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Phenotype and Susceptibility to HIV-infection of CD4⁺ Th17 Cells in the Human Female Reproductive Tract

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Abstract

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 Th17 cells represent a major subset in FRT tissues analyzed, and that Th17 cells were the main CD4⁺ T cell population expressing CCR5 and CD90. In pre-menopausal 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 post-menopausal women relative to pre-menopausal 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 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.

Keywords

Th17; HIV; female reproductive tract; post-menopausal; pre-menopausal; CD90

INTRODUCTION

Currently 34 million people are known to be infected with HIV and the pandemic continues to spread despite intensive preventive efforts¹. Worldwide, approximately half of these infections are women; however an imbalance between genders is found in areas like sub-Saharan Africa, where young women ages 15–24 are up to 8 times more likely to be infected than young men^{1–3}.

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DISCLOSURE

The authors declared no conflict of interest.

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 results⁴. 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). While 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 status⁷, as well as the direct modification of susceptibility to HIV infection as we recently demonstrated⁸. 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 infection⁹. Studies in non-human primates indicate that memory CD4⁺ T cells expressing CCR5 preferentially support HIV replication¹⁰. Work with human cervico-vaginal tissue explants also 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 phenotype¹¹.

Experiments in macaques demonstrated that MIP3 α (CCL20) was rapidly produced in response to SIV vaginal challenge and that blockade of this molecule resulted in the prevention of systemic dissemination of infection¹². MIP3 α 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 Th17 cells. In addition to CCR6 expression, Th17 cells are characterized by their production of IL-17 and expression of the transcription factor retinoic acid receptor-related orphan receptor C2 (RORC2), the human ortholog of ROR γ t 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 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 patients¹⁷⁻²⁰. 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 expression of

CD90 (Thy-1) in a subset of Th17 cells isolated from blood which was depleted in non-treated HIV infected patients²¹.

In the present study we characterized the distribution and phenotype of CD4⁺ T cells at different sites in the FRT from pre- and post-menopausal 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 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 amongst 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 if the menstrual status of the patients would affect T cell distribution in FRT tissues, we stratified samples according to pre- or post-menopausal status, as established by tissue histology. Menstrual status significantly influenced T cell distribution in EM, but not in END or ECT, with pre-menopausal women containing a higher percentage of CD3⁺CD4⁺ T cells (Figure 1c) as compared to post-menopausal women. Additionally, in post-menopausal 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 samples¹⁹. 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 higher than 90% (Figure 2a). RT-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 highest in END, followed by ECT and lowest in EM.

We next explored how END gene expression without *in vitro* stimulation compared to gene expression in PHA-activated CD4⁺ T cells from blood, which is known to upregulate Th17

markers²³. 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 down-regulated in tissue resident T cells¹⁵, was significantly lower in CD4⁺ T cells from END compared to blood. Similar patterns were seen when blood CD4⁺ cells were compared to EM and ECT cells (not shown).

CD4⁺ T cells from FRT express high levels of CCR6

Since 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 (Figures 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). Since 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.

We then analyzed the influence of menopausal status by stratifying the participants shown in Figure 3b into those corresponding to pre- (black dots) or post-menopausal (white dots) women (Figure 3f). The number of CCR6⁺ CD4⁺ T cells in EM from pre-menopausal women was significantly lower than that seen in post-menopausal women. Additionally, when EM, END and ECT pre-menopausal cells were compared, the number of CCR6⁺ cells in END from pre-menopausal women was significantly greater than those found in EM. No differences were found amongst post-menopausal 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 CCR5¹⁸⁻²⁰. 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), while 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 pre- and post-menopausal women revealed significantly increased levels of CCR5⁺ CCR6⁺ T cells in post-menopausal women with respect to pre-menopausal 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 between 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 blood²¹, but CD90 expression 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 to 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 6 different patients 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 (MOI=0.1) that gave detectable p24 and would help identify the most susceptible cells was chosen (supplementary Figure 1c). 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 publications²⁴.

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 if 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 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 to 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 4 quadrants of ECT infected cells to see if infection was preferential to a given cell phenotype. As expected, all p24⁺ cells were found in 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 pre- and post-menopausal women. Regarding differences in cell distribution between anatomical sites, CD4⁺ T cells in general and Th17 cells in particular were lowest in EM compared to END and ECT. With respect to comparisons between pre- and post-menopausal

tissues, post-menopausal 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. Additionally we found decreased susceptibility to infection in CD4⁺ T cell from EM compared to 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 ROR γ t, 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. While Th17 cells have been broadly studied in other mucosal surfaces¹⁶, to the best of our knowledge, our study is the first to compare Th17 subsets among FRT tissues. It is worth noting that CD4⁺ T cells from the FRT showed a unique phenotype and gene expression profile compared to 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 to 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 to blood, consistent with the known down-regulation of the chemokine receptor CCR7 during the development of tissue resident memory T cells²⁵.

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 to 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*, 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 SIV-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 6 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 CXCR3, 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 and HIV trans-infection by blood derived dendritic cells²⁷. We found that CD8⁺ T cell function, measured as CTL activity, was suppressed during the secretory phase of the menstrual cycle in EM, but not END or ECT²⁸. 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 less than 1% of T cells in peripheral blood²⁹. CD90 (Thy-1) is a glycosyl phosphatidylinositol (GPI)-anchored glycoprotein preferentially localized in lipid rafts²⁹. CD90 appears to mediate cell to cell interactions, participate in transferring of other GPI-anchored proteins, (such as CD55 and CD59), from one cell membrane to another²⁹ and was preferentially localized at the contact areas between infected and target cells³⁰. 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 might represent a highly susceptible T cell subset²¹. The role that CD90 may play 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 pre and post-menopausal 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 post-menopausal women, similar to previous findings in peripheral blood from post-menopausal women^{31–33}. Additionally, we found that menopausal status affected Th17 frequency in FRT tissues. In pre-menopausal 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 post-menopausal women, Th17 cells were significantly increased in EM with respect to pre-

menopausal women, suggesting selective regulation of different T cell subsets. Although in the present study we cannot demonstrate that the observed differences between pre- and post-menopausal women are due to hormonal changes, recent studies in mice demonstrated that estrogen deficiency promotes the differentiation of Th17 cells³⁴. Ovariectomized mice had increased numbers of Th17 cells and this effect could be reversed by estradiol supplementation. Down-regulation of Th17 cells in EM of pre-menopausal 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 loss³⁵

Two main findings in our study support the reported increase in HIV-susceptibility in post-menopausal women^{36, 37}. First, we observed increased CCR5 expression in Th17 cells from post-menopausal women in all tissues analyzed, suggesting that these cells would be more susceptible to infection than those from pre-menopausal women. Although we could not compare HIV-susceptibility between pre- and post-menopausal women in our cohort, we observed a trend for higher infection levels in cells from post-menopausal 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 post-menopausal 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.

METHODS

Study subjects

Studies were performed with Dartmouth College Institutional Review Board 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 (CPHS). 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 endometrium (endometrial dating). Post-menopausal status was characterized by an atrophic endometrium. 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 endometrium (EM), endocervix (END) and ectocervix (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 HBSS 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 1h at 37°C. Type IV collagenase was selected based on preliminary studies to ensure non-cleavage of surface markers (Supplementary Figure 1a). After digestion, cells were dispersed through a 250µm mesh screen (Small Parts, Miami Lakes, FL) and filtered through a 20µm 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 manufacturer 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, TCRγ/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 higher than 90% (see Figure 2A). Yield recovery of CD4⁺ T cells is shown in Supplementary Figure 1b. Following isolation, cells were analyzed by RT-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 (PHA) (2.5 µg/ml; Sigma, St Louis, MO) and IL-2 (50 U/ml) (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: Human rIL-2 from Dr. Maurice Gately, Hoffmann- La Roche Inc)³⁸ for 24h as described before⁸.

Flow cytometry

Mixed cell suspensions were stained for surface markers with combinations of the following antibodies: CD8-FITC, CD4-FITC, CD4-PE, CD3-APC, 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 previously⁸. 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 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 (MFI).

Intracellular staining

Detection of RORC2 and intracellular IL-17A was performed on mixed cell populations after dead cell removal. For RORC2, cells were surface stained first and then fixed and permeabilized with the Foxp3 Fixation/permeabilization concentrate and diluent (eBioscience) according to 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 (PMA) (Abcam) and ionomycin (Calbiochem) for 5h 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 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 before⁸. 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 previously⁸, but using RPL13A as housekeeping gene^{39, 40}. Results are presented as relative gene expression levels, calculated using the formula 2^{-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 2h at a MOI 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 ELISA (Advanced Bioscience Laboratories, Rockville, MD) following manufacturer's recommendations.

Statistics

Data analysis was performed using the GraphPad Prism 5.0 software. A two sided P value <0.05 was considered statistically significant. Comparison of two groups was performed

with the non-parametric test U-Mann Whitney 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

Refer to Web version on PubMed Central for supplementary material.

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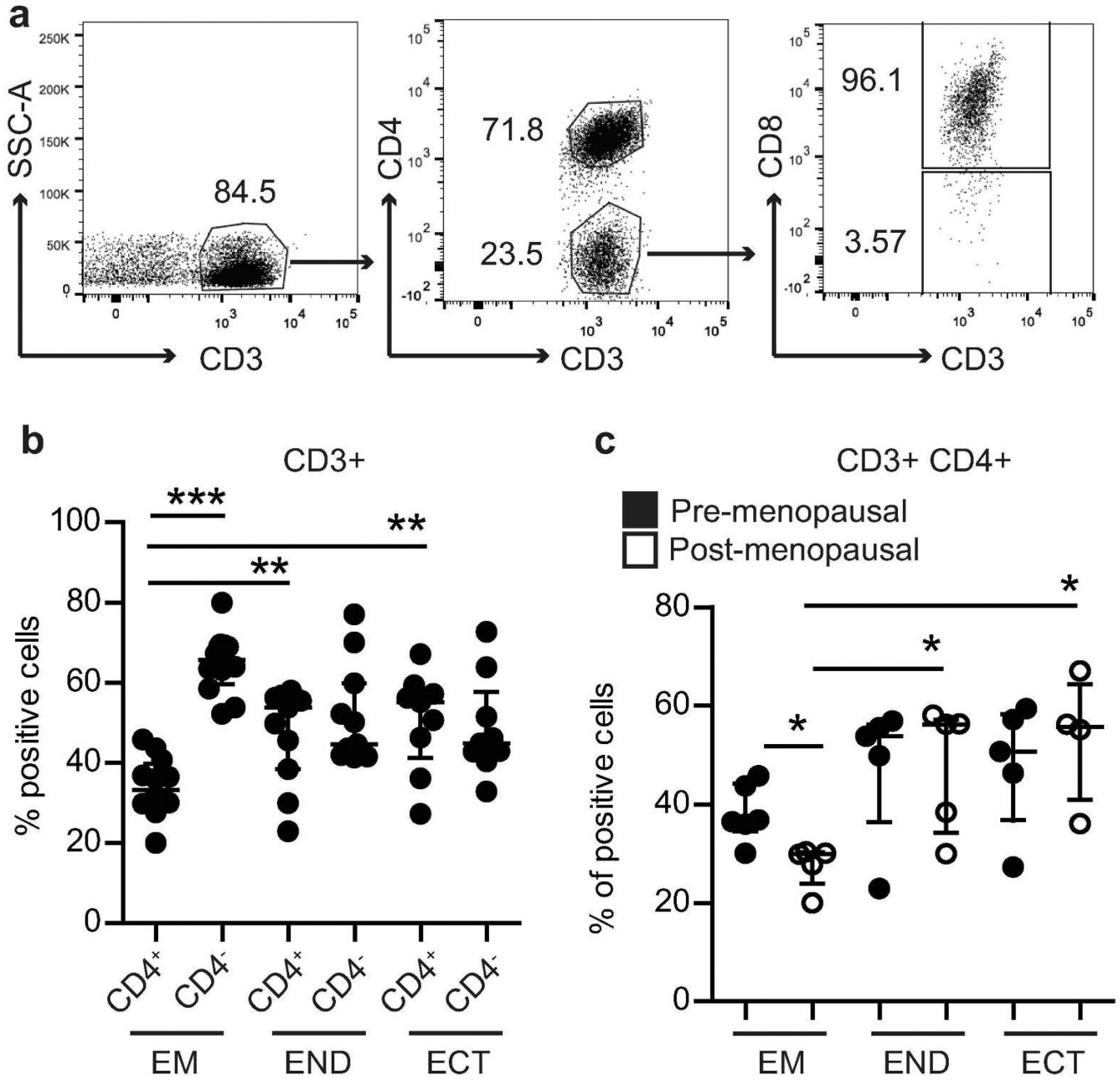


Figure 1. Distribution of CD4⁺ T cells in FRT tissues

(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 endometrium (EM) but not in endocervix (END) or ectocervix (ECT). (c) Distribution of CD3⁺CD4⁺ T cells in pre-menopausal (black dots; one in the secretory phase of the menstrual cycle) and post-menopausal (white dots) women. Each dot represents a single patient. Horizontal lines represent the median ± IQR. *p<0.05; **p<0.01; ***p<0.001

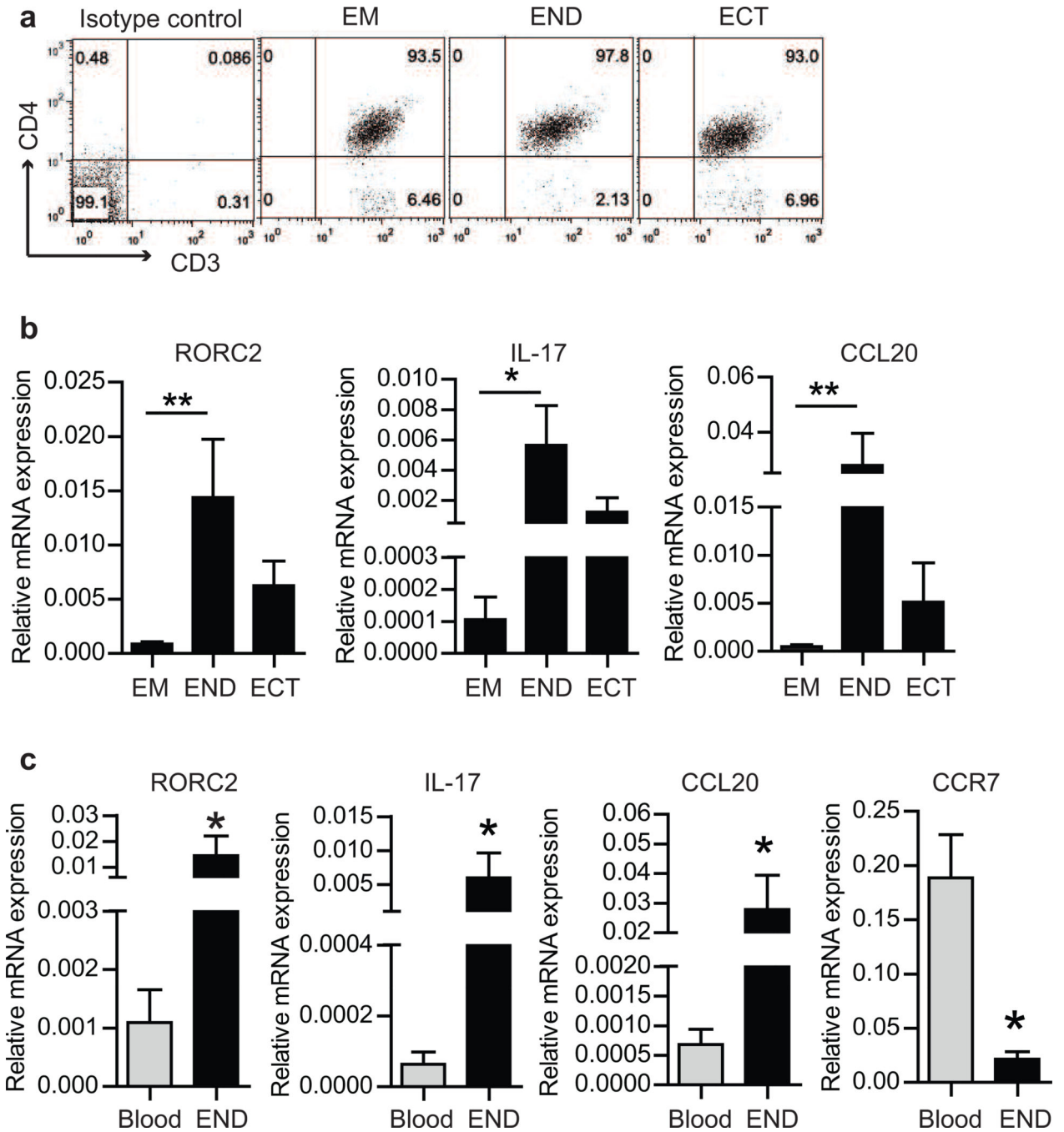


Figure 2. Expression of Th17 genes in FRT tissues

(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 (RORC2, Th17 and CCL20) in purified CD4⁺ T cells from FRT tissues. (c) Comparison of relative gene expression between PHA-activated CD4⁺ T cells isolated from blood and CD4⁺ T cells isolated from endocervix without in vitro activation (END). EM=endometrium; END=endocervix; ECT=ectocervix. Bars represent median \pm IQR from 5 independent experiments with 5 different donors, 4 pre-menopausal (2

in the secretory and 2 in the proliferative phase of the menstrual cycle) and 1 post-menopausal women. *p<0.05; **p<0.01

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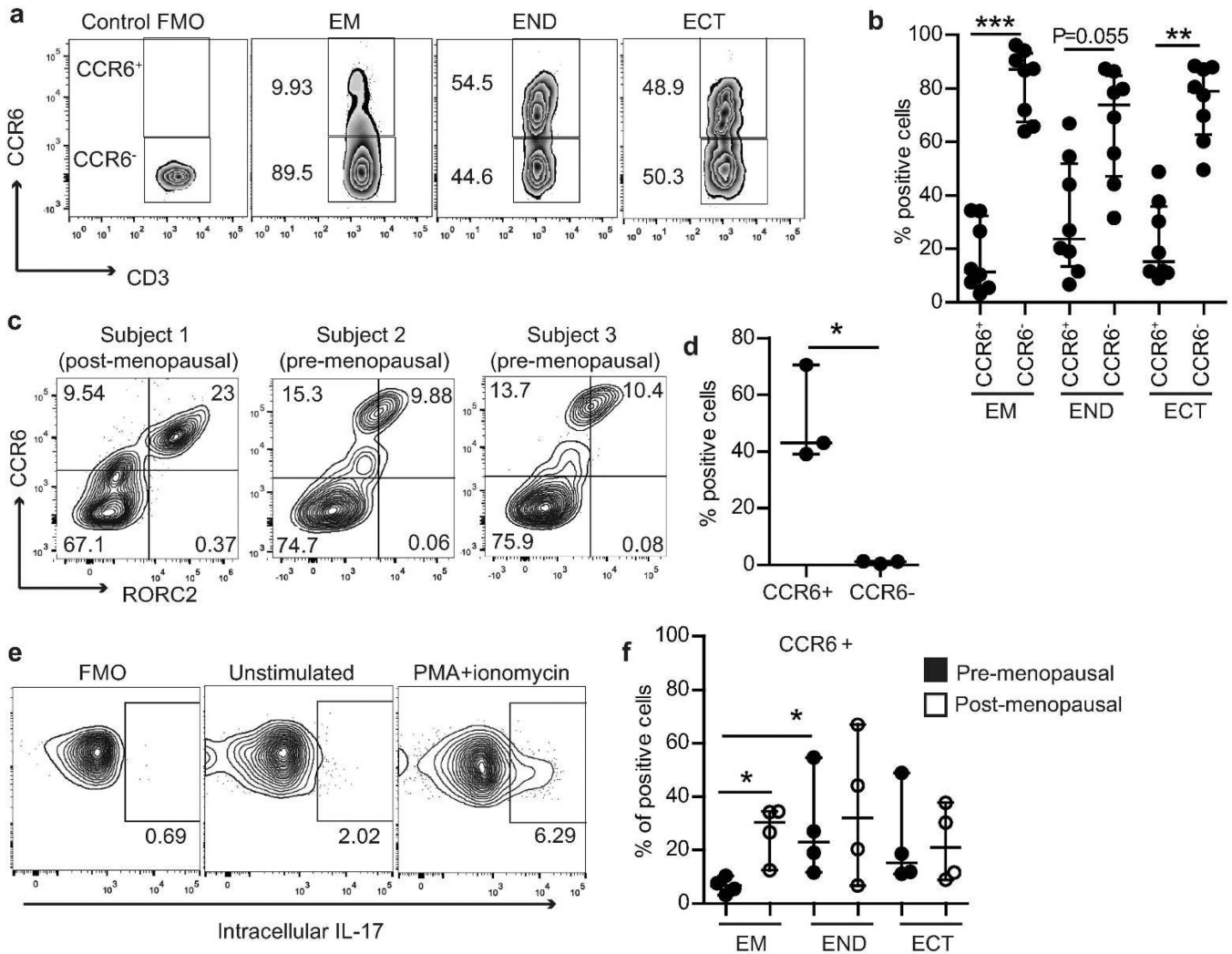


Figure 3. Expression of CCR6 in CD4⁺ T cells from FRT tissues

(a) Representative zebra plot showing percent of CCR6⁺ and CCR6⁻ cells within the expression in CD4⁺ T cell population (gated on CD3⁺CD4⁺). Negative control was established using fluorescence minus one (FMO). (b) Flow cytometric analysis of the percent of CCR6 positive and CCR6 negative 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 \pm IQR. ** $p < 0.01$; *** $p < 0.001$. (c) Contour plot and (d) graph showing intracellular staining in three different donors for 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 IL-17A in CCR6⁺ CD4⁺ T cells under unstimulated or PMA+ionomycin stimulated conditions. IL-17 production was induced after stimulation. (f) Stratification of the patients shown in figure 3b into pre-menopausal (black dots; proliferative phase of the cycle) and post-menopausal (white dots) women to compare CCR6 expression. Each dot represents a single patient.

Horizontal lines represent the median \pm IQR. * $p < 0.05$. EM=endometrium;
END=endocervix; ECT=ectocervix.

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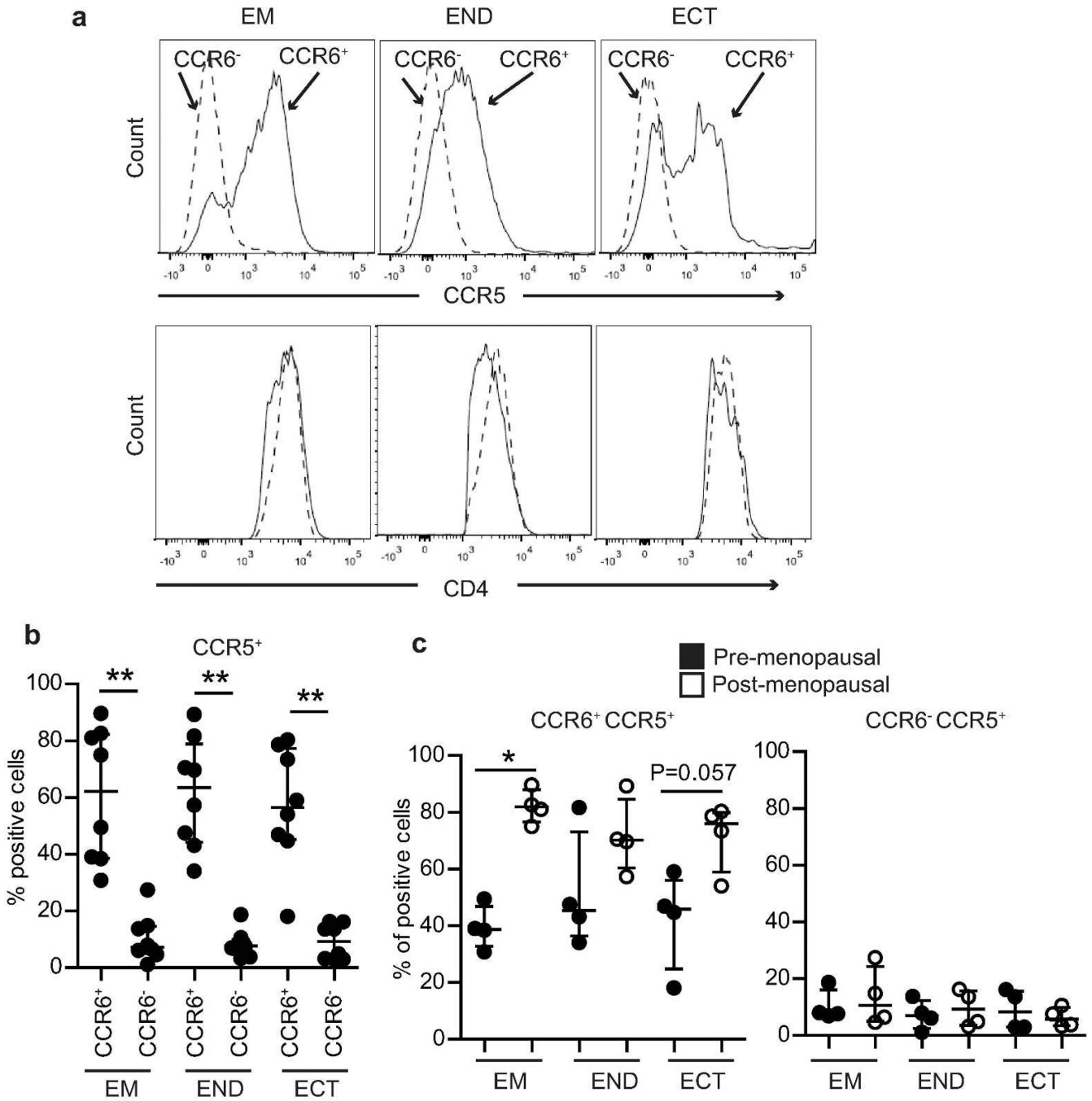


Figure 4. Expression of CCR5 in CCR6⁺CD4⁺ T cells

(a) Representative overlay of CCR6 negative (dashed line) and CCR6 positive (solid line) CD4⁺ T cell populations for the expression of CCR5 and CD4. The CCR6⁺ population expressed higher levels of CCR5 compared to the CCR6⁻ population. (b) Significantly increased expression of CCR5 in CCR6 positive cells with respect to the CCR6 negative CD4⁺ T cell population in the different FRT tissues. (c) Stratification of the patients shown in 4b into pre-menopausal (black dots; proliferative phase of the cycle) and post-menopausal (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 \pm IQR. * $p < 0.05$, ** $p < 0.01$.

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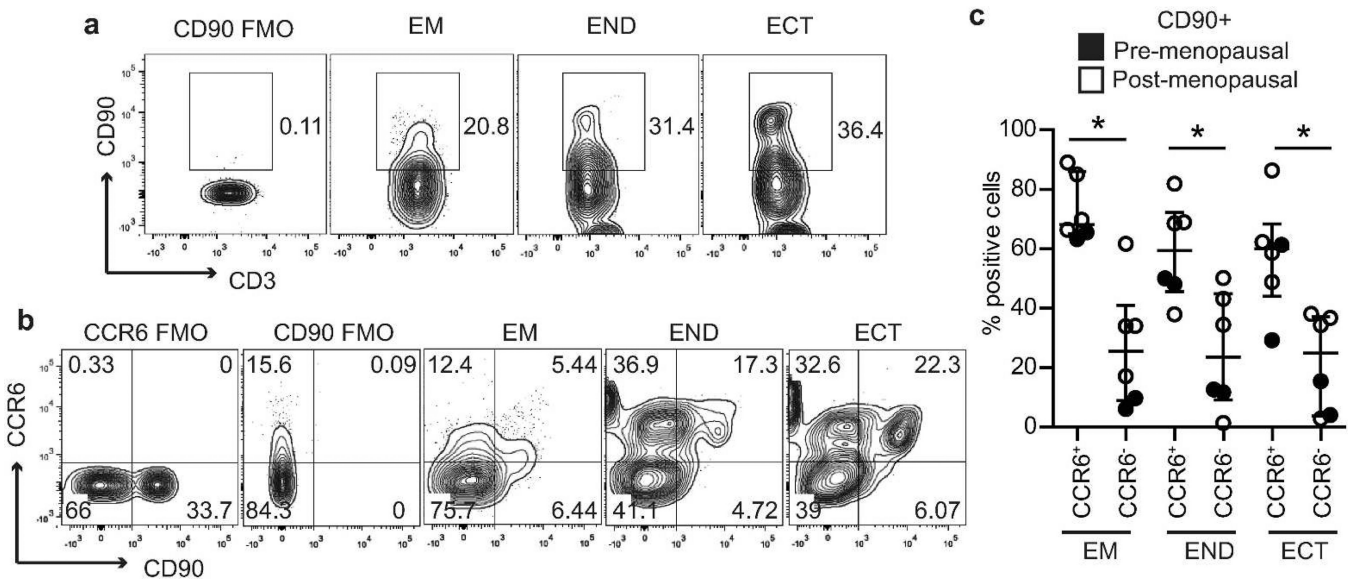


Figure 5. Expression of CD90 in CD4⁺ T cells from the FRT

(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 pre (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 ± IQR. *p<0.05.

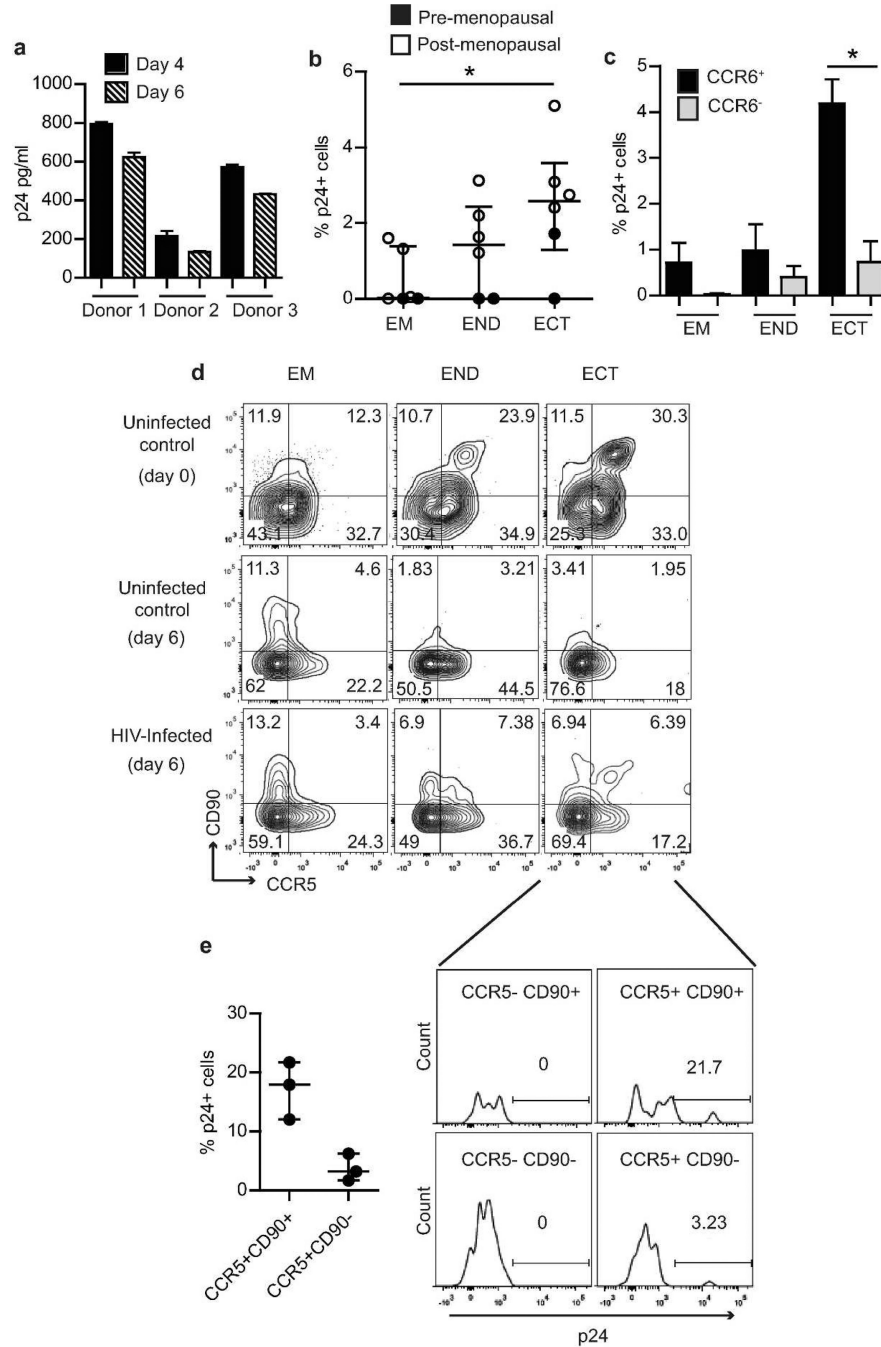


Figure 6. Susceptibility of CD4⁺ T cells to HIV-infection in FRT tissues

(a) Released p24 into the culture media 4 and 6 days after infection of cells from the ectocervix as detected by p24 ELISA. (b) Percent of intracellular p24+ CD4⁺ T cells 6 days after in vitro HIV-infection (n=6). Patients were identified as pre-menopausal (proliferative phase of the menstrual cycle; black dots) or post-menopausal (white dots). Each dot represents a different patient, matched tissues from EM, END and ECT were used. Horizontal lines represent the median ± IQR; *p<0.05. (c) Percent 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 \pm SEM from 5 independent donors. **(d)** Representative contour plot of 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 3 experiments with different donors. **(e)** Histograms showing intracellular p24 expression in each quadrant from the lower right panel in figure 5c (ECT; HIV-infected). Numbers show the percentage of p24⁺ cells in each population. The graph on the left represents the percent of p24⁺ cells within the CCR5⁺CD90⁺ and the CCR5⁺CD90⁻ populations in the 3 donors analyzed.

Table 1

Characteristics of the patients of the study

	Pre-menopausal	Post-menopausal
Number of donors	9	13
Age 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)	

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