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Phenotype and functionality of CD4+ and CD8+ T cells in the upper reproductive tract of healthy premenopausal women

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Abstract

Problem—The goal of this study was to investigate the phenotype and functional responsiveness of CD4+ and CD8+ T-cells in the upper reproductive tract of healthy premenopausal women. The lower reproductive tract is frequently studied as a site of sexually transmitted infections; however, the upper tract may also be a portal of entry and dissemination for pathogens, including HIV-1.

Method of Study—Endometrial biopsy, endocervical curettage, cytobrush and blood were collected during mid-luteal phase from 23 healthy women. T-cells were isolated and analyzed by flow cytometry.

Results—As compared to their counterparts in blood, endometrial and endocervical T-cells had enhanced CCR5 expression, and were enriched for activated, effector memory cells. Endometrial T-cells were more responsive to polyclonal stimuli, producing a broad range of cytokines and chemokines.

Conclusions—These findings underscore the responsiveness of endometrial T-cells to stimulation, and reveal their activated phenotype. These findings also suggest susceptibility of the upper reproductive tract to HIV-1 infection.

Keywords

T-cell; CTL; HIV; STD; Endometrium; Endocervix

INTRODUCTION

The female reproductive tract (FRT) is unique in that it must be immunologically tolerant of a semi-allogeneic conceptus, but must also retain the ability to respond to potential

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pathogens. The lower FRT includes the vagina and ectocervix; the upper FRT includes the endocervix, uterine endometrium and fallopian tubes. The mucosal lining in the lower FRT is composed of multi-layered stratified squamous epithelium[1], whereas the upper FRT is lined by single-layered columnar epithelium with tight junctions [2, 3]. The mucosal immune system in the FRT consists of epithelial cells, stromal cells and immune cells that migrate into the uterus, cervix and vagina [4] and provide a robust physical and immunological barrier against sexually transmitted infections [5]. Epithelial cells of the FRT express toll-like receptors [6] as well as intracellular pattern recognition receptors [7] and secrete a variety of cytokines, growth factors, chemokines and antimicrobial peptides that participate in host defense through interactions with immune cells that reside in the epithelium and the stromal layer [8, 9].

Lymphocytes and antigen presenting cells are present throughout the FRT. Leukocytes comprise 6–20% of total cells and are dispersed throughout the FRT tissues with the highest proportion seen in the uterine endometrium and fallopian tubes [10]. The major population of leukocytes consists of T-lymphocytes, including CD3+ lymphocytes. Granulocytes are also present, particularly in the fallopian tubes. B-lymphocytes and monocytes are dispersed throughout the FRT, but are not as prominent as T-lymphocytes and granulocytes [11]. In the upper FRT, reproductive hormone levels modulate immune functions including cytotoxic T lymphocyte (CTL) activity, secretion of immunoglobulins, cytokines, chemokines, growth factors and antimicrobial peptides over the course of the menstrual cycle [11–15]. In contrast to the upper FRT, CTL activity and the frequency and distribution of immune cells in the lower FRT (i.e., ectocervix and vagina) remain constant throughout the menstrual cycle [15–17].

Previous studies suggest that the upper FRT might also be a portal for the entry of HIV-1 following sexual intercourse [5, 18–20]. Susceptibility of the upper tract to infection likely varies throughout the menstrual cycle, with the highest susceptibility predicted at mid-luteal phase [5, 9, 21–24]. Here, we have studied the phenotype and functionality of fresh CD4+ and CD8+ T cells isolated from the endometrium, endocervix, and peripheral blood (PB), collected from healthy premenopausal women during the mid-luteal (secretory) phase of the menstrual cycle. T-cells of both endocervix and endometrium had a predominant activated, effector memory phenotype with increased expression of CCR5 as compared to blood T-cells. Notably, mid-luteal endometrial CD4+ T-cells were significantly enriched for a CCR5+, activated, effector memory phenotype as compared to their counterparts in endocervix, suggesting high susceptibility to HIV-1 infection. In addition, endometrial T-cells (both CD4+ and CD8+) were more responsive to polyclonal stimuli, producing pro-inflammatory cytokines and chemokines, compared to blood T-cells. Taken together, these results reveal that endometrial T-cells have a phenotype that may render them highly susceptible to infection by HIV-1 and other sexually transmitted pathogens.

MATERIALS AND METHODS

Study participants and specimen collection

Participants were enrolled in two clinical research studies approved by University of California, San Francisco (UCSF) Committee on Human Research (CHR). The exclusion criteria for both studies were pregnancy, a current genital herpes outbreak or   4 or more herpes outbreaks in the last year, irregular menses, an abnormal Pap test within the past 12 months, recent use of intravaginal or intrauterine contraceptives, use of exogenous sex hormones, abnormal vaginal discharge or bleeding, immunocompromised status or use of immunosuppressive medications, or daily use of non-steroidal anti-inflammatory drugs. Within 7–11 days post luteinizing hormone surge during the mid-luteal phase approximately 20 mL of PB were collected in vacutainer tubes coated with EDTA (BD Pharmingen, SA Jose, CA) and endometrial biopsies were obtained using 3 mm Miltex brand Softflex

endometrial biopsy cannula (1–2 passes). The cytobrush (Cytobrush Plus® cell collector, CooperSurgical, Trumbull, CT) and curettage specimens (using Kevorkian curette with basket square tip, CooperSurgical, Trumbull, CT) were obtained from the endocervix. FRT specimens were placed into R-15 medium (RPMI 1640 containing 15% fetal bovine serum, 100 IU/mL penicillin, 100 IU/mL streptomycin, 2 mM and glutamine). All specimens were transported on ice from UCSF within three hours of collection and processed immediately upon receipt.

Isolation of Mononuclear Cells

PBMC were isolated by Ficoll-Hypaque (Pfizer, New York, NY) density gradient centrifugation, and washed in phosphate-buffered saline (PBS). Cells contained in endocervical cytobrush samples were first dislodged from the brushes by rubbing the brushes together, then pipetting a stream of media on the brushes several times. After achieving a suspension, cells were passed through a 70-μM nylon cell strainer (Becton Dickinson Discovery Labware, Bedford, MA) followed by washing in R-15 medium. Cells from endocervical curettage samples were suspended by repeated pipetting, followed by straining and washing, as described above for cytobrush samples. Endometrial biopsies, being solid pieces of tissue, were subjected to two to three rounds of collagenase type II digestion (0.5 mg/mL; Sigma-Aldrich, St. Louis, MO). We previously tested collagenase for digestion of common T-cell surface markers and found it did not significantly affect detection of most markers used for routine T-cell phenotyping, with the exception of CD62L, a marker not included in the present study (C. Cox and B. Shacklett, unpublished data) [25, 26]. Collagenase digestion was followed by mechanical disruption using an 18-gauge blunt-end needle and passage through a 70-μM nylon cell strainer. The pooled cells were washed in R-15 medium. Red blood cell lysis was performed when required on PBMC, endometrium and endocervical (cytobrush and curettage) cells using ammonium chloride-potassium carbonate-EDTA (ACK).

Monoclonal Antibodies

Fluorochrome-labeled monoclonal antibodies used for the phenotypic and ICS assay include CD3 (clone UCHT1), CD4 (clone RPA-T4), CD8 (clone SK1), CCR7 (clone 3D12), CXCR4 (clone12G5), CCR5 (clone 2D7), CD8 (clone RPA-T8), IFNγ (clone B27), TNF-α (clone MAb11), MIP-1β (clone D21-1351) from Becton-Dickinson Pharmingen (San Diego, CA), CD45RA (2H4), CD4 (clone T4D11) from Beckman Coulter (Fullerton, CA), CD38 (clone HB7), CD107a (clone H4A3), and IL-10 (clone JES3-19F1) from BD Biosciences (San Jose, CA), HLADR (clone TU36), Aqua amino reactive dye from Invitrogen (Carlsbad, CA), CD66b (G10F5) Biolegend (San Diego, CA) and IL-17 (clone eBio64CAP17), IL-2 (clone MQ1-17H12), from eBioscience (San Diego, CA).

Phenotypic and Intracellular Cytokine Staining (ICS) and Flow Cytometry

PB, endometrial, and endocervical cells were stained the same day of isolation for cell surface phenotype. Fluorescence minus one (FMO) controls were included as needed [27]. PBMC and endometrial cells required for the ICS assay were rested overnight in R-15 medium. Briefly, $2–3×10^6$ cells in 200 μL complete medium were treated with anti-CD107a, monensin (1 μM GolgiStopTM, BD Biosciences), brefeldin A (5 μg/mL, Sigma), and staphylococcus enterotoxin B (SEB, 0.5 μg/mL) or phorbol myristate acetate (PMA, 50 ng/mL) and ionomycin (ION, 500 ng/mL). R-15 medium containing anti-CD107a, monensin (1 mM GolgiStopTM, BD Biosciences), and brefeldin A (5 mg/mL, Sigma) served as negative control. Following a 5 hour incubation, cells were incubated for 5 minutes in PBS/2% FCS/0.5 mM/EDTA, stained for surface markers and viable cells using aqua amino reactive dye in PBS/2% FCS for 20 min at 4°C, fixed in 4% formaldehyde, then permeabilized using FACS Perm 2 (BD Biosciences). Cells were then washed in PBS/2% FCS, stained for

intracellular cytokines and CD3, washed again, then stored at 4°C in PBS/1% formaldehyde until analysis within 24 hours. The expression of CD107a, IL-2, IL-10, IL-17, TNF-α, IFN-γ and MIP-1β were measured as described previously [28]. Flow cytometry data were acquired on an LSRII (BD Immunocytometry Systems, San Jose, CA) equipped with 405, 488, and 643 nm lasers and utilizing FACSDIVA software (BDIS).

Analysis of flow cytometry data was performed using FlowJo software (TreeStar, Ashland, OR). For phenotypic analysis of mucosal T-cells, a broad scatter gate was used to capture lymphocytes, followed by doublet discrimination, then selection of viable CD3+ cells. After excluding granulocytes using CD66b, CD4+ and CD8+ T-cells were identified and further analyzed. For analysis of cytokine production, CD4+ and CD8+ T-cells were gated using a similar strategy and then analyzed for individual responses. The cytokine response values obtained from cells stimulated with SEB or with PMA/ION were then background-corrected using the values obtained from cells treated with R-15 medium alone [29].

Statistical Analysis

CD4+ and CD8+ T-cell phenotypic markers between the tissues were compared using two-tailed Mann-Whitney test with Holms' sequential Bonferroni correction.[30] CD4+ and CD8+ T-cells cytokine production between endometrium and PBMC was compared using Wilcoxon matched pairs signed rank test. All analysis was done using GraphPad Prism (GraphPad Software, La Jolla, CA).

RESULTS

Characteristics of the study subjects

Twenty-three participants were enrolled in the study, of whom 43% were White/Caucasian, 35% were Black/African-American, 9% were Asian or Pacific Islander and mixed, 4% were Hispanic/Latino. The median age was 36 years (range 28–40).

Flow cytometric analysis of mucosal T-cells

CD4+ and CD8+ T-cells were analyzed by cell surface staining for expression of CD45RA, CCR7, CD38, HLA-DR, CCR5 and CXCR4 as shown in Figure 1A. These phenotypic markers were further analyzed through Boolean combinations of markers for activation (CD38, HLADR), maturation (CCR7, CD45RA) and trafficking (CXCR4, CCR5). In separate experiments, CD4+ and CD8+ T-cells were analyzed for responsiveness to stimulation with SEB or PMA/ION. Seven functional responses were measured: CD107a, IL-2, IL-10, IL-17, TNF-α, IFN-γ and MIP-1β, as shown in Figure 1B and described in Materials and Methods.

Enhanced expression of CCR5 on endometrial CD4+ and CD8+ T-cells

The percentages of T-cells from endometrium, endocervix and PB expressing chemokine receptors, CCR5 and CXCR4 were measured by flow cytometry (Fig. 2). As compared to PB, CD4+ T-cells from endometrium and endocervix displayed significantly higher percentages of CCR5-positive cells (Fig. 2A). In contrast, the percentage of endometrial and endocervical CD4+ and CD8+ T-cells expressing CXCR4 was significantly lower compared to PB (Fig. 2C & 2D). Interestingly, expression of CCR5 was significantly greater and CXCR4 expression was significantly lower on endometrial CD4+ (Fig. 2A & 2C) and CD8+ T-cells (Fig. 2B & 2D) compared to cytobrush T-cells. Furthermore, the CCR5 median fluorescence intensity (MFI) of endometrial CD4+ T-cells was significantly greater than for PB, ($P = 0.0082$, Fig. 3) suggesting greater CCR5 receptor density. Elevated expression of

CCR5 by CD4+ T-cells in the gastrointestinal tract has also been reported, and is believed to partly explain the high susceptibility of these cells to HIV-1 infection[31].

CD4+ and CD8+ T-cells from the endometrium display a memory phenotype

Resting and effector memory CD4+ T-cells have the greatest susceptibility to infection with CCR5-utilizing HIV-1[32, 33], and different memory/effector T-cell subsets display different effector functions *in vitro* [34]. We measured expression of CCR7 and CD45RA on endometrium, endocervix and PB T-cells to phenotypically characterize memory/effector T-cells from the upper FRT. Populations were defined as naïve (CCR7+CD45RA+), central memory (T_{CM}, CCR7+CD45RA−), effector memory (T_{EM}, CCR7−CD45RA−) and terminally differentiated effector memory (T_{EMRA}, CCR7−CD45RA+) CD4+ and CD8+ T-cells (Fig. 4) [35, 36].

Significantly lower percentages of naïve CD4+ and CD8+ T-cells (Fig. 4A, E) were detected in the endocervix and endometrium as compared to PB. Furthermore, the percentage of naïve CD4+ and CD8+ T-cells was significantly lower in endometrium than in endocervical curettage or cytobrush (Fig. 4A, E). Central memory CD4+ T-cells were least abundant in endometrium, but present at similar frequencies in other sites (Fig. 4B), and central memory CD8+ T-cell frequencies were similar at all sites (Fig. 4F). Consistent with a prior report [37], the proportions of T_{EM} CD4+ and CD8+ T-cells were greater in the endocervix compared to PB; we also found this to be true for endometrial cells (Fig. 4C, G). The frequencies of TEMRA CD4+ and CD8+ T-cells were significantly lower in endometrium compared to PB (Fig. 4D, H). The percentages of TEMRA CD4+ T-cells in endocervical samples did not differ significantly from endometrium or blood, but the frequency of TEMRA CD8+ T-cells in endocervix was similar to blood, and significantly greater than in endometrium (Fig. 4H).

Endometrial CD4+ and CD8+ T-cells are highly activated

Coexpression of CD38 and HLA-DR on T-cells is commonly used to assess T-cell activation; expression of these markers is a strong correlate of disease progression in HIV-1 infected individuals [38, 39]. In healthy individuals, coexpression of these markers is significantly elevated in T-cells of the gastrointestinal tract, compared to PB[31]. We measured expression of these markers both individually and in combination. Activated CD4+ and CD8+ T-cells co-expressing CD38/HLA-DR were present at significantly higher frequency in the endometrium and endocervix compared to PB. In addition, CD38/HLA-DR coexpression was significantly higher on endometrial CD4+ and CD8+ T-cells as compared to endocervical T-cells (Fig. 5).

CCR5-expressing T_{EM} and activated T-cells are enhanced in endometrium

CCR5 expression by CD4+ T-cells is associated with an activated, memory phenotype as well as susceptibility to HIV-1 infection. Indeed, activated, memory CD4+ T-cells have been associated with susceptibility to infection with R5-tropic virus and produce the majority of R5-tropic HIV-1[33, 40–43]. We measured the frequencies of CCR5+CD38+HLADR+ and CCR5+CCR7−CD45RA−(T_{EM}) CD4+ and CD8+ T-cells in endometrium, endocervix and PB (Fig. 6). The CCR5− expressing activated and effector memory (T_{EM}) CD4+ T-cell population was significantly more abundant in endometrium compared to endocervix and PB (Fig. 6A, C), suggesting that cells in endometrial tissue may be particularly susceptible to HIV-1 infection. CCR5-expressing T_{EM} CD4+ T-cells were also more abundant in the endocervix (curettage, $P = 0.010$) compared to PB (Fig. 6C). Most endometrial CD8+ T-cells were also CCR5-positive and expressed activation markers CD38 and HLA-DR, although no difference between tissues was seen in the percentage of CCR5-expressing T_{EM} CD8+ T-cells (Fig. 6B, D).

Endometrial CD4+ and CD8+ T-cell responses

A pro-inflammatory mucosal environment has been associated with an increased risk of HIV-1 acquisition [44–46]. We measured CD4+ and CD8+ T-cell responses by stimulating cells freshly isolated from PB and endometrial biopsy with SEB and PMA/ION and staining with fluorescently labeled monoclonal antibodies to measure the production of cytokines, chemokines and a marker of degranulation (CD107a). Functional analysis was not performed on endocervical tissue, as the numbers of cells obtained from cytobrush and curettage were insufficient for these assays. As compared to PBMC, endometrial CD4+ T-cells produced significantly higher levels of IL-2, IL-17, IFN-γ and MIP1-β (Fig. 7A, B) following stimulation with either PMA/ION or SEB, and higher levels of TNF-α after SEB stimulation (Fig. 7A). Endometrial CD8+ T-cells were significantly more responsive than PBMC to SEB in the production of IL-10, IFN-γ, IL-2 and TNF-α (Fig. 7A) and to PMA/ION in the production of IFN-γ, IL-2 and MIP1-β (Fig. 7B). Endometrial CD4+ T-cells also produced increased IL-10 relative to PBMC following SEB stimulation, and CD8+ T-cells produced increased CD107a relative to PBMC after PMA/ION stimulation; however, these trends did not reach significance.

DISCUSSION

The tissues of the upper FRT are rich in immune effector cells, including CD4+ and CD8+ T-cells; however, little is known of the phenotype or functionality of these cells due to the difficulties inherent in obtaining fresh tissue samples. The uterine endometrium and endocervix are lined by a single layer of columnar epithelium and may be readily exposed to agents deposited in the lower FRT; accordingly, the upper FRT may serve as a portal of entry for HIV-1 and other pathogens [18, 19]. Understanding the immunological milieu of upper FRT may therefore be important for the design of effective strategies to prevent sexually transmitted infections and for assessing the safety of future microbicide candidates. In the present study, we demonstrate that during the mid-luteal phase of menstrual cycle, T-cells from the endometrium and endocervix have enhanced expression of CCR5 and are predominantly of an activated, effector memory phenotype, compared to PB T-cells. Furthermore, in comparison to endocervix, T-cell expression of memory and activation markers, as well as the HIV-1 coreceptor CCR5, are enhanced in the endometrium. Endometrial T-cells are also more responsive to polyclonal stimulation than cells from PB, producing a wide range of pro-inflammatory cytokines and chemokines. These findings suggest that the upper FRT is rich in potential HIV-1 target cells and immune responsive effector cells. Further studies are warranted to determine the extent to which the endometrium is a site of HIV-1 replication during natural infection. Importantly, this study also demonstrates the feasibility of utilizing endometrial biopsy material to explore the phenotype and functionality of T-cell populations localized to the upper FRT.

Expression of CCR5 co-receptors is reportedly enhanced on endocervical T-cells during the secretory phase of the menstrual cycle [47–49]. The mid-luteal (secretory) phase of the cycle has also been proposed to represent a 'window of vulnerability' to sexually transmitted infections, notably HIV-1 [5]. This speculation is based on the hormone-dependent alterations including suppression of innate and adaptive immune responses, reduced epithelial barrier integrity and reduced viscosity of mucus, all of which contribute to optimizing conditions for embryo implantation [5, 9, 21–23]. In support of this hypothesis, susceptibility of reproductive tract to HIV-1 and SIV infection was reported to be high during the luteal phase of the menstrual cycle [50, 51] Previous studies have also shown that the use of progestin-based contraceptives increases susceptibility to HIV-1 acquisition in women [52], although this topic remains controversial [53, 54].

In the present analysis, during the mid-luteal phase of the menstrual cycle, both endometrium and endocervix had higher percentages of CCR5 expressing CD4+ T-cells compared to PB. The CCR5 receptor density was also higher in endometrial CD4+ T-cells compared to that of PB. While there are reports demonstrating increased CCR5 expression on CD4+ T-cells in endocervical cells compared to PB [49, 55, 56], to our knowledge the comparisons within the upper FRT presented herein have not been reported previously. CCR5 expression was elevated on endometrial compared to endocervical T-cells, and CXCR4 expression was enhanced in endocervical T-cells compared to endometrium. A recent study reported preferential binding of HIV-1 gp120 to cervical T-cells expressing CCR5[55]. Our finding that CD4+ T-cells from the endometrium and endocervix express high levels of CCR5 suggests that the upper FRT may be a site of transmission for R5 tropic HIV-1. The upper FRT could theoretically also be susceptible to X4 tropic HIV-1 due to increased CXCR4 expression on endocervical CD4+ T-cells compared to endometrium as observed in our analysis; however, levels of CXCR4 have not been associated with initial transmission of HIV-1, and preferential transmission of R5 over X4 tropic viruses has been well documented, although the precise mechanism remains unresolved [57]. Uterine epithelial cells also express CD4, CCR5 and CXCR4 depending on the stage of the menstrual cycle [58] and may play a role in HIV-1 transmission via transcytosis. However, given the reduction of epithelial barrier integrity that occurs during the mid-luteal phase [9], HIV-1 may also directly contact underlying CCR5-expressing target cells.

Increased T_{EM} and activated T-cells in mucosal tissues have been associated with increased HIV-1 infection and replication [32, 40, 42, 59]. Several studies of HIV-1 and SIV have shown that the initial burst of viral replication following transmission occurs in CCR5-expressing T_{EM} and activated CD4+ T-cells [33, 40–43]. In a recent study by Saba and colleagues,[51] the frequency of CCR5 expression was found to be similar on cervical T-cells from 'productive' (i.e., productively infected) vs 'non-productive' cervical tissue explants. Both cell types were of effector memory phenotype. Interestingly, T-cells from non-productive explants produced higher levels of CCR5-binding chemokines than T-cells from productive explants, suggesting a role for these chemokines in preventing HIV-1 infection [51].

In the present analysis, T_{EM} and activated CD4+ and CD8+ T-cells were elevated in the endometrium and endocervix of the upper FRT compared to PB. The observed increase in T_{EM} cells in endometrium and endocervix was accompanied by a decrease in naïve, T_{CM} and T_{EMRA} cells. Thus, not only do endometrial CD4+ T-cells express high levels of CCR5, but they also have the highly activated, effector memory phenotype associated with HIV-1 susceptibility in some studies. Previous studies have also demonstrated higher percentages of T_{EM} and activated CD4+ T-cells in the endocervix compared to PB [49, 60]. The reasons for the observed differences between endometrium and endocervix in the frequency of CCR5– expressing T-cells, T_{EM} and activated T-cells remain unclear, but are likely related to differential hormone-mediated regulation of immune cells in distinct regions of the FRT [15, 58, 61, 62].

A pro-inflammatory mucosal environment has consistently been associated with an increased risk of HIV-1 acquisition [44–46] by modulating the expression of HIV-1 receptors of available target cells [63, 64]. Elevated levels of pro-inflammatory cytokines and inflammatory immune cells have been demonstrated in the cervical fluid and biopsies compared to PB [44, 55, 65, 66]. With limited reports on T-cell responses in the endometrium [67], we have shown here that the endometrial CD4+ and CD8+ T-cells are more highly responsive than PBMC to PMA/ION and SEB stimulation in the production of pro-inflammatory cytokines and chemokines. The secreted pro-inflammatory cytokines and chemokines may be beneficial to the host by blocking infectivity of microbial pathogens; however, they may also contribute to pathogen dissemination by recruiting and/or activating

additional susceptible target cells. There is evidence that viral particles can ascend into the upper FRT through the endocervical canal [19, 68] and substances such as semen, sperm and microbicides reach the endometrium from the vagina within hours of deposition [69–72]. The fact that these agents introduced into the lower FRT can gain access to the upper FRT and the present observation that this site contains high levels of pro-inflammatory cytokines and HIV-1 target cells implies its inherent vulnerability to HIV-1 infection.

In summary, during mid-luteal phase T-cells from the endometrium and endocervix were observed to have a phenotype that is distinct from that of PB T-cells, with a predominance of highly activated, effector memory T-cells expressing CCR5. In comparison to endocervix, T-cell expression of memory and activation markers, as well as the HIV-1 coreceptor CCR5, are enhanced in the endometrium. In addition, compared to PBMC, endometrial T-cells were highly responsive to polyclonal stimuli with the production of pro-inflammatory cytokines and chemokines. Taken together, these studies demonstrate the feasibility of using biopsy samples to evaluate CD4+ and CD8+ T-cell populations, including antigen-specific T-cells, in the uterine endometrium. They also reveal that CD4+ T-cells in the endometrium have a phenotype associated with high susceptibility to HIV-1 infection. Further studies to evaluate the potential role of the upper FRT in HIV-1 transmission, viral replication and pathogenesis are warranted.

Acknowledgments

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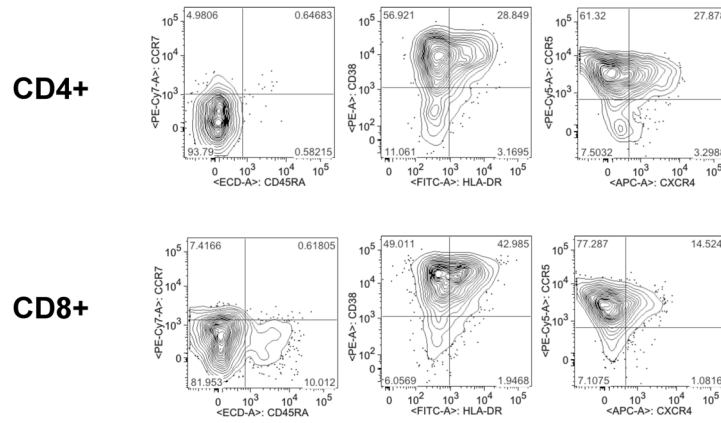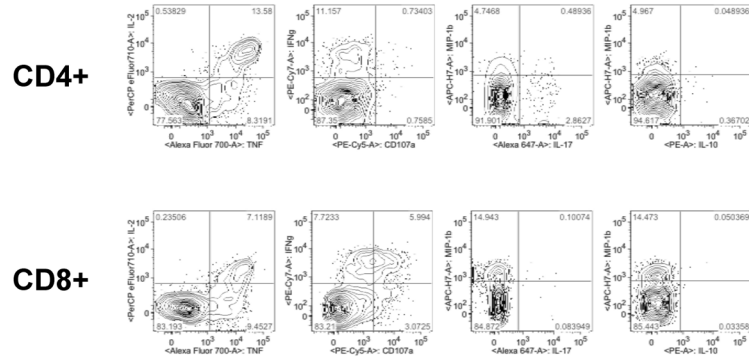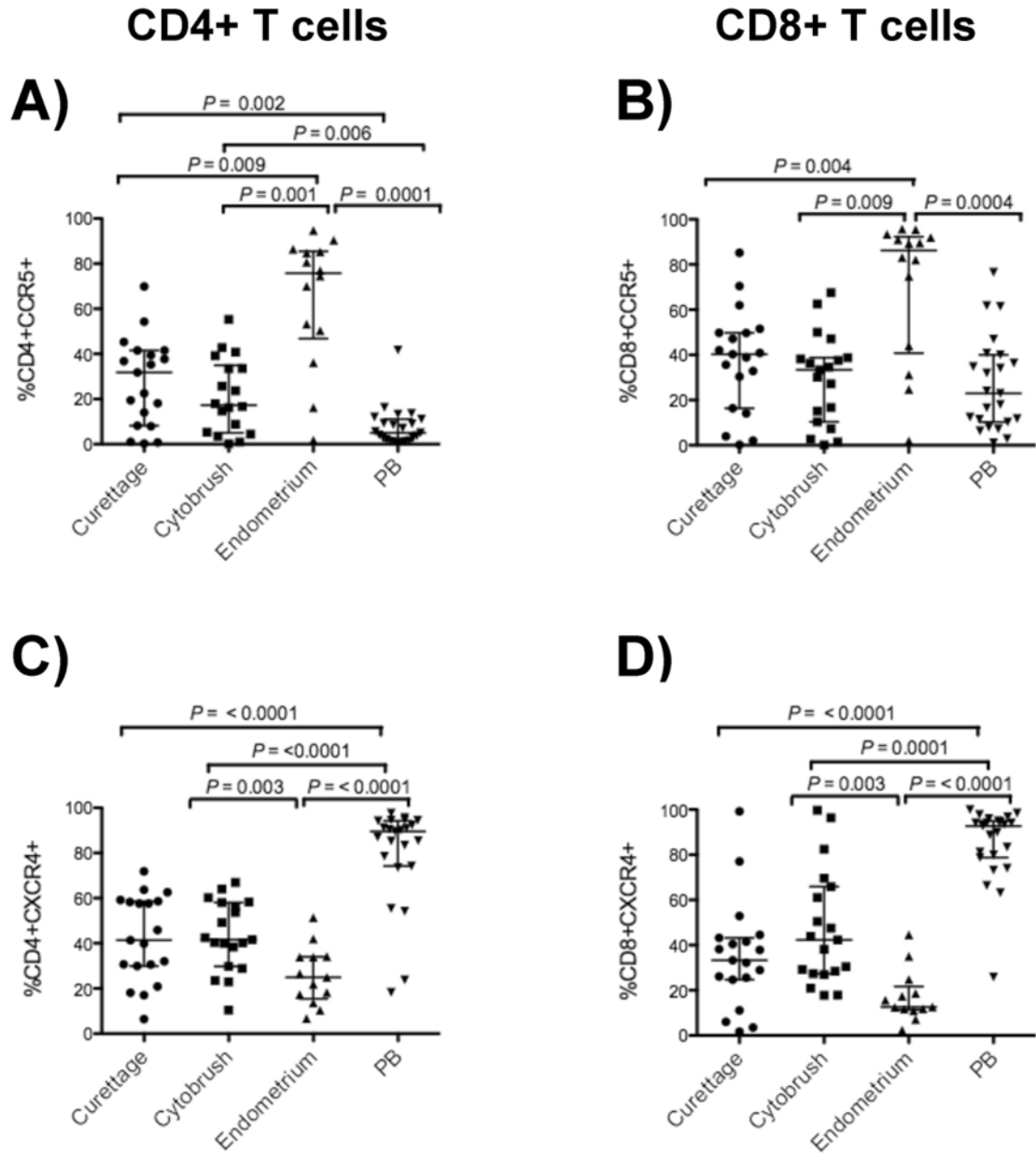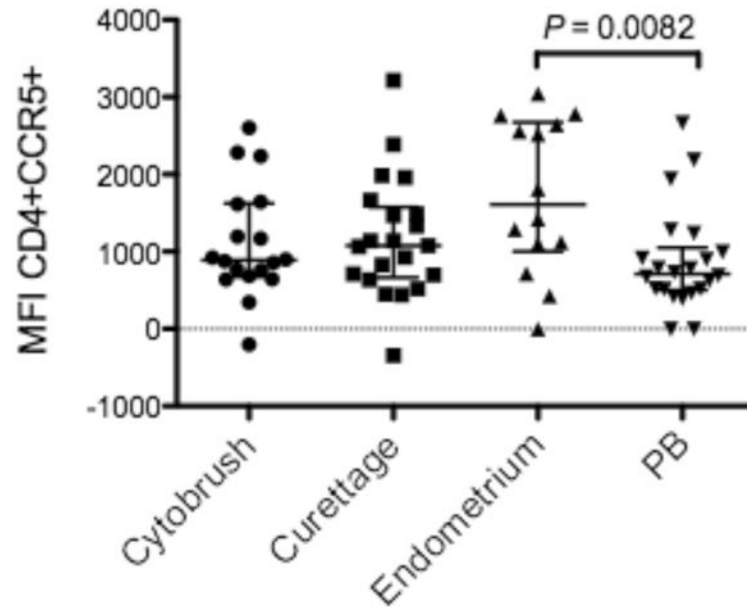
Figure 1A

Figure 1B

Figure 1. Flow cytometry gating used in the analysis of endometrial T-cell phenotype and function

Panel (A): For phenotypic analysis, after initial gating of endometrial lymphocytes and doublet discrimination, viable cells were gated for CD3+CD66b− (not shown) and then for CD4+ (top row) and CD8+ (bottom row) T-cells. The resulting populations were gated for CD45RA, CCR7, CD38, HLA-DR, CCR5 and CXCR4. **Panel (B):** For analysis of endometrial T-cell responses to polyclonal stimulation, seven functional responses (CD107a, IL-2, IL-10, IL-17, TNF-α, IFN-γ and MIP-1β) were measured. Initial gating was performed to identify lymphocytes and doublet discrimination (not shown), followed by gating of viable cells on CD4+ (top row) and CD8+ (bottom row) T-cells, and finally for individual responses.

CD4+ T cells

CD8+ T cells

Figure 2. CCR5-expressing T-cells were enriched in endometrium compared to endocervix and peripheral blood (PB)

The figures show the percentages of CD4+ (left panels A, C) and CD8+ T-cells (right panels B, D) from curettage, cytobrush, endometrium and peripheral blood (PB) expressing CCR5 and CXCR4. Horizontal lines correspond to the median values and the vertical lines and whiskers represent 25th and 75th percentiles. Two tailed Mann-Whitney tests and Holms' sequential Bonferroni corrections were performed.

Figure 3. Median fluorescence intensity (MFI) of CCR5 on CD4+ T-cells
CCR5 MFI was significantly higher on CD4+ T-cells in endometrium compared to PB. Similar trends were observed when comparing endocervical curettage CD4+ T-cells vs PB (P=0.09) and endometrial vs endocervical cytobrush CD4+ T-cells ($P = 0.05$). MFI was calculated using FlowJo software (Tree Star, Inc., Ashland, OR). Horizontal lines correspond to the median values; vertical lines and whiskers represent 25th and 75th percentiles. Two tailed Mann-Whitney tests and Holms' sequential Bonferroni corrections were performed.
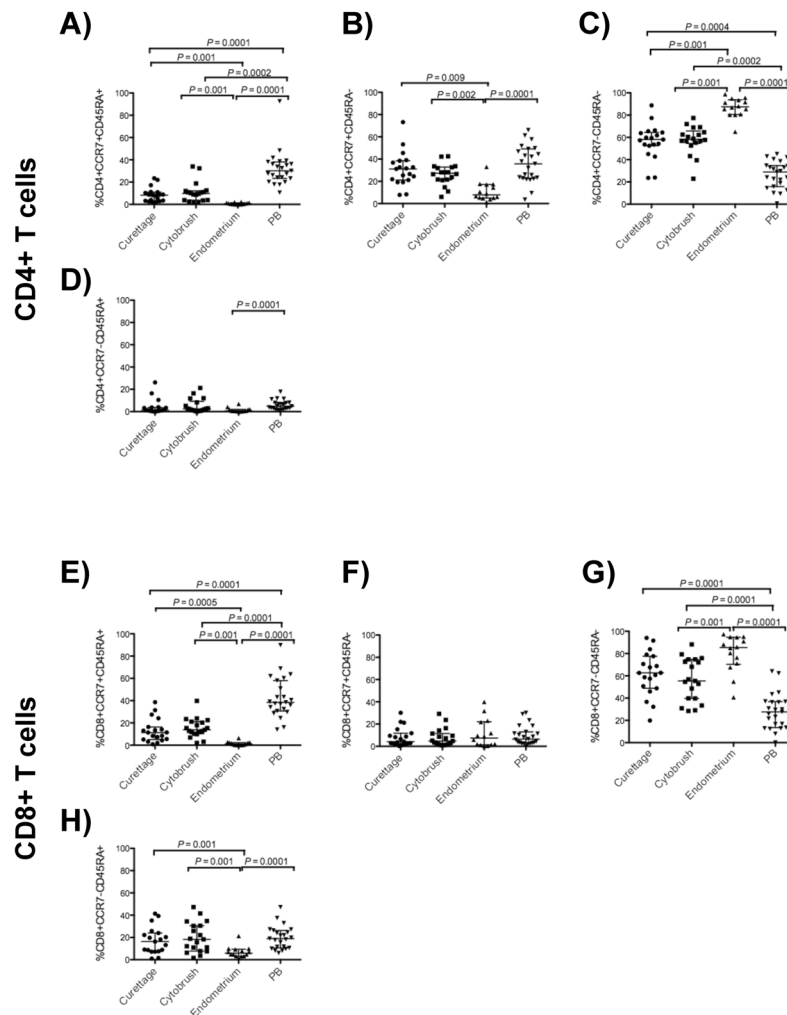
Figure 4. Endometrial CD4+ and CD8+ T-cells display a memory phenotype
The figure shows percentages of CD4+ (upper panels, A–D) and CD8+ (lower panels, E–H) T-cells with the phenotypes CCR7+CD45RA+ (naïve), CCR7+CD45RA− (central memory), CCR7−CD45RA− (effector memory), and CCR7−CD45RA+ (terminally differentiated memory) from curettage, cytobrush, endometrium and peripheral blood (PB). Horizontal lines correspond to median values; vertical lines and whiskers represent the 25th and 75th percentiles. For statistical analysis, two-tailed Mann-Whitney tests were performed with Holms' sequential Bonferroni corrections.
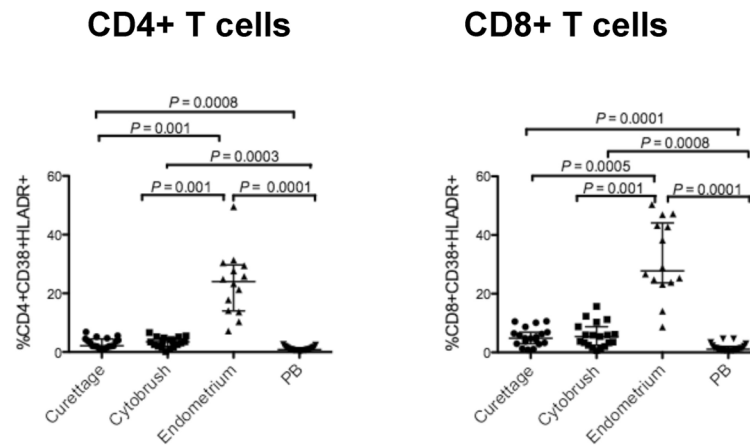
Figure 5. Endometrial CD4+ and CD8+ T cells are highly activated
The figure shows the percentage of CD4+ (left, A) and CD8+ (right, B) T-cells expressing CD38 and HLA-DR from curettage, cytobrush, endometrium and PB. Horizontal lines correspond to median values; vertical lines and whiskers represent the 25th and 75th percentiles. For statistical analysis, two-tailed Mann-Whitney tests and Holms' sequential Bonferroni corrections were performed.

CD4+ T cells
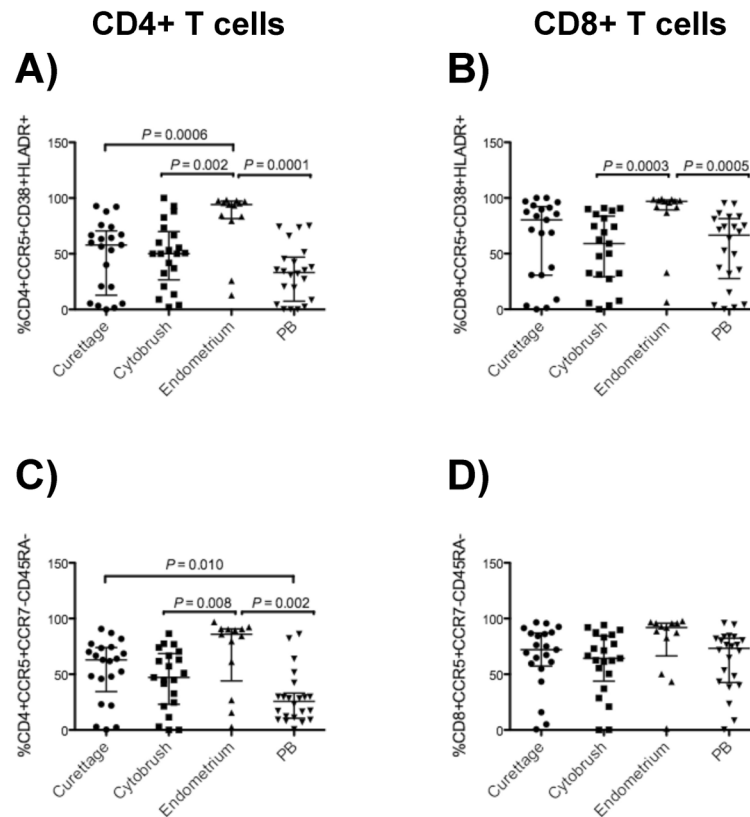
CD8+ T cells

Figure 6. Enhanced CCR5-expressing activated and effector memory CD4+ and CD8+ T-cells in endometrium
Shown here are the frequencies of CD4+ (left panels, A and C) and CD8+ (right panels, B and D) T-cells with the phenotypes CCR5+CD38+HLADR+ and CCR5+CCR7−CD45RA−. Horizontal lines correspond to median values; vertical lines and whiskers represent the 25th and 75th percentiles. Two-tailed Mann-Whitney tests and Holms' sequential Bonferroni corrections were performed for statistical analysis.
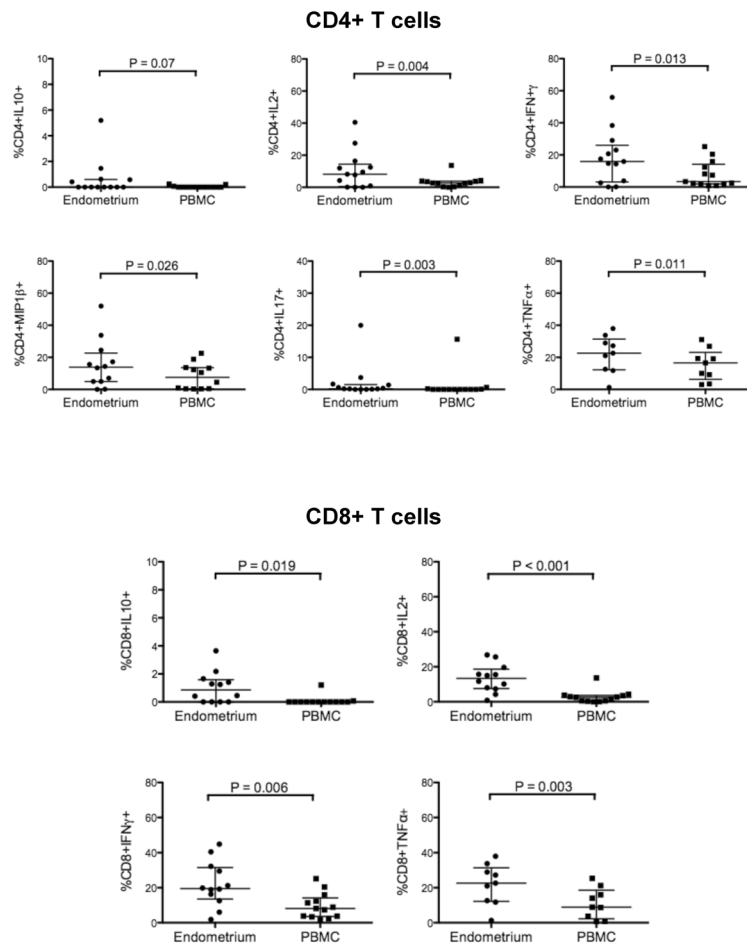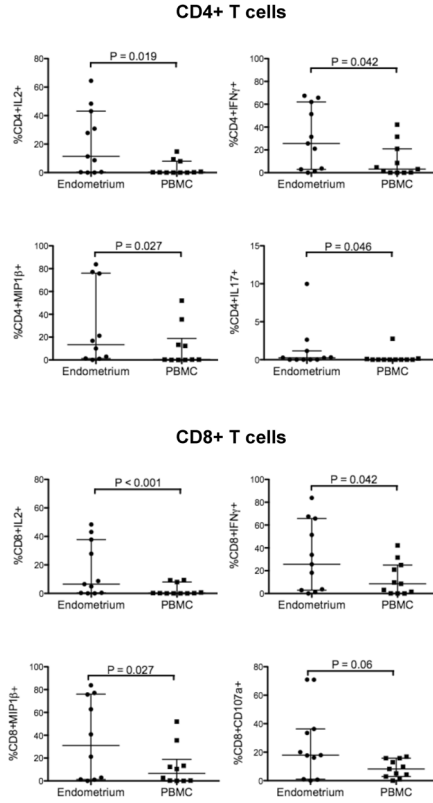
Figure 7A

Figure 7B

Figure 7. Endometrial T-cells are highly responsive to polyclonal stimulation
Figure 7A shows the percentage of endometrial and PB CD4+ (upper panels) and CD8+ T cells (lower panels) responding to staphylococcus enterotoxin B (SEB) stimulation. Figure 7B shows the percentage of endometrial and PB CD4+ (left) and CD8+ T-cells (right) responding to phorbol myristate acetate (PMA) and ionomycin (ION) stimulation. Horizontal lines correspond to median values; vertical lines and whiskers denote the 25th and 75th percentiles. Statistical analyses were performed using Wilcoxon-matched pairs signed rank test and *P* values   0.05 were considered significant.