ORIGINAL ARTICLE

Distribution of Immune Cells in the Human Cervix and Implications for HIV Transmission

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Problem
Knowledge of the mucosal immune cell composition of the human female genital tract is important for understanding susceptibility to HIV-1.

Method of study
We developed an optimized procedure for multicolor flow cytometry analysis of immune cells from human cervix to characterize all major immune cell subsets in the endocervix and ectocervix.

Results
Half of tissue hematopoietic cells were $CD14^+$, many of which were macrophages and about a third were $CD11c^+$, most of which were $CD103^-$ $CD11b^+$ $CX3CR1^+$ DC-$SIGN^+$ dendritic cells (DCs). The other dominant population were T cells, with more CD8 than CD4 cells. T cells (both CD8 and CD4) and B cells were more abundant in the ectocervix than endocervix of pre-menopausal women; however, $CD8^+$ T cell and B cell numbers declined in the ectocervix after menopause, while CD4 T cell counts remained higher. B, NK and conventional myeloid and plasmocytoid DCs each were a few percent of tissue hematopoietic cells. Although the ectocervix had more HIV-susceptible $CD4^+$ T cells, polarized endocervical explants supported HIV replication significantly better.

Conclusion
Due to their abundance in the genital tract, $CX3CR1^+$ DC-$SIGN^+$ DCs might be important in HIV transmission. Our data also suggest that the columnar epithelium of the upper genital tract might be a preferential site for HIV transmission.

Introduction

The female genital tract (FGT) mucosa is important in the defense against invasion by pathogenic microorganisms (reviewed in[1,2]). The mucosa of the upper FGT (uterus, fallopian tubes, and endocervical canal) is covered by a single-layered columnar epithelium. The endocervical canal glands produce mucus, which can trap microbial pathogens and prevent ascending infection. In contrast, the lower genital tract (ectocervix and vagina) is covered by a stratified squamous non-keratinized epithelium, which potentially provides a more effective barrier than the columnar epithelium. Immune cells are recruited to the mucosa and mediate innate and adaptive immune responses. Although our knowledge of

mucosal immunity at some sites, such as the intestinal tract and lung, has increased recently, less is known about immunity in the FGT which is the portal of entry for most new HIV infections. A few studies have looked at resident immune cells in the human cervical mucosa,[3–6] where most HIV sexual transmission is thought to occur. Many of these studies were performed using immunohistochemistry and flow cytometry before multicolor analysis became routine. In the human cervix, as other mucosal tissues, T lymphocytes are much more abundant than B cells, $CD8^+$ T cells outnumber $CD4^+$ T cells, and most T cells have an effector memory phenotype.[3–5] Although a few recent studies have quantified subsets of lymphocytes, no study has looked at the abundance of all the major lymphoid and myeloid cell types within the same sample and studied their localization in different anatomical regions and associated this with infectivity.

Current models of HIV sexual transmission are largely based on studies of SIV transmission in *Rhesus macaques*.[7–10] However, results in the macaque SIV model might not translate to human HIV transmission.[9–13] There is a need to investigate sexual transmission of HIV in human models. An *in vivo* model of sexual transmission in 'humanized' NOD/scid/$IL2R_\gamma^{-/-}$ mice transplanted with fetal $CD34^+$ cells, liver and thymus, was recently developed.[14–16] Although all subtypes of human immune cells may be present in these mice, they may be less abundant and could traffic differently in response to chemokines produced by mouse epithelial cells. As an alternative model, human cervical tissue explants have been used to study *ex vivo* mucosal transmission of sexually transmitted infections such as HIV-1.[5] An important consideration for judging how well the rhesus macaque or humanized mouse infection models might mimic transmission to women is defining how well the numbers and distribution of immune cells in the FGT of rhesus macaques or humanized mice recapitulates what is found in women. To begin to answer these questions, we used multicolor flow cytometry and immunohistochemical analysis to better define the human innate and adaptive immune cells in the endo- and ectocervix of healthy women, using cervical tissue samples from women undergoing hysterectomy for benign non-inflammatory conditions. We developed an optimized protocol to isolate and analyze by flow cytometry immune cells in the human cervix, which enabled us to detect all major immune cells types simultaneously. We also compared immune cell representation in the endocervix and ectocervix to identify differences that might be important for susceptibility to viral transmission.

We found that $CD14^+$ cells were the most abundant hematopoietic cells in the cervix, comprising about half of all hematopoietic cells. Although most of these were $CD11c^-$macrophages, about a third were $CD14^+$ $CD11c^+$ $CD11b^+$ $CD103^-$ cells, most likely DCs, that also expressed CX3CR1 (the fractalkine receptor) and DC-SIGN, both coreceptors for HIV. T cells (both $CD4^+$ and $CD8^+$) were more abundant in the ectocervix than endocervix of pre-menopausal women. However, $CD8^+$ numbers declined in the ectocervix after menopause, while $CD4^+$ numbers remained high. Although the ectocervix had more HIV-susceptible $CD4^+$ T cells, infection of polarized endocervical explants supported a higher level of HIV-1 replication than that of ectocervical explants.

Materials and methods

Human Cervical Tissue

Human cervical tissue was obtained from twenty-six women without cervical pathology and signs of cervical infection or inflammation, undergoing hysterectomy for benign conditions such as fibroids, at Massachusetts General Hospital with Institutional Review Board approval. In 13 cases, the tissue contained both ectocervix and endocervix, which were separated by anatomical localization and were confirmed on tissue sections used for immunohistochemistry. For these samples, we also obtained information on age, menopausal status (as identified by patient history by the treating gynecologist), hormone use, and parity from the patient medical records. Seven of these donors were pre-menopausal (41–52 years old) and six were post-menopausal women (50–77 years old). Menopausal status was defined as having no menses in 6 months. Because of the ages of the study subjects, some of the subjects classified as pre-menopausal could actually be peri-menopausal. The tissue was stored at 4°C in RPMI 1640 media (Cellgro, Manassas, VA, USA) containing 10% human serum (AB) (GemCell, West Sacramento, CA, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin sulfate (H10 medium) until processing within 24 hrs of surgery.

Cervical Tissue Digestion

To prepare a single cell suspension, cervical mucosa, separated from the underlying stroma by cutting 5 mm below the epithelial surface, was minced into 5×5 mm^3 pieces, placed in 5 mg/mL Collagenase IV (Invitrogen, Carlsbad, CA, USA) in H10 media, and subjected to mechanical dissociation for 1 min using mouse spleen 01.01 program of the gentleMACS Dissociator (MACS Miltenyi Biotec, Auburn, CA, USA). The sample was incubated for 30 min at 37°C with shaking at 120 rpm, followed by another cycle of mechanical dissociation. The suspension was filtered through a 100-μm cell strainer (BD Biosciences, San Jose, CA, USA), and cells were collected by centrifugation at 500 ***g*** for 5 min at 4°C. Liberase TL and Liberase DL enzyme blends (Roche Applied Science, Indianapolis, IN, USA) were tested during the optimization of the protocol.

Flow Cytometry

Immune cells were identified by staining with the following antibody cocktails – for T cells, NK cells and B cells: anti-CD3-APC (cl.OKT3), anti-CD4-PE-Cy7 (cl.OKT4), anti-CD56-PE (cl.HCD56), anti-CD16-AF488 (cl.3G8), anti-CD19-PerCP-Cy5.5 (cl.HIB19), anti-CD45-Pacific blue (cl.HI30); for phenotyping T cells: anti-CD3-PerCP-Cy5.5 (cl.UCHT1), anti-CD4-PE (cl.OKT4), anti-CD8-APC (cl.HIT8a), anti-CD45RA-PE-Cy7 (cl. HI100), anti-CD27 (cl. 0323), anti-CCR7-AF488 (cl.TG8/CCR7), anti-CD45-Pacific blue; for T cells, chemokine receptors: anti-CD3-PerCP-Cy5.5, anti-CD4-PE-Cy7, anti-CCR5-PE (cl.T21/8), anti-CXCR4-APC (cl.12G5), anti-CD45-Pacific blue; for APCs: anti-CD3 (cl.OKT3)/CD20 (cl.2H7)/CD56(cl.HCD56)/CD16 (cl.3G8) – all AF488 labeled, anti-CD14-APC (cl.HCD14), anti-HLA-DR-PerCP-Cy5.5 (cl.L243), anti-CD11c-PE-Cy7 (cl.3.9), anti-CD123-PE (cl.6H6), anti-CD45-Pacific blue. In some experiments, anti-CD68-PE (cl. Y1/82A), anti-DC-SIGN-APC (cl.9E9A8), anti-CD11b-APC (cl. ICRF44), anti-CD103-AF488 (cl. Ber-ACT8), anti-CX3CR1-PE (cl. 2A9-1), and anti-NKp44-APC (cl. P44-8) were used. All antibodies were from Biolegend (San Diego, CA, USA) except anti-DC-SIGN (R & D Systems, Minneapolis, MN, USA). The staining was performed in FACS buffer [Dulbecco's phosphate-buffered saline, 1 mM EDTA, 25 mM HEPES (all from Invitrogen) with 0.5% BSA (Sigma)]. Dead cells were excluded using Sytox blue dead stain dye (Invitrogen). Data were acquired using a FACSCantoII (BD Biosciences) and analyzed using FlowJo version 9-1.3.1 (Tree Star, Ashland, OR, USA).

Infection of cervical explants with HIV_{BaL}

Polarized cervical explants were prepared as previously described.[16] Briefly, the cervix was cut using 3-mm biopsy punches (Acuderm, Fort Lauderdale, FL, USA) and embedded with the epithelium facing up in 3% agarose in the upper chamber of a 24-well transwell system (Corning, Lowell, MA, USA). HIV_{Bal} (20 ng p24) was applied to the apical surface in the upper chamber for 48 hrs; following a wash, the explants were cultured for six additional days with medium replacement every 48 hrs. The levels of p24 antigen in culture media were measured using the HIV-1 p24 ELISA Kit (Perkin Elmer, Waltham, MA, USA). Absorbance was measured with a SpectraMax 190 Microplate Reader (Sunnyvale, CA, USA). HIV_{BaL} was obtained from the NIH AIDS Reagent Program, and viral stock was prepared as previously described.[16]

Immunohistochemistry

Cervical cryosections (8 μm), fixed in 4% paraformaldehyde (Sigma) and blocked with Protein Block (Biogenex, Fremont, CA, USA) and Biotin/Avidin Blocking Kit (Vector Laboratories, Burlingame, CA, USA), were stained with the following antibodies: anti-CD3 (cl.UCHT1), anti-CD4 (cl.RPA-T4), anti-CD14 (cl. M5E2), and anti-DC-SIGN (cl.DCN46) (all from BD Biosciences). Biotinylated anti-mouse immunoglobulins, alkaline phosphatase-conjugated streptavidin, and fast red substrate (all from Biogenex) were used to develop the reaction. Hematoxylin (Leica Biosystems, Richmond, IL, USA) was used for nuclear counterstain. Images were acquired using a Zeiss Axiovert 200M microscope and Slidebook software (Intelligent Imaging, Denver, CO, USA).

Statistical analysis

Data are presented as median value (the 50th percentile) and interquartile range (IQR). Wilcoxon matched pairs test was used to compare the percent of immune cells between ectocervix and endocervix and Mann–Whitney nonparametric test was used to compare pre-menopausal and post-menopausal cases. All statistical analysis was performed using GraphPad Prizm 4 software (GraphPad Software, San Diego, CA, USA).

American Journal of Reproductive Immunology **71** (2014) 252–264

Results

Immune cell composition of human ectocervix

To characterize cervical immune cells by flow cytometry, we first needed to optimize a protocol for liberating immune cells from cervical tissue, which is usually accomplished by treating tissue with collagenase and other proteases.[16–19] An optimal protocol needs to balance the opposing considerations of maximizing tissue digestion and immune cell yield with the danger of proteolytic digestion of cell surface receptors used to identify cell subtypes. To accomplish this goal, we compared yields and proportions of different immune subtypes in cervical tissue samples and peripheral blood mononuclear cells, the latter with or without protease digestion. Crude collagenase treatment interfered with the detection of some immune receptors, especially CD56, used to identify NK cells and substantially reduced the staining intensity of others, including CD4 (Figure S1). Treatment with purified enzyme blends Liberase TL or DL improved CD56 detection in PBMCs; however, the yield of cells from cervical tissue was unacceptably reduced (data not shown). We therefore selected an isolation protocol that treated tissue fragments with collagenase IV in 10% serum-containing medium.[20] This protocol did not completely protect CD56 in PBMCs (data not shown), but incubation of cervical tissue fragments with 5 mg/mL collagenase IV for 30 min in combination with gentle mechanical dissociation maintained CD56 staining in the readily detectable range (Fig. 1e).

This protocol was used to isolate cervical cells from twenty-six subjects. Viable immune cells were identified by gating on cells expressing the pan-hematopoietic cell marker CD45 (to exclude non-immune cells, such as epithelial cells and fibroblasts) that did not stain with Sytox Blue (to exclude dead cells) (Fig. 1a). On average, we obtained 3.7×10^5 $CD45^+$ cells/gm of tissue (range, $0.8–6.9 \times 10^5$). Cells were co-stained for CD3, CD4, CD56, CD16, CD19, CD14, HLA-DR, CD11c, CD123, DC-SIGN, CD103, CD11b, and CX3CR1 to identify different lymphocyte and antigen-presenting cell (APC) populations (Fig. 1). Neutrophils, which constituted approximately 10% of all tissue $CD45^+$ cells, were excluded by forward and side scatter gating (Fig. 1b). Data for individual ectocervical samples are compiled in Table S1.

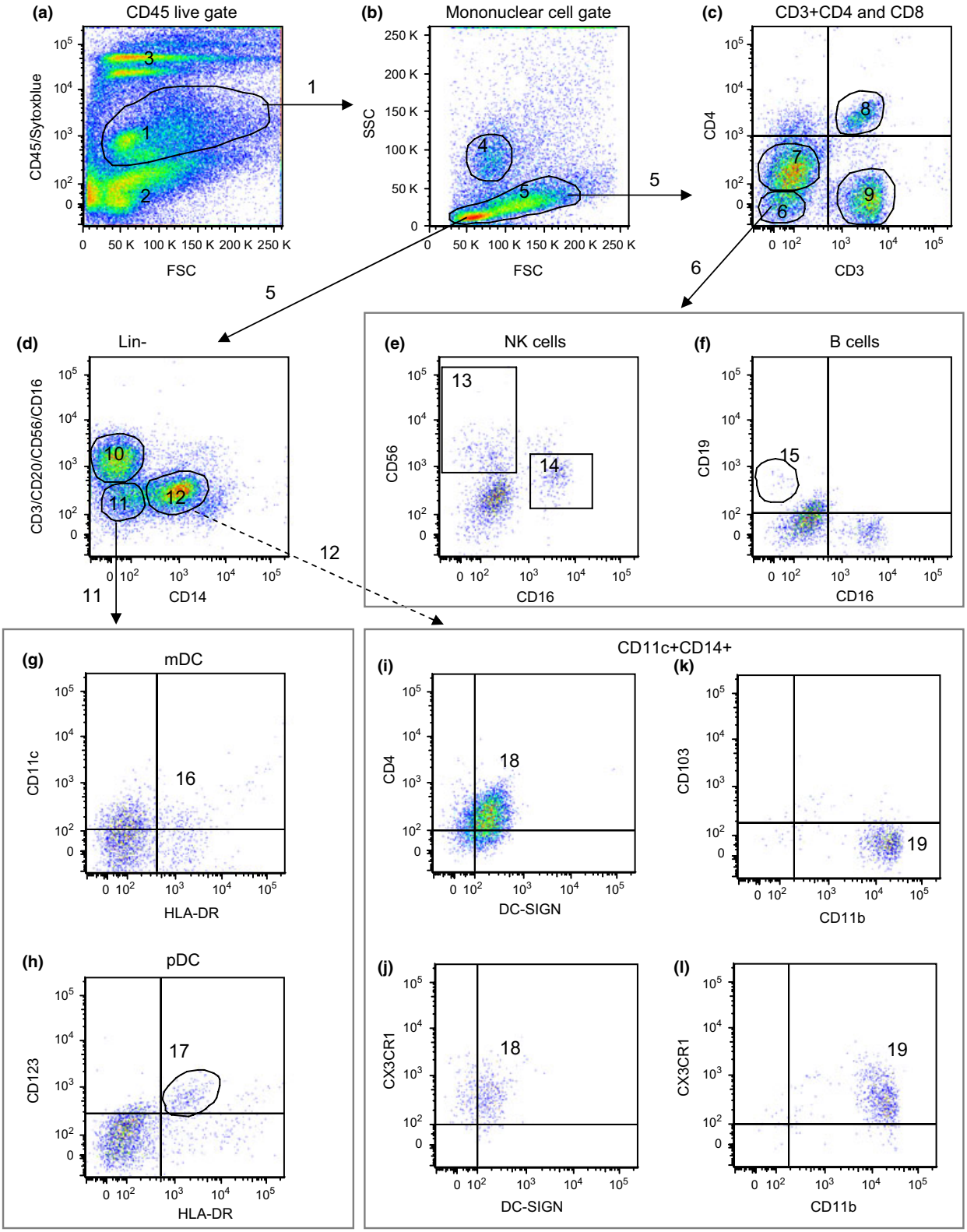
Lymphocyte subsets in human ectocervix

In the ectocervix, 35% of $CD45^+$ mononuclear cells were $CD3^+$ T cells (interquartile range (IQR) 23–43%, $n = 26$), of which on average 41% were $CD4^+$ and 59% were $CD8^+$ T cells (Fig. 2a). The median CD4/CD8 ratio was 0.7. However, in 9 of 26 cases, the cervical epithelia contained more $CD4^+$ T cells than $CD8^+$ T cells (Table S1). In contrast, in human blood, T cells are on average 70% of the mononuclear cells[21] and $CD4^+$ T cells are about twice as abundant as $CD8^+$ T cells (Fig. 2e). Within the $CD4^+$ T cells in the cervix, 70% were of the effector/memory ($CD27^-$ $CD45RA^-$) or effector phenotype ($CD27^-$ $CD45RA^+$) ($n = 4$) (Table S2), which mostly lack expression of CCR7 (data not shown). Within the $CD8^+$ T cells, the majority (more than 60%) expressed CD45RA, but not CD27, indicative of the effector cell phenotype ($n = 4$) (Table S2). The expression of the chemokine receptors CCR5 and CXCR4 varied greatly between donors. Median CCR5 expression was 28% in the $CD4^+$ T cells and 16% for $CD8^+$ cells (Table S2). CXCR4 was detected in 51% of $CD4^+$ and 44% of $CD8^+$ T cells ($n = 6$) (Table S2).

CD3-negative lymphocytes (Fig. 1c,e,f) in the cervical epithelium included $CD56^{dim}$ $CD16^+$ and $CD56^{bright}$ $CD16^-$ NK cells (on average, NK cells were 2.7% of $CD45^+$ mononuclear cells) and $CD19^+$ B cells (median 0.9%, range 0.2–1.8%) (Fig. 2b). These cell types were underrepresented relative to their frequencies in the blood, where NK cells and B cells each constituted as many as 10% of mononuclear cells.[21,22] The relative numbers of 'cytokine-producing' $CD56^{bright}$ $CD16^-$ and 'cytotoxic' $CD56^{dim}$ $CD16^+$ NK cells in the cervix varied between donors with $CD56^{bright}$ $CD16^-$ NK cells ranging between 0.8 and 3% (median 1.7%, $n = 18$) and $CD56^{dim}$ $CD16^+$ NK cells ranging between 0.6 and 3% (median 1%, $n = 18$) of all $CD45^+$ mononuclear cells (Fig. 2b and Table S1). In contrast, in the circulation, cytotoxic $CD16^+$ NK cells were the prevalent NK cell subset,[22] comprising up to 10% of mononuclear cells (Fig. 2e).

Antigen-presenting cells in human ectocervix

To analyze APC subtypes in the cervix, we co-stained for CD14 and lymphocyte lineage markers (CD3, CD20, CD56, and CD16) (Fig. 1d). Lin-negative $CD14^+$ cells were the largest population of immune cells in the cervix, ranging between 37 and 55% of $CD45^+$ mononuclear cells (median 42%, $n = 10$).



American Journal of Reproductive Immunology **71** (2014) 252–264

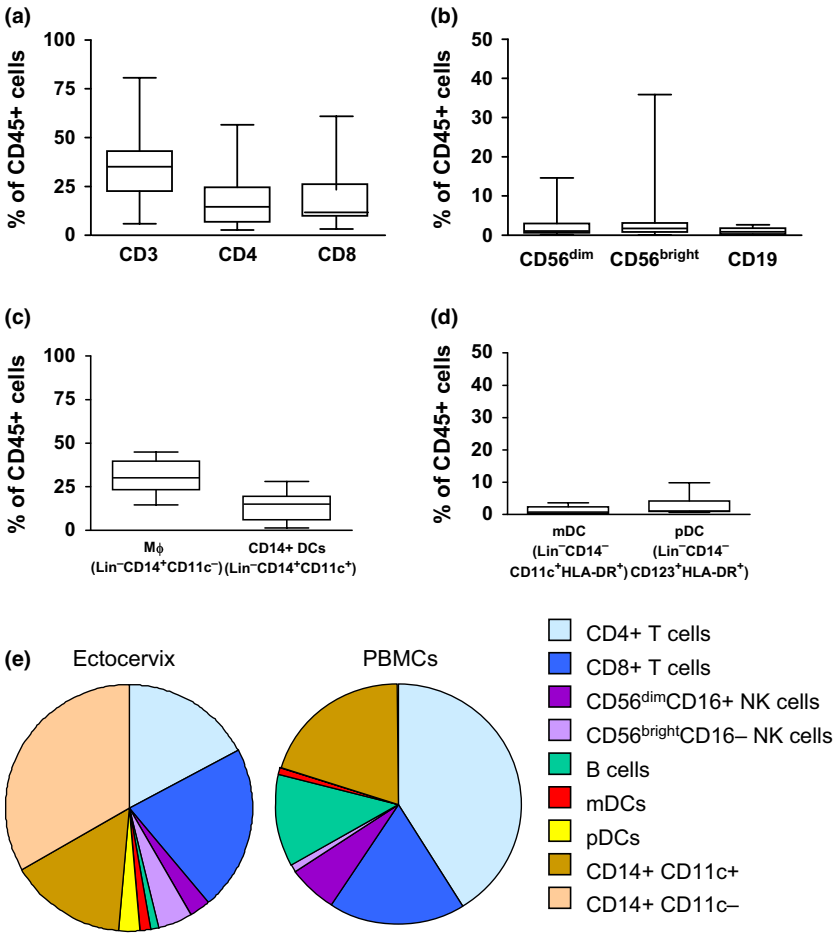


Fig. 2 Distribution of immune cell subsets in the human ectocervix. Box-and-whiskers graph showing the distribution of T cells (n = 26) (a), NK (n = 18) and B cells (n = 16) (b), macrophages ($CD14^+$ $CD11c^-$) and $CD14^+$ $CD11c^+$ DCs (n = 10) (c), mDCs (n = 12) and pDCs (n = 11) (d). The line in the middle of the box is the median; the box extends from the 25th to 75th percentiles, and the whiskers present minimum and maximum values. Pie charts (e) compare the median distribution of immune cells as a proportion of all $CD45^+$ cells in human ectocervical samples (n = 26) compared with peripheral blood mononuclear cells (PBMCs) of normal donors (n = 3).

The majority of $CD14^+$ cells were $CD11c^-$ macrophages, which was confirmed by CD68 staining (data not shown). Macrophages ranged between 23 and 40% (median, 30%) of all $CD45^+$ cells (Fig. 2c and Table S1). Approximately one-third of $CD14^+$ cells were also $CD11c^+$. These $CD14^+$ $CD11c^+$ cells may represent $CD14^+$ tissue DCs[23] or possibly recently migrated monocytes that have not completely differentiated into tissue macrophages (Fig. 2c). The percentage of $CD14^+$ $CD11c^+$ cells within tissue hematopoietic cells was similar to that of peripheral blood $CD14^+$ $CD11c^+$ monocytes (10–20% of mononuclear cells)[21] (Fig. 2e). Most of the $CD14^+$ $CD11c^+$ cells in the cervix expressed HIV-attachment receptor DC-SIGN and were also $CD4^{dim}$ (Fig. 1i). They also stained for the fractalkine receptor CX3CR1, which is another HIV-1 coreceptor (Fig. 1j). Most of the $CD14^+$ $CD11c^+$ cells were $CD103^-$ $CD11b^+$ $CX3CR1^+$ (60–90%, n = 3) (Fig. 1k,l).

Conventional dendritic cell subsets, defined as lymphocyte lin- and $CD14^-$, included $CD11c^+$ HLA-DR^+ myeloid DCs (mDCs) and $CD123^+$ HLA-DR^+ plasmacytoid DCs (pDCs) (Fig. 1g,h). Myeloid DCs ranged from 0.3 to 2.3% (median 0.8%, n = 12) and pDCs ranged from 0.9 to 4% (median 1%, n = 11) of cervical $CD45^+$ cells (Fig. 2d). In the tissue, DCs

Fig. 1 Representative flow cytometry of immune cells in the ectocervix. (a) $CD45^+$ cells (1) excluding $CD45^-$ (2) and Sytox blue-stained dead cells (3). (b) Mononuclear cells (5) and granulocytes (4) based on forward and side scatter (c) $CD3^+$ $CD4^+$ (8) and $CD3^+$ $CD4^-$ (9) T cells and $CD3^-$ cells (6), excluding $CD4^{dim}$ $CD3^-$ APCs (7). (d) Lin-$CD14^-$ (11) or lin-$CD14^+$ antigen-presenting cells (APCs) (12) excluding CD3/CD20/CD56/$CD16^+$ cells (10). (e) $CD56^{bright}$ $CD16^-$ (13) and $CD56^{dim}$ $CD16^+$ (14) NK cells (f) $CD19^+$ B cells (15) (g) $CD11c^+$ HLA-DR^+ mDCs (16) (h) $CD123^+$ HLA-DR^+ pDCs (17). Dotted line corresponds to $CD11c^+$ cells (i, j). DC-SIGN$^+$ $CX3CR1^+$ $CD4^{dim}$ (18) and (k, l) $CD103^-$ $CD11b^+$ $CX3CR1^+$ cells (19).

were slightly more abundant than in peripheral blood where they are normally <1% of mononuclear cells (Fig. 2e).

Comparison of immune cell subsets in endocervix and ectocervix

Thirteen of the cervical tissue samples contained both ectocervix and endocervix. There was no significant difference in the proportion of $CD45^+$ cells in the endocervix and ectocervix (data not shown). However, on average, $CD3^+$ T cells were about twice as abundant (P =0.0002) in the ectocervix (IQR 23–43% of $CD45^+$ cells, median 36%) as in the endocervix (IQR 10–27%, median 17%), (Fig. 3a). Patients were classified as pre-menopausal or post-menopausal based on their history by their treating gynecologist. The pre-menopausal ectocervical samples contained significantly more of both CD4 and CD8 T cells than their paired endocervical tissues, but in post-menopausal samples, only $CD4^+$ cells were significantly more abundant in the ectocervix (Fig. 3b). As a consequence, the CD4/CD8 ratio was significantly (P = 0.001) higher in the ectocervix of post-menopausal women (median 1.2, range 0.9–2.2) compared with pre-menopausal (median 0.66, range 0.6–0.7) (Fig. 3b).

There were significantly more B cells in the ectocervix (median 0.9%, IQR 0.23–1.75%) than the endocervix (median 0.4%, IQR 0.2–0.8%) (P = 0.04), which was predominantly in pre-menopausal women (Fig. 3c). There was no significant difference in either $CD56^{dim}$ $CD16^+$ or $CD56^{bright}$ $CD16^-$ NK cells comparing the ectocervix versus the endocervix (Fig. 3d). However, $CD56^{dim}$ $CD16^+$ cells were more abundant in pre-menopausal tissues, a difference that reached significance only in the endocervix (P = 0.03) (Fig. 3d). $CD14^+$ macrophages and DC subsets were similarly abundant in the ectocervix and endocervix (data not shown). Thus, the ectocervix of pre-menopausal women contained significantly more CD4 T, CD8 T, and B lymphocytes than the endocervix, but only CD4 T cells were more abundant in post-menopausal women. Among all subjects, only two pre-menopausal women reported hormone use (oral or skin patch contraceptive), and the cell distribution in these two samples was not significantly different from the rest of the samples, indicating that hormone use could not explain the observed differences. There were no significant differences in parity between the pre- and post-menopausal subjects (most donors reported two to four childbirths).

Recent interest has focused on innate lymphoid cells in mucosal tissues which have similar functions as corresponding subsets of adaptive immune cells. In particular, IL-22-secreting NK cells (NK-22 cells) are a subset of mucosal $CD3^-$ $CD56^{bright}$ $CD16^-$ unconventional NK cells, which enhance innate immune responses and inflammation at mucosal sites and are identified by expression of the NK activating receptor, NKp44.[24] We detected NK-22 cells both in the endocervix and in the ectocervix, but they were rare (median, 0.2% and 0.1% of the $CD3^-$ cells in the lymphocyte gate, respectively, n = 9) (Figure S2).

T-lymphocytes cluster on either side of the basement membrane, while APCs are mostly in the stroma

To compare the localization within the ectocervix and endocervix of cells potentially important in HIV transmission, frozen tissue sections (n = 4) were stained for CD3, CD4, CD14, and DC-SIGN. As previously reported,[4] $CD3^+$ T cells, including $CD4^+$ lymphocytes, concentrated close to the basement membrane in the lamina propria and within the stratified epithelium of the ectocervix (representative sections shown in Fig. 4a,b). They were also found within the columnar epithelium or close to the basement membrane in the endocervix (Fig. 4a, b). Lymphocyte aggregates were common in the ectocervix (Fig. 4a,b). $CD8^+$ cells had a similar distribution (data not shown). The abundance of APCs found by flow cytometry was confirmed by CD14 staining (Fig. 4c). DC-$SIGN^+$ cells were largely found within the stroma of the ectocervix and endocervix (Fig. 4d). Most DC-$SIGN^+$ cells were also $CD14^+$ as revealed by co-staining (data not shown).

HIV infection of polarized endocervix and ectocervix

It is unclear whether HIV transmission in humans occurs primarily at the endocervix or ectocervix. There are conflicting results from studies in rhesus macaque and human cervical explants. Here, we found an increased number of HIV-susceptible CD4 T cells in the ectocervix compared with the endocervix, but endocervical CD4 T cells were abundant within the epithelium and in the stroma close to the epithelial basement membrane. To compare the susceptibility of human endocervix and ectocervix to HIV-1 applied to the epithelial surface, we prepared polarized cervical explants in a transwell system. HIV_{BaL} was added to the upper chamber of the transwell to mimic mucosal infection. Viral replication

American Journal of Reproductive Immunology **71** (2014) 252–264

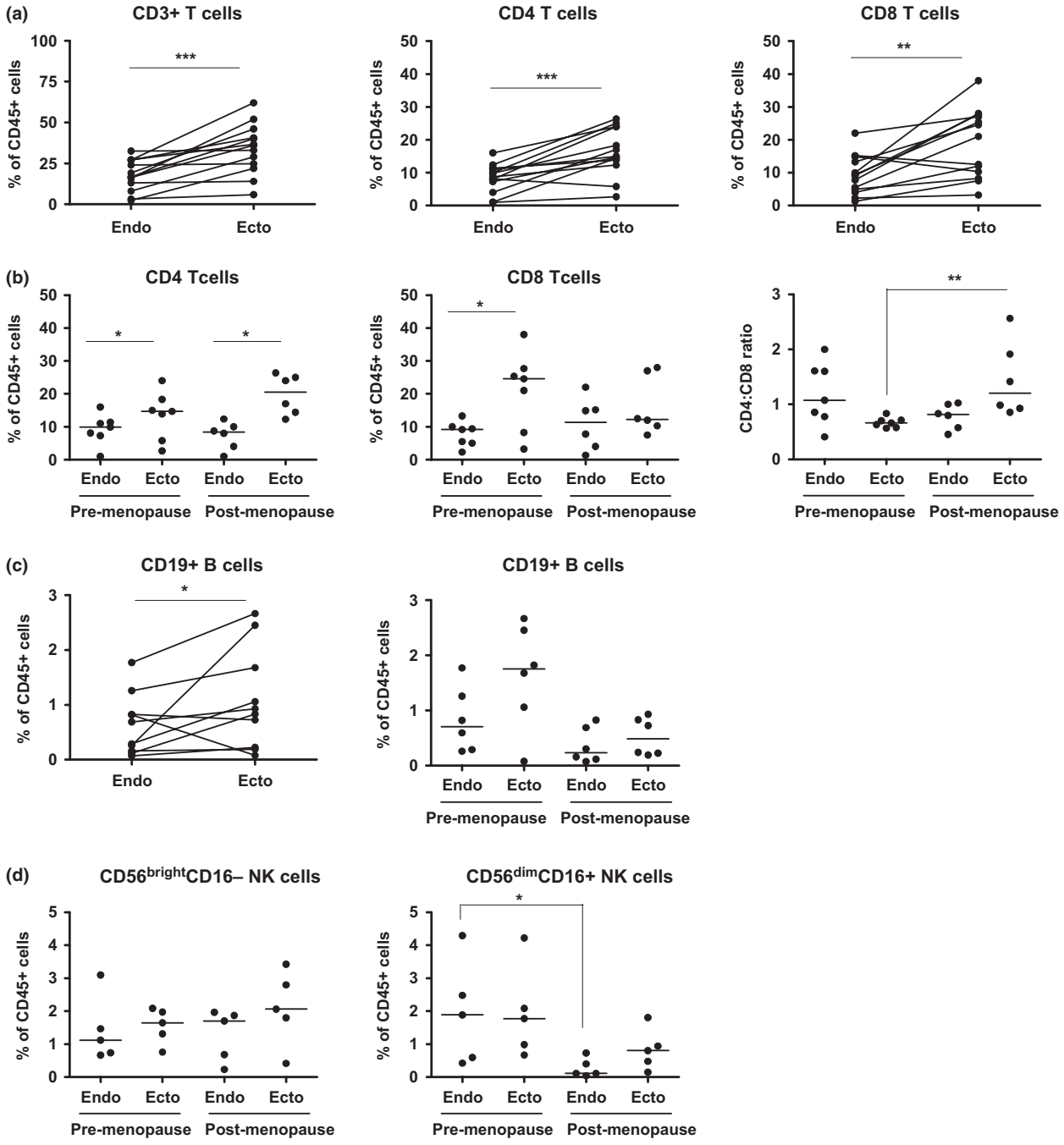


Fig. 3 Comparison of immune cell subsets in the human endocervix and ectocervix. Graphs show the percentage of T cell subsets (a, b), B cells (c), CD56^{bright} CD16^{-} and CD56^{dim} CD16^{+} NK cells (d) within all CD45^{+} cells (n = 13 in a, b; n = 12 in c; n = 10 in d). *P < 0.05, **P < 0.01, ***P < 0.001 by Wilcoxon matched paired test for comparing the endocervix and ectocervix and by Mann–Whitney test for comparing pre-menopausal (n = 7 in a, b; n = 6 in c; n = 5 in d) and post-menopausal subjects (n = 6 in a, b; n = 6 in c; n = 5 in d).

in the tissue was assessed by measuring secretion of p24 antigen by ELISA into the lower chamber of the transwell for 1 week post-infection, when the tissues remained largely viable. Two to four pieces of endocervix and ectocervix were polarized for each of six donors. Despite variations also between

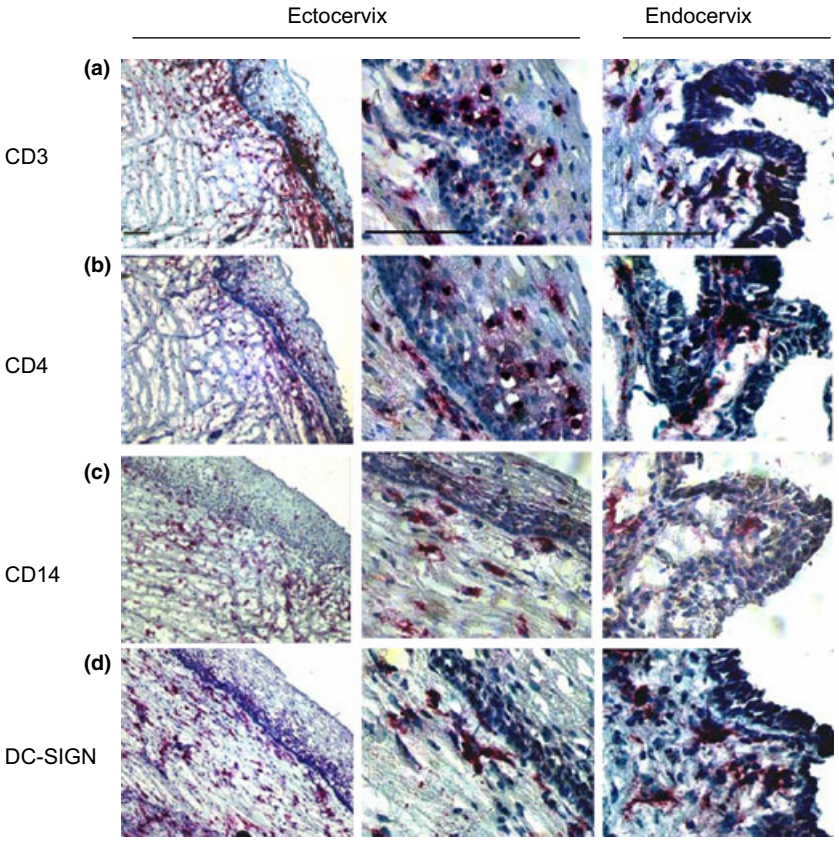


Fig. 4 Location of HIV-1-susceptible immune cells in the human ectocervix and endocervix by immunostain for T cell markers, CD3 (a) and CD4 (b), for $CD14^+$ antigen-presenting cells (APCs) (c) and for the stromal DC marker DC-SIGN (d) using fast red (red) and hematoxylin nuclear counterstain (blue), scale bar, 100 μm.

individual pieces of tissue from the same donor (Fig. 5a), viral replication on average was consistently higher in the endocervical fragments than in the ectocervix for each donor (Fig. 5b). The difference in p24 antigen secreted by endo- and ectocervix was statistically significant ($P < 0.05$ at each time point tested). Thus, although the endocervix has fewer susceptible $CD4^+$ T cells than the ectocervix, it is more susceptible to HIV-1 infection in polarized explant cultures.

Discussion

We present here the first study showing simultaneous analysis of all major types of immune cells in the human cervix, using an optimized way to retrieve immune cells from the mucosa that balances the conflicting requirements for efficient liberation from the tissue to enhance cell yields with the need to protect cell surface markers used for immunophenotyping from protease digestion. We also compared immune cell composition in the endocervix and ectocervix and in pre-menopausal and post-menopausal donors because hormonal status can influence the immune cell composition in the genital tract and it changes during menstrual cycle and pregnancy.[1] One surprising finding in our study was the relative abundance of $CD14^+$ cells, which represented on average about half of the hematopoietic cells in the tissue. Previous studies suggested that only ~10% of mononuclear cells in the cervix are macrophages.[1,5] This difference could be due to different methods of tissue digestion and efficiency of cell retrieval because myeloid cells might be more difficult to release from the stroma. The abundance of $CD14^+$ cells was confirmed by immunostaining. They were mostly found in the lamina propria. Most $CD14^+$ cells were conventional $CD11c^-$ macrophages, but an unexpectedly large subset (about a third) were $CD11c^+$, which may represent newly recruited

American Journal of Reproductive Immunology **71** (2014) 252–264

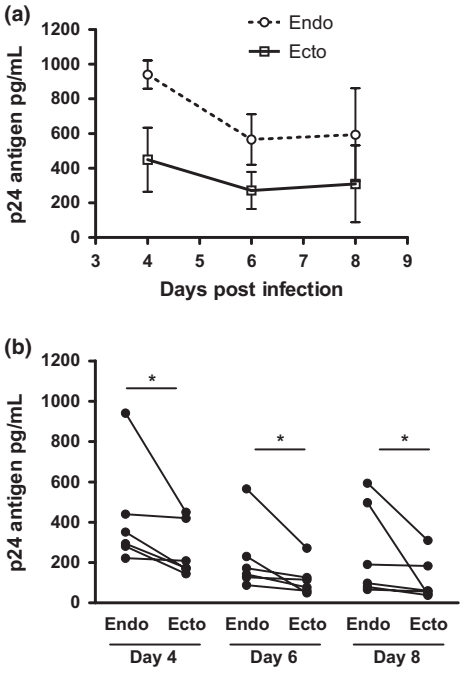


Fig. 5 HIV-1 infection of polarized human endo- and ectocervix. Polarized cervical explants were exposed to HIV_{Bal}, and viral replication was assessed by measuring p24 antigen secreted into the lower chamber post-infection. Graphs in (a) depict the mean and standard error of the mean for data from individual polarized pieces of endocervix (dotted line, $n = 3$) and ectocervix (solid line, $n = 3$) for a single representative donor and (b) shows the mean values data for all six donors. Lines in (b) connect the values for individual donors (*$P < 0.05$, Wilcoxon matched paired test).

monocytes and/or monocyte-derived tissue DCs. Most of these cells in the cervix were $CD103^-$ $CD11b^+$ $CX3CR1^+$ and likely correspond to the subset of lamina propria DCs described in the gut that are monocyte derived and play a role in sampling luminal antigens.[25,26] A recent study shows that trafficking to the lymph nodes of these $CX3CR1^+$ DCs in the gut is inhibited by microbiota in a MyD88-dependent manner to restrict responses to luminal antigens.[27] This subset of DCs had not been described before in the cervix, which is colonized by commensal bacteria, and it might play an important role in the FGT. The abundant population of $CD14^+$ $CD11c^+$ cells in the cervix also express the HIV-binding receptor DC-SIGN. This was confirmed by immunostaining, and previous studies also demonstrate that DC-SIGN was highly and broadly expressed on cells in the lamina propria of both the human endocervix and ectocervix.[28] This is important for HIV-1 transmission because DC-$SIGN^+$ DCs can mediate transfer of the virus to T cells and dissemination to lymphoid tissue.[29–31] Compared with the $CD14^+$ $CD11c^+$ cells, there were about 10-fold fewer lin-$CD14^-$ conventional mDCs and pDCs in the cervix. The numbers of mDCs and pDCs varied between samples, but rarely exceeded 1% of the hematopoietic cells.

The other major population was T cells, which on average comprised 36% of tissue mononuclear hematopoietic cells. We found more T cells in the ectocervix than the endocervix ($P < 0.001$). While CD4 T cells greatly outnumber CD8 T cells in the blood, in the cervix the CD4/CD8 ratio tended to be close to one or reversed, although there was much subject variability. An over-representation of tissue $CD8^+$ T cells has also been reported previously.[4] However, our results demonstrate that this finding was mostly limited to the ectocervix of pre-menopausal women. T cells accumulated on both sides of the basement membrane: many were intra-epithelial and many were organized in aggregates close to the epithelial papillae consistent with previous reports.[4,6] These T cell aggregates might be important sites of HIV amplification during transmission, because they probably represent aggregates of previously activated T cells.[32] Most of the T cells in the cervix were of memory or effector memory phenotype, which did not express CCR7 and expressed varying levels of the HIV coreceptors, CCR5 and CXCR4, consistent with previous studies.[5,33]

Both 'cytokine-producing' $CD56^{bright}$ $CD16^-$ and 'cytotoxic' $CD56^{dim}$ $CD16^+$ NK cells were present at low numbers. Previous reports suggested that $CD16^-$ NK cells are prevalent in the upper FGT where they have been associated with maintaining immune tolerance toward the developing fetus, while $CD16^+$ NK cells are mainly found in the lower FGT.[34] Interestingly, we found slightly more cytotoxic NK cells in the cervix of pre-menopausal compared with post-menopausal women, which together with the decreased CD8 T cell and B cell numbers suggest reduced immune defense in the cervical mucosa post-menopause. The presence of NK cells in the cervix is important as recent studies have demonstrated a role of NK cells in inhibiting HIV-1 infection.[35,36] Among the $CD16^-$ NK cells, a variety of innate lymphocytes that have a cytokine secretion profile and functional properties corresponding to different functional subsets of T cells have been recently described in the gut mucosa.[24] We looked in the cervix at the presence of NK-22 innate

lymphocytes, which secrete IL-22 and appear to be important in preventing bacterial invasion in the gut mucosa.[24] We detected NK-22 cells at low frequency among $CD3^-$ lymphocytes, but we did not find a difference between the endocervical compartment, which is largely free of bacteria, and the ectocervix, which is colonized by bacterial flora.

Despite the small sample size, we found significant differences in the distribution of T and B lymphocytes and NK cells in the endocervix and ectocervix based on menopausal status. A limitation of the study is that the women undergoing hysterectomy tend to be older and some of the subjects determined as pre-menopausal might actually be perimenopausal. Our data provide new findings that could be further investigated in a study including a larger number of samples collecting information on additional clinical factors, including hormonal status, sexual activity, sexually transmitted infections, tobacco use, and use of intra-uterine devices, which might influence immune cell composition in the genital tract.

One of our goals in this study was to obtain information that would be useful for understanding sexual transmission of HIV. The dominant model of HIV transmission comes from studies of SIV in non-human primates. However, results about transmission of SIV in Rhesus macaques may not translate into what happens during HIV transmission in women. One important difference between SIV and HIV is that HIV is restricted from replicating efficiently in resting T cells, macrophages, monocytes, and dendritic cells by the host factor SAMHD1,[37] but because the SIV vpx protein causes SAMHD1 degradation, SIV can bypass this restriction and infect these cells. Thus, the cells involved in the initial infection and the early innate immune response to the virus could be different. An unresolved issue in the mucosal transmission of HIV-1 is the role of myeloid cells. A recent study suggests that human intestinal DCs can migrate toward R5 HIV-1 and capture the virus.[38] Our results reveal the abundance of $CD103^-$ $CD11b^+$ $CX3CR1^+$ DCs in the cervix, which might be important for HIV-1 transmission, because the fractalkine receptor CX3CR1, which promotes the formation of dendrites projecting to the lumen,[39] is also an HIV-1 fusion coreceptor.[40] We also demonstrate that these cells express the HIV-1-binding receptor DC-SIGN and are $CD4^-$dim. Thus, these mucosal DCs could be important in the human FGT either in sensing HIV and inducing an adaptive immune response or in transferring virus to T cells in the tissue.

Another controversial issue in HIV transmission is the preferential site of HIV infection in the human FGT. Although the ectocervix is more subject to infection and trauma that could disrupt the integrity of the mucosal surface, it has a stratified epithelium that might provide a more effective barrier to infection than the single layer of epithelial cells in the endocervix. However, the endocervix is covered with mucus that could provide a physical barrier to trap pathogens and might also contain natural antimicrobial agents. In experimental SIV infection, atraumatic vaginal exposure initially leads to infection of CD4 T cells in the endocervix, which is more susceptible to transmission than the lower FGT.[7,8] A recent study using fluorescently labeled virus in rhesus macaque and unpolarized human cervical explants demonstrates, however, that HIV-1 penetrates at a similar frequency both the columnar epithelium of the endocervix and the squamous epithelium of the ectocervix and vagina.[41] This study also demonstrated that the virus can be bound within the mucus coating of the endocervix to prevent easy penetration of the thin columnar epithelium in rhesus macaque and in unpolarized human cervical explants, while it penetrates within the squamous epithelium to reach susceptible DCs and T cells.[41] Another study that compared HIV infection of unpolarized ectocervical and endocervical explants in solution found that the ectocervix was more susceptible to HIV infection.[42] Here, using polarized explants, which likely better mimic the *in vivo* situation, we found that HIV replicated better in the endocervix than in the ectocervix for all six donors tested. Our results would suggest that the nature of the physical barrier, rather than the numbers of susceptible cells, is the key determinant. In the ectocervix, HIV needs to pass through many layers of epithelial cells to reach susceptible cells, while in the endocervix, susceptible cells are very close to the surface.[43] Results obtained in explant studies, however, need to be interpreted cautiously, because explants are poor substitutes for the complex constellation of events that occur during *in vivo* transmission when other factors (including semen, vaginal flora, pH) come into play. Despite the limitations of the *ex vivo* explant model, our data clearly suggest that the columnar epithelium of the upper genital tract, including the endometrium, might be more vulnerable to infection

American Journal of Reproductive Immunology **71** (2014) 252–264

than the intact squamous epithelium of the lower genital tract.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Analysis of the effect of collagenase digestion in serum-free medium on cell surface staining of NK cells in representative human PBMCs and cervical samples. NK cell subsets were identified as $CD3^-$ $CD4^-$ (left) according to expression of CD56 and CD16 (right). Samples were from ectocervix (a) and untreated (b) or collagenase-treated (c) PBMCs. Collagenase treatment reduced CD4 fluorescence and eliminated CD56 staining.

Figure S2. Identification of NK-22 cells in human cervix. Representative flow cytometry analysis of NKp44 expression among $CD3^-$ $CD56^+$ cells in the human cervix (a). Graph represents NK-22 cells as a percentage of $CD3^-$ cells in the endocervix and ectocervix ($n = 9$). The box extends from the 25th to 75th percentiles and the whiskers present the maximum values (b).

Table S1. Immune cell populations in individual human ectocervical samples.

Table S2. Phenotype of CD4+ and CD8+ T cells ($n = 4$) and chemokine receptor expression ($n = 6$) in human ectocervix.