception use, no. of CSWs					Clients per week, no.	Douching, no. of CSWs		Vaginal discharge, no. of CSWs	STI status, no. of CSWs		
				None	DP	Oral	TL	IUCD		Postcoital	Daily		Ulcer	RPR	Gonorrhea
HIV-1–uninfected CSWs (n = 18)	35.2 ± 2.1 (37)	25.1 ± 4.9 (18)	7.4 ± 1.5 (4)	11	4	1	0	1	22.6 ± 2.7 (21)	3	14	2	1	3	1
HIV-1–resistant CSWs (n = 28)	41.8 ± 1.2 (43.5)	26.6 ± 3.9 (22)	12.6 ± 1.3 (11)	20	5	1	2	0	24.7 ± 3.6 (24.5)	14	8	2	0	0	0
HIV-1–infected CSWs (n = 24)	41.6 ± 1.1 (40.5)	17.7 ± 3.7 (14)	12.4 ± 1.6 (12)	15	5	0	3	0	24.4 ± 3.9 (21)	10	9	0	0	1	1

NOTE. Data are mean ± SE (median), unless otherwise noted. Data were collected via a questionnaire administered by a clinic nurse before samples were collected. Biological sampling was performed by the physician in charge without knowledge of the HIV-1 status of the CSWs, to avoid potential bias of results. Contraceptive use included Depo-Provera (DP), oral contraceptives (oral), tubal ligation (TL), and intrauterine contraceptive devices (IUCDs). Sexually transmitted infection (STI) status included an undefined ulcer (ulcer), being T palladium reactive by rapid plasma reagent test (RPR), and gonorrhea. Note that 2 HIV-1–infected and 2 HIV-1–resistant CSWs were removed from the menses data, because of menopause (defined as no menses for >84 days).

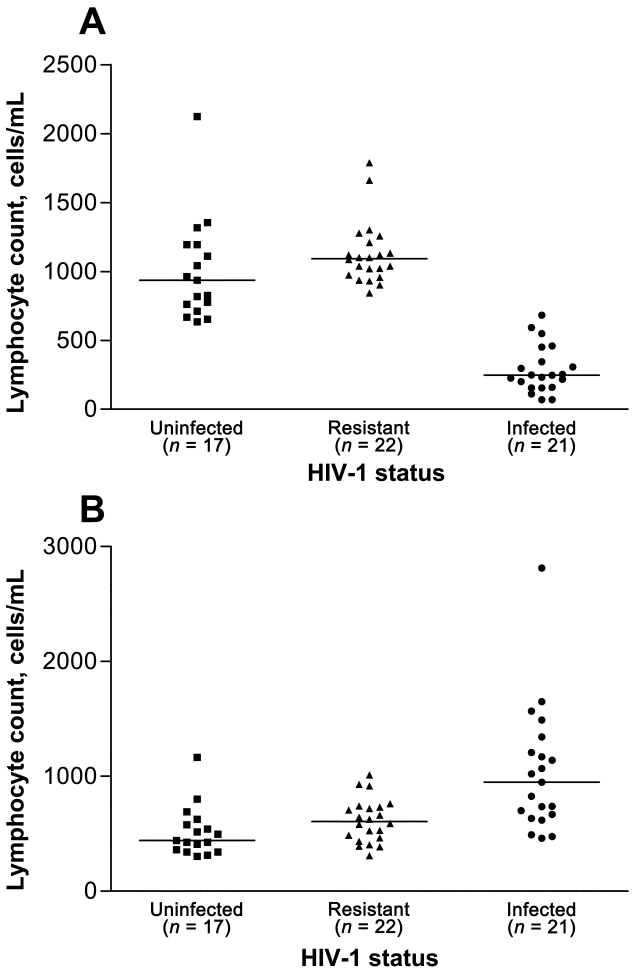

Figure 1. No significant differences in blood T cell counts between HIV-1–resistant commercial sex workers (CSWs) and HIV-1–uninfected CSWs. Blood samples were collected in EDTA tubes and were analyzed for $CD4^+$ and $CD8^+$ T cell counts by fluorescence-activated cell sorter (FACS) analysis for each woman who also had a cervicovaginal lavage sample collected. The HIV-1–infected CSWs had lower $CD4^+$ T cell counts *(A)* and higher $CD8^+$ T cell counts *(B)* than did both the HIV-1–uninfected ($P < .0001$ and $P < .0001$, respectively) and the HIV-1–resistant ($P = .002$ and $P < .0001$, respectively) CSWs. A trend toward elevated $CD8^+$ T cell counts in the HIV-1–resistant CSWs, compared with those in the HIV-1–uninfected CSWs, was noted ($P = .085$). Horizontal bars represent the median response for each group.

tected from challenge with SIVmac251 demonstrated that SIV-specific CTL responses correlate with protection [8]. A similar study demonstrated the induction of Gag-specific $CD8^+$ T cells in the vaginal and cervical lamina propria of infected macaques [5]. Interestingly, the frequency of the mucosal responses exceeded that detected in systemic compartments. Site-specific and HIV-1–specific neutralizing IgA and IgG responses elicited by intranasal immunization with a Nef gene–deleted SHIV were protective after challenge [9]. Although these studies demonstrate an important role for the immune system in the genital tract of monkeys, it remains unclear whether these phenomena are capable of preventing naturally acquired infection in humans.

In sub-Saharan Africa, the primary route of transmission of HIV-1 is through heterosexual contact [10]. Although HIV-1–infected individuals have specific immune responses, understanding protective responses against HIV-1 is best studied in individuals who are naturally resistant to infection. A number of genetic mechanisms have been described, such as the *CCR5Δ32* HIV-1 coreceptor mutation [11] and associations with specific HLA alleles, such as A2/6802 and DRB1*01 [12]. However, genetic mechanisms do not exclusively account for resistance to HIV-1 infection in all individuals. Studies of populations of naturally resistant individuals who are highly exposed to HIV-1 suggest that immunological mechanisms may confer HIV-1 resistance.

In Kenya, a group of repeatedly exposed commercial sex workers (CSWs) have been identified as HIV-1 resistant [13]. Studies of these women have suggested that HIV-1–specific protective immune responses exist, including systemic and genital CTL [14, 15] and IgA responses [16]. These antibodies have been shown to be neutralizing and to prevent HIV-1 transcytosis in vitro [17, 18]. Further characterization of these responses in these women may help to unravel the potential immune mechanisms of HIV-1 resistance.

The purpose of the present study was to characterize lymphocyte populations and the immunoregulatory environment in the genital tract of HIV-1–resistant CSWs. We compared HIV-1–resistant CSWs to both HIV-1–uninfected and HIV-1–infected CSWs and to a non–CSW population at low risk for exposure. T cell counts as well as cytokine and chemokine levels were assessed. We report that the HIV-1–resistant CSWs had significantly elevated T cell counts in the genital tract, compared with the susceptible women. This difference was not reflected systemically, indicating that elevated T cell counts were present at the mucosal level only. Further, chemokine-expression patterns and HIV-1 coreceptor levels were altered in the HIV-1–resistant CSWs.

SUBJECTS, MATERIALS, AND METHODS

Reagents and antibodies. Antibodies and isotype controls for fluorescence-activated cell sorter (FACS) analysis (anti–human CD3–fluorescein isothiocyanate [FITC], CD4–Cy-C, CD8-phycoerythrin [PE], CXCR4-PE, and CCR5-FITC) as well as antibodies for ELISAs were purchased from BD Biosciences. Recombinant interleukin (IL)–2, IL-10, IL-13, macrophage inflammatory protein (MIP)–1α (CCL3), MIP-1β (CCL4), and RANTES (CCL5) were supplied by PeproTech Canada. Additional reagents were purchased from Gibco BRL Laboratories.

Study populations. Women of similar socioeconomic status were enrolled from a CSW cohort and a non–CSW cohort at low risk for exposure in Nairobi, Kenya. HIV-1 status was as-

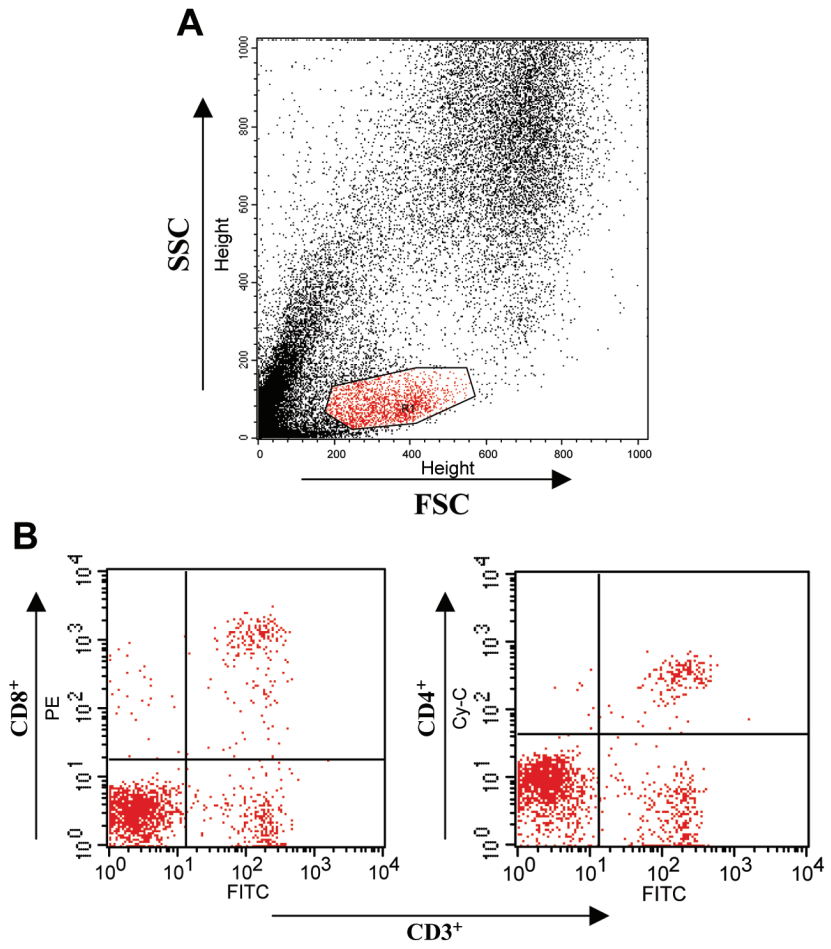

Figure 2. Representative fluorescence-activated cell sorter panels for $CD4^+$ and $CD8^+$ T cell populations in the cervical-cell samples. Cervical leukocytes were collected from a cytobrush and Ayres spatula by a brief vortex, were washed, and were isolated by ficoll-hypaque density centrifugation. The cells were stained with CD3–fluorescein isothiocyanate (FITC) antibody and either CD4–Cy-C or CD8-phycoerythrin (PE) fluorescent antibody. Samples were analyzed by use of a flow cytometer, and 100,000 events were acquired per sample. *A,* Representative panel of the forward scatter (FSC) vs. side scatter (SSC) plot, with the lymphocyte population gated. *B,* Dot plots of $CD8^+$ *(left panel)* and $CD4^+$ *(right panel)* T cells.

sessed by serological testing and was confirmed by polymerase chain reaction (PCR) [19]. Cervicovaginal lavage (CVL) samples, cell scrapings, and blood samples were obtained from HIV-1–uninfected ($n = 18$), HIV-1–resistant ($n = 28$) and HIV-1–infected ($n = 24$) CSWs from the Pumwani area. HIV-1–resistant CSWs were defined as active CSWs who remained HIV-1 seronegative and PCR negative for >3 years of follow-up [13]. Clinical and epidemiological data, such as days from last menses and contraceptive use, were recorded. The HIV-1–uninfected non-CSW control subjects at low risk for exposure ($n = 10$) were enrolled from a mother-and-child health-care clinic at the Pumwani district hospital [20]. Women were excluded from the study if <18 years old, menstruating, or pregnant. This study was approved by the Universities of Manitoba and Nairobi human research ethics boards, and all subjects provided informed consent.

Mucosal sample collection and transport. Previous studies of mucosal immune responses in humans have been impeded by low cell recovery [3, 4]. We established a collection method ideal for resource-poor clinical settings that minimized blood contamination. CVL samples were collected as described elsewhere [21]. Briefly, 3 Weck-Cel sponges (Medtronic Xomed) were used to collect fluid from the posterior fornix of the vagina after a 2-mL sterile PBS lavage. Next, an Ayres spatula was used to collect cells from the ectocervix, and a cytobrush was used to collect cells from the endocervix. Samples were collected from the CSWs by a single physician and from the non-CSWs by a different but similarly trained physician. Samples were placed in PBS and kept on ice until processed.

Sample processing and cell analysis. Blood was collected for HIV-1 serological testing, and T cells were counted by use of a Coulter Counter (Beckman Coulter). $CD4^+$ and $CD8^+$ T cell counts were assessed by FACS analysis, as described elsewhere [15]. CVL samples with visible blood contamination were

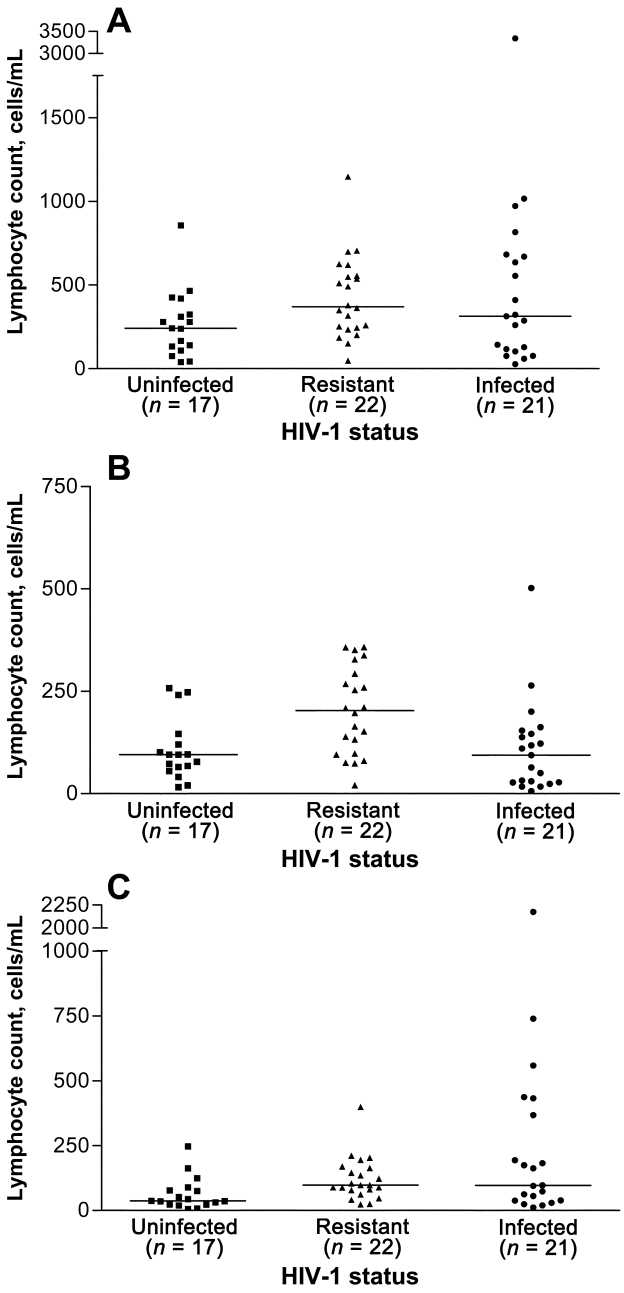

Figure 3. Elevated cervical T cell counts in HIV-1–resistant commercial sex workers (CSWs). *A,* Cervical $CD3^+$ T cell counts for each CSW group. Total T cell counts were significantly higher in the HIV-1–resistant CSWs than in the HIV-1–uninfected CSWs ($P = .021$). *B,* Significantly elevated $CD4^+$ T cell counts in the HIV-1–resistant CSWs, compared with those in the HIV-1–infected ($P = .003$) and the HIV-1–uninfected ($P = .002$) CSWs. *C,* Significantly elevated $CD8^+$ T cell counts in the HIV-1–resistant CSWs, compared with those in the HIV-1–uninfected CSWs ($P = .003$). In addition, $CD8^+$ T cell counts were elevated in the HIV-1–infected CSWs, compared with those in the HIV-1–uninfected non-CSW control subjects at low risk for exposure ($P = .018$). Horizontal bars represent the median response for each group.

excluded from analysis (1 from an HIV-1–uninfected CSW, 6 from HIV-1–resistant CSWs, and 3 from HIV-1–infected CSWs). Cervical leukocytes were collected from the cytobrush and Ayres spatula by a brief vortex, were washed, and were isolated by ficoll-hypaque density centrifugation. Isolated cells were stained with CD3, CD4, CD8, CXCR4, and CCR5 antibodies for 30 min, were washed, and were fixed by use of 1% paraformaldehyde. Acquisition and analysis were performed by a single operator blinded to sample-group identity, by use of a FACS flow cytometer (Becton Dickinson) and CellQuest Pro software (version 4.0; Becton Dickinson).

Chemiluminescent ELISAs. CVL fluid was eluted from Weck-Cel sponges, as described elsewhere [21]. Chemiluminescent ELISAs for human IL-2, IL-10, IL-13, MIP-1α, MIP-1β, and RANTES were performed on selected samples on the basis of sample availability, as described elsewhere [22].

Statistical analysis. To avoid bias, both sample collection and data analysis were conducted blinded to subject status. Statistical analysis was performed by use of SPSS for Windows (version 10.1; SPSS). Regression analysis was conducted in concert with the University of Manitoba Biostatistical Consulting Unit. Epidemiological data were analyzed by Student's t test for ordinal data and by the χ^2 test for categorical data. Immunological data were analyzed by the Mann-Whitney U test. $P < .05$ was considered to be statistically significant.

RESULTS

Study subjects. Clinical information was collected from the CSWs to address the issue of confounding variables for mucosal immune responses. Study groups were equivalent with respect to age, days from last menses, and average number of clients per week (table 1). Duration of sex work was equivalent between the HIV-1–resistant and the HIV-1–infected CSWs; however, the HIV-1–uninfected CSW group was composed of recent cohort enrollees and had a shorter duration of sex work. Although the HIV-1–uninfected CSWs reported a median duration of sex work of 4 years, they were not defined as resistant because we could not assess a sufficient level of exposure that would meet our epidemiological definition [13].

Gynecological exams were performed by a physician, who assessed the presence of genital-tract infections and identified evidence of bacterial and viral sexually transmitted infections (STIs). Subclinical infections of chlamydia, *Neisseria gonorrhoea, Treponema pallidum, Haemophilus ducreyi,* and herpes simplex virus were diagnosed by PCR and serological and bacteriological testing. The HIV-1–uninfected CSWs had a higher number of STIs than did the HIV-1–resistant CSWs; however, the difference was not statistically significant ($P = .11$). Exclusion of CSWs with a concurrent STI from subsequent analysis did not alter our findings, nor did their inclusion in linear regression analysis. All CSWs reported similar use of condoms

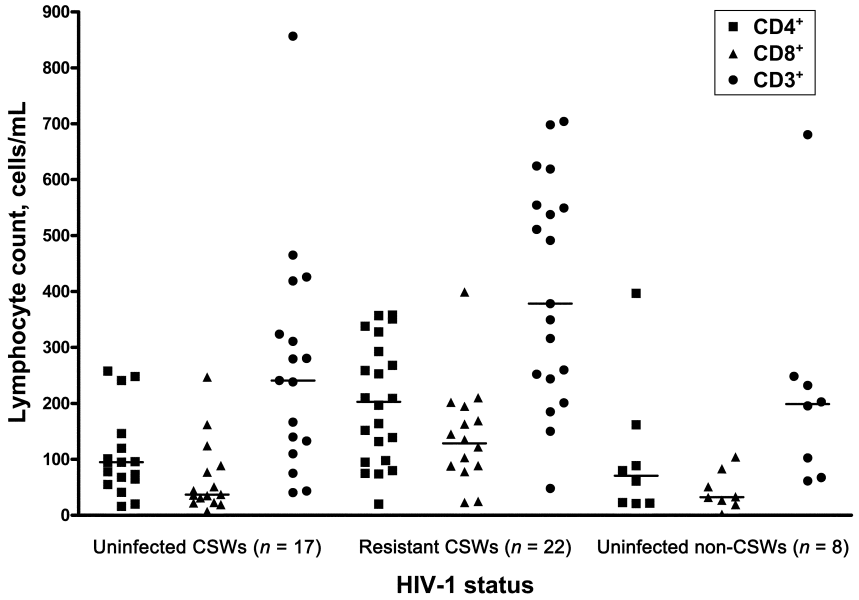

Figure 4. Elevated T cell counts in the HIV-1–resistant commercial sex workers (CSWs), compared with those in the HIV-1–uninfected CSWs and the HIV-1–uninfected non-CSW control subjects at low risk for exposure. T cell counts in the CSWs were compared with those in the non-CSWs as a control for high-risk behavior. T cell counts (for $CD4^+$, $CD8^+$, and total $CD3^+$ T cells) were comparable between the HIV-1–uninfected CSWs and the low-risk HIV-1–uninfected non-CSWs. Comparisons between the HIV-1–resistant CSWs and both the HIV-1–uninfected non-CSWs and the HIV-1–uninfected CSWs showed significantly elevated T cell counts in the former ($P < .05$). Horizontal bars represent the median response for each group.

and spermicide (data not shown), hormonal contraception (oral and Depo-Provera), and other contraception methods (tubal ligation and intrauterine devices). The majority of CSWs reported douching postcoitally and/or daily with soap and water. The HIV-1–uninfected CSWs who reported douching did so predominantly daily, whereas the HIV-1–resistant and the HIV-1–infected CSWs reported mostly postcoital douching ($P = .005$ and $P = .032$, respectively).

Systemic $CD4^+$ and $CD8^+$ T cell counts. Blood $CD4^+$ and $CD8^+$ T cell counts were obtained for all of the CSWs (figure 1). The HIV-1–infected CSWs had lower $CD4^+$ T cell counts and higher $CD8^+$ T cell counts than did both the HIV-1–uninfected ($P < .0001$ and $P < .0001$, respectively) and the HIV-1–resistant ($P = .002$ and $P < .0001$, respectively) CSWs. No significant differences were observed in $CD4^+$ and $CD8^+$ T cell counts between the HIV-1–uninfected and the HIV-1–resistant CSWs, although a trend toward a higher $CD8^+$ T cell count was noted ($P = .085$).

Cervical $CD4^+$ and $CD8^+$ T cell counts in CSWs. Cervical mononuclear cells were isolated, and the lymphocyte population was gated on the basis of forward- versus side-scatter profiles (figure 2). Representative panels are shown in figure 2*B*. $CD4^+$ and $CD8^+$ T cell populations were differentiated by $CD3^+CD4^+$ and $CD3^+CD8^+$ T cell markers and isotype controls that defined background and quadrant settings. Backgating was used to confirm the identity of dual-positive cells.

When total cervical T cell counts were compared, it was observed that the HIV-1–resistant CSWs had elevated levels of $CD3^+$ T cells relative to the HIV-1–uninfected CSWs ($P = .021$) but not the HIV-1–infected CSWs (figure 3*A*). When levels of the $CD3^+CD4^+$ T cell subset were examined, it was observed that the HIV-1–resistant CSWs had increased $CD4^+$ T cell counts, compared with both the HIV-1–uninfected ($P = .002$) and the HIV-1–infected ($P = .003$) CSWs (figure 3*B*). The $CD8^+$ T cell counts in the HIV-1–resistant CSWs were also increased in relation to the HIV-1–uninfected CSWs ($P = .003$) but not the HIV-1–infected CSWs (figure 3*C*). Similar to what was observed systemically, the HIV-1–infected CSWs also had elevated cervical $CD8^+$ T cell counts, compared with the HIV-1–uninfected CSWs ($P = .018$). To control for the potential effects of confounding epidemiological variables, such as days from last menses, contraceptive use, and STIs, we conducted stepwise linear regression analysis of these factors and their effects on differences in cervical $CD4^+$ and $CD8^+$ T cell counts. The stepwise addition of these variables, whether individually or in combination, did not affect the differences between these counts (for the differences in cervical $CD4^+$ and $CD8^+$ T cell counts between the HIV-1–resistant and the HIV-1–uninfected CSWs with 3 variables added, $P = .044$ and $P = .029$, respectively).

Comparison of the CSWs to HIV-1–uninfected non-CSW control subjects at low risk for exposure. To control for the effect that sex work has in contributing to elevated $CD4^+$ and $CD8^+$ T cell counts, we tested HIV-1–uninfected non-CSWs as a low-risk control group. This group had cervical $CD3^+$, $CD4^+$,

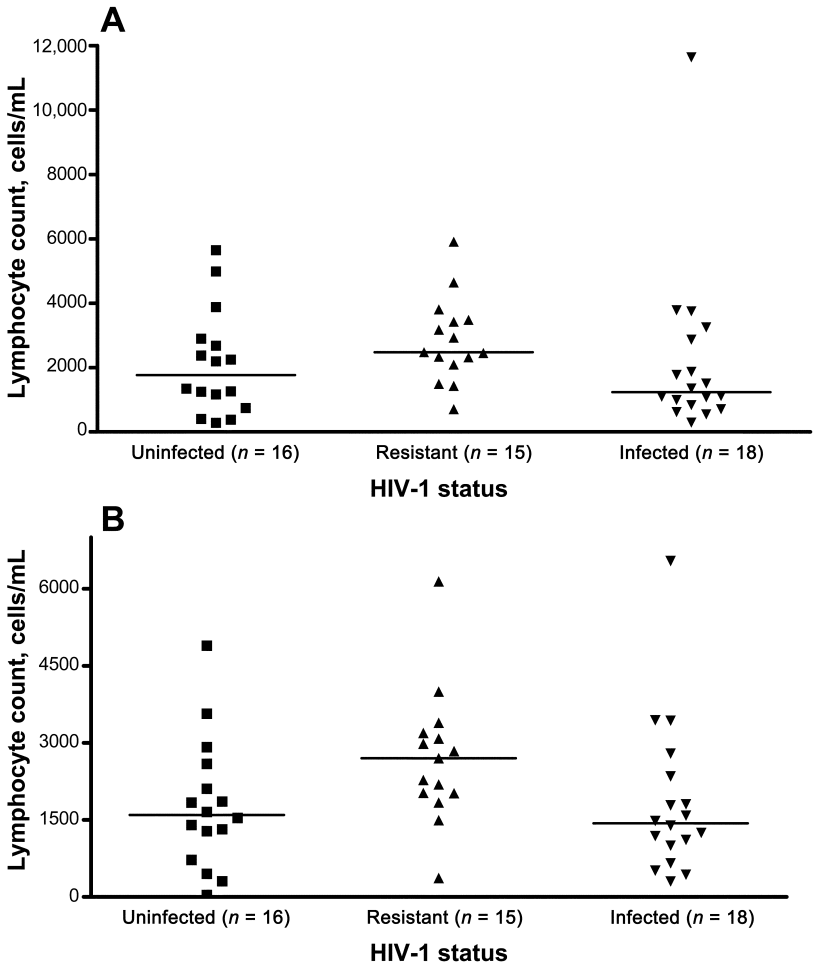

Figure 5. Increased expression of HIV-1 coreceptors in the cervical tissue of HIV-1–resistant commercial sex workers (CSWs). CCR5 and CXCR4 expression was assessed in $CD4^+$ T cell–gated populations for each of the CSW groups. *A,* $CD4^+CCR5^+$ T cell counts. Comparison between the HIV-1–resistant and the HIV-1–infected CSWs showed a significant elevation in $CD4^+CCR5^+$ T cell counts in the former ($P = .014$), and comparison between the HIV-1–resistant and the HIV-1–uninfected CSWs showed a strong trend ($P = .056$). *B,* $CD4^+CXCR4^+$ T cell counts. The HIV-1–resistant CSWs demonstrated a significant increase in $CD4^+CXCR4^+$ T cell counts, compared with the HIV-1–uninfected CSWs ($P = .012$). In addition, the HIV-1–resistant CSWs showed higher $CD4^+CXCR4^+$ T cell counts, compared with the HIV-1–infected CSWs ($P = .003$). Horizontal bars represent the median response for each group.

and $CD8^+$ T cell counts that were similar to those of the HIV-1–uninfected CSWs (figure 4). Again, the HIV-1–resistant CSWs demonstrated elevated cervical $CD3^+$, $CD4^+$, and $CD8^+$ T cell counts, compared with the non-CSW control subjects ($P = .036$, $P = .035$, and $P = .0054$, respectively).

HIV-1 coreceptor expression in cervical cells. In a subset of women who had enough cervical cells available with which to do a second FACS panel, we assessed the number of $CD4^+$ T cells that were also chemokine receptor positive in the cervix, to determine the prevalence of HIV-1–susceptible target cells (figure 5). The HIV-1–resistant CSWs trended toward elevated $CD4^+CCR5^+$ T cell counts, compared with both the HIV-1–uninfected and the HIV-1–infected CSWs ($P = .014$ and $P = .056$, respectively) (figure 5*A*). $CD4^+CXCR4^+$ T cell counts in the HIV-1–resistant CSWs were significantly increased, compared with those in both the HIV-1–uninfected and the HIV-1–infected CSWs ($P = .012$ and $P = .003$, respectively) (figure 5*B*).

Cytokine and chemokine expression in the genital mucosa. On the basis of sample availability, we assessed the levels of a number of immunoregulatory cytokines in women from the CSW and non-CSW groups, to compare mucosal cytokine expression. IL-2, IL-10, and IL-13 levels were similar in the HIV-1–uninfected, HIV-1–infected, and HIV-1–resistant CSWs and the HIV-1–uninfected non-CSW control subjects (table 2). However, β chemokine levels were significantly different between the 3 CSW groups. The HIV-1–uninfected CSWs had higher levels of MIP-1α than did the HIV-1–infected CSWs ($P = .029$), and the HIV-1–resistant CSWs had significantly higher levels of RANTES than did the HIV-1–uninfected CSWs ($P = .005$).

Table 2. Mucosal cytokine and chemokine levels for interleukin (IL)–2, IL-10, IL-13, macrophage inflammatory protein (MIP)–1α, MIP-1β, and RANTES in commercial sex workers (CSWs) and in HIV-1–uninfected non-CSW control subjects at low risk for exposure.

Group	IL-2	IL-10	IL-13	MIP-1α	MIP-1β	RANTES
CSWs						
HIV-1 uninfected	3.3 ± 1.7 (1.0)	20.9 ± 17.0 (4.3)	134.6 ± 30.6 (117.2)	**199.5 ± 31.1 (214.9)**[a]	125.4 ± 38.7 (75.6)	**5.0 ± 1.9 (2.2)**[b]
HIV-1 infected	1.9 ± 0.6 (1.1)	4.9 ± 2.3 (1.3)	119.6 ± 18.3 (101.7)	**105.5 ± 24.4 (116.2)**[a]	76.9 ± 27.1 (46.8)	29.9 ± 13.0 (11.1)
HIV-1 resistant	2.8 ± 1.3 (1.0)	3.0 ± 1.0 (2.9)	146.3 ± 28.7 (127.8)	171.4 ± 27.4 (168.3)	149.2 ± 46.5 (93.6)	**53.7 ± 30.9 (16.1)**[b]
HIV-1–uninfected non-CSW control subjects	5.7 ± 2.1 (1.0)	12.7 ± 7.2 (1.0)	89.7 ± 21.0 (88.2)	155.9 ± 57.0 (184.4)	156.9 ± 50.0 (119.4)	12.6 ± 5.6 (2.0)

NOTE. Data are mean ± SE (median) and are expressed in picograms per milliliter. Expression levels were assayed by a chemiluminescent-based ELISA; $n = 9$ samples for each group for the cytokine data, and $n = 10$ samples for each group for the chemokine data. Statistically significant values are boldfaced.

[a] $P = .029$

[b] $P = .005$.

DISCUSSION

In sub-Saharan Africa, HIV-1 primarily spreads via heterosexual contact [10]; therefore, initial virus-host contact and transmission occurs in the genital tract. Although most studies of immune responses to HIV-1 have been performed in the systemic compartment, these may not reflect mucosal responses, which are important in sexual transmission of HIV-1. Previous studies have demonstrated differences between lymphocyte populations in the genital mucosa and blood-derived cells [5, 23] and have described HIV-1–specific CTLs in the genital tracts of HIV-1–infected women [24–26] and exposed but uninfected men [27]. We have demonstrated that the HIV-1–resistant CSWs in the Pumwani cohort have HIV-1–specific IgA in their genital tracts in the absence of systemic IgA [17] and that HIV-1–specific CTLs are found in the cervix of resistant women at a frequency higher than that in blood [15]. Such data emphasize that studies performed on 2% of the systemic lymphocytes of the body may not accurately reflect mucosal lymphocyte responses [28].

Here we have demonstrated elevated T cell counts in the genital mucosa of HIV-1–resistant women, compared with control subjects. We have also shown that, similar to what is observed systemically, $CD8^+$ T cell counts in the genital tract of HIV-1–infected women are elevated, compared with those in HIV-1–uninfected women. Previous studies of cervical-biopsy samples from HIV-1–infected women have also shown elevated $CD8^+$ T cell counts, rather than elevated $CD4^+$ T cell counts [29], suggesting a role for $CD8^+$ T cells in mucosal immune responses to HIV-1. It is important to note that the HIV-1–resistant CSWs in our study who had elevated cervical $CD8^+$ T cell counts, compared with the HIV-1–uninfected control subjects, were free of HIV-1 disease. Along with previous data showing high levels of HIV-1–specific $CD8^+$ T cells in the cervix [30], this suggests a role for mucosal $CD8^+$ T cell responses in mediating protection against HIV-1 infection. Perhaps most interesting, mucosal $CD4^+$ T cell counts were also found to be elevated in the HIV-1–resistant CSWs, compared with the HIV-1–infected and the HIV-1–uninfected CSWs. These differences were not reflected systemically, suggesting that the HIV-1–resistant CSWs are immunologically unique at the mucosal level, which may be related to their ability to escape HIV-1 infection. Because lymphocytes are critical to the regulation of humoral and cellular immune responses, this has important implications for our understanding of immunity to HIV-1 infection. A previous study by Biasin et al. [31] demonstrated similar unique mucosal immune responses in a population of HIV-1–exposed women who were uninfected, although the authors did not examine protein levels or mucosal T cell counts directly.

Many factors—sexual behavior, hygienic practices, genetic influences, and environment—may account for elevated $CD4^+$ T helper cell counts. Previous studies have demonstrated that HIV-1–uninfected women in Thailand had elevated genital-tract T helper cell counts, compared with North American women [32]. To control for the confounding effects of sex work, we compared HIV-1–uninfected CSWs to HIV-1–uninfected non-CSWs. No differences in mucosal T cell counts were observed, ruling out sex work alone as an explanation. Because these women had similar genetic and socioeconomic backgrounds, it is likely that elevated T helper cell counts in resistant women represent a distinct biological difference and not an environmental effect. Analyses of stratified data show that these women were equivalent with respect to age, days from last menses, average number of clients per week, and hormonal contraception use (table 1). We enrolled women sequentially as they attended our clinics; therefore, we were unable to control for these factors directly. However, using multiple linear regression models, we have demonstrated that differences in T cell counts were independent of these factors.

The longer duration of sex work reported by the HIV-1–resistant CSWs may account for the differences in T cell counts, with exposure and immune response to HIV-1 infection and other STIs being responsible for the elevated counts rather than a reflection of a mechanism of resistance. To address this possibility, we are currently conducting a prospective study to determine whether elevated T cell counts are predictive of immunity to HIV-1 infection or are a by-product of immune stimulation. Our data suggest that immune stimulation alone does not explain the elevated T cell counts in the HIV-1–resistant CSWs, because the HIV-1–uninfected non-CSW control subjects experienced more STIs than did the HIV-1–resistant CSWs yet still had lower T cell counts.

Cellular HIV-1 infection requires CD4 and either CCR5 or CXCR4 [33]. We found a trend toward increased $CD4^+CCR5^+$ T cell counts and a marked increase in $CD4^+CXCR4^+$ T cell counts in the genital mucosa of the HIV-1–resistant CSWs, compared with those in the HIV-1–uninfected CSWs. Analysis of mean fluorescent intensity showed that this increase was attributed to increased $CD4^+$ T cell counts rather than increased receptor expression per cell, demonstrating, if anything, that the HIV-1–resistant CSWs would be more likely to become HIV-1 infected.

The immunoregulatory milieu of the genital mucosa is important in determining the response to antigenic challenge. We quantified levels of IL-2, IL-10, and IL-13 as indicators of Th1 versus Th2 responses; however, no differences were detected. We assessed levels of RANTES, MIP-1α, and MIP-1β, because these can inhibit HIV-1 infection in vitro and are elevated in some exposed yet uninfected women [34]. The HIV-1–infected women in the present study had lower levels of MIP-1α than did the HIV-1–uninfected CSWs, likely a result of perturbation of the immune system due to HIV-1 infection. Interestingly, the HIV-1–resistant CSWs had elevated levels of RANTES in mucosal secretions, suggesting a potential mechanism of pro-

tection against HIV-1 infection. Higher expression of RANTES would block CCR5 on susceptible cells, preventing infection from occurring. It has been shown that HIV-1–resistant women have equivalent systemic RANTES levels, again emphasizing the significance of studies conducted at the mucosal site [19]. We confirmed that the subgroups of women tested for cytokines and chemokines were epidemiologically similar to the overall groups, as is shown in table 1. It should be noted that other HIV-1 inhibitory factors, such as stroma cell–derived factor 1 or α and β defensins, may have played a role in resistance to infection in this group. Perhaps the most interesting possibility is the role that RANTES plays as a chemoattractant, rather than as an inhibitor of HIV-1. RANTES is a potent chemoattractant that is instrumental in the recruitment of T cells to tissue sites [35]. This may explain the increased mucosal T cell counts in the HIV-1–resistant CSWs; however, further studies are required.

There are several potential weaknesses or limitations to the present study. One is that we were unable to determine exposure to HIV-1 before entry into our cohort. Although the HIV-1–uninfected CSWs reported a median of 4 years of sex work (table 2), we could not ascertain sufficient exposure to define these women as resistant, because they were new enrollees; the HIV-1–resistant CSWs were defined as those who had been active CSWs for >3 years under our observation. Even with strong intervention strategies for safer sexual practices, we know that, if sufficiently exposed, the HIV-1–uninfected CSWs will become infected, unless they fall into our resistant phenotype. This makes identification of immunological differences between these groups less likely but strengthens any positive associations identified. In addition, variability in human immune responses can make differences between groups difficult to detect; thus, studies such as ours need to be interpreted cautiously. Another limitation is that we analyzed confounding epidemiological variables by use of linear regression, which can be sensitive to outlying variables. Ideally, we would control for this at the outset of the study; however, this was not possible, because of random patient recruitment. Furthermore, the corrections necessary for analyzing ≥ 2 factors makes data interpretation difficult—that is, if we applied the Bonferroni correction to our cytokine and chemokine data, the level of significance would need to reach $P < .0014$ to be statistically valid. This is difficult to achieve in human studies of modest size. Analytical variability, such as that in the gating strategy used to determine cervical T cell counts, may also have affected the results; however, to control for this potential error as best as possible, this analysis was performed blinded, and backgating was used. Clearly, the best approach to control for these issues is to corroborate these data in prospective studies or to replicate these findings in other exposed yet uninfected cohorts.

It is imperative to determine whether elevated mucosal T helper cell counts in HIV-1–resistant women represent effector or central memory cell phenotypes, because these are the initial target cells infected by HIV-1 [36]. We are currently developing techniques to determine the HIV-1 specificity of cervical lymphocytes in HIV-1–resistant women. Because no reliable marker for genital-tract lymphocyte recruitment has been defined in humans, it is difficult to determine whether elevated T cell counts in HIV-1–resistant women are due to increased recruitment from the systemic circulation or are the result of localized proliferation. The mucosal marker CD103 ($\alpha E\beta 7$) has been used to identify genital mucosa–derived lymphocytes [30]; however, it is not specific for genital-tract cells, because it has been found on the surface of cells from other mucosal sites [37, 38].

In summary, we have characterized T cells, cytokines, and chemokines in the genital tracts of HIV-1–resistant CSWs. The HIV-1–resistant CSWs had elevated T cell counts and RANTES expression in the genital mucosa, phenomena not apparent in the systemic circulation. Importantly, these investigations were performed in women epidemiologically resistant to HIV-1 infection, and, although only associations, our observations provide clues as to what may constitute protective mucosal immune responses to HIV-1. Unlike previous studies that examined doomed HIV-1–specific responses during infection, the present study examined what likely are effective immune responses to HIV-1. The elevated RANTES expression observed in the HIV-1–resistant CSWs may be an immunological mechanism whereby resistance to infection is conferred. Determination of the basis of natural immunity to HIV-1 will positively impact the development of the HIV-1 vaccines necessary to combat this infection.

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