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Coexpression of CCR5 and IL-2 in Human Genital But Not Blood T Cells: Implications for the Ontogeny of the CCR5$^+$ Th1 Phenotype[1]

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Memory T cells that home to inflamed tissues typically express the β-chemokine receptor CCR5 and exhibit a Th1 cytokine profile. The migration of these cells into the genital tract following antigenic exposure has particular relevance to acquisition of HIV-1 infection, because CCR5 functions as the coreceptor for most sexually transmitted HIV-1 strains. We recently established methodology to purify and culture mononuclear cells from the female reproductive tract, and here we analyzed the phenotype, CCR5 expression, and cytokine production of cervicovaginal T cells in up to 16 donors. The proportion of mucosal T cells expressing CCR5 was markedly expanded as compared with peripheral blood (mean 88% vs 24% in 13 donors), but the receptor density on individual CCR5$^+$ T cells was only slightly increased (mean 5837 vs 4191 MEPE (molecules of equivalent PE) units in 6 of 7 donors). Intracellular costaining for IL-2, IFN-γ, IL-4, and IL-5 revealed a Th1-type pattern in cervical T cells, with significantly higher percentages of IL-2- and IFN-γ-producing T cells in the mucosa than in blood (mean 67% vs 29%). Coexpression of surface CCR5 with intracellular IL-2 and IFN-γ was observed only among T cells in the mucosa, but not among those in circulation. Thus, we postulate that T cell homing to the genital mucosa leads to differentiation into the combined CCR5$^+$ Th1 phenotype. Moreover, the predominance of CCR5$^+$ Th1-type T cells in normal cervical mucosa provides targets accessible for the efficient transmission of macrophage-tropic HIV-1 variants in women following sexual exposure. ***The Journal of Immunology*****, 1999, 163: 2306–2313.**

Leukocyte migration is controlled by the combined actions of various adhesion molecules (1, 2) as well as chemotactic cytokines (chemokines) and their receptors (3–6). As the mechanisms of chemokine action on circulating leukocytes unfold (7), it appears that individual chemokines attract particular leukocytes based on ligand specificity and expression patterns of the relevant receptors. For instance, the chemokine receptor CCR5 is expressed on activated and memory T cells (8), and the proportion of CCR5-expressing T cells is markedly increased in certain inflammatory lesions as compared with peripheral blood (9–11). Thus, CCR5 expression and local chemokine production may regulate recruitment of CD45RO$^+$ memory T cells to inflammatory reactions such as vaginitis or cervicitis, colitis, and rheumatoid arthritis, as well as tissues that frequently encounter a variety of environmental Ags. Moreover, expression of CCR5 may direct Ag-specific memory T cells to genital, intestinal, and airway mucosae where control of infection and prevention of diseases depend on continuous replenishment with immune effector cells.

Both in mice and humans, Th cells have been subdivided into Th1 cells secreting IL-2 and IFN-γ; Th2 cells secreting IL-4, IL-5, and IL-10, and Th0 cells secreting various cytokine combinations of the two subtypes (12–14). Like CCR5$^+$ T cells, Th1 cells are believed to preferentially home to inflammatory sites, with the exception of parasite-induced and allergic infiltrates where Th2 cells predominate (15, 16). The assumption that CCR5$^+$ and Th1 T cells share homing properties and represent an identical cell type is supported by the recent finding that Th1 clones or T cells polarized in vivo toward the Th1 phenotype characteristically express high levels of CCR5 (17, 18). Concomitant expression of CCR5 and secretion of Th1 cytokines by T cells may occur during circulation or after homing to tissue. However, the sequence of events leading to the differentiation of the CCR5$^+$ Th1 T cell phenotype remains unclear. Thus, one of the goals of this study was to understand this process by comparing the phenotypes of blood and genital mucosal T cells.

Regulation of the CCR5$^+$ Th1 phenotype in the genital tract has particular relevance to acquisition of HIV-1 infection by sexual contact. CCR5 functions as the major coreceptor for HIV-1 strains transmitted sexually (19–22). Moreover, HIV-1 preferentially infects CD4$^+$ T cells with the Th1 phenotype (23, 24). Thus, infiltration into the genital mucosa of CD4$^+$ Th1 cells which bear the HIV-1 coreceptor as a result of local environmental stimuli as well as sexually transmitted infections provides susceptible target cells for HIV-1 infection. To better understand host factors associated with HIV-1 transmission, here we present an extensive phenotypic analysis of T cells isolated from normal human cervicovaginal mucosa. By correlating CCR5 expression and cytokine production on a single cell level and comparing those findings with T cells from peripheral blood, we provide evidence that the link between CCR5 expression and Th1 phenotype is only established when T cells home to the genital mucosa and does not exist in peripheral blood.

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Materials and Methods

Isolation of mucosal and blood mononuclear cells

Blood and tissue blocks containing portions of ectocervix or vagina were obtained from women undergoing hysterectomy or vaginal repair operation at the University of Washington Medical Center and affiliated hospitals. Subjects were either at low risk for HIV-1 infection or tested HIV-1-negative and had no cervicovaginal inflammation when examined preoperatively. The University of Washington Human Subjects Committee approved the study, and volunteers provided written consent before the procedure.

Immediately after sampling, the tissue was placed in a sterile specimen container holding 50 ml of culture medium (RPMI 1640 supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2.5 μg/ml amphotericin B, 2 mM L-glutamine (BioWhittaker, Walkersville, MD), and 10% FBS (Gemini, Calibasas, CA)) and transported on ice to the laboratory. Processing began within 2 h of collection. After washing the sample five times in cold PBS, a maximum of 2×2 cm (cervix) or 4×4 cm (vagina) of mucosa per donor were dissected from the underlying tissue. The mucosa was then placed in culture medium containing 1μM DTT (Sigma, St. Louis, MO) for 10 min at 37°C to dissolve mucus and cleave off loose epithelial cells. The mucosa was placed in sterile water for 30 s to lyse contaminating blood cells remaining on the surface of the tissue, extensively washed in cold PBS, and cut into pieces <1 mm with a sterile razor. Tissue pieces were transferred to T75 flasks (Costar, Cambridge, MA) and washed five times to remove loose epithelial cells and remaining contaminating blood cells by adding cold PBS. After the last wash, medium was added very gently, the flasks were transferred to an incubator, carefully avoiding agitation to prevent liberation of epithelial cells, and the tissue was incubated at 37°C and 5% CO_2. After 36 h, Collagenase D (2 mg/ml, Boehringer Mannheim, Indianapolis, IN) was added for 10 min at 37°C to reduce trapping of emigrated cells within collagenous debris. The flasks were slowly tilted, the tissue pieces were allowed to settle for ~10 min, and the supernatant containing the emigrated cells, both intraepithelial and lamina propria, was carefully harvested. The cell suspension was then underlayered with Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) and centrifuged for 20 min at $895 \times g$. Cells collected at the interface were washed three times and counted by trypan blue (Sigma) exclusion under light microscopy. Lymphocyte yields were $0.3–3 \times 10^6$ for cervical epithelium and $1–10 \times 10^6$ for vaginal epithelium with >95% viability and <5% contamination with epithelial cells. PBMC from the same donors were isolated by Ficoll-Hypaque density centrifugation, and washed, and counted as above.

Flow cytometry analysis of phenotypic markers

To define phenotypic properties of the mucosal lymphocytes, cells were reacted in V-bottom 96-well plates (Costar) with combinations of the following mAbs: anti-CD3 PE-Cy5, anti-CD4 FITC, anti-CD8 PE-Cy5, and anti-CD19 PE (all from Sigma); anti-CD4 PE, anti-CCR5 FITC, anti-CXCR3 PE, and anti-CCR5 PE (all from PharMingen, San Diego, CA); anti-CD49d and anti-CD62L PE (both from Coulter, Hialeah, FL); anti-CD25 PE (Biosource, Camarillo, CA); anti-CD28 PE, anti-CD38 PE, anti-CD44 FITC, anti-CD69 PE, anti-TCR$\alpha\beta$ FITC, and anti-TCR$\gamma\delta$ PE (all from Becton Dickinson, San Jose, CA); anti-CD45RA PE, anti-CD45RO FITC, anti-CD45RO PE, and anti-CD103 FITC (all from Dako, Glostrup, Denmark); and anti-CCR3 (clone 7B11, obtained through the AIDS Research and Reference Reagent Program from LeukoSite, Cambridge, MA). For conjugated Abs, a minimum of 4×10^4 cells were incubated for 20 min at 4°C in 100 μl of PBS supplemented with 1% BSA (Sigma) and 0.1% sodium azide (Sigma) (FACS buffer) containing 10% normal mouse serum (Sigma) and a combination of three Abs conjugated to three different fluorochromes (each 5–10 μg/ml) or matching isotype controls (Becton Dickinson or Coulter). Cells were washed twice with FACS buffer to remove unbound Abs and fixed in 0.5% para-formaldehyde (Baker, Phillipsburg, NJ). For the two unconjugated Abs, cells were first reacted for 20 min at 4°C in FACS buffer containing 10 μg/ml of either anti-CD49d, anti-CCR3, or matching isotype control and washed twice. Cells were then resuspended in 100 μl of FACS buffer containing 10% normal goat serum and 5 μg/ml PE-labeled goat anti-mouse IgG (Fc) $F(ab')_2$ (Coulter), incubated for another 20 min at 4°C, and washed twice. Finally, cells were resuspended in FACS buffer containing 10% normal mouse serum and a combination of two Abs or isotype controls (5–10 μg/ml) conjugated to FITC and PE-Cy5, washed twice, and fixed for analysis. Cells were analyzed on a Calibur flow cytometer (Becton Dickinson) for three-color fluorescence. Scatter gates were chosen to acquire only small cells with low granularity, which were >95% lymphocytes, and to exclude larger and more granular cells, which were predominantly nonlymphoid mononuclear cells and epithelial cells. Isotype controls were used to define background staining and quadrant markers. Cells stained with only one specific Ab were used to compensate for overlapping signals between different fluorescence channels.

Quantification of surface molecule expression by flow cytometry

Cells were incubated for 40 min at 4°C in FACS buffer containing 10% normal mouse serum and a saturating amount of anti-CCR5 PE (PharMingen) or matching isotype control in combination with anti-CD3 PE-Cy5, washed twice to remove unbound Abs, and fixed in 0.5% para-formaldehyde. Receptor density per cell, designated as molecules of equivalent PE (MEPE),[3] was calculated by comparing staining of samples to a standard curve. Individual standard curves were established for each experiment by analysis of a mixture of six calibrated bead populations containing six different MEPE levels/bead (RCP-30-5, Spherotech, Libertyville, IL), plotting the fluorescence intensity of each bead population against its designated MEPE value, and interpolating between points by linear regression. Differences between MEPE values of circulating and mucosal T cells were tested for significance using the student *t* test for paired samples.

Intracellular cytokine staining

Mucosal cells and PBMC were stimulated immediately after isolation with 50 ng/ml PMA and 1 μM ionomycin (both from Sigma) in the presence of 2 μM monensin (GolgiStop; PharMingen) in culture medium for 4 h at 37°C and 5% CO_2. After stimulation, cells were stained for surface markers using anti-CD3 PE-Cy5, anti-CD4 PE-Cy5 (PharMingen), anti-CCR5 PE, and isotype controls as described above, then fixed and permeabilized (Cytofix/Cytoperm Kit, PharMingen). Cells were then reacted in 100 μl of FACS buffer for 30 min at 4°C with various combinations of anti-IL-2 FITC (2 μg/ml), anti-IFN-γ FITC (2 μg/ml), anti-IFN-γ PE (1.5 μg/ml), anti-IL-4 PE (1.3 μg/ml), anti-IL-5 PE (1.5 μg/ml), and matching isotype controls (PharMingen). Fluorescence was analyzed on a Calibur flow cytometer as described above. Cells treated with monensin, but not PMA and ionomycin, were always incubated in parallel as controls and never stained positive for IL-2 and only occasionally for IL-4 within the $CD3^-$ but not $CD3^+$ compartment. As previously reported (25), frequencies of IL-4-producing T cells were generally lower than IL-2-producing T cells. However, the utility of the anti-IL-4 Ab used in this analysis was demonstrated by positive staining in the majority of bronchoalveolar mast cells isolated from an allergic control individual.

Results

Lymphocytes isolated from the human cervicovaginal mucosa are predominantly TCR$\alpha^+\beta^+$, CD45RO$^+$ T cells

Following isolation and staining for flow cytometry, two distinct cell populations from the cervicovaginal mucosa were observed in the forward and side scatter plots (data not shown). The larger cells with higher granularity mainly consisted of nonlymphoid mononuclear cells and contaminating epithelial cells (data not shown), whereas the smaller cells with lower granularity represented >90% $CD3^+$ T lymphocytes (Fig. 1*A*). Nearly all T cells expressed TCR$\alpha\beta$ (data not shown) rather than the $\gamma\delta$ TCR (Fig. 1*A*; Table I). $CD8^+$ T cells were generally more common than $CD4^+$ T cells, with a mean CD4/CD8 ratio of 0.83 in 15 patients (Fig. 1*B*; Table I). A small fraction of T cells coexpressed both the CD4 and the CD8 receptor (Fig. 1*B*). The remaining small cells with lower granularity were $CD16^+$ NK cells, ranging from 0 to 19.8% (median 0.6%, $n = 12$), and $CD19^+$ B lymphocytes, ranging from 0.1 to 2.5% (median 0.6%, $n = 12$) (Fig. 1*C*; Table I). Cells isolated from the vaginal and the ectocervical mucosa were not phenotypically different (data not shown).

When analyzed for coexpression of CD45RO and CD45RA, the majority of mucosal T cells expressed only the memory cell marker CD45RO. However, subpopulations of $CD45RA^+$ naive T cells and $CD45RA^+$ $CD45RO^+$ T cells were also consistently found (Fig. 1*D*; Table I). $CD4^+$ T cells were more clearly dominated by the $CD45RO^+$ memory subtype than $CD8^+$ T cells (Fig. 1, *E* and *F*; Table I).

[3] Abbreviation used in this paper: MEPE, molecules of equivalent PE.

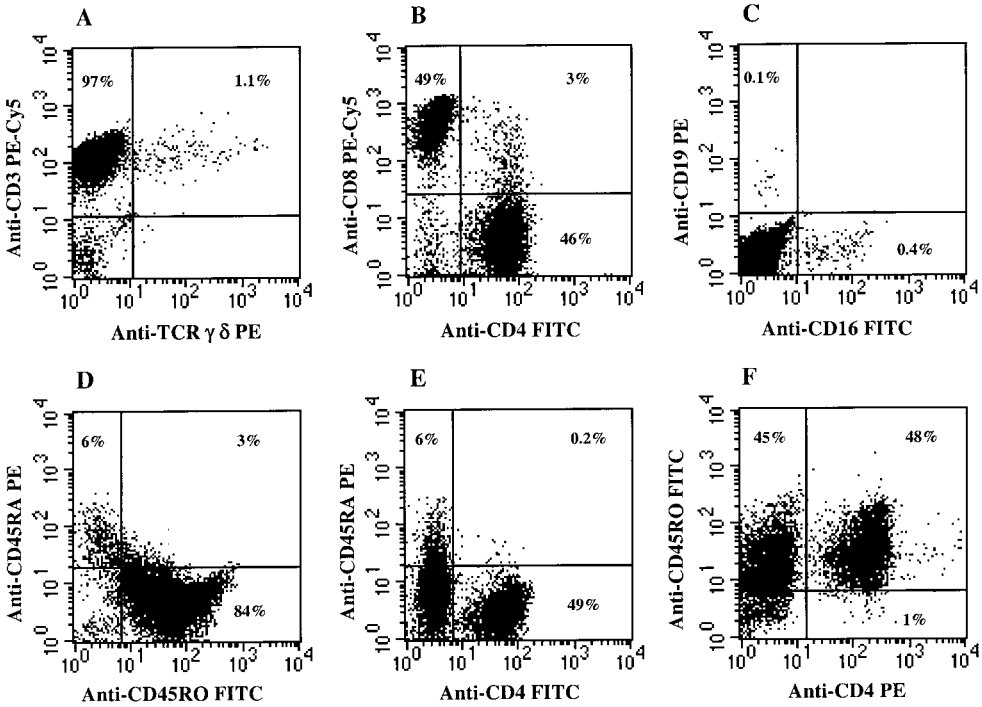

FIGURE 1. T cells with TCR$\alpha\beta$ and memory phenotype predominate in the human female genital mucosa. By flow cytometry, small cells with low granularity corresponding to lymphocytes were gated on the forward and side scatter plot and analyzed for the displayed surface markers. Quadrants were set according to the staining of each isotype control Ab. Percentages of the total cells gated are designated within relevant quadrants. The staining patterns with the indicated fluorescent-labeled Abs are representative of at least four tissue donors.

Most cervicovaginal T cells express early activation markers (CD69$^+$) and mucosal homing receptors

Although the majority of mucosal T cells stained positive for CD69 (mean 94%) and HLA-DR (mean 68%), only a fraction expressed the IL-2 receptor (CD25) and CD38, indicating early but to a lesser extent, late T cell activation (Fig. 2, *A–D*; Table I). Of note, only 12% of CD4$^+$ T cells from peripheral blood expressed HLA-DR (data not shown). The costimulatory molecule CD28 was expressed on almost all mucosal CD4$^+$ cells, but only on about two-thirds of CD8$^+$ T cells (Table I).

When examined, all mucosal T cells expressed CD29, the β_1 subunit of the very late activation (VLA) integrins and CD49d, the α_4 subunit which together with the β_1 subunit forms VLA-4 (Fig. 2, *E* and *F*; Table I). About two-thirds of CD8$^+$ and one-third of CD4$^+$ mucosal T cells also stained positive for CD103, the α_E subunit of the $\alpha_E\beta_7$ integrin (Fig. 2*G*; Table I). In addition, CD44

Table I. *Phenotype of T lymphocytes isolated from the human cervicovaginal mucosa*

	Mean Percentage of Ab-Reactive T Cells[a]					
	CD3$^+$		CD4$^+$		CD8$^+$	
Surface-Marker	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD	*n*
TCR$\gamma\delta$	1.3 ± 0.6	12	ND		ND	
CD4	44 ± 12	15	NA		NA	
CD8	52 ± 15	15	NA		NA	
CD45RA	25 ± 22	11	6 ± 7	4	29 ± 10	4
CD45RO	89 ± 15	10	96 ± 5	7	85 ± 6	7
CD25	19 ± 16	11	25 ± 8	4	9 ± 6	4
CD38	23 ± 21	4	20 ± 23	4	27 ± 20	4
CD69	94 ± 5	7	96 ± 2	4	98 ± 1.7	4
HLA-DR	68 ± 24	8	79 ± 6	2	83 ± 10	2
CD28	79 ± 20	10	96 ± 5	6	67 ± 11	6
CD29	99 ± 0.7	2	98		100	
CD44	98 ± 8	4	97 ± 1.4	4	99 ± 0.6	4
CD49d	97 ± 6	8	96 ± 6	5	98 ± 4	5
CD62L	13 ± 5	9	17 ± 7	6	8 ± 2	6
CD103	52 ± 13	12	32 ± 17	6	63 ± 12	6
CCR5	87 ± 5	16	85 ± 3	4	85 ± 3	4
CCR3	0.5 ± 0.8	4	0.5 ± 0.6	2	0.3 ± 0.4	2

[a] ND, not done; NA, not applicable.

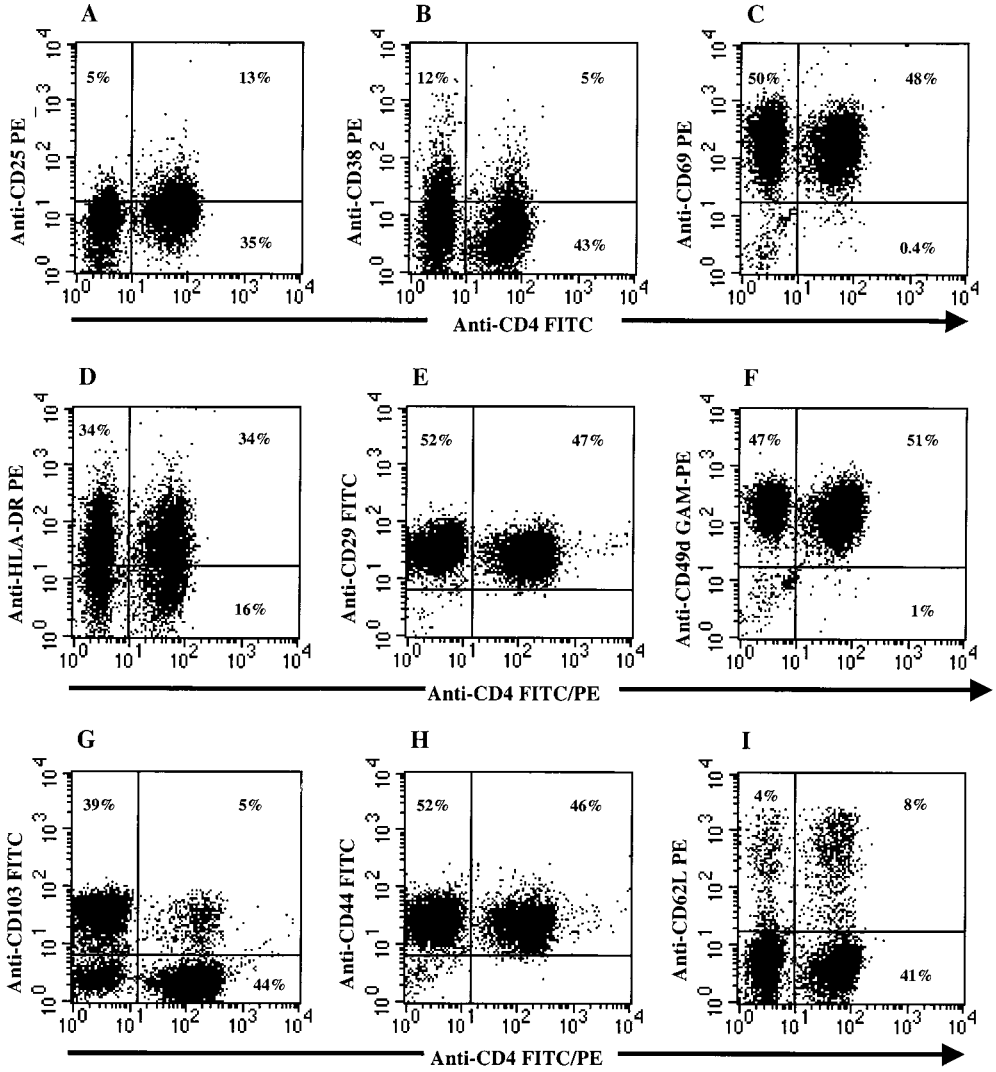

FIGURE 2. Expression of activation and homing receptors on cervicovaginal T cell subpopulations. Expression of various surface markers is displayed on the *y*-axis against expression of CD4 on the *x*-axis. The cell population gated on the forward and side scatter plot was 98% $CD3^+$. Except for CD29 ($n = 2$), the staining shown was representative for at least four tissue donors, and the percentages of total cells gated are designated within relevant quadrants.

expression was abundant (Fig. 2*H*; Table I). These integrins are typically expressed on memory T cells homing to sites of Ag-exposure (VLA-4, CD44) and more specifically to mucosal tissues (CD44, CD103) (26, 27). In contrast, L-selectin (CD62L), preferentially expressed by naive T cells homing to peripheral lymphoid organs (28), was found on only very few of the cervicovaginal T lymphocytes (Fig. 2*I*; Table I). The mucosal T cells were clearly distinguishable from peripheral blood T cells, which expressed high CD62L ligand and low or absent CD103 (data not shown).

The frequency of CCR5-expressing T cells but not the receptor density on individual cells is markedly increased in the genital mucosa as compared with peripheral blood

The percentages of mucosal T cells expressing CCR5 ranged from 78 to 97% (mean 87%) in the 16 tissue donors tested, and no difference was apparent between $CD4^+$ and $CD8^+$ cells in 4 donors tested (Fig. 3*A*; Table I). Both HLA-DR^+ and HLA-DR^- $CD4^+$ mucosal T cells expressed CCR5 (Fig. 3*A*), and nearly all (mean, 87%) $CCR5^+$ $CD4^+$ mucosal T cells were $CD45RO^+$ memory cells (Fig. 3*B*). In contrast, expression of CCR3 on cervicovaginal T cells was not observed (Table I). The proportion of T cells expressing CCR5 was markedly lower in peripheral blood (range 4.2–55%, mean 24%) than in the mucosal compartment (range 81–97%, mean 88%) in 13 individuals ($p < 0.001$) (Fig. 3*C*). We also examined in two donors the expression of the chemokine receptor CXCR3, a T cell marker associated with inflammatory reactions. In these analyses, 20–25% of the mucosal $CD4^+$ T cells expressed CXCR3, and of these, 95–96% coexpressed CCR5 (data not shown).

To examine the possibility that CCR5 expression on mucosal T cells is up-regulated over the 36-h period required to isolate the emigrated cells, we compared receptor expression on peripheral blood T cells just following isolation and after 36 h in culture in five of the tissue donors. CCR5 was significantly down-regulated on blood T cells during culture from a mean of 33% (range 21–55%) to a mean of 13% (range 4–21%) (29), indicating that the high proportion of CCR5-expressing mucosal T cells is unlikely a consequence of in vitro events. We also compared the effect of fetal bovine serum vs human AB serum on expression of CCR5 on mucosal T cells and found no significant differences (data not shown).

Our findings indicate a preponderance of $CCR5^+$ T cells in the lower female genital tract, but it was unclear whether these cells also have a higher density of CCR5 on their surface. To address

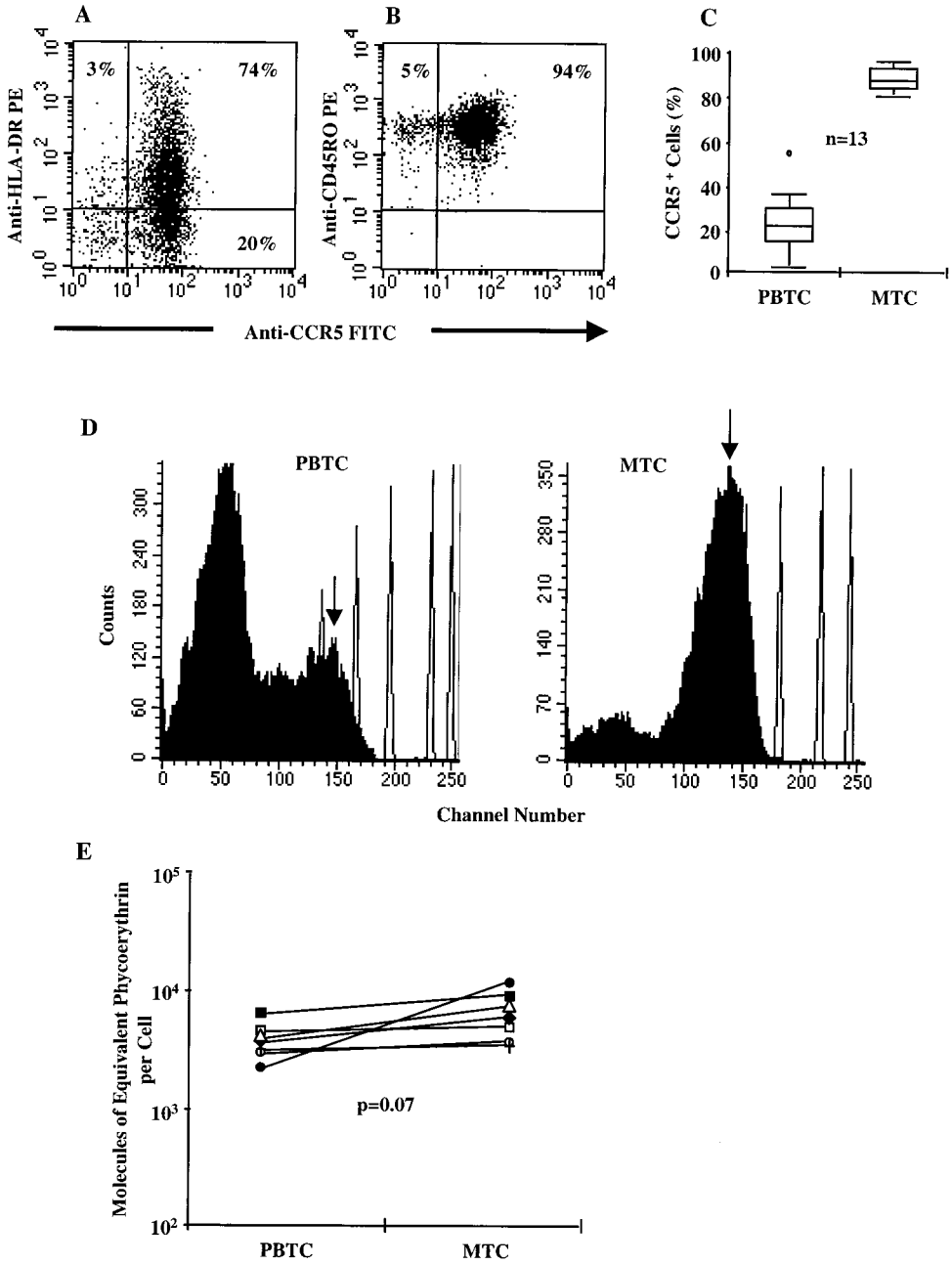

FIGURE 3. CCR5 expression on peripheral blood T cells (PBTC) in comparison to mucosal T cells (MTC). Three color flow cytometry of $CD4^+$ cervical T cells for HLA-DR and CCR5 (*A*) and CD45RO and CCR5 (*B*). *C*, Percent of T cells expressing CCR5 as determined by two color flow cytometry analysis for CD3 and CCR5 in 13 individuals. Box plots depict median (horizontal line within the box), interquartile range (upper and lower limits of box), highest and lowest values (upper and lower horizontal bars outside the box), and one outlier (○). *D*, To determine relative CCR5 receptor density, cells were simultaneously stained with anti-CD3 PE-Cy5 and saturating amounts of anti-CCR5 PE, and a mixture of calibrated PE bead populations (MEPE) were run in parallel. Histograms show the fluorescence intensity for binding of anti-CCR5 PE to CD3-gated T cells in one representative individual. Black arrows indicate the fluorescence intensities for which MEPE values were calculated (see text). Histograms depicting the bead mixture are overlayed as black lines and were used to establish a standard curve for calculation of MEPE values. *E*. MEPE values for CD3-gated T cells in seven individuals tested. The lines connect values from the two sites, peripheral blood and genital mucosa, in each donor.

this, we estimated the CCR5 receptor density on mucosal and peripheral blood T cells from seven donors by comparing their fluorescence intensities with calibrated beads containing known quantities of PE molecules (MEPE) (Fig. 3, *D* and *E*). As shown in one representative donor, anti-CCR5 PE binding curves and peaks for the six bead populations (with six different levels of PE fluorescence) were overlaid, and the arrows depict the level of anti-CCR5 PE binding for which MEPE units per T cell were calculated (Fig. 3*D*). Anti-CCR5 binding curves usually had a broader shape in blood than in mucosal T cells and the MEPE units were calculated within the last distinct peak of the curve. This strategy quantifies CCR5 expression on T cells with high receptor density rather than calculating an average for all CCR5 positive T cells. In comparison to circulating T cells, CCR5 surface receptor density was not significantly up-regulated on mucosal T cells ($p = 0.07$) (Fig. 3*E*). Mean MEPE units were 4191 (range 3009–6468) for circulating T cells and 5837 (range 3511–9365) for cervical T cells in six of seven tissue donors tested. However, one individual

demonstrated a pronounced up-regulation from 2291 MEPE units on circulating T cells to 12050 MEPE units on cervical T cells.

The potential to produce IL-2 and IFN-γ is significantly increased in cervical T cells as compared with peripheral blood

To determine whether T cells infiltrating the human genital mucosa preferentially exhibit the Th1 or Th2 phenotype, we analyzed their ability to synthesize IL-2 and IFN-γ or IL-5 and IL-4, respectively, upon stimulation with ionomycin and PMA (see *Materials and Methods*). One representative comparison is shown in Fig. 4. On the forward and side scatter plots, a small percentage of mucosal T cells (Fig. 4*B*, right arrow) were larger, more granular, and phenotypically activated than the predominant population which we further analyzed here. Among six donors tested, the mean percentage of $CD4^+$ T cells producing IL-2 was clearly higher in the cervical mucosa (mean 61%, range 43–83%) than in peripheral blood (mean 35%, range 17–58%) (Fig. 4, *C* and *D*). Of the larger, more granular mucosal T cells, practically all produced IL-2 (data not shown). Similarly, of the $CD4^+$ T cells derived from the genital mucosa in three of these donors examined, an average of 61% (range 55–70%) produced IFN-γ, in contrast to 12% (range 6–20%) from peripheral blood (Fig. 4*E*). In addition, a mean of 43% (range 32–49%) of $CD4^+$ T cells in the genital mucosa simultaneously produced both, and a mean of 87% (range 77–93%) at least one of the Th1 cytokines (Fig. 4*E*, and data not shown). By contrast, IL-4-producing $CD4^+$ T cells from the six donors were infrequent in both mucosal (mean 7.6%, range 2.6–18%) and blood-derived $CD4^+$ T cells (mean 4%, range 1.1–6.6%) (Fig. 4*F*, and data not shown). Of note, the majority of IL-4-producing mucosal $CD4^+$ T cells were coproducing IL-2 (mean 53%, range 38–64%) and thus corresponded to the Th0 rather than the Th2 phenotype (data not shown). Production of IL-5 never exceeded 2% in both the mucosal and blood derived $CD4^+$ T cells of three donors tested.

IL-2 production and CCR5 expression are correlated in mucosal but mutually exclusive in circulating T cells

Approximately two-thirds of T cells isolated from the genital mucosa either produce IL-2 or IFN-γ or express CCR5, suggesting relatively tight correlation of both traits. However, it is not clear whether differentiation to the combined IL-2^+, $CCR5^+$ or IFN-γ^+, $CCR5^+$ phenotype occurs during circulation or when homing to the genital tract. To address this, we first investigated coexpression of CCR5 and IL-2 (six donors) or IFN-γ (three donors) in peripheral blood T cells (Fig. 4*C*). Interestingly, although appreciable numbers of circulating $CD4^+$ T cells produced IL-2 (mean 35%, range 17–58%) coexpression of intracellular IL-2 and CCR5 was infrequently observed (Fig. 4*C*). On average, only 3% (range 1–6%) of IL-2-producing $CD4^+$ T cells simultaneously expressed CCR5. Similarly, in three donors, only 17% (range 3.5–29%) of the IFN-γ-producing peripheral blood cells coexpressed CCR5. By contrast, examining the mucosal T cells from the same donors, the majority of IL-2- and IFN-γ-producing mucosal $CD4^+$ T cells also expressed CCR5 (mean 88% (range 80–94%) and 82% (range 79–85%), respectively) (Fig. 4*D*). Approximately 100% of the larger and more granular lymphocytes coexpressed intracellular IL-2 and CCR5. Thus, production of Th1-type cytokines and CCR5 expression are usually mutually exclusive in circulating T cells but tightly coupled in mucosal T cells. This finding indicates that differentiation to the complete $CCR5^+$ Th1 phenotype does not occur while in circulation but during or after homing of T cells to the genital mucosa.

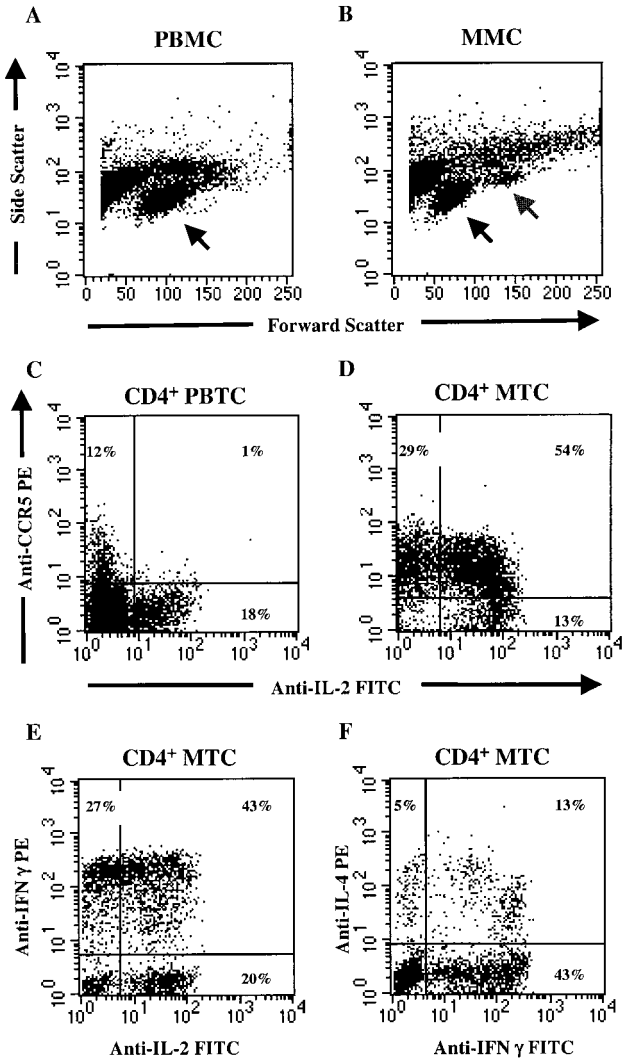

FIGURE 4. Correlation of intracellular cytokine and surface CCR5 expression by flow cytometry in peripheral blood (PBTC) and cervical T cells (MTC) for one representative donor. Flow cytometry forward and side scatterplots of PBMC (*A*) and mucosal mononuclear cells (MMC) (*B*). The arrow (left in *B*) points to the lymphocyte populations gated for the analysis in plots *C–F*. The right arrow points to a separate mucosal lymphocyte population in lower frequency, larger, and more granular. *C* and *D*, Three color flow cytometry analysis for intracellular IL-2 on the *x*-axis and CCR5 on the *y*-axis in $CD4^+$-gated peripheral blood T cells (*C*) and in $CD4^+$-gated mucosal T cells (*D*). *E*, Three color analysis for intracellular IL-2 on the *x*-axis and intracellular IFN-γ on the *y*-axis in $CD4^+$-gated mucosal T cells. *F*, Three color analysis for intracellular IL-4 on the *y*-axis and intracellular IFN-γ on the *x*-axis in $CD4^+$-gated mucosal T cells. Quadrants were established by staining patterns of cells treated with monensin but not PMA and ionomycin, as well as cells reacted with isotype control Abs. Both controls resulted in comparable negative staining patterns. Percentages of $CD4^+$-gated T cells are provided within relevant quadrants.

Discussion

Immune surveillance requires the coordinated interactions of leukocytes with vascular endothelial cells and involves a stepwise procedure wherein engagement of adhesion and signaling receptors allows both Ag nonspecific and specific cells to enter sites of infection or injury. The homing specificities associated with recruitment of lymphocytes to extralymphoid sites such as the gut or cutaneous tissues are well characterized, but as yet less is known

about those associated with the genital tract, particularly in humans. To a large extent, the inability to purify sufficient cell numbers and maintain their functional properties has precluded identification of the phenotype and homing properties of lymphoid populations within the genital mucosa. We believe our approach presented here offers a breakthrough in this respect. Our results demonstrate that the large majority of T cells accessing the human genital mucosa are memory cells exhibiting early but not late activation markers. Thus, these cells may acquire Ag specificity in the genital mucosa while circulating through this microenvironment, but are as yet not fully activated or differentiated to an effector phenotype. T cells in the genital mucosa express the integrins $\alpha_E\beta_7$ and $\alpha_4\beta_1$ that are commonly involved in T cell recruitment to other extralymphoid mucosal sites. In addition, chemokines and their receptors as well as other intercellular adhesion molecules may be involved in T cell trafficking to the genital mucosa.

Of note, our findings indicate that T cells expressing the chemokine receptor CCR5 are abundant in the human female genital mucosa, which stands in marked contrast to the low numbers of CCR5-expressing T cells in the circulation. One possible explanation for these findings is that CCR5 is up-regulated during the 36-h interval used to acquire emigrated T cells from the cervicovaginal tissue in vitro. We believe this is unlikely for several reasons. The time period employed was short, our medium lacks stimulatory agents such as IL-2, PHA, or anti-CD3, the application of similar conditions to isolate peripheral blood T cells actually resulted in down-regulation of CCR5, and only prolonged activation of T cells over many days led to CCR5 up-regulation in vitro in a recent report (10). Thus, our data suggest that memory T cells expressing CCR5 are either selectively recruited to the genital mucosa or they up-regulate CCR5 during extravasation or once localized within the mucosa. That the peak density of CCR5 receptors on individual cells was not significantly different between circulating and mucosal T cells in six of seven individuals argues for a mechanism that selectively recruits CCR5$^+$ T cells, and favorably those with higher receptor densities, to the genital mucosa. In addition, interactions between locally secreted chemokines and the CCR5 receptor may retain T cells in the mucosa during antigenic challenge.

In support of recent reports that chemokine receptors are differentially expressed in Th1 and Th2 cells, our findings in the human genital tract link CCR5 expression with IL-2 and IFN-γ rather than IL-4 and IL-5 production. The frequency of IL-2- and IFN-γ-producing cells paralleled CCR5 expansion and was significantly higher in cervical than in circulating T cells. CCR5 expression in genital tract T cells was therefore coupled with a Th1 (and infrequently Th0) rather than the Th2 phenotype. This was further substantiated by the demonstration that individual mucosal CCR5$^+$ T cells consistently displayed concomitant production of IL-2 and IFN-γ, and that expression of CCR3, associated with the Th2 phenotype (16, 18), was absent. By contrast, CCR5-expressing and Th1 cytokine-producing T cells constitute two separate populations in peripheral blood of the same individuals. This result suggests that differentiation to the Th1 phenotype is coupled with CCR5 up-regulation during or after homing of T cells to the genital mucosa.

If CCR5 is involved in leukocyte recruitment to inflammatory sites, as has been suggested previously (10, 11, 17, 18), then the CCR5$^+$ IL-2$^-$ rather than CCR5$^-$ IL-2$^+$ T cells are more likely to be recruited to the genital mucosa. Local events such as secretion of cytokines and chemokines may subsequently drive differentiation into the combined CCR5$^+$ Th1 phenotype. CCR5 may play a role in this process, which is supported by findings in a murine model system that macrophage inflammatory protein-1α (MIP-1α), a natural ligand of CCR5, drives differentiation from Th0 to Th1 cells (30). Once the link between CCR5 expression and Th1 phenotype is established, it may be maintained by autocrine loops whereupon continued expression of CCR5 depends on the presence of IL-2 (31), and synthesis of MIP-1α, MIP-1β, and RANTES retains CCR5$^+$ cells in the mucosa (32).

The high frequency of CCR5$^+$ T cells in the female genital mucosa may explain the relatively high efficiency of infection of these cells with macrophage-tropic HIV-1 isolates, particularly when conjugated to mucosal dendritic cells. Thus, these T cells may serve as early targets of infection soon after HIV-1 exposure and contribute to the selective expansion of macrophage- and CCR5-tropic HIV-1 strains. Th1-dominated immune responses to coinfections or tissue damage may further enhance the infection locally, which is supported by studies demonstrating increased risk of HIV-1 transmission among women with genital ulcer disease and other sexually transmitted infections (33, 34).

To extend these findings, additional studies now are needed to identify the chemokine and cytokine milieu that regulate chemotaxis and differentiation of these CCR5-expressing, IL-2- and IFN-γ-producing T cells, as well as the induction of Ag-specific immune repertoire in the genital mucosa. Our work establishes the feasibility to address these issues, and clearly new insights may have utility in understanding acquired immunity to HIV-1 and other sexually transmitted diseases.

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