Phenotype and susceptibility to HIV infection of CD4$^+$ Th17 cells in the human female reproductive tract

M Rodriguez-Garcia[1], FD Barr[1], SG Crist[1], JV Fahey[1] and CR Wira[1]

Prevention of sexual acquisition of HIV in women requires a substantial increase in our knowledge about HIV-target cell availability and regulation in the female reproductive tract (FRT). In this study, we analyzed the phenotype and susceptibility to HIV infection of CD4$^+$ T cell in the endometrium (EM), endocervix (END), and ectocervix (ECT) of the FRT. We found that T helper type 17 (Th17) cells represent a major subset in FRT tissues analyzed and that Th17 cells were the main CD4$^+$ T-cell population expressing C-C motif chemokine receptor 5 (CCR5) and CD90. In premenopausal women, CD4$^+$ T cells and Th17 cells, in particular, were significantly lower in EM relative to END and ECT. Th17 cells were elevated in EM from postmenopausal women relative to premenopausal tissues but not changed in END and ECT. Susceptibility of CD4$^+$ T cells to HIV infection measured as intracellular p24 was lowest in the EM and highest in the ECT. Additionally, we found that Th17 cells co-expressing CCR5 and CD90 were the most susceptible to HIV infection. Our results provide valuable information for designing preventive strategies directed at targeting highly susceptible target cells in the FRT.

INTRODUCTION

Currently, 34 million people are known to be infected with HIV and the pandemic continues to spread despite intensive preventive efforts.[1] Worldwide, approximately half of these infected persons are women; however, an imbalance between genders is found in areas like sub-Saharan Africa, where young women aged 15–24 years are up to eight times more likely to be infected than young men.[1–3]

A combination of behavioral and biological factors may be involved in the differential incidence of infection between men and women. Intensive effort is being invested to try to prevent HIV infection in women, with mixed and disappointing results.[4] To develop successful approaches to prevent sexual acquisition of HIV in women, a considerable improvement in our knowledge is necessary regarding HIV-target cell distribution and regulation in the female reproductive tract (FRT). Although the pathogenesis of HIV infection is being widely studied in immune cells from blood, we are only starting to understand and unravel the complex interactions between HIV and the immune system in the FRT.

A unique characteristic of the FRT immune system is the dual task of protection against infection while allowing reproduction. Immune cells in the FRT are directly regulated by sex hormones throughout the menstrual cycle and indirectly by the mucosal microenvironment, which is also responsive to hormonal fluctuations.[5,6] The direct effects of hormones on immune cells include changes in phenotype and activation status,[7] as well as the direct modification of susceptibility to HIV infection as we have recently demonstrated.[8] In that study, we found that estradiol treatment of CD4$^+$ T cells isolated from blood and monocyte-derived macrophages before HIV challenge reduced infection levels in a dose-dependent manner. Therefore, hormonal influence should be taken into account when analyzing HIV-target cells in the FRT.

CD4$^+$ T cells are one of the main targets for HIV infection; however, not all CD4$^+$ T cells are equally susceptible to infection.[9] Studies in non-human primates indicate that memory CD4$^+$ T cells expressing C-C motif chemokine receptor 5 (CCR5) preferentially support HIV replication.[10] Work with human cervico-vaginal tissue explants also

[1]Department of Physiology and Neurobiology, Geisel School of Medicine at Dartmouth, Lebanon, New Hampshire, USA. Correspondence: M Rodriguez-Garcia (Marta.Rodriguez.Garcia@dartmouth.edu)

Received 23 August 2013; accepted 13 March 2014; published online 23 April 2014. doi:10.1038/mi.2014.26

demonstrated that HIV infection occurs preferentially with R5 viruses and that the majority of $CD4^+$ T cells present in these explants display an effector memory phenotype.[11]

Experiments in macaques demonstrated that macrophage-inflammatory protein 3α (C-C motif chemokine ligand 20 (CCL20)) was rapidly produced in response to simian immunodeficiency virus vaginal challenge and that blockade of this molecule resulted in the prevention of systemic dissemination of infection.[12] Macrophage-inflammatory protein 3α is a high-affinity ligand for CCR6 and will attract $CCR6^+$ cells to the infectious focus. Cells expressing CCR6 include immature dendritic cells, B cells, and a subset of $CD4^+$ T cells identified as T helper type 17 (Th17) cells. In addition to the CCR6 expression, Th17 cells are characterized by their production of interleukin (IL)-17 and expression of the transcription factor retinoic acid receptor-related orphan receptor C2 (RORC2), the human ortholog of RORγt expressed in the mouse.[13,14] Th17 cells are mainly found in tissues, especially those in contact with the microbial environment, where they mediate immune responses to fungi and are important in maintaining the integrity of epithelial surfaces.[15,16] Although Th17 cells have been broadly characterized in mucosal tissues such as the gut, lungs, or skin, with the exception of the endocervix,[16,17] relatively little is known about their presence and distribution in other FRT tissues.

Several reports indicate that Th17 cells display high susceptibility to HIV infection and disappear early from the gut and cervix of HIV-infected patients.[17–20] Th17 cells express higher levels of CCR5 and possess a memory phenotype, which may account for their increased HIV susceptibility.[18,19] Furthermore, a recent study demonstrated the expression of CD90 (Thy-1) in a subset of Th17 cells isolated from blood that was depleted in non-treated HIV-infected patients.[21]

In the present study, we characterized the distribution and phenotype of $CD4^+$ T cells at different sites in the FRT from premenopausal and postmenopausal women and compared their susceptibility to HIV infection. Understanding which cells are readily available to be infected, where they are located in the FRT, and the extent to which these cells are regulated by sex hormones and the mucosal tissue environment are essential to achieving successful prevention of sexual acquisition of HIV in women.

RESULTS

$CD4^+$ T-cell distribution in FRT tissues

Hysterectomy tissues were digested as described in Methods to obtain mixed cell suspensions. Differences in the distribution of $CD4^+$ T cells between endometrium (EM), endocervix (END), and ectocervix (ECT) were investigated analyzing CD3, CD4, and CD8 expression levels by flow cytometry (**Figure 1a**). In EM, within the total T-cell population ($CD3^+$) (**Figure 1b**), $CD4^+$ T cells accounted for approximately 35% of the $CD3^+$ cells present and were significantly lower than the $CD3^+CD4^-$ population ($P<0.0001$), which corresponds to $CD8^+$ T cells (**Figure 1a**). In contrast, END and ECT $CD4^+$ T cells accounted for approximately 50% of the total $CD3^+$ cells (**Figure 1b**). Comparison of $CD3^+CD4^+$ T cells among the three FRT tissues showed significantly lower numbers of $CD4^+$ T cells in EM relative to those present in END and ECT (**Figure 1b**).

To investigate whether the menstrual status of the patients would affect T-cell distribution in FRT tissues, we stratified samples according to premenopausal or postmenopausal status, as established by tissue histology. Menstrual status significantly influenced T-cell distribution in EM, but not in END or ECT, with premenopausal women containing a higher percentage of $CD3^+CD4^+$ T cells (**Figure 1c**) as compared with postmenopausal women. Additionally, in postmenopausal women, the percentage of $CD4^+$ T cells was lower in EM with respect to END or ECT (**Figure 1c**).

Th17 cells constitute a major T-cell subset in the FRT

A previous report indicated that Th17 cells constitute an important T-cell subset in cervical cytobrush samples.[19] Therefore, we further characterized the immunophenotype of $CD4^+$ T cells in our hysterectomy samples by investigating the presence of Th17 subsets in the different FRT compartments. $CD4^+$ T cells were purified by negative selection to obtain an untouched pure population. After selection, EM, END, and ECT $CD4^+$ T-cell purity was $>90\%$ (**Figure 2a**). Reverse transcriptase–PCR analysis demonstrated that purified $CD4^+$ T cells from EM, END, and ECT express both RORC2 and IL-17, which identify Th17 cells[14,15,22] (**Figure 2b**). Additionally, $CD4^+$ T cells express CCL20, also described to be produced by Th17 cells (**Figure 2b**). Gene expression of RORC2, IL-17, and CCL20 was the highest in END, followed by ECT, and the lowest in EM.

We next explored how END gene expression without *in vitro* stimulation compared with gene expression in phytohemagglutinin-activated $CD4^+$ T cells from blood, which is known to upregulate Th17 markers.[23] As seen in **Figure 2c**, gene expression of RORC2, IL-17, and CCL20 was significantly higher in cervical samples relative to that seen in blood $CD4^+$ T cells. Additionally, gene expression of CCR7, which is known to be downregulated in tissue-resident T cells,[15] was significantly lower in $CD4^+$ T cells from END compared with blood. Similar patterns were seen when blood $CD4^+$ cells were compared with EM and ECT cells (not shown).

$CD4^+$ T cells from FRT express high levels of CCR6

As CCR6 is a defined surface marker for Th17 subsets,[13,22] we analyzed expression of CCR6 in mixed cell suspensions from the different FRT sites. Expression of CCR6 in $CD3^+$ $CD4^+$ T cells was detected in all FRT tissues, but expression varied among anatomical sites (**Figure 3a,b**). Median values for percentage of $CCR6^+$ cells were 11.3% in EM, 23.7% in END, and 15.2% in ECT (**Figure 3b**). As Th17 cells express the transcription factor RORC2 and produce IL-17, we performed intracellular staining to confirm that these cells were Th17 cells. The $CCR6^+$ population expressed RORC2 (**Figure 3c,d**) and produced IL-17 in response to stimulation (**Figure 3e**), corresponding to the Th17 subset.

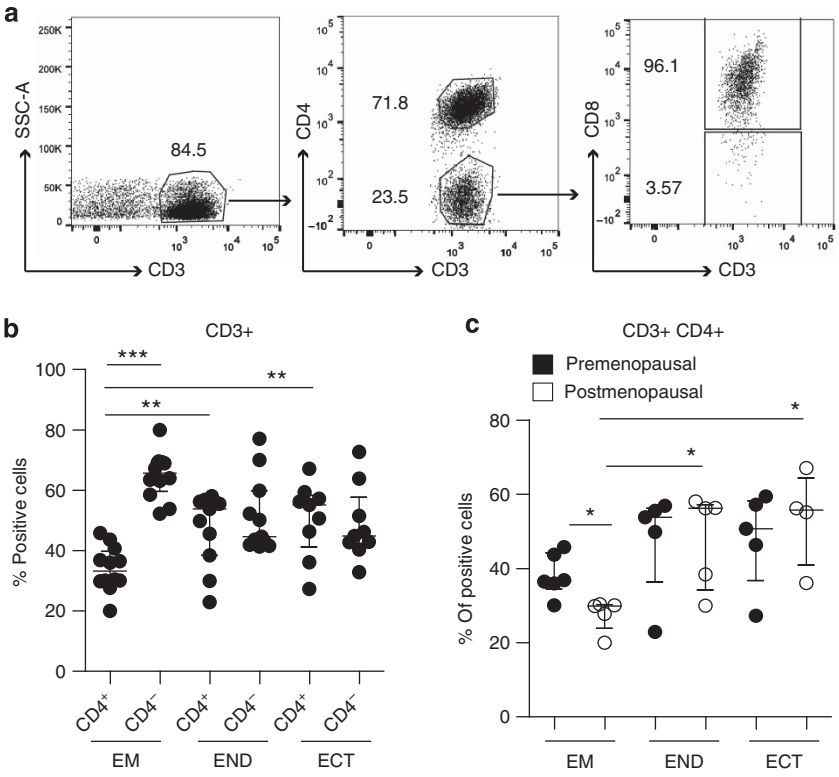

Figure 1 Distribution of $CD4^+$ T cells in female reproductive tract tissues. (**a**) Dot plots showing the gating strategy to select T-cell populations. $CD3^+CD4^-$ T cells correspond to $CD8^+$ T cells. (**b**) Comparison between $CD4^+$ and $CD4^-$ (CD8) T-cell populations within each tissue after gating on $CD3^+$ T cells. $CD8^+$ T cells ($CD4^-$) were significantly increased with respect to $CD4^+$ T cells in the endometrium (EM) but not in the endocervix (END) or ectocervix (ECT). (**c**) Distribution of $CD3^+CD4^+$ T cells in premenopausal (black dots; one in the secretory phase of the menstrual cycle) and postmenopausal (white dots) women. Each dot represents a single patient. Horizontal lines represent the median ± interquartile range. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$.

We then analyzed the influence of menopausal status by stratifying the participants shown in **Figure 3b** into those corresponding to premenopausal (black dots) or postmenopausal (white dots) women (**Figure 3f**). The number of $CCR6^+CD4^+$ T cells in EM from premenopausal women was significantly lower than that seen in postmenopausal women. Additionally, when EM, END, and ECT premenopausal cells were compared, the number of $CCR6^+$ cells in END from premenopausal women was significantly greater than those found in EM. No differences were found among postmenopausal tissues (**Figure 3f**).

$CCR6^+CD4^+$ T cells express CCR5

Th17 cells are more susceptible to HIV infection, and it has been hypothesized that this is due to expression of higher levels of CCR5.[18–20] Therefore, we measured expression of CCR5 in $CCR6^+$ and $CCR6^-CD4^+$ T-cell subsets from the FRT. As seen in **Figure 4a**, an overlay of $CCR6^+$ and $CCR6^-CD4^+$ T cells demonstrated that $CCR6^+$ T cells (solid line) co-express higher levels of CCR5 than $CCR6^-$ T cells (dashed line), whereas no differences in CD4 expression were found between both populations. As seen in **Figure 4b**, analysis of different donors confirmed that CCR5 expression was significantly higher in $CCR6^+$ than in $CCR6^-CD4^+$ T cells in the three FRT compartments analyzed. Subsequent stratification of the individuals shown in **Figure 4b** into premenopausal and postmenopausal women revealed significantly increased levels of $CCR5^+CCR6^+$ T cells in postmenopausal women with respect to premenopausal women in EM. The same trend was observed in END and ECT although these differences did not reach statistical significance (**Figure 4c**, left graph). No differences among $CCR5^+CCR6^-CD4^+$ T cells were evident for either group (**Figure 4c**, right graph).

CD90 is highly expressed in $CD4^+$ T cells from the FRT

CD90 was recently described in a small fraction of Th17 cells from blood,[21] but CD90expression is unknown in cells from the FRT. Therefore we investigated CD90 expression in $CD4^+$ T cells from the FRT. In a representative sample (**Figure 5a**), CD90 was expressed on $CD4^+$ T cells from EM, END, and ECT, with 20-36% of positive cells. Analysis of co-expression of CD90 and CCR6 in $CD4^+$ T cells from the same donor (**Figure 5b**) showed that the majority of $CD90^+$ cells were found within the $CCR6^+$ population in the three FRT compartments analyzed (**Figure 5b**). This pattern was maintained when $CD4^+$ T cells from six different patients

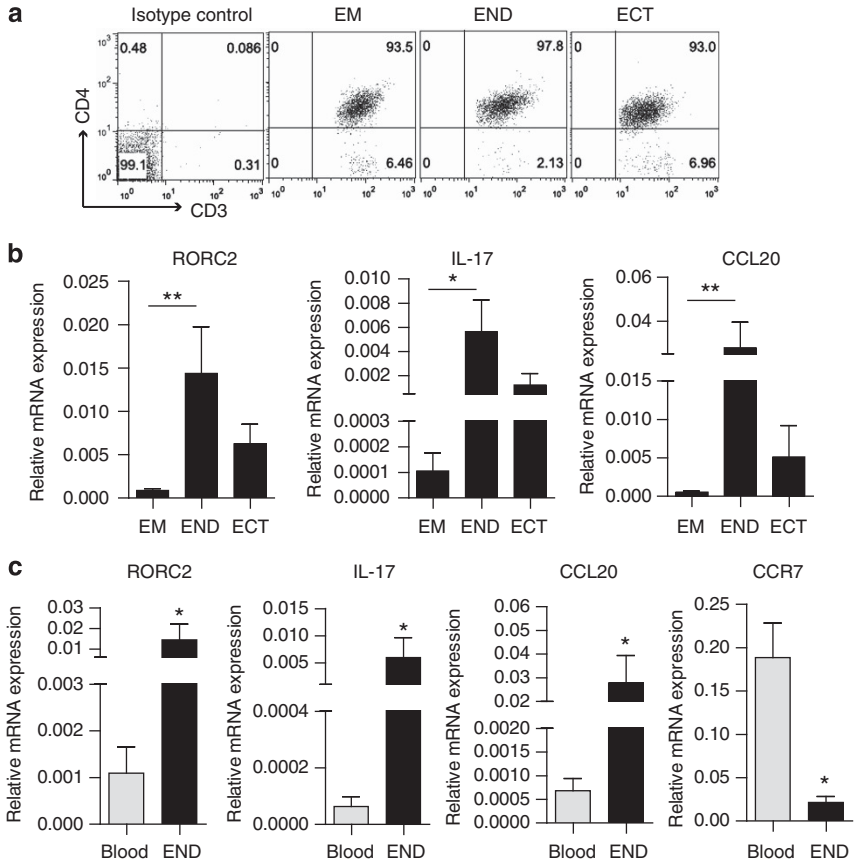

Figure 2 Expression of T helper type 17 (Th17) genes in female reproductive tract (FRT) tissues. (**a**) Representative example of a dot plot showing the purity of $CD3^+CD4^+$ T cells after isolation from FRT tissues with magnetic beads. (**b**) Gene expression relative to house-keeping gene of Th17 markers (retinoic acid receptor-related orphan receptor C2, Th17, and C-C motif chemokine ligand 20) in purified $CD4^+$ T cells from FRT tissues. (**c**) Comparison of relative gene expression between phytohemagglutinin-activated $CD4^+$ T cells isolated from blood and $CD4^+$ T cells isolated from endocervix without *in vitro* activation (END). Bars represent median ± interquartile range from five independent experiments with five different donors, four premenopausal (two in the secretory and two in the proliferative phase of the menstrual cycle), and one postmenopausal women. $^*P<0.05$; $^{**}P<0.01$. ECT, ectocervix; EM, endometrium; END, endocervix.

were analyzed (**Figure 5c**), with significantly higher expression of CD90 in $CCR6^+$ cells than in $CCR6^-$ cells.

Differential susceptibility of $CD4^+$ T cells to HIV infection within FRT tissues

We hypothesized that differences in $CD4^+$ T-cell populations would result in differential susceptibility to HIV infection among FRT tissues and that $CCR6^+$ cells would be more susceptible to infection. To test this hypothesis, mixed cell suspensions from EM, END, and ECT were prepared and infected *in vitro* with HIV (BaL) as detailed in Methods. A low viral dose (multiplicity of infection = 0.1) that gave detectable p24 and would help identify the most susceptible cells was chosen (see **Supplementary Figure S1c** online). Secreted p24 was detected in the supernatants at low levels (**Figure 6a**), as expected with the low viral input used, and infections were not productive, consistent with previous publications.[24]

To identify the $CD4^+$ T cells producing p24, intracellular p24 was measured by flow cytometry 6 days after infection. The highest proportion of p24+ T cells was found in ECT (**Figure 6b**). In contrast, p24 was detected only in two of the six EM samples analyzed. Next we asked whether $CCR6^+$ $CD4^+$ T cells were more susceptible to HIV infection. As seen in **Figure 6c**, irrespective of the site analyzed, more $CCR6^+CD4^+$ T cells were p24+ than their corresponding $CCR6^-$ population.

We then explored the possibility that $CD90^+$ cells were more susceptible to infection. Following 6 days in culture, CD90 expression was decreased in uninfected controls relative to the initial values on day 0 (**Figure 6d**). In infected cells (**Figure 6d**, bottom row), CD90 expression was also reduced relative to day 0; however, expression was approximately 60% higher than the values measured in uninfected controls for END and ECT, with no changes in EM. Compared with uninfected controls on day 6, the $CD4^+$ T-cell population co-expressing CCR5 and CD90 doubled in HIV-infected samples from END (7.38% vs. 3.21%) and tripled in ECT (6.39% vs. 1.95%), with no changes in EM. We then analyzed p24 expression in each of the four quadrants of ECT-infected cells to see if infection was preferential to a given cell phenotype. As expected, all p24+ cells were found in

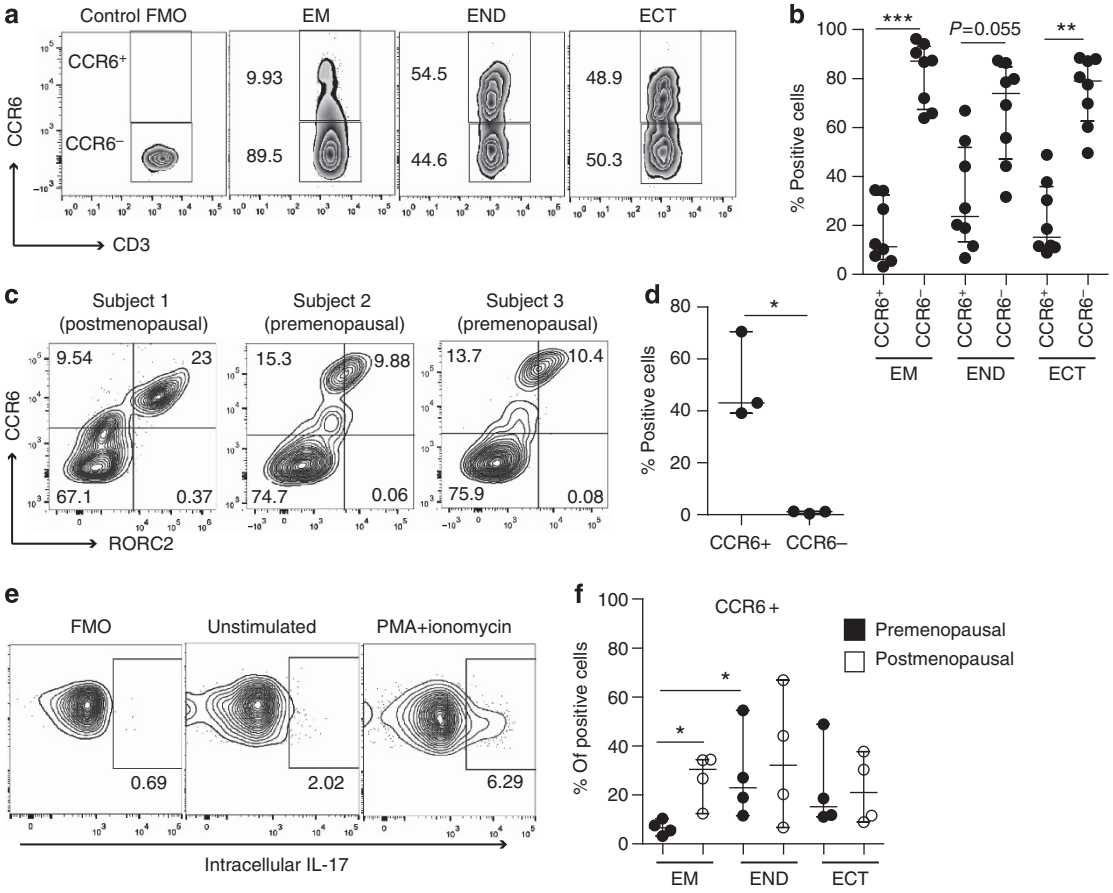

Figure 3 Expression of C-C motif chemokine receptor 6 (CCR6) in CD4$^+$ T cells from female reproductive tract (FRT) tissues. (**a**) Representative zebra plot showing percentage of CCR6$^+$ and CCR6$^-$ cells within the CD4$^+$ T-cell population (gated on CD3$^+$CD4$^+$). Negative control was established using fluorescence minus one (FMO). (**b**) Flow cytometric analysis of the percentage of CCR6$^+$ and CCR6$^-$ cells found in each FRT tissue (gated on CD3$^+$CD4$^+$ T cells). Each dot represents a different patient; matched endometrium (EM), endocervix (END), and ectocervix (ECT) from each patient were analyzed in parallel. Horizontal lines represent median ± interquartile range (IQR). $^{**}P<0.01$; $^{***}P<0.001$. (**c**) Contour plot and (**d**) graph showing intracellular staining in three different donors for retinoic acid receptor-related orphan receptor C2 (RORC2) in the CCR6$^+$ population after gating on CD3$^+$CD4$^+$ T cells. RORC2 is exclusively expressed in the CCR6$^+$ population but not in CCR6$^-$ cells (**e**) Intracellular staining of interleukin (IL)-17A in CCR6$^+$ CD4$^+$ T cells under unstimulated or phorbol 12-myristate 13-acetate (PMA) + ionomycin stimulated conditions. IL-17 production was induced after stimulation. (**f**) Stratification of the patients shown in panel **b** into premenopausal (black dots; proliferative phase of the cycle) and postmenopausal (white dots) women to compare CCR6 expression. Each dot represents a single patient. Horizontal lines represent the median ± IQR. $^*P<0.05$. ECT, ectocervix; EM, endometrium; END, endocervix.

cells expressing CCR5 (**Figure 6e**; upper and lower right panels); however, the highest percentage of p24+ cells was found within the population co-expressing CCR5 and CD90 (**Figure 6e**; upper right panel and graph).

DISCUSSION

The characterization of the target cells in the FRT involved in sexual acquisition of HIV remains a major gap in our knowledge. In the present study, we compared phenotype, distribution, and susceptibility to HIV infection of CD4$^+$ T cells from EM, END, and ECT in premenopausal and postmenopausal women. Regarding differences in cell distribution between anatomical sites, CD4$^+$ T cells in general and Th17 cells in particular were lowest in EM compared with END and ECT. With respect to comparisons between premenopausal and postmenopausal tissues, postmenopausal women had reduced CD4$^+$ T cells, increased CD8$^+$ T cells, and increased Th17 cells in EM and increased CCR5 expression in Th17 cells from EM, END and ECT. In addition, we found decreased susceptibility to infection in CD4$^+$ T cells from EM compared with END and ECT.

Gene expression analysis of purified CD4$^+$ T cells from FRT tissues revealed expression of IL-17 and CCL20, cytokines produced by Th17 cells, and RORC2, the human ortholog of mouse RORγt, which is the master transcription factor involved in Th17 cell development.[14,15] Consistent with gene expression profiles, a high proportion of CD4$^+$ T cells in the FRT had surface expression of CCR6, a marker for Th17 cells, intracellular RORC2, and produced IL-17 in response to stimulation. Although Th17 cells have been broadly studied in other mucosal surfaces,[16] to the best of our knowledge, our study is the first to compare Th17 subsets among FRT tissues. It is worth

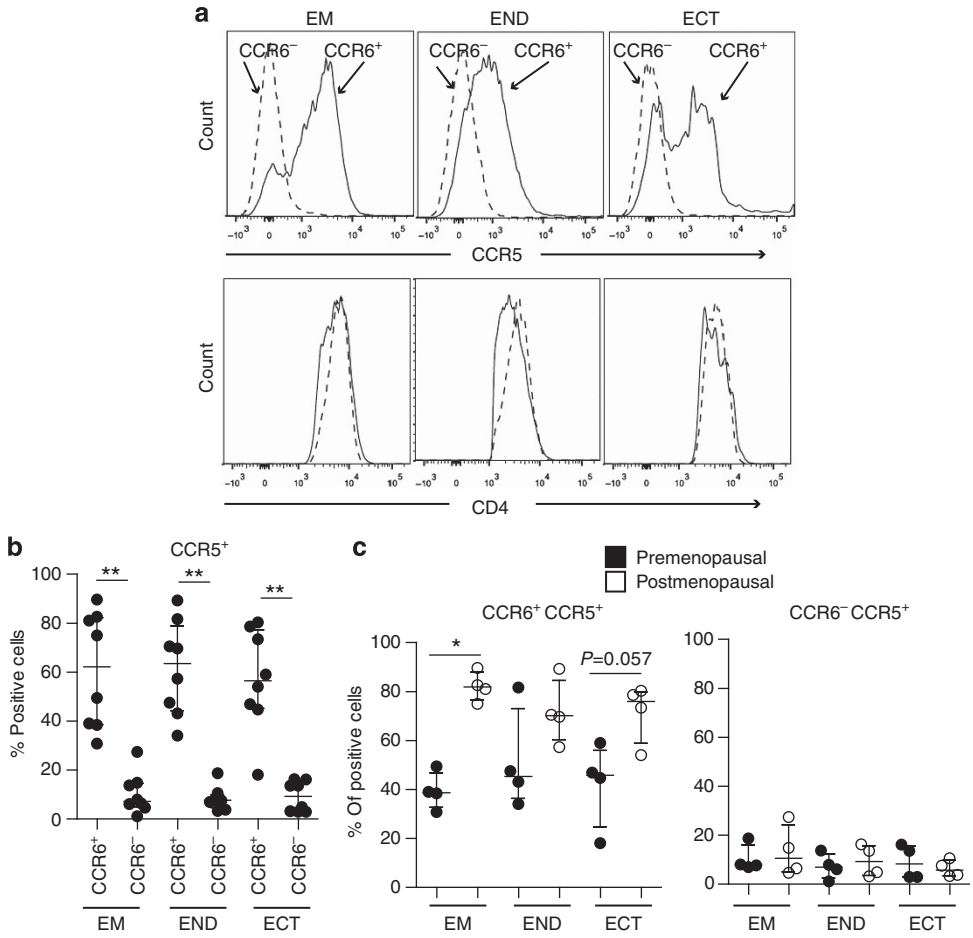

Figure 4 Expression of C-C motif chemokine receptor 5 (CCR5) in CCR6$^+$CD4$^+$ T cells. (**a**) Representative overlay of CCR6$^-$ (dashed line) and CCR6$^+$ (solid line) CD4$^+$ T-cell populations for the expression of CCR5 and CD4. The CCR6$^+$ population expressed higher levels of CCR5 compared with the CCR6$^-$ population. (**b**) Significantly increased expression of CCR5 in CCR6$^+$ cells with respect to the CCR6$^-$ CD4$^+$ T-cell population in the different FRT tissues. (**c**) Stratification of the patients shown in panel **b** into premenopausal (black dots; proliferative phase of the cycle) and postmenopausal (white dots) women to compare CCR5 expression. Each dot represents a different patient; matched endometrium (EM), endocervix (END), and ectocervix (ECT) from each patient were analyzed in parallel. Horizontal lines represent the median ± interquartile range. *$P<0.05$, **$P<0.01$.

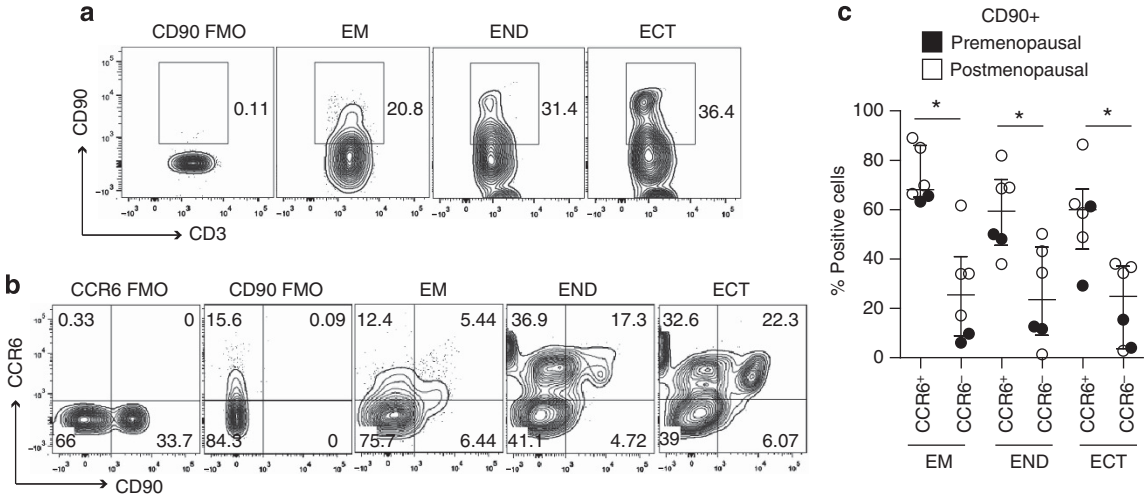

Figure 5 Expression of CD90 in CD4$^+$ T cells from the female reproductive tract. (**a**) Representative contour plot of CD90 expression in CD4$^+$ T cells. Negative control was established using fluorescence minus one (FMO). (**b**) Representative contour plot of expression of CD90 in CCR6$^+$ and CCR6$^-$ CD4$^+$ T cells (after gating on CD3$^+$ CD4$^+$) T cells. CD90 was preferentially expressed in CCR6$^+$ cells. (**c**) Comparison of CD90 expression within the CCR6$^+$ and the CCR6$^-$ CD4$^+$ T-cell populations. Patients are identified as premenopausal (black dots; proliferative phase of the cycle) or postmenopausal (white dots). Each dot represents a single patient ($n=6$). Matched endometrium (EM), endocervix (END), and ectocervix (ECT) from each patient were analyzed in parallel. Horizontal lines represent the median ± interquartile range. *$P<0.05$.

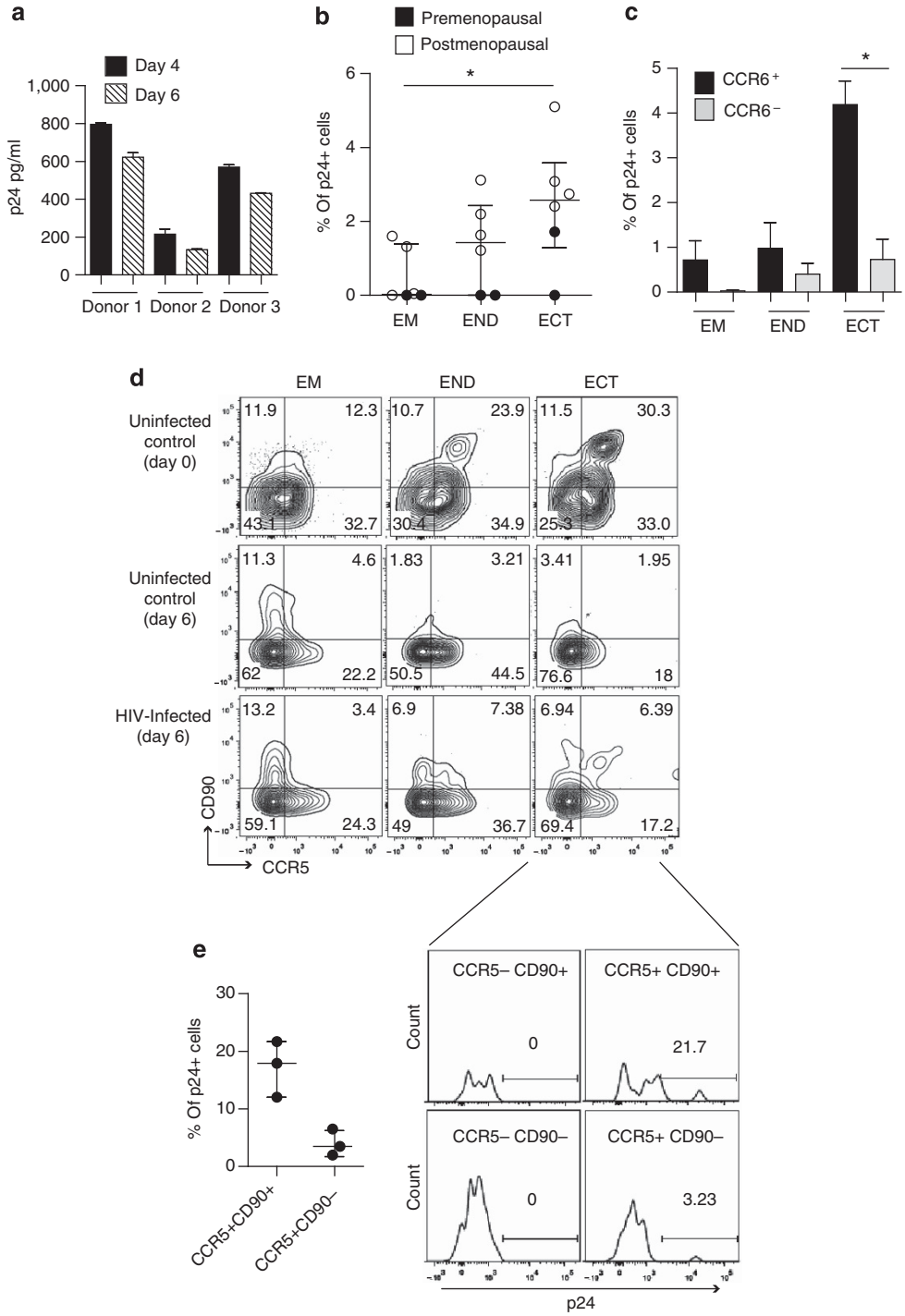

Figure 6 Susceptibility of CD4$^+$ T cells to HIV infection in female reproductive tract tissues. (**a**) Released p24 into the culture media 4 and 6 days after infection of cells from the ectocervix as detected by p24 enzyme-linked immunosorbent assay. (**b**) Percentage of intracellular p24+ CD4$^+$ T cells 6 days after *in vitro* HIV infection ($n=6$). Patients were identified as premenopausal (proliferative phase of the menstrual cycle; black dots) or postmenopausal (white dots). Each dot represents a different patient, matched tissues from endometrium (EM), endocervix (END), and ectocervix (ECT) were used. Horizontal lines represent the median ± interquartile range; *$P<0.05$. (**c**) Percentage of intracellular p24+ cells within the CCR6$^+$ CD4$^+$ T-cell population (black bars) and the CCR6$^-$ CD4$^+$ T-cell population (grey bars). Bars represent mean ± s.e.m. from five independent donors. (**d**) Representative contour plot of C-C motif chemokine receptor 5 (CCR5) and CD90 expression in CD4$^+$ T cells from EM, END, and ECT 6 days after *in vitro* HIV infection. Top row shows the uninfected controls before *in vitro* infection, middle row shows the uninfected control after 6 days in culture, and bottom row shows the infected controls. Infected and uninfected samples were maintained in culture under identical conditions except for the absence of virus in the uninfected control. Representative of three experiments with different donors. (**e**) Histograms showing intracellular p24 expression in each quadrant from the lower right panel in **d** (ECT; HIV-infected). Numbers show the percentage of p24+ cells in each population. The graph on the left represents the percentage of p24+ cells within the CCR5$^+$CD90$^+$ and the CCR5$^+$CD90$^-$ populations in the three donors analyzed.

noting that $CD4^+$ T cells from the FRT showed a unique phenotype and gene expression profile compared with their blood counterparts. The proportion of T cells that we found in EM, and to a lesser extent in END and ECT, was markedly different than the levels found in peripheral blood, where $CD3^+CD4^+$ T cells account for approximately 60–70% of the total T cells. Additionally, expression of IL-17, CCL20, and RORC2 was increased in $CD4^+$ T cells from END compared with blood, suggesting that $CD4^+$ T cells from each compartment may have unique innate and adaptive immune functions. As expected, CCR7 gene expression was reduced in $CD4^+$ T cells from the tissue compared with blood, consistent with the known downregulation of the chemokine receptor CCR7 during the development of tissue-resident memory T cells.[25]

McKinnon *et al.*[19] recently characterized $CD4^+$ T cells collected by cytobrush, which recovers cells located on the surface of the cervical mucosa. In their study, Th17 cell frequency in cervix was enhanced compared with blood, and Th17 cells co-expressed CCR5. Our study complements these findings by analyzing the cells present within FRT tissues. We also found that Th17 cells were abundant in the FRT and constituted the main T-cell subset co-expressing CCR5. Additionally, our study indicates that within each tissue, Th17 cells are more susceptible to *in vitro* HIV infection than the rest of $CD4^+$ T cells. This finding is in agreement with preferential gp120 binding by Th17 cells described by McKinnon *et al.*[19] and with other studies previously indicating that Th17 cells from blood have increased susceptibility to HIV infection.[18,20]

Our studies demonstrate that $CD4^+$ T cells from END and ECT are more readily infected by HIV than cells from EM. Others have shown in non-human primates that the cervix is the first site of detectable simian immunodeficiency virus infection.[10,12] Our results extend these findings, suggesting that in the human the cervix is the initial site of detectable HIV infection and demonstrating that not all $CD4^+$ T cells are equally susceptible to HIV infection. In contrast to END and ECT, HIV infection was detected in two out of six patients in EM. Reduced numbers of $CD4^+$ T cells, and in particular of Th17 cells, in EM with respect to END and ECT could partially explain the reduced susceptibility of this tissue to HIV infection. Further studies are needed to carefully identify the mechanisms in EM that restrict infection in $CD4^+$ T cells; this information could lead to the development of new preventive approaches.

Interestingly, our studies indicated that Th17 cells were more frequent in END than in ECT, but highest number of infected cells was found in ECT. Just why such differences were seen at adjacent sites remains to be determined. Plasticity and flexibility and the existence of intermediate subsets of Th17 cells have been reported, with alterations in lineage commitment that is regulated by the mucosal environment.[9,26] In our studies, we measured expression of CCR6 but did not analyze additional chemokine receptors such as CCR4 and C-X-C motif chemokine receptor 3, which allow sub-classification into Th17 and Th1/Th17 subsets, respectively. Both subsets express RORC2, but their cytokine profiles are different and may display differential susceptibility to HIV infection. Another possible explanation is that upon entry into a given FRT tissue, the local environment influences phenotype and function of immune cells. Previously, we found that basolateral secretions from EM epithelial cells suppress DC-SIGN (dendritic cell–specific intercellular adhesion molecule-3-grabbing non-integrin) and HIV trans-infection by blood-derived dendritic cells.[27] We found that $CD8^+$ T-cell function, measured as cytotoxic T lymphocyte activity, was suppressed during the secretory phase of the menstrual cycle in EM but not END or ECT.[28] Our findings in the present study demonstrate site phenotype specificity for $CD4^+$ T cells as well as selective susceptibility to HIV infection. Further studies are needed to understand the impact of the tissue environment on T-cell function in the FRT.

Here we describe for the first time that CD90 was highly expressed on $CD4^+$ T cells from the FRT, in particular in Th17 cells. Although CD90 is expressed in the majority of T cells in mice, CD90 expression in T cells from humans has only been reported in a small population of cortical thymocytes and $<1\%$ of T cells in peripheral blood.[29] CD90 (Thy-1) is a glycosyl phosphatidylinositol-anchored glycoprotein preferentially localized in lipid rafts.[29] CD90 appears to mediate cell-to-cell interactions, participate in transferring of other glycosyl phosphatidylinositol-anchored proteins, such as CD55 and CD59, from one cell membrane to another[29] and was preferentially localized at the contact areas between infected and target cells.[30] We found a higher percentage of p24 + cells in the population co-expressing CCR5 and CD90 than in cells expressing solely CCR5, which may suggest increased susceptibility to infection. Our data are in agreement with CD90 detection in a subset of Th17 cells from peripheral blood that was depleted during HIV infection, suggesting that this might represent a highly susceptible T-cell subset.[21] The role that CD90 may have in HIV acquisition remains unexplored, and future studies with purified cell populations need to determine their involvement in HIV acquisition. Although the physiological functions of this cell subset remain to be elucidated, selective targeting of $CCR5^+CD90^+CD4^+$ T cells may be an attractive strategy for preventing sexual acquisition of HIV in women.

Our studies also demonstrate that T-cell subsets in FRT tissues vary with menopausal status. Differences in $CD4^+$ T-cell distribution between premenopausal and postmenopausal women were found in EM but not in the other two compartments, suggesting regulation of T-cell subsets in the uterus. The CD4:CD8 T-cell ratio was decreased in EM from postmenopausal women, similar to previous findings in peripheral blood from postmenopausal women.[31–33] Additionally, we found that menopausal status affected Th17 frequency in FRT tissues. In premenopausal women, the proportion of Th17 cells was lower in EM than in END, likely contributing to the reduced HIV susceptibility of T cells from EM. In postmenopausal women, Th17 cells were significantly

increased in EM with respect to premenopausal women, suggesting selective regulation of different T-cell subsets. Although in the present study we cannot demonstrate that the observed differences between premenopausal and postmenopausal women are due to hormonal changes, recent studies in mice demonstrated that estrogen deficiency promotes the differentiation of Th17 cells.[34] Ovariectomized mice had increased numbers of Th17 cells, and this effect could be reversed by estradiol supplementation. Downregulation of Th17 cells in EM of premenopausal women may be physiologically necessary for successful fertilization, implantation, and pregnancy, considering the reported Th17 cell increase in peripheral blood and decidual tissues from women with recurrent pregnancy loss.[35]

Two main findings in our study support the reported increase in HIV susceptibility in postmenopausal women.[36,37] First, we observed increased CCR5 expression in Th17 cells from postmenopausal women in all tissues analyzed, suggesting that these cells would be more susceptible to infection than those from premenopausal women. Although we could not compare HIV susceptibility between premenopausal and postmenopausal women in our cohort, we observed a trend for higher infection levels in cells from postmenopausal women. Second, we demonstrate that menopausal status correlates with the presence of HIV-susceptible $CD4^+$ T-cell populations in EM. The increase in Th17 cells in EM from postmenopausal women found in our study suggests that the EM is also a portal of entry for HIV in these women. Further research is needed to determine whether hormonal fluctuations throughout the menstrual cycle and hormonal shifts that occur with menopause directly modify the susceptibility of $CD4^+$ T cells to HIV infection in the FRT.

In conclusion, we demonstrate that Th17 cells are abundant in the FRT, that they display increased susceptibility to HIV infection, and that differential distribution and regulation of these cells may be responsible for the differential predisposition to infection of different FRT tissues. Our studies contribute important information for the development of preventive strategies oriented to target highly susceptible cells in the FRT.

Table 1 Characteristics of the patients of the study

	Premenopausal	Postmenopausal
Number of donors	9	13
Age, years, median (range)	46 (38–53)	56 (44–72)
Menstrual stage		
Proliferative	66.7% (6/9)	
Secretory	33.3% (3/9)	
Atrophic		100% (13/13)
Surgical indication		
Prolapse	22.2% (2/9)	100% (13/13)
Fibroids	55.5% (5/9)	
Menorrhagia	22.2% (2/9)	

METHODS

Study subjects. Studies were performed with Dartmouth College Institutional Review Board's approval. HIV-negative women undergoing hysterectomies at Dartmouth-Hitchcock Medical Center (Lebanon, NH) gave written informed consent before surgery. Approval to use tissues was obtained from the Committee for the Protection of Human Subjects. Indications for surgery were benign conditions and tissue samples distal from the sites of pathology and without pathological lesions were selected as determined by a pathologist. Women were not on oral contraceptives before hysterectomy. Menopausal status was determined by a pathologist based on the histological evaluation of sections of the EM (endometrial dating). Postmenopausal status was characterized by an atrophic EM. Age, surgical indication and menopausal status of each patient are shown in **Table 1**. Information regarding genital infections was not obtained.

Tissue processing. Tissues obtained from hysterectomies included EM, END and ECT and were transferred to the laboratory immediately after surgery. Vaginal tissues were not available. Most experiments were performed with matching tissues from the same patient. Tissues were rinsed with Hank's Balanced Salt Solution and minced under sterile conditions into 1–2-mm fragments and digested using an enzyme mixture containing 0.05% collagenase type IV (Sigma-Aldrich, St Louis, MO) and 0.01% DNAse (Worthington Biochemical, Lakewood, NJ) for 1 h at 37 °C. Type IV collagenase was selected based on preliminary studies to ensure non-cleavage of surface markers (see **Supplementary Figure S1a**). After digestion, cells were dispersed through a 250-μm mesh screen (Small Parts, Miami Lakes, FL) and filtered through a 20-μm mesh screen (Small Parts) to separate epithelial cells from stromal cells. Stromal cells were then washed and counted and dead cells removed using the Dead cell removal kit (Miltenyi Biotec, Auburn, CA) according to the manufacturer's instructions to obtain a mixed cell suspension for flow cytometric analysis and HIV infection.

$CD4^+$ T-cell isolation. Following removal of dead cells, $CD4^+$ T cells were isolated using negative magnetic bead selection with the $CD4^+$ T-cell isolation kit (Miltenyi Biotec) following instructions with minor modifications. This negative selection process depletes CD8, CD14, CD16, CD19, CD36, CD56, CD123, T-cell receptor γ/δ, and CD235a and delivers untouched $CD3^+CD4^+$ T cells. Additionally, anti-fibroblast microbeads (Miltenyi Biotec) were added in combination with the microbeads supplied with the kit to ensure depletion of stromal fibroblasts present in the mixed cell suspension. After two rounds of negative selection, purity of the $CD4^+$ T-cell population was > 90% (see **Figure 2a**). Yield recovery of $CD4^+$ T cells is shown in **Supplementary Figure S1b**. Following isolation, cells were analyzed by reverse transcriptase–PCR as described below, without *in vitro* stimulation.

$CD4^+$ T cells from blood were isolated with the $CD4^+$ T-cell isolation kit (Miltenyi Biotec) following standard ficoll centrifugation and activated *in vitro* with Phytohemagglutinin (2.5 μg ml^{-1}; Sigma, St Louis, MO) and IL-2 (50 U ml^{-1}) (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr Maurice Gately, Hoffmann–La Roche)[38] for 24 h as described before.[8]

Flow cytometry. Mixed cell suspensions were stained for surface markers with combinations of the following antibodies: CD8-FITC (fluorescein isothiocyanate), CD4-FITC, CD4-PE (phycoerythrin), CD3-APC (allophycocyanin), CD3-APC-Cy7 (eBioscience, San Diego, CA), CCR5-PE-Cy7 (BD Biosciences, San Jose, CA), CCR5-PE (R&D Systems, Minneapolis, MN), CCR6-PerCp-Cy5.5, CD90 Alexa

Fluor-647 (Biolegend, San Diego, CA). For the HIV-infection experiments, following infection for 6–7 days, intracellular levels of p24 were analyzed as described previously.[8] Briefly, after surface staining, cells were washed, fixed, and permeabilized (20 min) following instructions provided in the Cytofix/Cytoperm Plus kit (BD Biosciences) and stained for intracellular p24 with KC57-FITC antibody (Beckman Coulter, Danvers, MA) for 30 min. Analysis was performed on BD FACSCalibur or BD FACSCanto flow cytometers (BD Biosciences) using FACSdiva software, and data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Expression of surface markers was measured by the percentage of positive cells and the mean fluorescence intensity.

Intracellular staining. Detection of RORC2 and intracellular IL-17A was performed on mixed cell populations after removal of dead cells. For RORC2, cells were surface stained first and then fixed and permeabilized with the Foxp3 Fixation/permeabilization concentrate and diluent (eBioscience) according to the instructions. Intracellular staining of RORC was done using the anti-human/mouse ROR gamma (t)-APC antibody (eBioscience). For IL-17A staining, cells were activated with phorbol 12-myristate 13-acetate (Abcam, Cambridge, MA) and ionomycin (Calbiochem, Billerica, MA) for 5 h in the presence of Brefeldin A (BD GolgiPlug protein transport inhibitor, BD Biosciences), surface stained and fixed and permeabilized with the BD Cytofix/Cytoperm kit (BD Biosciences) according to the instructions. Intracellular staining of IL-17A was performed using the anti-human IL-17A-PE antibody (BD Biosciences).

RNA isolation and real-time PCR. Total RNA was extracted from purified $CD4^+$ T cells using the micro RNeasy kit (QIAGEN, Valencia, CA). DNase digestion was done on-column with the RNase-Free DNase set (Qiagen). After RNA quantification, cDNA was generated with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed in duplicates using the 5′ fluorogenic nuclease assay with TaqMan chemistry on the ABI 7300 Prism real-time PCR instrument (Applied Biosystems, Carlsbad, CA) as described before.[8] Primers and probe sets were obtained from Applied Biosystems assays-on-demand (RPL13A, RORC2, IL-17, CCL20, CCR7). Amplification conditions and analyses were performed as described previously[8] but using RPL13A as housekeeping gene.[39,40] Results are presented as relative gene expression levels, calculated using the formula $2^{-\Delta C_t}$.[41] For each gene, a difference in C_t values (ΔC_t) was calculated by subtracting the mean C_t for the housekeeping gene RPL13A from the mean C_t of each gene of interest.

HIV infection. Mixed cell suspensions were infected with HIV-1 BaL for 2 h at a multiplicity of infection of 0.1 after which residual virus was washed away. The same input number of cells was used for EM, END, and ECT. Uninfected controls were incubated with medium without the virus for the same amount of time. After incubation, cells were plated in round bottom ultra low attachment 96-well plates (Corning, Corning, NY). Cell cultures were maintained for 6–7 days, with half of the well media collected and replaced with fresh media on day 3. At the end of the infection time, cells were washed, stained for surface markers as indicated, and levels of p24 measured intracellularly by flow cytometry (KC57-FITC; Beckman Coulter). Additionally, released p24 into the culture media was measured by p24 enzyme-linked immunosorbent assay (Advanced Bioscience Laboratories, Rockville, MD) following the manufacturer's recommendations.

Statistics. Data analysis was performed using the GraphPad Prism 5.0 software (GraphPad Software, Inc., La Jolla, CA). A two-sided P value < 0.05 was considered statistically significant. Comparison of the two groups was performed with the non-parametric Mann–Whitney U-test or Wilcoxon paired test. Comparison of three or more groups was performed applying the non-parametric Kruskal–Wallis test followed by Dunns post-test.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

ACKNOWLEDGMENTS
This study was supported by NIH contract HHSN272201000001C and NIH grants AI102838 and AI071761 (C.R.W.). We thank the study participants, pathologists, Obstetrics and Gynecology surgeons, operating room nurses, and support personnel at Dartmouth-Hitchcock Medical Center. Flow cytometric analysis was carried out in DartLab, the Immunoassay and Flow Cytometry Shared Resource at the Geisel School of Medicine at Dartmouth. We thank Richard Rossoll for technical assistance.

DISCLOSURE
The authors declared no conflict of interest.

REFERENCES

1. WHO. Progress report 2011: Global HIV/AIDS response (2011).
2. NIAID. HIV infection in Women (2010).
3. UNAIDS. Women, girls, gender equality and HIV (2010).
4. Hankins, C.A. & Dybul, M.R. The promise of pre-exposure prophylaxis with antiretroviral drugs to prevent HIV transmission: a review. *Curr. Opin. HIV AIDS* **8**, 50–58 (2013).
5. Wira, C.R., Fahey, J.V., Ghosh, M., Patel, M.V., Hickey, D.K. & Ochiel, D.O. Sex hormone regulation of innate immunity in the female reproductive tract: the role of epithelial cells in balancing reproductive potential with protection against sexually transmitted pathogens. *Am. J. Reprod. Immunol.* **63**, 544–565 (2010).
6. Rodriguez-Garcia, M., Patel, M.V. & Wira, C.R. Innate and adaptive anti-HIV immune responses in the female reproductive tract. *J. Reprod. Immunol.* **97**, 74–84 (2013).
7. Hickey, D.K., Patel, M.V., Fahey, J.V. & Wira, C.R. Innate and adaptive immunity at mucosal surfaces of the female reproductive tract: stratification and integration of immune protection against the transmission of sexually transmitted infections. *J. Reprod. Immunol.* **88**, 185–194 (2011).
8. Rodriguez-Garcia, M. *et al.* Estradiol reduces susceptibility of CD4(+) T cells and macrophages to HIV-infection. *PLoS One* **8**, e62069 (2013).
9. McKinnon, L.R. & Kaul, R. Quality and quantity: mucosal CD4+ T cells and HIV susceptibility. *Curr. Opin. HIV AIDS* **7**, 195–202 (2012).
10. Zhang, Z. *et al.* Sexual transmission and propagation of SIV and HIV in resting and activated CD4+ T cells. *Science* **286**, 1353–1357 (1999).
11. Saba, E. *et al.* HIV-1 sexual transmission: early events of HIV-1 infection of human cervico-vaginal tissue in an optimized ex vivo model. *Mucosal Immunol.* **3**, 280–290 (2010).
12. Li, Q. *et al.* Glycerol monolaurate prevents mucosal SIV transmission. *Nature* **458**, 1034–1038 (2009).
13. Acosta-Rodriguez, E.V. *et al.* Surface phenotype and antigenic specificity of human interleukin 17-producing T helper memory cells. *Nat. Immunol.* **8**, 639–646 (2007).
14. Annunziato, F., Cosmi, L., Liotta, F., Maggi, E. & Romagnani, S. Defining the human T helper 17 cell phenotype. *Trends Immunol.* **33**, 505–512 (2012).
15. Sallusto, F., Zielinski, C.E. & Lanzavecchia, A. Human Th17 subsets. *Eur. J. Immunol.* **42**, 2215–2220 (2012).
16. Weaver, C.T., Elson, C.O., Fouser, L.A. & Kolls, J.K. The Th17 pathway and inflammatory diseases of the intestines, lungs, and skin. *Annu. Rev. Pathol.* **8**, 477–512 (2013).
17. Chege, D. *et al.* Blunted IL17/IL22 and pro-inflammatory cytokine responses in the genital tract and blood of HIV-exposed, seronegative female sex workers in Kenya. *PLoS One* **7**, e43670 (2012).
18. Gosselin, A. *et al.* Peripheral blood CCR4+CCR6+ and CXCR3+ CCR6+CD4+ T cells are highly permissive to HIV-1 infection. *J. Immunol.* **184**, 1604–1616 (2010).
19. McKinnon, L.R. *et al.* Characterization of a human cervical CD4+ T cell subset coexpressing multiple markers of HIV susceptibility. *J. Immunol.* **187**, 6032–6042 (2011).

20. Monteiro, P. *et al.* Memory CCR6 + CD4 + T cells are preferential targets for productive HIV type 1 infection regardless of their expression of integrin beta7. *J. Immunol.* **186**, 4618–4630 (2011).
21. Guillot-Delost, M. *et al.* Human CD90 identifies Th17/Tc17 T cell subsets that are depleted in HIV-infected patients. *J. Immunol.* **188**, 981–991 (2012).
22. Annunziato, F. *et al.* Phenotypic and functional features of human Th17 cells. *J. Exp. Med.* **204**, 1849–1861 (2007).
23. Su, C.C., Lin, H.C., Lin, Y.P., Shan, Y.S. & Yang, B.C. Expression of Th17-related genes in PHA/IL-2-activated human T cells by Fas signaling via caspase-1- and Stat3-dependent pathway. *Cell. Immunol.* **281**, 101–110 (2013).
24. Saba, E. *et al.* Productive HIV-1 infection of human cervical tissue ex vivo is associated with the secretory phase of the menstrual cycle. *Mucosal Immunol.* **6**, 1081–1090 (2013).
25. Sheridan, B.S. & Lefrancois, L. Regional and mucosal memory T cells. *Nat. Immunol.* **12**, 485–491 (2011).
26. Huff, K.R., Akhtar, L.N., Fox, A.L., Cannon, J.A., Smith, P.D. & Smythies, L.E. Extracellular matrix-associated cytokines regulate CD4 + effector T-cell responses in the human intestinal mucosa. *Mucosal Immunol.* **4**, 420–427 (2011).
27. Ochiel, D.O., Ochsenbauer, C., Kappes, J.C., Ghosh, M., Fahey, J.V. & Wira, C.R. Uterine epithelial cell regulation of DC-SIGN expression inhibits transmitted/founder HIV-1 trans infection by immature dendritic cells. *PLoS One* **5**, e14306 (2010).
28. White, H.D. *et al.* CD3 + CD8 + CTL activity within the human female reproductive tract: influence of stage of the menstrual cycle and menopause. *J. Immunol.* **158**, 3017–3027 (1997).
29. Haeryfar, S.M. & Hoskin, D.W. Thy-1: more than a mouse pan-T cell marker. *J. Immunol.* **173**, 3581–3588 (2004).
30. Rudnicka, D. *et al.* Simultaneous cell-to-cell transmission of human immunodeficiency virus to multiple targets through polysynapses. *J. Virol.* **83**, 6234–6246 (2009).
31. Giglio, T. *et al.* Immune cell circulating subsets are affected by gonadal function. *Life Sci.* **54**, 1305–1312 (1994).
32. Kumru, S., Godekmerdan, A. & Yilmaz, B. Immune effects of surgical menopause and estrogen replacement therapy in peri-menopausal women. *J. Reprod. Immunol.* **63**, 31–38 (2004).
33. Ghosh, M., Rodriguez-Garcia, M. & Wira, C.R. The immune system in menopause: pros and cons of hormone therapy. *J. Steroid Biochem. Mol. Biol.* **S0960-0760**, 00163–00165 (2013).
34. Tyagi, A.M., Srivastava, K., Mansoori, M.N., Trivedi, R., Chattopadhyay, N. & Singh, D. Estrogen deficiency induces the differentiation of IL-17 secreting Th17 cells: a new candidate in the pathogenesis of osteoporosis. *PLoS One* **7**, e44552 (2012).
35. Lee, S.K., Kim, J.Y., Lee, M., Gilman-Sachs, A. & Kwak-Kim, J. Th17 and regulatory T cells in women with recurrent pregnancy loss. *Am. J. Reprod. Immunol.* **67**, 311–318 (2012).
36. Comparison of female to male and male to female transmission of HIV in 563 stable couples. European Study Group on heterosexual transmission of HIV. *BMJ* **304**, 809–813 (1992).
37. Meditz, A.L. *et al.* CCR5 expression is elevated on endocervical CD4 + T cells in healthy postmenopausal women. *J. Acquir. Immune Defic. Syndr.* **59**, 221–228 (2012).
38. Lahm, H.W. & Stein, S. Characterization of recombinant human interleukin-2 with micromethods. *J. Chromatogr.* **326**, 357–361 (1985).
39. Mane, V.P., Heuer, M.A., Hillyer, P., Navarro, M.B. & Rabin, R.L. Systematic method for determining an ideal housekeeping gene for real-time PCR analysis. *J. Biomol. Tech.* **19**, 342–347 (2008).
40. Biswas, N. *et al.* Effect of tenofovir on nucleotidases and cytokines in HIV-1 target cells. *PLoS One* **8**, e78814 (2013).
41. Rodriguez-Garcia, M. *et al.* Increased alpha-defensins 1-3 production by dendritic cells in HIV-infected individuals is associated with slower disease progression. *PLoS One* **5**, e9436 (2010).