THE JOURNAL OF IMMUNOLOGY

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J Immunol 2011; 187:6032-6042; Prepublished online 2 November 2011;
doi: 10.4049/jimmunol.1101836
http://www.jimmunol.org/content/187/11/6032

The Journal of Immunology is published twice each month by
The American Association of Immunologists, Inc.,
9650 Rockville Pike, Bethesda, MD 20814-3994.

Print ISSN: 0022-1767 Online ISSN: 1550-6606.

Characterization of a Human Cervical CD4$^+$ T Cell Subset Coexpressing Multiple Markers of HIV Susceptibility

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The HIV pandemic disproportionately affects women, with most infections acquired through receptive vaginal sex. Although the target cells by which HIV establishes infection in the female genital tract remain poorly defined, it is known that immune activation results in CD4$^+$ T cells with enhanced susceptibility, as does expression of the mucosal integrin α4β7 and the HIV coreceptor CCR5. Blood and cervical cytobrush specimens were collected from female sex workers (FSWs) in Nairobi, Kenya. Genital infection diagnostics were performed, T cell populations were defined by multiparameter flow cytometry based on their expression of surface receptors relevant to mucosal homing and/or HIV acquisition, and cytokine production was assayed by intracellular cytokine staining. The integrin α4β7 was expressed on 26.0% of cervical CD4$^+$ T cells, and these cells were more likely to express both the HIV coreceptor CCR5 ($p < 0.0001$) and the early activation marker CD69 ($p < 0.0001$) but not CXCR4 ($p = 0.34$). Cervical Th17 frequencies were enhanced compared with blood (7.02 versus 1.24%; $p < 0.0001$), and cervical IL-17A$^+$ CD4$^+$ T cells preferentially coexpressed α4β7 and CCR5. Expression of IFN-γ and IL-22 was greater in cervical Th17 cells than in blood Th17 cells. In keeping with the hypothesis that these cells are preferential HIV targets, gp120 preferentially bound CCR5$^+$ cervical T cells, and cervical Th17 cells were almost completely depleted in HIV$^+$ FSWs compared with HIV$^-$ FSWs. In summary, a subset of Th17 CD4$^+$ T cells in the cervical mucosa coexpresses multiple HIV susceptibility markers; their dramatic depletion after HIV infection suggests that these may serve as key target cells during HIV transmission. *The Journal of Immunology*, **2011, 187: 6032–6042.**

There were >1.8 million new HIV infections in sub-Saharan Africa in 2009, almost three quarters of the global total, and most of these infections were in women (1). Despite the scale of the epidemic, the probability of HIV transmission during a single act of unprotected receptive penile-vaginal sex with an HIV$^+$ man is <0.2% (2). The fact that our natural mucosal immune defenses are >99% protective is very fortunate from the perspective of epidemic spread, but this very low per-act transmission probability makes it difficult to define the mucosal immune basis of virus acquisition or altered host susceptibility (3).

HIV transmission within stable, serodiscordant couples is associated with a higher HIV RNA virus level in both the blood and the genital tract of the infected partner (4, 5). In an analogous fashion, HIV susceptibility could also be enhanced by factors that increase the number of potential HIV target cells in the genital mucosa of the uninfected partner, which is one of several proposed mechanisms to explain the increase in HIV transmission associated with infection by herpes simplex type 2 (6). These observations suggest that two thresholds must be overcome for HIV transmission to occur. First, there may be a threshold level of infectious virus needed in the genital secretions of the infected partner; second, there may be a threshold number and/or density of susceptible target cells required in the genital mucosa of the uninfected partner (7). Nonhuman primate models support this concept, documenting initial foci of infected mucosal CD4$^+$ T cells that expand prior to systemic viremia, and pilot studies demonstrated that a microbicide blocking mucosal CD4$^+$ T cell recruitment prevented SIV infection (8). A better definition of the initial mucosal cells present in the female genital tract that are targeted by HIV during human sexual transmission might assist in the design of better biomedical prevention strategies.

In addition to functional heterogeneity, not all CD4$^+$ T cells are equally susceptible to HIV infection (9). The near-uniform transmission during sex of HIV strains that use the CCR5 coreceptor (R5 tropic strains) implies that HIV preferentially infects mucosal target cells that express CCR5 (10). It was also demonstrated that HIV replicates preferentially within activated T cells (11). The integrin α4β7, induced on T cells after priming by mucosally derived dendritic cells, mediates T cell trafficking to gut mucosal sites via a cascade of receptor interactions on gut endothelial cells that includes the addressin MadCAM (12). α4β7 was shown to bind HIV gp120 directly and correlates with

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Received for publication June 22, 2011. Accepted for publication September 15, 2011.

This work was supported by the Canadian Institutes of Health Research (CIHR MOP 89983) and the Bill and Melinda Gates Foundation. L.R.M. is funded by a Canadian Institutes of Health Research fellowship and the International Infectious Diseases and Global Health Training Program. J.A., C.C., and K.E.B. were supported by the Intramural Research Program of the National Institutes of Health. R.K. is a Canada Research Chair in HIV.

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Abbreviations used in this article: ART, antiretroviral therapy; BD, Becton Dickinson; BV, bacterial vaginosis; CMC, cervical mononuclear cells; FSW, female sex worker; IQR, interquartile range; qPCR, qualitative real-time PCR.

www.jimmunol.org/cgi/doi/10.4049/jimmunol.1101836

enhanced virus infection and cell–cell spread (13, 14). In addition, $\alpha4\beta7^+$ $CD4^+$ T cells were reported to be preferentially infected during acute SIV infection in nonhuman primates (15).

The $CD4^+$ Th17 subset is defined by the production of IL-17A and plays a key role in antimicrobial defense at mucosal sites, as well as in some autoimmune diseases (16). Overactivity of Th17 cells is associated with the gastrointestinal pathology observed in Crohn's disease (17), whereas Th17 deficiency underpins chronic mucocutaneous candidiasis (18, 19). As might be expected from their inflammatory nature, HIV replicates preferentially within Th17 cells (20). Not surprisingly, these cells are depleted during HIV infection (21), particularly in the gut mucosa (22), but are maintained in HIV^+ individuals with an undetectable viral load (23). However, the presence and correlates of Th17 cells within the genital tract have not been examined and neither has their potential role as an HIV target cell at this mucosal site.

In this article, we demonstrate that a subset of activated cervical $CD4^+$ T cells express α4β7, CCR5, IL-17A, and IFN-γ. We further show that these cells preferentially bind HIV-1 gp120 in vitro and are almost entirely depleted from the cervix in vivo during HIV infection. The presence of multiple susceptibility markers on a subset of genital tract $CD4^+$ T cells suggests that these cells may play a key role as HIV targets during sexual transmission.

Materials and Methods

Ethics statement

Informed written consent was obtained prior to participation, and Institutional Review Boards at Kenyatta National Hospital (Nairobi, Kenya) and Universities of Manitoba and Toronto (Canada) approved the study. The described studies were conducted according to the principles expressed in the Declaration of Helsinki.

Study participants

Participants were recruited for the study from existing female sex worker (FSW) cohorts located in Nairobi, Kenya. Each participant was tested for HIV-1 serology, peripheral CD4 counts, bacterial vaginosis (BV) by Gram stain, *Chlamydia trachomatis* and *Neisseria gonorrhea* by PCR (Roche), and *Trichomonas vaginalis* by InPouch. Demographic and behavioral data were also collected. HIV^+ participants were said to be in the early stage of infection if sampled <6 mo from the suspected infection date (midpoint between last HIV^- and first HIV^+ visit).

Sample collection

Blood was collected by venipuncture using heparin as an anticoagulant. Cervical sampling included the collection of samples in the following order: urine; cervicovaginal lavage using 2 ml sterile PBS; two Dacron swabs for assessment of cervicovaginal viral shedding, collected into viral-transport medium; a vaginal swab for BV; and cervical cytobrush and cell scraper for collection of cervical immune cells. The present study focused on cytobrush samples to define cell populations. Cytobrushes were inserted into the cervical os and rotated 360˚, followed by a gentle scraping of the ectocervix and a mini-lavage to collect cells. Cytobrushes were transferred to a conical vial containing PBS and transported on ice to the laboratory for processing.

Sample processing

Cervical immune cells were obtained from the cytobrush and filtered through a 100-μm filter (Becton Dickinson [BD]). Tubes were vortexed first several times to remove as many cells as possible from each cytobrush. After filtering, cells were washed in warm RPMI 1640 10% FBS 1% antibiotic/antimycotic and used in the assays described below.

Flow cytometry Abs

We used the following flow cytometry Abs for staining PBMC and cervical cells in a number of experiments: Act-1–PE (α4β7, National Institutes of Health AIDS Reagent program), CD3-Alexa Fluor 700, CD4-ECD (Beckman Coulter), CCR9-Alexa Fluor 647, CD103-FITC, CCR5–PE–Cy5, allophycocyanin-Cy7, CXCR4–PE–Cy5, CD69–PE–Cy7, IL-17A–FITC, IL-22–allophycocyanin, IFN-γ–PE–Cy7, and TNF-α–Alexa Fluor 700. All Abs were from BD, unless otherwise indicated.

Surface staining

Washed cervical cells were stained for surface markers, including pretitrated amounts of Vivid (Invitrogen) to discriminate dead from live cells, for 20 min at room temperature. Samples were acquired on a BD LSRII Flow Cytometer configured for detection of 10 colors. All events were collected for cervical samples.

Intracellular cytokine staining

Cells were resuspended in IMDM supplemented with 10% Serum replacement factor (Invitrogen) 1% antibiotic/antimycotic and then stimulated with PMA (1 ng/ml) and ionomycin (1 μg/ml) for 6 h. After the first hour, protein secretion was inhibited through addition of pretitrated amounts of BD GolgiStop and GolgiPlug. Surface staining was carried out as above, followed by washing, permeabilization using BD Fix/Perm, and staining with a mixture of Abs to intracellular markers for 15 min at room temperature. Cells were then washed and fixed in 1% paraformaldehyde and acquired as described above. Results are reported following background subtraction.

RNA extraction, reverse transcription, and quantitative real-time PCR

Samples were processed and assayed via quantitative real-time PCR (qPCR) as previously described (24). RNA was extracted from cell pellets, and genomic DNA was eliminated using the Qiagen RNeasy Plus Kit (Qiagen). cDNA was created by adding 10 ng RNA to the RT master mix and was reverse transcribed using the Superscript III Kit (Invitrogen). Genomic DNA standards were isolated from purified placental tissue and used as a universal standard over a 7-log dilution range to calculate relative gene-expression levels (25). Single intraexon gene-specific primers were generated using Primer Express Software (Perkin Elmer Applied Biosystems) or OligoPerfect (Invitrogen). Specific primers were designed to target IL-17A (forward, 5′-CATGAACTCTGTCCCCATCC-3′; reverse, 5′-CCCACGGACACCAGTATCTT-3′) and IL-22 (forward, 5′-TGCATTTGACCAGAGCAAAG-3′; reverse, 5′-AGTTTGGCTTCCCATCTTCC-3′) mRNA induction. SYBR green fluorescent dye was used to detect amplification under previously published amplification conditions (24). All reactions were run in triplicate. Quantitative PCR values crossing threshold were obtained during the exponential amplification phase using SDS 2.3 Software (Applied Biosystems). The housekeeping genes GAPDH and β-actin were used to assess target gene expression in the cervix and blood, respectively (24). Gene quantities were calculated from standard curves in arbitrary units and normalized by dividing the amplified gene target by the housekeeping gene for each sample and reported as the normalized gene induction ratio. All kits were used per the manufacturer's instructions.

Protein quantification

Cell culture supernatants were saved following the same stimulation as above for mRNA quantification. The Searchlight chemiluminescent multiplex-ELISA assay (Aushon Biosystems, Billerica, MA) was used to quantify IL-17A and IL-22 cytokines and was run per the manufacturer's instructions.

HIV gp120-binding assays

Cervical cells were collected and isolated as described and resuspended in buffer either containing divalent cations ($Mn^{++}Ca^{++}$) or EDTA. Following blocking with mouse and human IgG, unlabeled SK3 mAb was added to all wells to block CD4 binding. The CD4-blocked wells contained divalent buffers, as this is shown to facilitate integrin binding. EDTA was used as an $integrin^-$ control (i.e., α4β7 block). Two to five micrograms of biotinylated gp120 Z205F (26) was incubated with the cells for 25 min, followed by washing and incubation with pretitrated amounts of neutravidin-PE and surface Abs for 20 min at room temperature. Cells were washed again, fixed using 1% paraformaldehyde, and acquired on the LSRII. Results are presented as gated on $CD3^+$ $CD8^-$ cells (CD4 is blocked).

Statistical analyses

Coexpression of markers was assessed using paired Student *t* tests. Differences in levels of expression between groups was determined by the Mann–Whitney *U* test. Correlations were determined using Spearman rank correlation. All statistical tests were run on SPSS v. 17.0 for Mac. Flow cytometry data were analyzed in FlowJo v. 8.6.6. and Microsoft Excel prior to statistical testing.

Results

Study participants

Convenience samples were obtained from HIV$^-$ and HIV$^+$ FSW participants recruited through two research clinics in Nairobi, Kenya between July and December 2009. Twenty HIV$^-$ FSWs were enrolled for characterization of surface marker expression. An additional 56 women were studied for cytokine responses following stimulation (demographics below). Given the limited cell yield obtained from a cervical cytobrush (27), it was necessary to perform the ex vivo and stimulation studies on different groups of participants. For the surface-expression study (n = 20), the median age, duration of sex work, and clients/wk for these participants was 30.8 y, 5.2 y, and 12, respectively. All screened negative for *C. trachomatis*, *N. gonorrhea*, *T. vaginalis*, and syphilis; 63% were BV-negative on Gram's stain. For the cytokine studies, the median age for HIV$^-$ (n = 41) participants was 30.3 y, with 2.9 y in sex work and 8.2 clients/wk. Sexually transmitted infections were rare (3/41, 7%), and included one case of *N. gonorrhea*, one case of syphilis, and one case of *T. vaginalis*. Most participants (66%) were BV-negative.

Expression of surface markers, including α4β7, on cervical CD4$^+$ T cells

We performed surface phenotyping of CD4$^+$ T cells from the blood and cervix, examining ex vivo expression of a number of surface receptors after gating on live CD3$^+$ CD4$^+$ T cells derived from cervical cytobrush and blood samples. These included the mucosal homing markers CCR9, α4β7, and CD103; the early immune activation marker CD69; and the HIV coreceptors CCR5 and CXCR4 (Fig. 1*A*). There was a wide range of α4β7 expression on cervical CD4$^+$ T cells (median 26.0, IQR 15.5–39.6%). The majority of α4β7 expression (median 91.8% of α4β7$^+$ T cells) was intermediate in intensity (Fig. 1*B*), as has been described in ex vivo studies of intestinal tissue (28), with the remainder being α4β7^{hi}. Expression of the integrin CD103 (αEβ7) was less common (median 9.6%), corroborating a previous report by our group (27), but was significantly higher in the cervix than in peripheral blood (0.6%, $p < 0.0001$). Moderate levels of the gut homing marker CCR9 expression were expressed (median, 17.9%; interquartile range [IQR], 13.1–33.3%, compared with 5.3% in blood). Although CCR9 and α4β7 have both been described as gut-homing markers, their expression levels did not correlate (p = 0.46), and coexpression analysis demonstrated that these molecules were often expressed on different cells (data not shown). Relatively high levels of CXCR4, CCR5, and CD69 were observed on cervical CD4$^+$ T cells (medians of 38.7, 81.5, and 53.6%, respectively), all of which were at higher levels in the cervix compared with blood (all $p < 0.01$; Fig. 1*A*). CCR5 was particularly overexpressed in the cervix compared with the blood (>10-fold higher); understanding further the phenotypic characteristics of these CD4$^+$ T cells has obvious implications for HIV transmission, given that CCR5-tropic strains are responsible for establishing HIV infection during male–female vaginal transmission.

Coexpression of selected markers on cervical CD4$^+$ T cells

To determine coexpression patterns of various susceptibility markers on the same cervical CD4$^+$ T cells, we assessed the expression of various markers in conjunction with α4β7 staining. Gating on CD3$^+$ CD4$^+$ T lymphocytes, we found high levels of double-positive populations for most of the markers assessed (Fig. 2*A*). Compared with α4β7$^-$ cells, expression of CCR5 and CD69 was increased on α4β7$^+$ CD4$^+$ cervical T cells (Fig. 2*B*). The median CCR5 expression on α4β7$^+$ CD4$^+$ cervical T cells was 81% (IQR, 61–93%), compared with 62% (IQR, 46–81%) on α4β7$^-$ CD4$^+$ T cells ($p < 0.0001$). Furthermore, the mean fluorescence intensity of α4β7 on CD4$^+$ T cells correlated with the levels of CCR5 expression on those cells (r = +0.565, p = 0.009, Fig 2*C*). Similarly, the median CD69 expression on α4β7$^+$ CD4$^+$ T cells was 53.4% (IQR, 33–79%), compared with 46.2% (IQR, 25–62%) on α4β7$^-$ CD4$^+$ T cells ($p < 0.0001$). Because each of

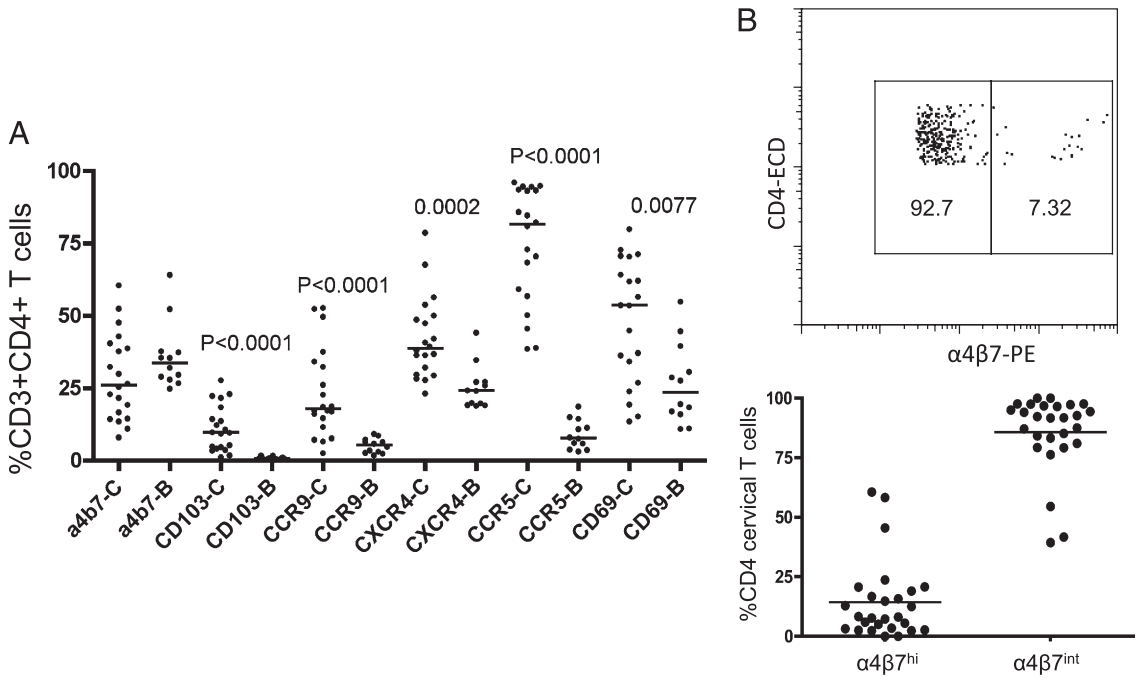

FIGURE 1. Expression of selected cell surface markers on cytobrush-derived cervical and blood CD3$^+$ CD4$^+$ T lymphocytes isolated ex vivo from HIV$^-$ FSWs from Nairobi, Kenya (n = 20). *A*, Ex vivo surface staining of α4β7, CD103, CCR9, CCR5, CXCR4, and CD69 in the blood (B) and cervix (C) of matched participants. *B*, Intensity of α4β7 staining (right gate, α4β7^{hi} versus left gate, α4β7^{int}) on α4β7$^+$ cervical CD4$^+$ T cells is shown for one representative participant (*top panel*) and for all participants (*bottom panel*). Horizontal lines indicate the medians.

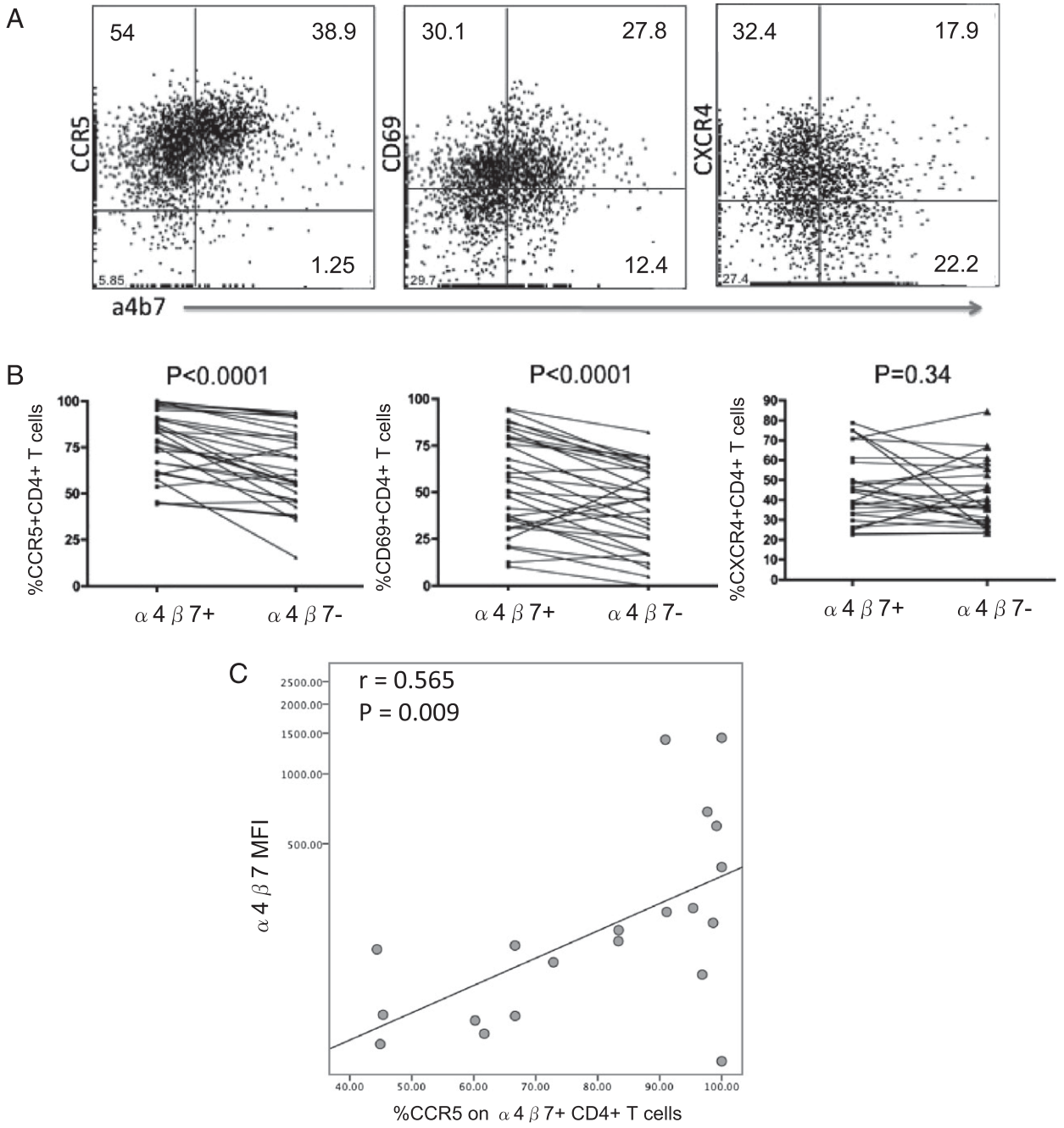

FIGURE 2. Coexpression of CCR5, CD69, and CXCR4 on α4β7$^+$ versus α4β7$^-$ cervical CD3$^+$ CD4$^+$ T cells. *A*, Representative flow cytometry plots of ex vivo surface staining are shown for CCR5, CD69, and CXCR4, with α4β7 on the *x*-axis. *B*, Relative expression of each marker on α4β7$^+$ and α4β7$^-$ cervical CD4$^+$ T cells is shown for matched participants (n = 20). *C*, Correlation between mean fluorescence intensity of α4β7 staining and percentage of CCR5 expression on cervical α4β7$^+$ CD4$^+$ T cells.

these markers defines a T cell subset that is preferentially infected by HIV in vitro (29), their coenrichment on the surface of the same cervical CD4$^+$ cells suggests that this coexpression may represent an important mucosal target cell during sexual HIV transmission. Conversely, CXCR4 was not preferentially expressed on α4β7$^+$ CD4+ T cells compared with α4β7$^-$ CD4$^+$ T cells (median, 44.3 versus 37.6%, p = 0.34, Fig. 2*B*).

Enhanced expression of α4β7, CCR5, and IFN-γ by cervical IL-17A$^+$ CD4$^+$ (Th17) cells

Although Th17 and Th22 cells are gaining prominence for their roles in mucosal immunology of the gut and skin (30, 31), these cell subsets have not been described in the female genital tract. We went on to characterize the frequency and phenotypes of Th17 cells in blood and cervix of HIV$^-$ FSWs (n = 41). Representative gating for Th17 responses in the blood and cervix is shown in Fig. 3*A* and for IL-22, IFN-γ, and TNF-α is shown in Fig. 3*B*. Following PMA/ionomycin stimulation, IL-17$^+$ CD4$^+$ T cells coproduced a range of additional cytokines, and we compared coexpression patterns between the blood and cervix (Fig. 3*C*). Coexpression of IL-17 and IL-22 in the cervix was fairly modest (median, 22.2% of IL-17$^+$ cells also expressed IL-22), and cervical Th17 cells tended to produce more IL-22 than did those in the blood (22.2 versus 12.0%; p = 0.07). We also found modest frequencies of the IFN-γ$^+$ IL-17$^+$ subset (Th1 Th17) in the female

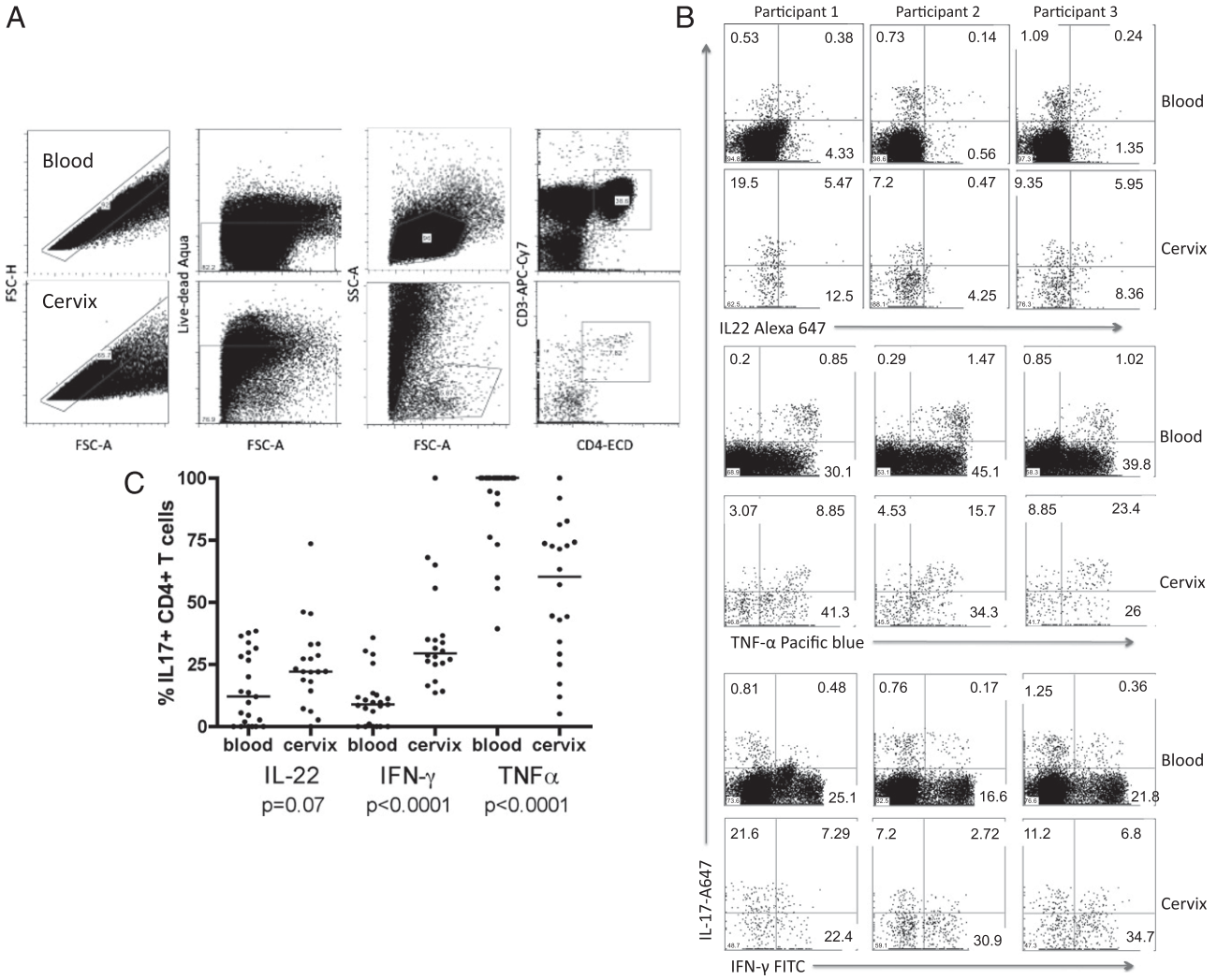

FIGURE 3. Phenotypic characteristics of IL-17–producing $CD3^+$ $CD4^+$ T cells in the cervix compared with blood. *A*, Representative IL-17A responses from the blood (*top panels*) and cervix (*bottom panels*), gating on (from *left* to *right*): singlets, live cells, lymphocytes, and $CD3^+$ $CD4^+$ T cells. *B*, IL-17A responses from the cervix of three participants, with IL-17A on the *y*-axis and IL-22 (*top rows*), TNF-α (*middle rows*), and IFN-γ (*bottom rows*) on the *x*-axes. Cells were stimulated with PMA/ionomycin for 6 h and gated as described. *C*, Frequencies of IL-17A cells that simultaneously coproduced IL-17A and each indicated cytokine (upper right quadrant of *B*) compared with matched samples from the blood. The horizontal lines represent the medians.

genital tract, and coexpression of IFN-γ^+ was three times higher in the cervix than the blood (median, 29.5 versus 8.9%, $p < 0.0001$). TNF-α was highly expressed in cervical Th17 cells (median 60.2%, Fig. 3*D*), albeit with significant heterogeneity between participants, but the proportion of Th17 cells coexpressing TNF-α was substantially higher in the blood than the cervix (median 100 versus 60.2%, $p < 0.0001$). These data demonstrated substantial functional differences in Th17 populations derived from the blood and genital mucosa.

Next, we tested the hypothesis that Th17 cells from the cervix would preferentially express α4β7 and CCR5, characteristics that would be expected to enhance their susceptibility to HIV infection. CCR5 expression was substantially increased on Th17 cells from the cervix compared with blood samples from the same participants (Fig. 4*A*, 4*B*). The median expression of CCR5 on cervical IL-17^+ T cells was 68.7% compared with 10.8% in the blood ($p < 0.0001$, Fig. 4*B*). This was not merely a reflection of higher overall expression of CCR5 in the cervix; indeed, when we compared the IL-$17A^+$ subset with other cervical $CD4^+$ T cells, there was an increased expression of CCR5 ($p = 0.026$, Fig. 4*C*). We then extended this analysis to IL-17^+ $CD4^+$ T cells expressing α4β7. Consistent with our data on total α4$\beta7^+$ $CD3^+$ $CD4^+$ T cells (Fig. 2), the majority of cervical IL-17^+ $CD4^+$ T cells coexpressed both α4β7 and CCR5 (67.2–75.2%, Fig. 4*D*).

Preferential HIV gp120 binding to cervical CD4$^+$ T cells expressing CCR5

Given that several susceptibility markers cluster on the same subset of cervical $CD4^+$ T cells, one hypothesis is that HIV is able to target these cells preferentially over other $CD4^+$ T cells from this site. We tested this by assessing ex vivo binding of biotinylated gp120 cells. We focused on CCR5 to represent the subset of optimal target cells, because this marker coincided with the others and was expressed at high levels, aiding detection despite low cell recovery from the cervix; low cell numbers precluded using markers, such as cytokines, which require cell permeabilization. α4β7–gp120 binding was implied by inhibition experiments in buffer that precludes integrin activation. Using an Env strain that had been isolated very soon after (and therefore relevant to) HIV transmission, we combined surface marker staining with gp120-binding ex vivo, after blocking CD4 with a mAb (Fig. 5). In two HIV^- participants, 78 and 83% of $CD3^+$ $CD8^-$ T cells that bound gp120 also expressed CCR5 compared with 53 and 62% of $CD3^+$ $CD8^-$ T cells that were $gp120^-$. Increased CCR5 expression was

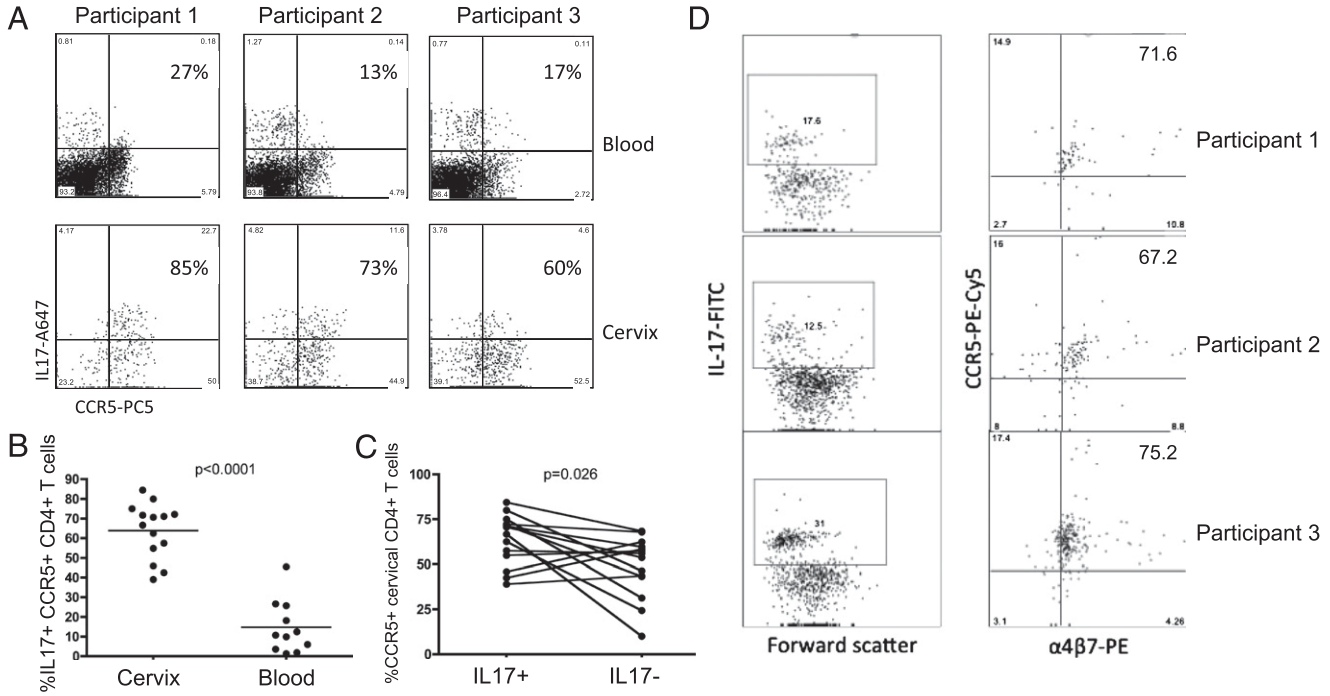

FIGURE 4. CCR5 and α4β7 expression on cervical IL-17$^+$ CD3$^+$ CD4$^+$ cells. *A*, Representative CCR5 expression on IL-17A$^+$ CD4$^+$ T cells from the blood (*top panel*) and cervix (*bottom panel*) from three representative HIV$^-$ participants. *B*, Relative expression of CCR5 on IL-17$^+$ CD4$^+$ T cells from the cervix compared with the blood. *C*, Expression of CCR5 on cervical IL-17$^+$ CD4$^+$ T cells compared with IL-17$^-$ CD4$^+$ T cells. *D*, The combination of α4β7 and CCR5 expression on cervical IL-17$^+$ CD4$^+$ T cells in three participants. The *left panels* show gating on CD3$^+$ CD4$^+$ IL-17A$^+$ cells, whereas the *right panels* show the expression of α4β7 and CCR5 on CD3$^+$ CD4$^+$ IL-17A$^+$ cells. All assays were performed as in Fig. 3. The horizontal lines represent the medians.

particularly evident in the gp120^{hi} cells, which also held true in experiments in which both CD4 and α4β7 were blocked (Fig. 5*A*, *right panels*). In support of this, we observed a strong correlation between the mean fluorescence intensities of gp120 binding and CCR5 staining in cervical T cells from HIV$^-$ participants in conditions where CD4–gp120 binding was blocked (n = 6, r = +0.89, p = 0.033, Fig. 5*B*). Finally, to determine the extent to which α4β7 binding might account for the preferential binding, we inhibited α4β7 and measured the decrease in gp120$^+$ staining. In participant 1, this blockade led to a 43.3% decrease in gp120 binding; in participant 2, a 32.8% decrease in gp120 binding was observed (Fig. 5*C*). These data showed that HIV gp120 preferentially bound to CCR5$^+$ cervical CD8$^-$ T cells that coexpressed multiple susceptibility markers (above) and that this preference was partly due to α4β7 binding.

Th17 cells are depleted from the cervix of HIV$^+$ FSWs

Given these in vitro data, we next hypothesized that a subset of CD4$^+$ T cells expressing multiple susceptibility markers would be depleted from the cervix of HIV$^+$ individuals. Although there are conflicting data regarding the depletion of CD4$^+$ Th17 cells from the blood during HIV infection (32), this cell subset is consistently depleted from the gut mucosa of HIV$^+$ participants and SIV$^+$ primates (22, 33–35). To determine Th17 frequency at another mucosal site, we examined the impact of HIV infection status on the frequency and magnitude of the Th17 subset in the cervix and blood. Nearly all HIV$^-$ subjects (23/24) had detectable responses in the blood, at a median frequency of 1.24% of CD3$^+$ CD4$^+$ T cells (Fig. 6*A*). Responses were detected less often in the cervix (16/41, 39%), but at a higher median frequency of 7.02% of CD3$^+$ CD4$^+$ T cells (in those with a positive assay, Fig. 6*C*). This lower frequency of detection may be associated with higher background, unstimulated cytokine levels in the cervix compared with the blood (median 6.2 versus 0.2% CD4$^+$ IL-17$^+$ T cells, $p < 0.0001$). Despite the differences in magnitude, there was a strong correlation between blood and genital tract IL-17 responses (r = +0.536, p = 0.001, data not shown). Similar data were observed for IL-22; although responses could be detected in the blood of 14/18 HIV$^-$ participants, only 14/41 cervical samples were IL-22$^+$ after background subtraction (Fig. 6*D*). Similar to IL-17, the magnitude of IL-22 responses was higher in the cervix than in the blood (4.04 versus 0.61%, Fig. 6*B*, 6*D*).

To determine whether these CD4$^+$ T cell subsets were depleted from the cervix during HIV infection, we tested HIV$^+$ FSWs (n = 16) from the same FSW cohorts for IL-17$^+$ CD4$^+$ T cells in cervical and blood samples, as above. Participants were HIV$^+$ chronic antiretroviral therapy (ART) naive (n = 11), HIV$^+$ on ART (n = 3), and HIV$^+$ early infection (n = 2). IL-17 responses were common in the blood, detected in 12/12 HIV-infected participants at a median magnitude of 0.99% of CD3$^+$ CD4$^+$ T cells; this was comparable to the Th17 frequencies observed in HIV$^-$ participants (median 1.24%, p = 0.9). In contrast, only 1/16 (6%) of HIV$^+$ subjects had a detectable IL-17 response in the cervix (mean compared with HIV$^-$, p = 0.034, Fig. 6*C*), and this response was low in magnitude (0.97% of CD4$^+$ T cells). Similarly, although CD4$^+$ T cell IL-22 responses were common in blood CD4$^+$ T cells from HIV$^+$ participants (9/12), only 1/16 participants (6%) had detectable IL-22 responses in the cervix (p = 0.099, Fig. 6*D*). Different HIV$^+$ participants made cervical IL-17 and IL-22 responses (ART naive and ART experienced, respectively).

Decreased cervical IL-17 and IL-22 gene and protein expression during HIV infection

To confirm the observation that Th17 cells were preferentially depleted from the cervix during HIV infection, we recruited 44 HIV$^+$ sex workers and 24 HIV$^-$ nonsex worker controls and

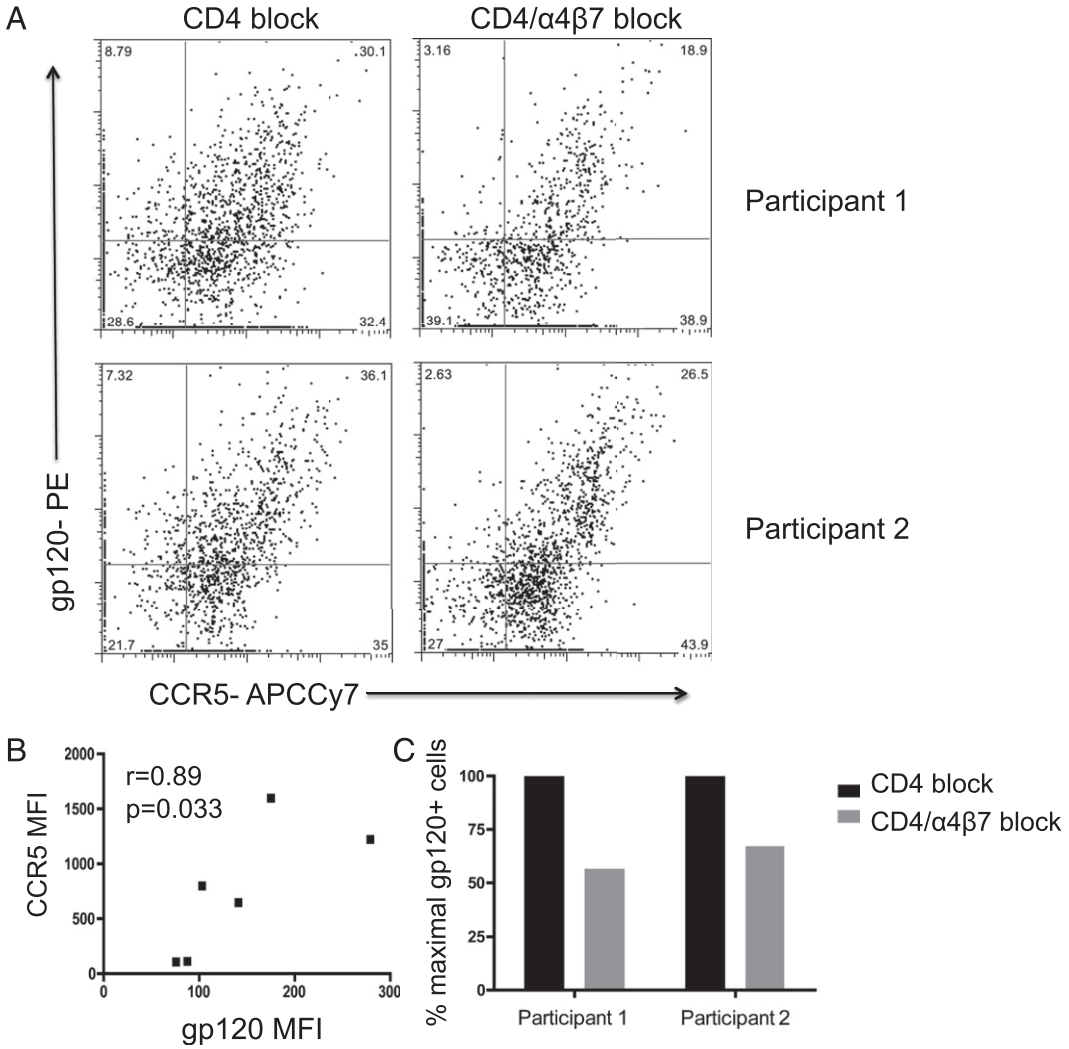

FIGURE 5. In vitro binding of cervical $CD3^+$ T cells to biotinylated founder gp120, costained with CCR5, in the presence and absence of α4β7 blockade. *A*, Coexpression of gp120-PE and CCR5–allophycocyanin–Cy7 on cervical $CD3^+$ $CD8^-$ T cells from two representative participants, in the presence of CD4 blockade (via mAb, *left panels*) or both CD4 and α4β7 blockade (α4β7 blockade via buffer containing EDTA, which inhibits integrin activation, *right panels*). *B*, Correlation between the mean fluorescence intensities of gp120-PE and CCR5–allophycocyanin–Cy7 staining on $CD3^+$ $CD8^-$ T cells from six matched participants. *C*, gp120 binding to cervical $CD3^+$ $CD8^-$ T cells in the presence (gray bars) or absence (black bars) of α4β7 inhibition. The bars indicate the total of the upper quadrants (i.e., all $gp120^+$ cells) displayed in *A*.

measured the expression of IL-17A and IL-22 mRNA by qPCR in cell pellets and protein expression in culture supernatants using multiplex ELISA in the cervix and blood after *Staphylococcus* enterotoxin B stimulation. Cervical analysis was only performed on samples with cell counts $>2 \times 10^5$. Compared with HIV^- participants, HIV^+ participants had lower expression of both blood and cervical IL-17A mRNA (blood, $p = 0.029$; cervical mononuclear cells [CMC], $p = 0.035$), as well as protein (blood, $p = 0.001$; CMC, $p = 0.032$, Fig. 7). Similarly, there was lower expression of IL-22 mRNA (blood, $p < 0.0001$; CMC, $p = 0.006$) and protein (blood, $p = 0 < 0.0001$; CMC, $p = 0.001$, Fig. 7) in HIV^+ participants.

Discussion

A better understanding of the early events of HIV-1 sexual transmission, as well as the identification of the cells targeted at this stage, remains an important area of HIV research. It was demonstrated in nonhuman primate models that mucosal $CD4^+$ T cells are key initial targets (36). Given that HIV is not transmitted during most sexual exposures, more work is needed to characterize the $CD4^+$ T cell subsets present in the female genital tract of high-risk populations, as well as how these relate to HIV susceptibility. We described a subset of cervical $CD4^+$ T cells that simultaneously expresses several cellular markers of HIV susceptibility, including CCR5, α4β7, IL-17, and IFN-γ. One hypothesis that accounts for the heterogeneity in the relative risk for HIV transmission during a given exposure is that a certain threshold of target cells is required to overcome innate defenses and establish HIV infection. Because the rate at which HIV infects and replicates in $CD4^+$ T cells is heavily dependent on the type and activation state of the target cell in question (37), this threshold may differ depending on the concentration of optimal HIV targets, not only the number of possible target cells. Our data demonstrated that multiple HIV susceptibility factors are expressed in tandem on the same T cells, raising the hypothesis that this population may play an important role in establishing HIV infection. Further work is required to prove this hypothesis; such work could provide a possible new avenue for HIV microbicide research. The relevance of these cells as a highly HIV-susceptible subpopulation is supported by their near-complete depletion from the cervix of HIV^+ individuals, as well as their preferential binding to gp120 in vitro. One important extension of

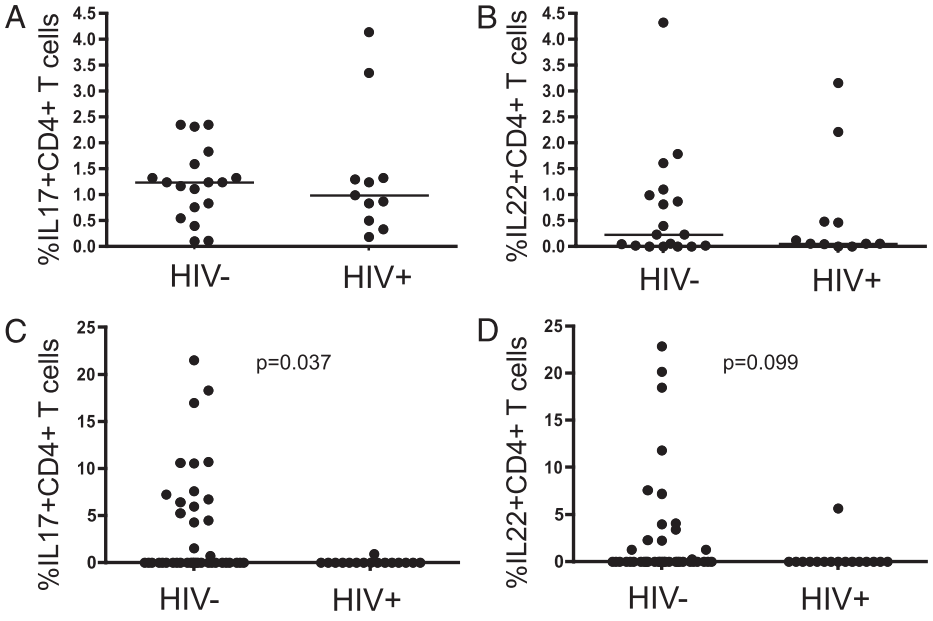

FIGURE 6. Magnitude of IL-17A– and IL-22–secreting $CD3^+$ $CD4^+$ T cells from the cervix and blood of HIV^+ FSWs versus HIV^- FSWs. *A*, The frequencies of IL-17A responses from the blood. *B*, The frequencies of IL-22 responses in the blood. *C*, The frequencies of IL-17A responses in the cervix. *D*, The frequencies of IL-22 responses in the cervix. All plots compare HIV^+ and HIV^- participants, and all stimulations and gating were as per Fig. 3. Presented values are the background-subtracted percentages of $CD3^+$ $CD4^+$ T cells from each compartment. Horizontal lines represent the medians.

these studies, although technically difficult for a number of reasons, is demonstration of preferential infection of these cells in vitro. At the moment, we are basing our assessment of susceptibility on a large body of previous research on cell susceptibility to HIV and measuring these factors ex vivo in a mucosal surface highly important for male-to-female transmission of HIV.

To our knowledge, this is the first study to describe $CD4^+$ T cells in the human genital tract that produce IL-17 and IL-22. Previous work showed that HIV coreceptors, such as CCR5 and $\alpha4\beta7$, are also expressed on Th17 cells in nonhuman primates (38), and our study confirmed this in humans. We showed that these cells are common in the cervix, where they display important phenotypic differences compared with the analogous populations in the peripheral blood. In particular, the dramatically increased expression of CCR5, in parallel with a relative expansion of the IFN-γ^+ IL-17^+ subset, provides a plausible explanation as to why this population would be rapidly and consistently depleted in HIV infection, because these cellular characteristics were found to associate with preferential HIV infectivity (39). However, in contrast to some previous reports (32), we found a depletion of IL-17^+ or IL-22^+ subsets in the blood of some, but not all, HIV^+ individuals. Although the reason for this difference is unclear, it may relate to the relatively early stage of HIV infection of our participants; of note, our group showed that Th17 depletion occurs very early in the gut mucosa but only at more advanced HIV clinical stages in the blood (22). Another possibility is differences in assay conditions and cohorts studied between this and previous studies.

Emerging evidence suggests that mucosal Th17 depletion plays an important role in HIV pathogenesis (40). These cells are prominent in mucosal surfaces, including the gut, where substantial $CD4^+$ T cell depletion has been observed very early in HIV infection (41–43). In nonhuman primates, depletion of Th17 cells following SIV infection was associated with loss of *Salmonella typhimurium* control, leading to systemic leakage of bacterial components that may enhance immune activation and disease progression (33). Furthermore, the balance of Th1 and Th17 cells in the gut was able to predict the development of disease in SIV^+ monkeys (34), and Th17 cells were depleted from the gut mucosa in pathogenic, but not in nonpathogenic, SIV infection models (35). In the current study, we used three independent experimental approaches to show that T cells producing IL-17 and IL-22 are also dramatically reduced in the cervix of HIV^+ women. The clinical impact of this mucosal depletion is not clear, but because Th17 cells are critical mediators of protection against mucosal fungal and bacterial infection (19, 44, 45), this suggests that the lack of Th17 and Th22 cells in the cervix may contribute to the increased frequency of candidiasis and other genital tract infections that is seen in HIV^+ women (46).

Although we demonstrated that Th17 and Th22 are preferentially depleted from the female genital tract during HIV infection, because these subsets were defined by the production of IL-17 and IL-22, respectively, the possibility remains that these cells were present in the cervix but functionally impaired. Although this is doubtful given the potency of PMA/ionomycin as a cell stimulant, further studies will need to examine this possibility, either by using phenotypic markers of the Th17 subset, such as CCR6 and/or CD161, and/or by directly demonstrating enhanced HIV susceptibility in vitro.

In addition to showing that cervical Th17 cells are physically depleted ex vivo during HIV infection, we demonstrated that HIV-1 gp120, isolated from a patient with acute infection, preferentially bound to cervical $CD4^+$ T cells expressing CCR5. On a per-cell basis, gp120 bound to cervical T cells with the highest level of CCR5 expression. Moreover, approximately half of this binding could be ablated in conditions that block binding to the integrin $\alpha4\beta7$. Blood CCR6-expressing Th17 cells were demonstrated to be preferentially infected by HIV, independent of $\alpha4\beta7$ (47), and our results suggested that one important reason for this may be the expression of multiple other HIV susceptibility markers by these cells.

The gut-homing integrin $\alpha4\beta7$ binds to HIV Env directly and correlates with enhanced susceptibility of $CD4^+$ T cells to HIV infection (13, 14). In this article, we demonstrated intermediate $\alpha4\beta7$ expression on the same cells that express several other markers important for cellular HIV susceptibility. Because MAdCAM-1 and VCAM-1, the ligands for $\alpha4\beta7$, display variable expression in the genital tract (48), it is unknown whether $\alpha4\beta7$ expression on cervical T cells represents active homing or whether these are cells that are homing to this site through other means. In a mouse model of *Chlamydia* infection, MAdCAM-1, VCAM-1, and ICAM-1 are temporarily induced in genital tracts of infected, but not uninfected, mice, suggesting active recruitment of

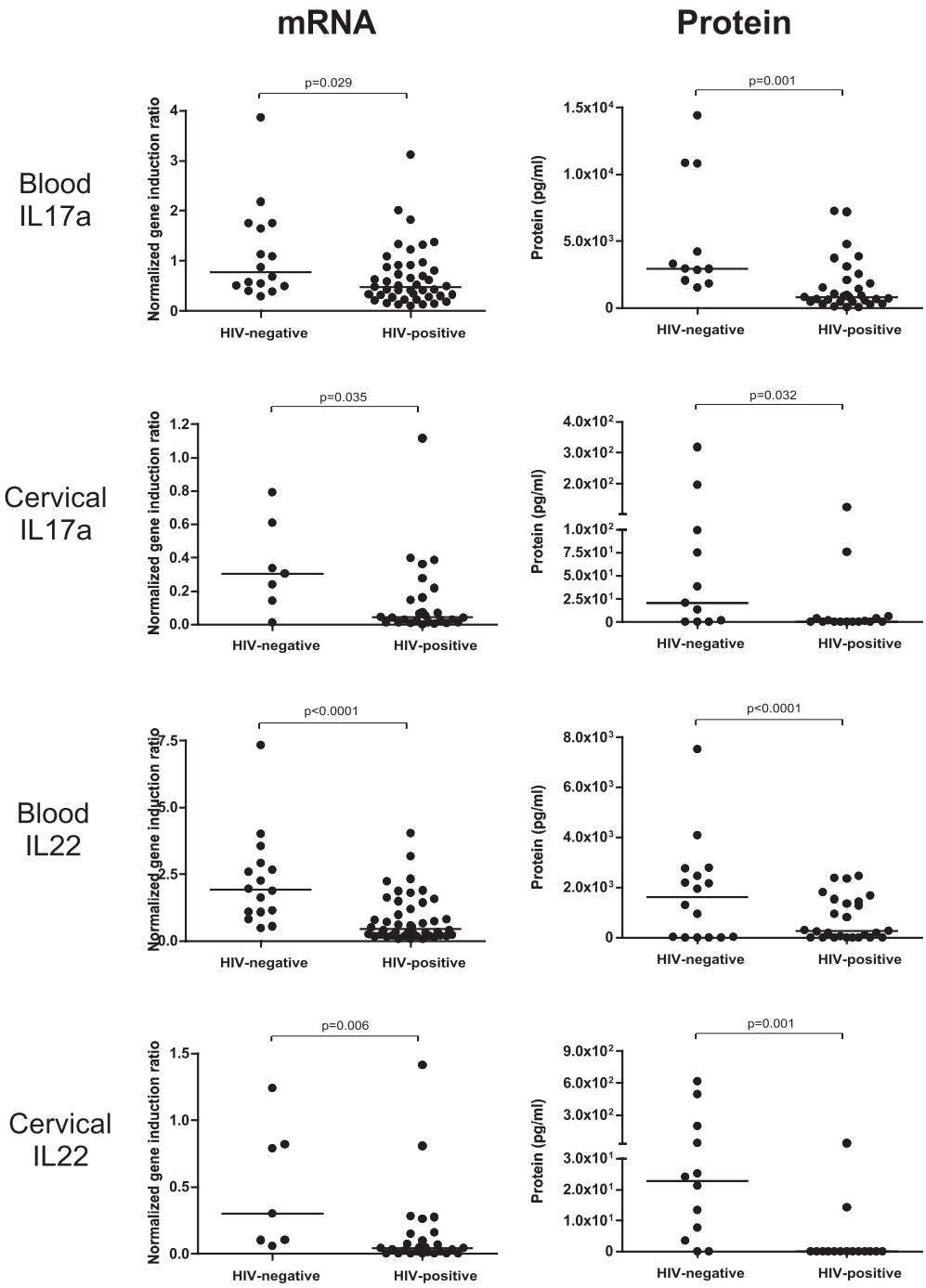

FIGURE 7. Levels of IL-17A and IL-22 mRNA and protein induction following a 6-h *Staphylococcus* enterotoxin B stimulation of blood and cervical mononuclear cells obtained from HIV^+ and HIV^- participants. Fold induction of mRNA levels in the blood and cervix are shown for IL-17A (*top left panels*) and IL-22 (*bottom left panels*), as well as cytokine protein levels for IL-17A (*top right panels*) and IL-22 (*bottom right panels*). All plots compare HIV^+ with HIV^- participants. mRNA was measured from cell pellets using qPCR, and protein levels were assayed from culture supernatants using multiplex Searchlight ELISA.

CD4 cells to the genital tract via α4β7 (49). Given the potential role of α4β7 in enhancing HIV spread, and its coexpression with other susceptibility markers in our study, blockade of this molecule might represent a possible prophylactic modality against HIV. Blockage of α4β7 has shown promise in the treatment of Crohn's disease, presumably by blocking recruitment of inflammatory T cells to the gut (50). In a nonhuman primate SIV model, α4β7 blockade was shown to delay the entry of SIV into gut tissues and reduce the SIV set point (51).

Our study is focused on FSWs; therefore, it will be important to look at non-FSW populations because of the possibility that the relatively high frequency of activated target cells observed might relate to increased rates of genital infections or trauma associated with sex work. Also, future work will need to determine the potential role that these T cell subsets may play in mucosal immune defense against genital pathogens, particularly because most sexually transmitted infections have demonstrated effects on increasing HIV transmission risk (52–54).

In conclusion, we defined a subset of $CD4^+$ T cells from the cervix of FSWs that appears to be highly susceptible to HIV, with a coalescence of expression of multiple HIV-enhancing factors, such as CCR5, α4β7, CD69, and IFN-γ, within the Th17 cervical

subset. Understanding the clinical associations of this highly HIV susceptible T cell subset, as well as the role that it plays in both HIV acquisition and in host defense against genital bacterial/fungal infections, may lead to novel strategies for HIV prevention.

Acknowledgments

We thank the staff at the Kenya AIDS Control Project who contributed to patient care, data management, administration, and laboratory diagnostics. We also thank the study participants for participation in this study.

Disclosures

The authors have no financial conflicts of interest.

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