

# HIV-1 sexual transmission: early events of HIV-1 infection of human cervico-vaginal tissue in an optimized *ex vivo* model

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Infection and dissemination of human immunodeficiency virus (HIV)-1 through the female body after vaginal intercourse depends on the activation/differentiation status of mucosal CD4 T cells. In this study, we investigated this status and the susceptibility to HIV-1 infection of human cervico-vaginal tissue *ex vivo*. We found that virtually all T cells are of the effector memory phenotype with broad CC chemokine receptor 5 (CCR5) expression. As it does *in vivo*, human cervico-vaginal tissue *ex vivo* preferentially supports the productive infection of R5 HIV-1 rather than that of X4 HIV-1 in spite of the broad expression of CXC chemokine receptor 4 (CXCR4). X4 HIV-1 replicated only in the few tissues that were enriched in CD27<sup>+</sup>CD28<sup>+</sup> effector memory CD4 T cells. Productive infection of R5 HIV-1 occurred preferentially in activated CD38<sup>+</sup>CD4 T cells and was followed by a similar activation of HIV-1-uninfected (bystander) CD4 T cells that may amplify viral infection. These results provide new insights into the dependence of HIV-1 infection and dissemination on the activation/differentiation of cervico-vaginal lymphocytes.

## INTRODUCTION

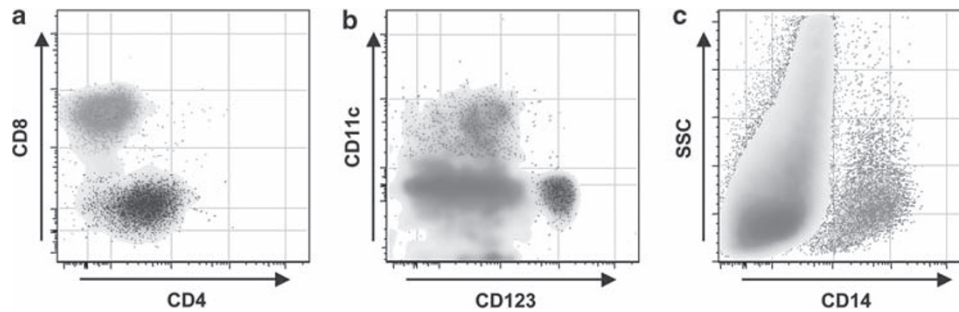
Heterosexual transmission of human immunodeficiency virus (HIV)-1 occurs predominantly through vaginal intercourse,<sup>1</sup> rendering the female lower genital tract mucosa an important gateway for virus entry. At this mucosal site, HIV-1 encounters its target cells, and after an initial local amplification eventually disseminates to the draining lymph nodes, establishing a systemic infection.<sup>2</sup> However, not all HIV-1 variants are capable of establishing such infection. For example, although both CCR5-tropic (R5) and CXCR4-tropic (X4) HIV-1 variants are present in the semen, only the virus using CCR5 but not CXCR4 is apparently transmitted and dominant in the early stages of HIV-1 disease.<sup>3,4</sup> Moreover, data have been recently published on the genetic and phenotypic characteristics of “founder” R5 HIV-1 variants that are capable of being efficiently transmitted and of establishing systemic infection.<sup>5</sup> As the cervico-vaginal tissue is the first gateway for HIV-1 during heterosexual transmission, the lower genital tract may serve as the first “gatekeeper,” conferring protection from at least some HIV-1 variants. Understanding of the “gatekeeping” mechanisms of HIV-1 transmission is critically important for the development of effective HIV-1-preventive measures (microbicides,

virustats, and vaccines). Such an understanding requires a robust knowledge of the phenotypes, and functional features of the cells which in the lower genital mucosa are implicated in the early events of HIV-1 transmission.<sup>6</sup> It seems that CD4 T cells are the predominant target for HIV-1 infection at the mucosal invasion sites.<sup>2,7</sup> Although macrophages and various dendritic cells (DCs) are important for the transmission of HIV-1, in particular by presenting infectious virions to CD4 T cells, the latter seem to be the cells that predominantly support productive HIV infection.<sup>5,8–11</sup> Thus, investigating the pattern of HIV-1 infection in CD4 T cells and characterizing cells that are major viral targets is crucial for our understanding of the mechanisms of HIV-1 transmission in the female genital tract.

In other anatomical sites that are critical for HIV-1 pathogenesis and transmission (lymph nodes and rectal mucosa), cell activation, cell differentiation, and expression of HIV-1 coreceptors on CD4 T-cell subsets have been identified as the most important factors that determine the efficiency of HIV-1 production. In spite of the critical importance of the cervico-vaginal tissue to HIV-1 infection, these key characteristics remain largely unknown for cervico-vaginal T cells. In this study, we report data on these characteristics.

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**Figure 1** Identification of T lymphocytes, dendritic cells, and macrophages in human cervico-vaginal tissue. Cell suspension from digested fresh cervico-vaginal tissue blocks was stained for markers of T lymphocytes, dendritic cells (DCs) (plasmacytoid DCs (PDCs) and myeloid DCs (MDCs)), and macrophages and analyzed using flow cytometry. Bivariate dot plots of the distribution of (a) CD4 and CD8 T cells, (b) PDCs and MDCs, (c) and macrophages are presented. CD4- and CD8-positive T cells were identified gating on CD3<sup>+</sup> lymphocytes. PDCs (CD123<sup>+</sup>) and MDCs (CD11c<sup>+</sup>) were identified gating on cells negative for CD3, CD14, CD16, CD56, CD19, CD20, and positive for HLA-DR. Macrophages (CD14<sup>+</sup>) were identified gating on CD3<sup>-</sup>, CD56<sup>-</sup>, CD19<sup>-</sup>, CD20<sup>-</sup> cells. Presented plots are representative of experiments with cervico-vaginal tissues from 5–12 donors. It must be noted that T lymphocytes, PDCs, MDCs, and macrophages that are involved in human immunodeficiency virus (HIV-1) infection are present in human cervico-vaginal tissue.

We found that in the cervico-vaginal tissue, CD4 and CD8 T cells are predominantly of the highly differentiated effector memory phenotype with a variable pattern of expression of activation markers. Using an *ex vivo* system of cervical tissue<sup>12</sup> that we have now developed further, we identified the phenotype as well as the activation and differentiation status of CD4 T cells that are preferentially infected by HIV-1. We also found that R5 HIV-1 replication in the cervico-vaginal tissue increases the fraction of activated cells that are preferentially infected, creating a vicious circle between R5 HIV-1 replication and the number of cell targets that support this infection. Finally, investigating the gatekeeping mechanism that selects between R5 and X4 HIV-1 infection we found that, in *ex vivo* cervico-vaginal tissue, susceptibility to productive R5 HIV-1 infection is much higher than that to X4 HIV-1. We linked this difference to the particular phenotypic and activation patterns of the CD4 T cells in this tissue.

## RESULTS

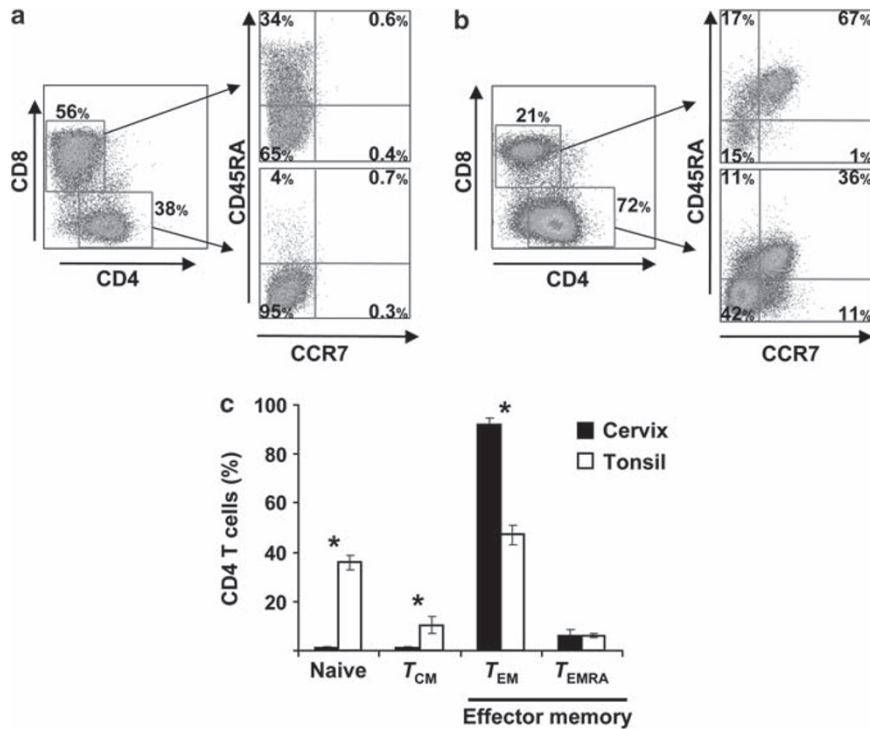
We analyzed various cell populations in human cervico-vaginal tissue. In particular, we focused on T cells which are located in the epithelial layer, lamina propria, or submucosa, and therefore are among the first cells that encounter HIV-1 in vaginal intercourse.<sup>13</sup> Toward this goal, in fresh surgical samples of this tissue, we identified lymphocytic populations from their expression of membrane antigens CD3, CD4, CD8, CCR7, CD45RA, CD27, and CD28, their activation status from their expression of CD25, CD38, CD57, CD69, CD95, and human leukocyte antigen DR (HLA-DR), as well as their potential susceptibility to HIV-1 infection from the expression of HIV-1 coreceptors CCR5 and CXCR4. To study HIV-1 infection in this tissue, we developed an original protocol for its long-term culture. We infected this tissue with R5 and X4 HIV-1, monitored viral replication, identified HIV-1-infected cells and their activation status, as well as the activation status of uninfected (bystander) cells, and evaluated cellular depletion.

### T-cell subsets in human cervico-vaginal tissue

First, using flow cytometry, we evaluated the distribution of cells of various subtypes in the cervico-vaginal tissue (**Figure 1a–c**). On average, T (CD3<sup>+</sup>) cells constitute  $82 \pm 5.4\%$  of total lymphocytes, whereas B cells, as identified from the expression of CD19 or CD20, constitute  $31.5 \pm 1.5\%$  of CD3<sup>-</sup> cells. On average, the cervico-vaginal tissue contains  $684 \pm 100$  T cells (CD3<sup>+</sup>) per milligram of wet tissue ( $n = 4$ ). In addition, we identified plasmacytoid DCs and myeloid DCs and calculated that for per milligram of wet tissue, there are on average  $96.1 \pm 22$  and  $80.7 \pm 39$  ( $n = 5$ ) of these, respectively. In addition, we found that there are on average  $235 \pm 92$  ( $n = 5$ ) macrophages per milligram of wet tissue (**Figure 1b and c**).

We compared the distribution of T cells in the cervico-vaginal tissue with that in lymphoid (tonsillar) tissue. Both CD4 and CD8 T cells accounted for almost the entire CD3<sup>+</sup> cell subset in the cervico-vaginal tissue ( $46 \pm 4.5\%$  and  $52 \pm 3.8\%$ , respectively,  $n = 12$ ), with an average CD4:CD8 ratio of  $1.1 \pm 0.1$  (**Figures 1a and 2a**). In contrast, in tonsillar tissue, there were  $133,747 \pm 32,457$  T cells (CD3<sup>+</sup>) per milligram of wet tissue. B cells constituted  $97.7 \pm 0.3\%$  of CD3<sup>-</sup> cells. CD4 T cells represented the predominant T-cell sub-population, with a CD4:CD8 ratio of  $4.4 \pm 0.5\%$  ( $n = 5$ ).

Second, to characterize the phenotypes of CD4 and CD8 T-cell sub-populations in the cervico-vaginal tissue, we investigated the distributions of naive (CCR7<sup>+</sup>/CD45RA<sup>+</sup>), central memory (T<sub>CM</sub>; CCR7<sup>+</sup>/CD45RA<sup>-</sup>), effector memory (T<sub>EM</sub>; CCR7<sup>-</sup>/CD45RA<sup>-</sup>), and terminal effector memory (T<sub>EMRA</sub>; CCR7<sup>-</sup>/CD45RA<sup>+</sup>) cells<sup>14</sup> (**Figure 2a**). We found that in the cervico-vaginal tissue,  $92 \pm 3\%$  of total CD4 T cells were T<sub>EM</sub> and  $6 \pm 2.4\%$  were T<sub>EMRA</sub>, whereas  $0.8 \pm 0.5\%$  and  $1 \pm 0.3\%$  were naive and T<sub>CM</sub> ( $n = 11$ ), respectively (**Figure 2c**). Similar fractions of naive and T<sub>CM</sub> cells were found among CD8 T cells ( $1.3 \pm 0.6\%$  and  $0.5 \pm 0.2\%$ , respectively;  $P > 0.05$ ), whereas the fractions of T<sub>EM</sub> and T<sub>EMRA</sub> among CD8 T cells ( $69 \pm 5.5\%$  and  $29 \pm 5\%$ , respectively) were significantly different from those in CD4 T cells ( $n = 11$ ,  $P < 0.0003$ ). In the tonsillar tissue, naive, T<sub>CM</sub>, T<sub>EM</sub>, and T<sub>EMRA</sub> cells constituted  $35.8 \pm 3.1\%$ ,  $10.3 \pm 3.4\%$ ,

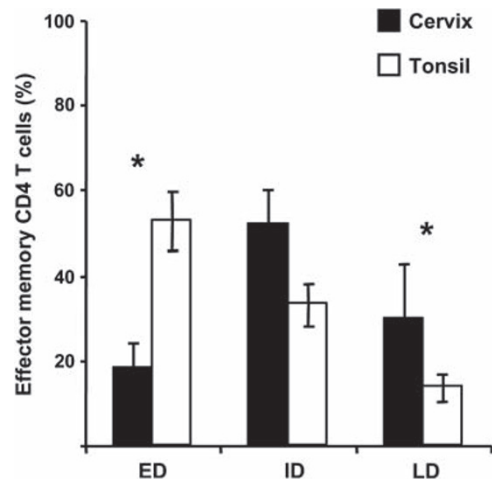


**Figure 2** T-lymphocyte sub-populations in human cervico-vaginal and tonsillar tissues. Cell suspension was stained for lymphocytic markers and analyzed using flow cytometry. Bivariate dot plots of the distribution of different T-lymphocyte sub-populations for (a) cervico-vaginal and (b) tonsillar tissues are presented. For each tissue, the distribution of CD4 and CD8 T cells is displayed in the left panel. The distribution of naive, T<sub>CM</sub>, T<sub>EM</sub>, and T<sub>EMRA</sub> sub-populations among CD4 and CD8 T cells is displayed in the upper right panel and in the lower right panel, respectively. Presented plots are representative of experiments with cervico-vaginal tissues obtained from 11 donors and tonsillar tissues from 5 donors. (c) Mean±s.e.m. of the fractions of naive, T<sub>CM</sub>, T<sub>EM</sub>, and T<sub>EMRA</sub> among CD4 T cells from these tissues are presented. \*Indicates a statistically significant difference with *P*<0.05. It must be noted that the distribution of naive, T<sub>CM</sub>, and T<sub>EM</sub> CD4 T cells in cervico-vaginal tissues is different from that in tonsillar tissues.

47±5.8%, and 6±0.7%, respectively, of CD4 and 62.8±3.2%, 5±0.4%, 20.2±4.6%, and 13.8±3.6%, respectively of CD8 T cells (*n*=5) (Figure 2b). Thus, in the CD4 T-cell subset, the fractions of naive, T<sub>CM</sub>, and T<sub>EM</sub> cells were significantly different between the cervico-vaginal and tonsillar tissues (*P*<0.03) (Figure 2c).

As we found that the majority of T cells in the cervico-vaginal tissue were of the effector memory phenotype (T<sub>EM</sub>, T<sub>EMRA</sub>), we analyzed their expression of the costimulatory molecules CD27 and CD28, which define cell differentiation toward specialized effector functions.<sup>15,16</sup> Among CD4 T cells, 21.2±5.3% were of early-differentiated effector phenotype (ED; CD27<sup>+</sup>CD28<sup>+</sup>), 47.7±5% were of intermediate effector phenotype (ID; CD27<sup>+</sup>CD28<sup>-</sup> or CD27<sup>-</sup>CD28<sup>+</sup>), and 31.2±8.4% were of late-differentiated effector phenotype (LD; CD27<sup>-</sup>CD28<sup>-</sup>) (*n*=10) (Figure 3). Similar results were obtained for CD8 T cells in the cervico-vaginal tissue, in which phenotypes ED, ID, and LD constituted 16.4±4, 52.6±4, and 30.9±2.9% of CD8 T cells, respectively (*n*=10).

The distribution of these cell sub-populations among effector memory cells (T<sub>EM</sub>, T<sub>EMRA</sub>) in the lymphoid tissue (tonsils) was significantly different from that in the cervico-vaginal tissue (*P*<0.05). Among CD4 T cells, the ED, ID, and LD sub-populations constituted 53±6.9%, 33.2±5.1%, and 13.7±3.3%,



**Figure 3** Distribution of early- (ED), intermediate- (ID), and late-differentiated (LD) effector memory CD4 T cells in human cervico-vaginal and tonsillar tissues. ED, ID, and LD effector memory CD4 T cells (T<sub>EM</sub>, T<sub>EMRA</sub>) were identified on the basis of the expression of CD27 and CD28 costimulatory molecules. Mean±s.e.m. of the fractions of ED, ID, and LD effector memory CD4 T cells in cervico-vaginal (*n*=10) and tonsillar (*n*=5) tissues are presented. \*Indicates a statistically significant difference with *P*<0.05. It must be noted that the distribution of ED and LD effector memory CD4 T cells in cervico-vaginal tissues is different from that in tonsillar tissues.

respectively ( $n=5$ ), whereas these subsets constituted  $38.6\pm6\%$ ,  $49\pm1.6\%$ , and  $12.2\pm4.5\%$ , respectively among CD8 T cells ( $n=5$ ) (Figure 3).

#### Expression of activation markers and HIV-1 coreceptors on T cells in human cervico-vaginal tissue

As for several types of nongenital tissues it has been shown that productive infection by HIV-1 occurs mainly in activated T cells,<sup>17</sup> we evaluated the expression of activation markers on T cells in fresh cervico-vaginal tissue. In both CD4 and CD8 T cells, CD69 and CD95 were expressed on  $>90\%$  of cells (Table 1). In contrast, CD57, CD38, and HLA-DR were expressed on a smaller fraction of T cells, and differentially in CD4 and CD8 T cells:  $10.6\pm2.7\%$ ,  $19.8\pm4\%$ , and  $3.3\pm1\%$  of CD4 T cells, respectively, expressed these markers, whereas

among CD8 T cells, these fractions constituted  $36.5\pm5.2\%$ ,  $30.6\pm6.2\%$ , and  $7.4\pm2.4\%$ , respectively ( $P<0.05$ ,  $n=5$ ,  $n=12$ ,  $n=9$ , respectively). CD25 expression was similar on CD4 and CD8 T cells ( $7.6\pm2\%$  and  $6.7\pm4.6\%$ , respectively;  $P=0.2$ ,  $n=10$ ) (Table 1).

The expression of activation markers in the cervico-vaginal tissue was different from that in the lymphoid tissue: in particular, on CD4 and CD8 T cells from the cervico-vaginal tissue, there was a higher expression of CD95 and CD69 than in the lymphoid tissue ( $P<0.05$ ,  $n=6$ ). The expression of CD38 was higher on CD4 T cells in the lymphoid than in the cervico-vaginal tissue ( $P=0.0003$ ,  $n=12$ ). There was no significant difference in the expression of CD25 and HLA-DR on CD4 and CD8 T cells between lymphoid and cervico-vaginal tissues ( $P>0.05$ ,  $n=9$ ), whereas the expression of CD57 on CD8 T cells was lower in the lymphoid than in the cervico-vaginal tissue ( $P=0.005$ ,  $n=5$ ) (Table 1).

Furthermore, we also evaluated the expression of the HIV-1 coreceptors CCR5 and CXCR4. We found that  $44\pm1.6\%$  of CD4 T lymphocytes expressed both CXCR4 and CCR5, and  $44\pm1.5\%$  of CD4 T cells expressed CXCR4 exclusively, whereas  $7\pm0.8\%$  expressed CCR5 exclusively ( $n=3$ ). The distribution of the expression of these HIV-1 coreceptors was different from that in the lymphoid tissue (Figure 4). In this tissue,  $15\pm4\%$  of CD4 T cells coexpressed CCR5 and CXCR4,  $4\pm1\%$  expressed CCR5 exclusively, and  $71\pm4.5\%$  expressed only CXCR4.<sup>3</sup>

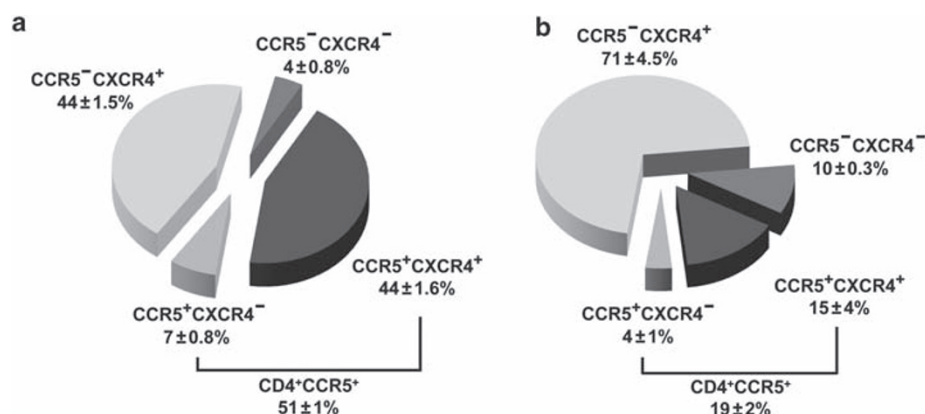
**Table 1** Expression of activation markers on CD4 and CD8 T cells from cervico-vaginal and tonsillar tissue

	Cervix (%)	Tonsil (%)
<b>CD4</b>		
CD69	91.7±7.7	73.3±4.3*
CD25	7.6±2	4.5±0.9
HLA-DR	3.3±1	6.6±1.2
CD38	19.8±4	45.8±3.8*
CD57	10.6±2.7	12±1.2
CD95	92.1±3	66.3±4.8*
<b>CD8</b>		
CD69	97.1±1.5	47±5.2*
CD25	6.7±4.4	7.5±2.1
HLA-DR	7.4±2.4	5.2±1
CD38	30.6±6.2	33.1±2
CD57	36.5±5.2	11.1±2.6*
CD95	96±1.4	31.5±7.4*

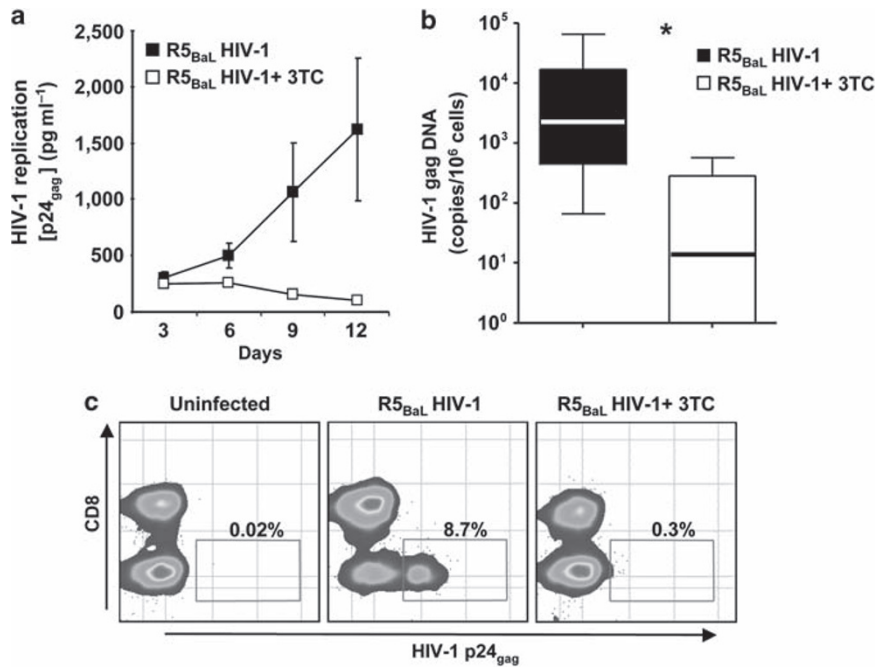
\*Indicates a statistical significant difference with  $P<0.05$ .

#### Development of a raft-based culture system of human cervico-vaginal tissue that efficiently supports HIV-1 infection *ex vivo*

Culture on collagen sponge gel rafts at the liquid–air interface has been described for several tissues as a system that preserves tissue viability, cytoarchitecture, and functions for at least 2–3 weeks.<sup>12,18–20</sup> In this study, we used such a system to culture cervico-vaginal tissues and infect it with HIV-1 under controlled laboratory conditions.



**Figure 4** Expression of human immunodeficiency virus (HIV)-1 coreceptors on CD4 T cells from human cervico-vaginal and tonsillar tissues. Cells isolated from fresh tissue blocks were stained for lymphocytic markers and analyzed using flow cytometry. Mean±s.e.m. of the fractions of total CD4 T cells expressing CCR5 and CXCR4 in cervico-vaginal (a,  $n=4$ ) and tonsillar (b,  $n=3$ ) tissues are presented. It must be noted that the fraction of CD4 T cells expressing CCR5 is higher in the cervico-vaginal tissue than in the tonsillar tissue, whereas the fraction of CD4 T cells expressing CXCR4 is similar.



**Figure 5** R5<sub>BaL</sub> human immunodeficiency virus (HIV)-1 infection of human cervico-vaginal tissues. Donor-matched blocks of human cervico-vaginal tissue were inoculated *ex vivo* with R5<sub>BaL</sub> HIV-1 and were treated with or without lamivudine (3TC) (5  $\mu$ M). **(a)** Kinetics of release of p24<sub>gag</sub> by inoculated tissues. Mean  $\pm$  s.e.m. for tissues from 16 donors are presented. For each donor, each datum point represents pooled viral release from 16 tissue blocks over 3-day periods. **(b)** The amount of HIV-1 gag complete reverse transcripts at day 12 post infection. Medians and the interquartile ranges are presented; the whiskers of the graph show the largest and smallest values. **(c)** The distribution of HIV-1-infected cells in donor-matched tissue blocks: uninfected, inoculated with R5<sub>BaL</sub> HIV-1, inoculated with R5<sub>BaL</sub> and treated with 3TC (5  $\mu$ M). The amounts of HIV-1 p24<sub>gag</sub>-positive cells are expressed as percentage of total CD3<sup>+</sup> T cells. Bivariate density plots of a representative experiment with cervico-vaginal tissues from 1 out of 13 donors are presented. \*Indicates a statistically significant difference with  $P < 0.05$ . It must be noted that p24<sub>gag</sub> accumulation in the culture medium measured by cytometric bead assay, production of HIV-1 complete reverse transcripts measured by real-time PCR, and identification of HIV-1-infected cells by flow cytometry confirm productive infection of human cervico-vaginal tissues by R5<sub>BaL</sub> HIV-1.

Cervical tissue obtained from surgery was dissected into tissue blocks and cultured on collagen sponge gel rafts for 12 days. Phenotypic analysis of T cells in these tissue blocks showed that the distributions of all the T-cell populations described above as well as the fractions of cells expressing activation markers and HIV-1 coreceptors did not change between day 0 and day 12 of culture ( $n = 8$ ,  $P > 0.5$ ). Cervico-vaginal tissue blocks were infected with HIV-1 (16 blocks per experimental condition), and viral replication was evaluated from the release of p24<sub>gag</sub> into the culture medium. In tissue blocks inoculated with R5<sub>BaL</sub> HIV-1, viral replication became evident on day 6 post inoculation and increased during the entire course of the experiment up to day 12. At day 12 post infection, the release of p24<sub>gag</sub> into the supernatant was  $1,619 \pm 635$  pg/ml (Figure 5a). On average, the total cumulative release of p24<sub>gag</sub> into the culture medium between day 6 and day 12 post inoculation was  $3,191 \pm 1,158$  pg/ml of p24<sub>gag</sub> ( $n = 16$ ). Over this period, each block of tissue produced, on average,  $199 \pm 72$  pg.

Potentially, some of the p24<sub>gag</sub> measured in the medium can reflect the slow release of viral inoculum adsorbed on the tissue rather than *de novo* HIV-1 production. To discriminate between these two processes, we conducted control experiments in which in donor-matched tissues, we prevented *de novo* viral production with an inhibitor of HIV-1 reverse transcriptase (lamivudine (3TC), 5  $\mu$ M). In tissues treated with 3TC, there was an  $89.7 \pm 5.3\%$  reduction in p24<sub>gag</sub> release on day 12 of culture compared with

matched tissues not treated with 3TC ( $P = 0.02$ ,  $n = 16$ ) (Figure 5a). These data were confirmed in experiments with another HIV-1 inhibitor, L-Thia-Pro, L- $\alpha$ -cyclohexyl-Gly-N $\alpha$ -nonanoyl-Regulated upon Activation, Normal T cell Expressed and Secreted (PSC-RANTES) (which downregulates R5 HIV-1 coreceptor CCR5).<sup>21</sup> In these tissues, there was an  $80.5 \pm 10.2\%$  reduction in p24<sub>gag</sub> release into the medium ( $n = 2$ ). Hence, the amount of p24<sub>gag</sub> in the medium bathing inoculated tissue largely reflects productive HIV-1 infection.

To confirm the productive infection by R5<sub>BaL</sub> HIV-1 in the cervico-vaginal tissue, we quantified the complete reverse transcripts using real-time PCR.<sup>22</sup> In R5<sub>BaL</sub> HIV-1-inoculated tissues, the median amount of complete reverse transcripts on day 12 post infection was 2,288 copies/10<sup>6</sup> cells (interquartile range (IQR) 432–716,869), whereas in 3TC-treated cultures, it was reduced to 13.9 copies per 10<sup>6</sup> cells (IQR 0–286) ( $P = 0.0001$ ,  $n = 14$ ) (Figure 5b).

Finally, flow cytometry of tissue cells stained for p24<sub>gag</sub> confirmed productive R5<sub>BaL</sub> HIV-1 infection. Among CD3<sup>+</sup> cells on day 12 post inoculation, the fraction of p24<sub>gag</sub>-expressing cells was  $6.6 \pm 1.4\%$  ( $n = 13$ ). Treatment with 3TC reduced this fraction to  $0.8 \pm 0.3\%$  ( $n = 13$ ,  $P = 0.002$ ). Gating on CD3<sup>+</sup> CD8<sup>-</sup> cells (to include T lymphocytes that downregulated CD4 because of HIV-1 infection<sup>23</sup>) showed that on average, in R5<sub>BaL</sub> HIV-1-inoculated tissue on day 12 post inoculation,  $14.1 \pm 3.3\%$  ( $n = 13$ )

of CD8<sup>-</sup> T cells expressed p24<sub>gag</sub>. Treatment with 3TC reduced the fraction of HIV-1-infected cells on average to 1.5±0.8% ( $P=0.002$ ,  $n=13$ ) (Figure 5c). Among CD3<sup>+</sup>CD8<sup>+</sup> cells, the fraction of p24<sub>gag</sub>-positive cells was 0.3±0.07, and this fraction was not significantly reduced by 3TC treatment (0.2±0.07;  $n=13$ ,  $P=0.3$ ). In addition, we evaluated the fraction of HIV-infected cells on day 12 post HIV-1<sub>BaL</sub> infection in the CD3<sup>-</sup> cell subset. In tissues infected with HIV-1<sub>BaL</sub>, 0.45±0.1% of CD3<sup>-</sup> cells expressed p24<sub>gag</sub>, whereas in tissues infected and treated with 3TC, 0.36±0.06% ( $n=13$ ) of CD3<sup>-</sup> cells expressed p24<sub>gag</sub>. The difference between these values was not statistically significant ( $P=0.31$ ).

Thus, the cervico-vaginal tissue *ex vivo* preserves different lymphocyte populations and supports productive HIV-1 infection in CD3<sup>+</sup>CD8<sup>-</sup> T cells but not in CD3<sup>+</sup>CD8<sup>+</sup> T cells or in CD3<sup>-</sup> cells.

#### T-cell activation and depletion in R5 HIV-1-infected human cervico-vaginal tissue

To evaluate depletion in various T-cell sub-populations in human cervico-vaginal tissue infected *ex vivo* with R5<sub>BaL</sub> HIV-1, we enumerated, using flow cytometry, the numbers of total and CCR5-expressing CD8<sup>-</sup> T cells. On day 12 post infection, 50.9±8.1% of CD8<sup>-</sup> T cells were depleted ( $n=16$ ). Among CD8<sup>-</sup>CCR5<sup>+</sup> T-cells, this depletion was 63.6±7.9%. Productive infection by R5<sub>BaL</sub> HIV-1 was the main cause for CD4 T-cell depletion, as the suppression of R5<sub>BaL</sub> HIV-1 infection by 3TC decreased the number of depleted cells by 80.4±7.6% ( $P<0.05$ ,  $n=16$ ). Similar results were obtained when R5<sub>BaL</sub> HIV-1 infection was prevented by PSC-RANTES: in tissues treated with this inhibitor, the depletion of CD4 T-cells by R5<sub>BaL</sub> HIV-1 was reduced by ~80% compared with untreated tissues. To evaluate the role of apoptosis in cell depletion, we measured the expression of the early apoptotic marker APO2.7 in HIV-1-infected and uninfected T cells from the cervico-vaginal tissue. On day 12 post infection with R5<sub>BaL</sub> HIV-1, 34±8.8% of the apoptotic CD4 (CD8<sup>-</sup>) T cells were p24<sub>gag</sub><sup>+</sup>, whereas among nonapoptotic CD4 (CD8<sup>-</sup>) T cells, 19.3±5.7% expressed p24<sub>gag</sub> ( $P=0.01$ ;  $n=6$ ).

To study the relationship between T-cell activation status and HIV-1 infection in the cervico-vaginal tissue, we evaluated the fractions of activated and nonactivated CD8<sup>-</sup> T cells in control and HIV-1-infected tissues. In addition, we evaluated the fractions of activated cells among productively infected and bystander cells. HIV-1-infected cells were identified by intracellular staining for p24<sub>gag</sub>; activated cells were identified from the expression of the activation markers CD25, CD38, CD57, CD69, CD95, and HLA-DR. We found that on day 12 after R5<sub>BaL</sub> HIV-1 inoculation into cervico-vaginal tissue blocks, the expression of CD38 was higher among HIV-1-infected T cells (21.9±3.3%) than among bystander (uninfected) T cells (15.8±2.4%;  $P=0.01$ ,  $n=12$ ). In addition, the fraction of activated cells expressing CD38 among bystander T cells in HIV-1-inoculated tissue was higher than the fraction of activated CD8<sup>-</sup> T cells in donor-matched uninfected controls (15.8±2.4% vs. 12.7±1.3%;  $P=0.02$ ,  $n=12$ ).

Except for CD38, there was no difference in the expression of other tested activation markers (HLA-DR, CD25, CD57, CD69, and CD95) between infected and bystander CD8<sup>-</sup> T cells in the HIV-1-infected tissue. Similarly, no difference was observed between the expression of these activation markers on bystander CD8<sup>-</sup> T cells from HIV-1-infected and from donor-matched uninfected tissues. No increased expression of activation markers, including CD38, was observed on CD8 T cells in HIV-1-infected compared with donor-matched uninfected tissues.

Thus, R5<sub>BaL</sub> HIV-1 preferentially infects CD4 T cells of activated (CD38<sup>+</sup>) phenotype, and the number of such cells among uninfected bystander CD4 T cells is increased upon R5<sub>BaL</sub> HIV-1 infection of human cervico-vaginal tissue.

#### Infection of human cervico-vaginal tissue *ex vivo* with the X4 HIV-1 variant

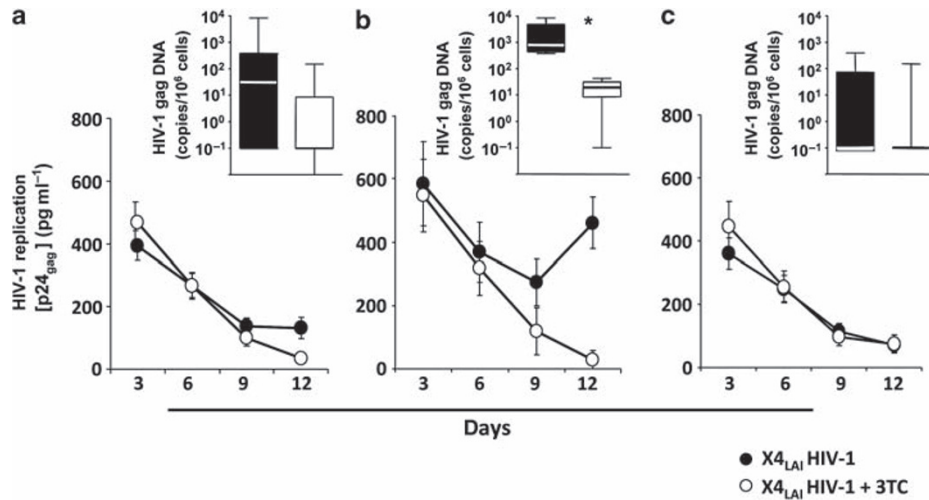
To compare the ability of human cervico-vaginal tissue to support productive infection by HIV-1 variants of different coreceptor tropisms, we inoculated tissue blocks with X4<sub>LAI</sub> HIV-1 and R5<sub>BaL</sub> HIV-1. On average, X4<sub>LAI</sub> HIV-1 replicated less efficiently than did R5<sub>BaL</sub> HIV-1. For X4<sub>LAI</sub> HIV-1, the average cumulative p24<sub>gag</sub> release between day 6 and day 12 post inoculation was 536±86 pg/ml: 64.6±5.3% less than that for R5<sub>BaL</sub> HIV-1 ( $P=0.03$ ,  $n=27$ ). Moreover, in contrast to the production of R5<sub>BaL</sub> HIV-1, which constantly increased over the 12 days of infection, there was a steady decrease in the release of X4<sub>LAI</sub> HIV-1 through the entire culture period (Figure 6a).

To evaluate the contribution of *de novo* production to the amount of the X4<sub>LAI</sub> HIV-1 released, we suppressed viral replication with 3TC. In contrast to R5<sub>BaL</sub> HIV-1-infected tissues, in tissues infected with X4<sub>LAI</sub> HIV-1, 3TC did not decrease the release of p24<sub>gag</sub> compared with donor-matched untreated tissues (the average cumulative p24<sub>gag</sub> release between day 6 and day 12 of culture was 403±81 vs. 536±86 pg/ml;  $n=27$ ,  $P>0.05$ ) (Figure 6a). In addition, there was no significant difference in the median numbers of complete reverse transcripts between X4<sub>LAI</sub> HIV-1-infected tissues untreated and treated with 3TC (31.1 copies per 10<sup>6</sup> cells (IQR 0–384) and 0 copies per 10<sup>6</sup> cells (IQR 0–8.6), respectively;  $P=0.1$ ,  $n=14$ ). Thus, apparently on average, no replication of X4<sub>LAI</sub> HIV-1 occurred in inoculated tissues, but rather the release of viral inoculum.

However, out of the 27 tissues reported above, in 4 of them inoculation with X4<sub>LAI</sub> HIV-1 resulted in a productive infection, although with slower kinetics than R5<sub>BaL</sub> HIV-1 (Figure 6b).

In these four cultures, the average cumulative p24<sub>gag</sub> release into the medium between day 6 and day 12 post inoculation was 1,102±238 pg/ml ( $n=4$ ), whereas for the rest of the tissues it was 438±77 pg/ml ( $n=23$ ). Moreover, in tissues obtained from these four donors, there was a 61.8±9% reduction in p24<sub>gag</sub> release after treatment with 3TC (465±183 pg/ml) ( $P=0.005$ ) in contrast to the 23 tissues in which 3TC did not significantly reduce p24<sub>gag</sub> release (23.3±5.7%;  $P=0.1$ ) (Figure 6c).

The analysis of complete reverse transcripts with real-time PCR confirmed the productive infection of these four tissues by

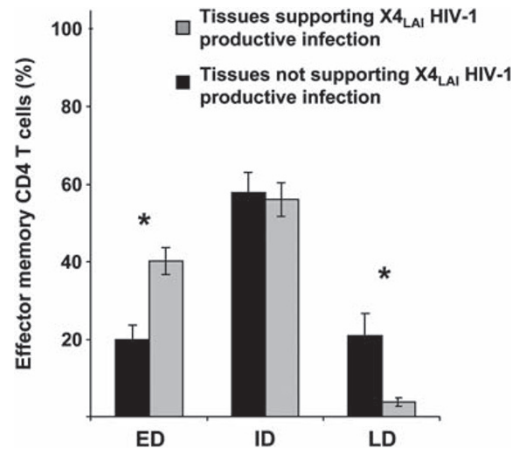


**Figure 6** X4<sub>LAI</sub> human immunodeficiency virus (HIV)-1 infection of human cervico-vaginal tissues. Donor-matched blocks of human cervico-vaginal tissue were inoculated *ex vivo* with X4<sub>LAI</sub> HIV-1 and were treated with or without lamivudine (3TC) (5 μM). **(a)** Kinetics of release of p24<sub>gag</sub> by tissues from 27 donors. **(b)** Kinetics of release of p24<sub>gag</sub> by tissues from 4 donors. These tissues were selected from the 27 presented in panel a based on the increase in p24<sub>gag</sub> release during culture. **(c)** Kinetics of release of p24<sub>gag</sub> by tissues from the remaining 23 donors. Each datum point for each tissue reflects p24<sub>gag</sub> produced by 16 tissue blocks over 3-day periods. Mean±s.e.m is presented. Insets: The amount of HIV-1 gag complete reverse transcripts measured in tissue blocks at day 12 post infection. The medians and interquartile ranges are presented. The whiskers of the graphs show the largest and smallest values. \*Indicates a statistically significant difference with *P*<0.05. An arbitrary value of 0.1 copies per ml was assigned to samples negative for HIV-1 gag complete reverse transcripts to allow graphical representation of the data. It must be noted that in human cervico-vaginal tissues inoculation with X4<sub>LAI</sub> HIV-1 resulted in a productive infection in 4 out of 27 tested tissues as demonstrated by comparison of p24<sub>gag</sub> release and complete reverse transcripts in tissues treated with or without 3TC.

X4<sub>LAI</sub> HIV-1: on day 12 post infection, the median amount of complete reverse transcripts was 437.5 copies per 10<sup>6</sup> cells (IQR 239.4–4,428) whereas in the other 23 tissues, there were 0 copies per 10<sup>6</sup> cells (IQR 0–172.1). In these four tissues, 3TC decreased the number of complete reverse transcripts by 97.6±1.3% (to the level of 19.1 copies per 10<sup>6</sup> cells, IQR 8.5–31.7; *P*=0.01), confirming productive infection by X4<sub>LAI</sub> HIV-1.

We investigated whether the ability to support productive X4<sub>LAI</sub> HIV-1 infection in these four tissues was related to a particular distribution of various T-cell subsets or to their activation status. There was no significant difference in the fractions of naive, T<sub>CM</sub>, T<sub>EM</sub>, T<sub>EMRA</sub>, and activated cells (CD25, CD38, CD57, CD69, CD95, and HLA-DR) as evaluated in matched uninfected tissues on day 12 post inoculation between tissues that support and those that do not support X4<sub>LAI</sub> HIV-1 infection (*P*>0.05). However, we found that in tissues that supported productive X4<sub>LAI</sub> HIV-1 infection, there were significantly more ED (CD27<sup>+</sup>CD28<sup>+</sup>) effector memory CD4 T cells than in tissues that did not support productive X4<sub>LAI</sub> HIV-1 infection (40.2±3.4%, *n*=4, vs. 19.9±3.7%, *n*=14; *P*=0.007) (Figure 7). Accordingly, in tissues that supported productive X4<sub>LAI</sub> HIV-1 infection, there were significantly fewer LD (CD27<sup>-</sup>CD28<sup>-</sup>) effector memory CD4 T cells than in tissues that did not support productive X4<sub>LAI</sub> HIV-1 infection (3.6±1.1%, *n*=4, vs. 21.2±5.6%, *n*=14; *P*=0.01). There was no statistical difference in the fraction of ID CD4 T cells between tissues that supported and those that did not support X4<sub>LAI</sub> HIV-1 infection (*P*=0.7) (Figure 7).

Thus, in contrast to R5 HIV-1, which efficiently replicated in all tested cervico-vaginal tissues, X4 HIV-1 typically did not replicate. However, in 4 out of 27 tested tissues, X4 replicated, although with slower kinetics than R5 HIV-1. These tissues



**Figure 7** X4<sub>LAI</sub> human immunodeficiency virus (HIV)-1 infection of human cervico-vaginal tissue and distribution of early-, intermediate-, and late-differentiated effector memory CD4 T cells. Mean±s.e.m. of the fractions of ED, ID, and LD effector memory CD4 T cells in cervico-vaginal tissues that supported (*n*=4) or did not support (*n*=23) productive infection by X4<sub>LAI</sub> HIV-1 are presented. \*Indicates a statistically significant difference with *P*<0.05. It must be noted that these tissues differ in the distribution of ED and LD effector memory CD4 T cells.

differed from the rest by a higher fraction of effector memory CD4 T cells expressing CD27 and CD28, but not by the distribution of other tested T-cell phenotypes or by the activation status of these cells.

**DISCUSSION**

In principle, the vaginal and cervical mucosa constitute a reliable natural barrier for HIV-1 transmission.<sup>4,12,24</sup> However, recent

epidemiological data have shown a disproportionate increase in female infections, predominantly through vaginal intercourse.<sup>1</sup> It has been suggested that this route of HIV-1 transmission is facilitated by microabrasions occurring during the intercourse, to lesions due to sexually transmitted diseases, and to environmental factors that breach the integrity of the cervico-vaginal epithelial barrier.<sup>4</sup> Upon penetration of this barrier, HIV-1 interacts with mucosal cells, CD4 T cells, macrophages, and various DCs. Although all these cells may have an important role in the early stages of HIV-1 infection, it seems that CD4 T cells are crucial to the establishment and dissemination of the infection.<sup>25,26</sup> Macrophages are productively infected *ex vivo* but do not seem to be the main target for the initial SIV (simian immunodeficiency virus)/HIV infection *in vivo*.<sup>11,27–29</sup> On the other hand, even though intraepithelial or subepithelial DCs efficiently incorporate virions by endocytosis, it remains controversial whether they are productively infected.<sup>26,27</sup> Similarly, although in the cervico-vaginal tissue, various DCs may contribute to the establishment of HIV infection (e.g., by presenting infectious virus to T lymphocytes as well as by the dissemination of newly produced virus), our *ex vivo* results show that CD4 + T lymphocytes are the prevalent HIV-1-infected cells. Therefore, in this study, we focused on cells of this subtype.

Analysis of CD4 T cells isolated from other tissues relevant to HIV-1 pathogenesis and transmission has shown that the CD4 T-cell population is not homogeneous in its ability to be infected and to support viral replication: this ability greatly depends on whether the cell is a memory or a naive cell, activated or non-activated, and on the pattern of expression of HIV-1 coreceptors.<sup>30,31</sup> These features may also contribute to the ability of the tissue to differentially support productive infection of different HIV-1 variants (“gatekeeping”). Surprisingly, with regard to the cervico-vaginal tissue, which serves as an important gateway for HIV-1, there are few data on the status of T cells and on its relation to HIV-1 infection and production.

In this study, we report data on these phenomena. We investigated the status of CD4 T cells in the cervico-vaginal tissue and their ability to selectively support productive infection with R5 but not with X4 HIV-1. For these experiments, we further developed a previously described technique for the culture of cervical explants.<sup>32,33</sup> We maintained cervico-vaginal tissue blocks on top of gel rafts at the liquid–air interface, instead of submerging them in the culture medium. In a raft-based culture, collagen support and oxygenation are sufficient to preserve the cytoarchitecture and thus to delay tissue deterioration.<sup>18</sup> Under these *ex vivo* conditions, cervico-vaginal tissue blocks readily support HIV-1 replication, as evidenced by the presence of complete reverse transcripts and of p24-positive T cells, and by the release of p24<sub>gag</sub> in the culture medium. Tissue treatment with 3TC almost completely prevented formation of the complete reverse transcripts, as well as the emergence of p24-positive T cells and the release of p24<sub>gag</sub>, thus confirming *de novo* viral production in the inoculated tissues. The residual viral infection in 3TC-treated tissue blocks is negligible and may reflect the extent of the first round of infection before the drug fully penetrates the tissue.

As with *in vivo* tissue, HIV-1 replication resulted in the depletion of CD4 T cells. We found that the apoptotic CD4 + T-cell population was enriched in HIV-infected cells, suggesting that induction of apoptosis contributes to the depletion of CD4 T cells in *ex vivo* cervico-vaginal tissue. The quantitative relationship between CD4 T-cell apoptosis and their depletion is difficult to establish, as the number of apoptotic cells represents a “snapshot,” whereas cell depletion is a cumulative parameter.

In addition, as a raft-based culture system has been used to maintain lymphoid (lymph nodes or tonsils) and rectal tissues,<sup>20</sup> we were able to compare the abilities of cervico-vaginal, rectal, and lymphoid tissues to support replication of HIV-1 and to investigate the relationship of these abilities to the distribution of T cells of various phenotypes in these tissues.

An important factor that may control HIV-1 replication is the functional status of T cells.<sup>34</sup> It has been shown that HIV-1 replication predominantly occurs in effector memory CD4 T cells.<sup>31</sup> Therefore, we analyzed the sub-population of such T cells in the cervico-vaginal tissue using flow cytometry, and found that virtually all T cells are of the effector memory phenotype (T<sub>EM</sub> and T<sub>EMRA</sub>), with an almost complete absence of naive and central memory T cells. In rectal mucosa, naive, central memory, and effector memory T cells are differently distributed in Peyer’s patches and in the lamina propria: in the former, the great majority of T lymphocytes are of naive and central memory phenotype, whereas in the latter, it is almost exclusively effector memory T cells that are present.<sup>35</sup> In contrast, in the lymphoid tissue, naive T cells constitute >60% of the T-cell population.

The prevalence of effector memory cells among cervico-vaginal T cells is critical for guarding against various incoming pathogens. The ability of the cervico-vaginal tissue to defend against pathogens is complemented by the high prevalence of CD8 T cells. We found that in the cervico-vaginal tissue, the fraction of CD8 T cells was 3–4 times larger than in rectal, tonsillar, or lymph node tissues.<sup>3,34</sup> Besides their direct cytolytic function, CD8 T cells are the major source of soluble factors<sup>36</sup> that suppress HIV-1 infection of CD4 T cells; thus, the higher prevalence of CD8 T cells in the cervico-vaginal tissue may contribute to the higher resistance of this tissue to HIV-1 transmission compared with the highly efficient transmission through the rectal mucosa. Moreover, in the cervico-vaginal tissue, CD8 T cells had a higher proportion of T<sub>EMRA</sub> than did CD4 T cells. This difference was accompanied by a higher expression of the activation markers CD38, HLA-DR, and CD57 in CD8 than in CD4 T cells. However, these defense mechanisms are not sufficient enough to prevent HIV-1 infection, and HIV-1 circumvents some of them for its benefit. Indeed, the prevalence of effector memory CD4 T cells of activated phenotype at the site of infection provides HIV-1 with more cell targets, thus creating a favorable environment for its efficient replication.<sup>31</sup>

As, unlike isolated cells, cells in *ex vivo* tissues support HIV-1 replication without exogenous stimulation and activation, this experimental system is particularly useful for the study of the effect of HIV-1 on cell activation. We found that in the cervico-vaginal tissue, R5 HIV-1 replication occurred preferentially in CD4 T cells that express the activation marker CD38.



Surprisingly, this increase in activation of HIV-1-infected cells was associated with a concomitant increase in the expression of CD38 in HIV-1-uninfected (bystander) CD4 T cells, but not in CD8 T cells. No other tested activation markers were upregulated in the cervico-vaginal tissue upon HIV-1 infection. Thus, it seems that HIV-1 replication in the cervico-vaginal tissue is associated with specific activation of CD4 bystander T cells. Although HIV-1 viral proteins such as Tat, Nef, and gp120<sup>37</sup> may be directly involved in this process, it is also possible that host soluble mediators (such as type I interferon or other cytokines)<sup>34,38</sup> produced by HIV-1-infected and uninfected cells may mediate the upregulation of CD38 on bystander T cells. A similar phenomenon, although with different activation markers (CD25<sup>+</sup> HLA-DR<sup>+</sup>), has been reported for lymphoid tissues.<sup>17</sup> Thus, it seems that in both lymphoid and cervico-vaginal tissues, HIV-1 specifically upregulates surface molecules that are characteristic of the cells that are preferentially infected by this virus. This phenomenon may expand the local pool of cells that are preferentially infected by HIV-1. Such an interpretation is consistent with a recent report, based on work on SIV-infected rhesus macaques, which showed that viral infection in the genital tract spreads from a few isolated CD4 T cells. These cells trigger a local inflammatory response that induces the recruitment of other CD4 T cells. Thus, the infection of a few isolated CD4 T cells rapidly expands into the infection of clusters of numerous CD4 T cells, eventually favoring dissemination of the virus to the draining lymph node.<sup>25</sup> The bystander activation of local CD4 T cells that were observed in the HIV-1-infected human cervico-vaginal tissue may represent a similar mechanism that facilitates the expansion of infection. Such bystander activation may in turn further amplify HIV-1 replication, and this vicious circle of infection–activation–infection would favor the systemic dissemination of the virus.

Although such a vicious circle seems to be general for both lymphoid and cervical tissues, in the former, it can be generated by both R5 and X4 HIV-1, whereas the cervico-vaginal tissue predominantly supports productive infection by R5 rather than X4 HIV-1. One of the factors that may contribute to this phenomenon is the high prevalence, shown in this study, of CCR5 expression among cervico-vaginal CD4 T lymphocytes. However, the ability of this tissue to support R5 rather than X4 HIV-1 infection is not explained solely by the pattern of HIV-1 coreceptor expression, as although the expression of CCR5 on CD4 T cells is high, almost all CD4 T cells express CXCR4 as well.

Other restriction factors that “gatekeep” against X4 HIV-1 infection should be investigated.<sup>39,40</sup> We identified the differentiation status of effector memory CD4 T cells as one such factor. On average, inoculation with X4<sub>LAI</sub> HIV-1 did not result in significant infection of cervico-vaginal explants: the amount of p24<sub>gag</sub> released into the medium from X4<sub>LAI</sub> HIV-1-inoculated tissue was similar to that released from 3TC-treated cultures inoculated with X4<sub>LAI</sub> HIV-1 or R5<sub>BAL</sub> HIV-1. However, in tissues from 4 out of 27 donors, we documented a productive infection by X4<sub>LAI</sub> HIV-1 as evaluated from the release of p24<sub>gag</sub> in the culture supernatant, from detection of p24<sub>gag</sub>-positive cells

with flow cytometry, and from production of complete reverse transcripts measured with real-time PCR.

What makes these four tissues different from the rest? It is not the presence of the CXCR4 coreceptor, as in tissues that did not support X4 HIV-1 replication CXCR4 was expressed on almost 90% of CD4<sup>+</sup> T cells. A phenotypic analysis of the lymphocytic population in the four tissues that supported X4 HIV-1 replication showed their enrichment in effector memory CD4 T cells of an early-differentiated phenotype (CD27<sup>+</sup> CD28<sup>+</sup>) associated with a smaller fraction of effector memory CD4 T cells of a late-differentiated phenotype (CD27<sup>-</sup> CD28<sup>-</sup>). Possibly, the cellular machinery of early-differentiated memory T cells is better equipped to support X4 HIV-1 replication than is that of more differentiated cells. Alternatively, a smaller fraction of late-differentiated CD4 T cells may be associated with a reduced production by these or other cells of X4 HIV-1-inhibitory soluble factors.<sup>41</sup> Although further studies are required to investigate the exact mechanisms by which the equilibrium between early- and late-differentiated effector memory CD4 T cells controls X4 HIV-1 productive infection, our results show that the susceptibility to X4 or R5 HIV-1 infection is not defined by the mere expression of specific coreceptors and can be restricted by innate immunological mechanisms operating in the human genital mucosa.

In summary, we analyzed the phenotypes of the lymphocytic populations in the cervico-vaginal tissue and found that CD4 T cells, the major HIV-1 cell targets, are in this tissue mostly in an activated and differentiated state; moreover, the majority of CD4 T cells express CCR5, thus rendering this tissue particularly susceptible to R5 HIV-1 infection. We confirmed this in actual experiments on human cervico-vaginal tissue *ex vivo*, a system we further developed. We found that R5 HIV-1 replication causes activation of uninfected bystander CD4 T cells, resulting in expansion of the pool of preferentially infected cells. This mechanism can promote the local expansion of infection that precedes the establishment of a systemic infection.

In addition, we found that in contrast to R5, replication of X4 HIV-1 was negligible except in a few tissues. In these tissues, a significant fraction of effector memory CD4 T cells coexpressed the markers CD27 and CD28, which characterize early-differentiated cells. Thus, it seems that in the cervico-vaginal tissue, expression of CXCR4 is not enough to support productive infection of X4 HIV-1. It has been suggested earlier that the “gatekeeping” mechanism is a superimposition of various incomplete restrictive barriers in the course of HIV-1 transmission, rather than a unique complete barrier.<sup>4</sup> Identification of the factors associated with restriction of X4 HIV-1 infection in the cervico-vaginal tissue will reveal mechanisms for these “gatekeeping” barriers.

In conclusion, our results provide new insights into the relationship between HIV-1 infection and the activation/differentiation status of the lymphocytic population in the cervico-vaginal tissue, thus contributing to a better understanding of the early events of sexual transmission of HIV-1. Such an understanding may lead to the development of new anti-HIV-1 strategies, in particular the expansion of the “gatekeeping”

mechanisms to all HIV-1 variants and the disruption of the vicious circle of HIV-1-triggered T-cell activation that facilitates HIV-1 dissemination.

## METHODS

**Tissue histoculture and HIV-1 infection.** Tonsillar tissues from routine tonsillectomy were obtained from the Children's National Medical Center (Washington, DC). Cervico-vaginal tissues were obtained from routine hysterectomy through the National Disease Research Interchange (Philadelphia, PA). All tissues were obtained according to an Institutional Review Board-approved protocol. Tonsillar tissues were dissected into 2-mm<sup>3</sup> blocks and digested for flow cytometry applications as described below. Culture of the cervico-vaginal tissue was set up as previously described, with minor modifications.<sup>20</sup> Briefly, the mucosal epithelium and the underlying stroma of both the ectocervix and the endocervix were separated from the muscular tissue, dissected into ~2-mm<sup>3</sup> blocks, and infected with the HIV-1 variant BaL (R5<sub>BaL</sub>) or LAI.04 (X4<sub>LAI</sub>).

Both viral preparations were obtained in the Virology Quality Assurance Laboratory at the Rush University (Chicago, IL). Viral stocks were obtained from the medium of peripheral blood mononuclear cell cultures inoculated with either BaL (R5<sub>BaL</sub>) or LAI.04 (X4<sub>LAI</sub>), originally received from the NIH AIDS Reagent Program. p24 stock concentrations were 49±3 and 53±3 ng/ml for BaL (R5<sub>BaL</sub>) and LAI.04 (X4<sub>LAI</sub>), respectively. Viral inocula were adjusted to obtain similar replication levels in the lymphoid tissue.<sup>42</sup>

For each experiment, 32 cervical blocks were transferred into two 1.5-ml conical tubes (16 blocks per tube), each containing 0.5 ml of viral stock. Simultaneously, lamivudine (3TC) at the dose of 5 μM was added to one of these tubes. After 2 h of incubation at 37°C, tissue blocks were gently washed three times with phosphate-buffered saline, placed on top of a collagen sponge gel into a 12-well plate (8 blocks per well), and cultured for 12 days, with a change of medium every third day. To the tissue blocks that were incubated with 3TC, the drug was added at each medium change.

**Flow cytometry.** Tonsillar and cervico-vaginal tissue blocks were digested with Collagenase IV (Invitrogen, Carlsbad, CA) at 5 mg/ml in RPMI 5% calf serum for 30 and 90 min, respectively. Single-cell suspensions were washed in staining buffer (phosphate-buffered saline supplemented with 2% normal mouse serum; Gemini Bioproducts, West Sacramento, CA). To characterize tissue lymphocytes, cell suspensions were stained with different combinations of the monoclonal antibodies, anti-CD3, anti-CD19, anti-CD20, anti-CD4, anti-CD8, anti-CD45RA, anti-CCR7, anti-CD27, anti-CD28, anti-CD25, anti-CD38, anti-CD57, anti-CD69, anti-CD95, anti-HLA-DR, anti-CCR5, and anti-CXCR4, coupled to a combination of the fluorochromes Qdot 605, Qdot 655, Pacific Blue, phycoerythrin (Pe), Pe-Cy5, Pe-Cy5.5, Pe-Cy7, APC, APC-Cy5.5, and APC-Cy7. To identify plasmacytoid DCs and myeloid DCs, cell suspensions were stained with combinations of the monoclonal antibodies anti-HLA-DR (Pe-Cy5-labeled), anti-CD123 (Pe), and anti-CD11c (APC), as well as with fluorescein isothiocyanate-labeled anti-CD3, anti-CD14, anti-CD16, anti-CD56, anti-CD19, and anti-CD20. We identified DCs as positive for HLA-DR but negative for CD3, CD14, CD16, CD56, CD19, and CD20; among this population, we identified plasmacytoid DCs as CD123+ and myeloid DCs as CD11c+. To identify macrophages, cell suspensions were stained with combinations of anti-CD14 (APC-Cy7-labeled) and fluorescein isothiocyanate-labeled anti-CD3, anti-CD56, anti-CD19, and anti-CD20 monoclonal antibodies. Macrophages were identified as positive for CD14 and negative for CD3, CD56, CD19, and CD20. All the antibodies were purchased from Invitrogen and BD Biosciences (San Jose, CA).

After surface staining, cells were permeabilized with Fix&Perm (Invitrogen) and stained with an anti-HIV-1 p24<sub>gag</sub> monoclonal antibody (KC57) conjugate with fluorescein isothiocyanate (Beckman Coulter, Fullerton, CA). Data were acquired on an LSRII flow cytometer

(BD Biosciences) equipped with 355, 407, 488, 532, and 638 nm laser lines using DIVA 6.1.2 software (BD Biosciences), and analyzed with FlowJo version 8.7.3 (Tree Star, Ashland, OR). We identified and excluded dead cells from the analysis using the LIVE/DEAD fixable Blue Dead Cell Stain kit (Invitrogen) and identified lymphocytes according to their light-scattering properties. Lymphocyte depletion was quantified by addition of TruCount beads (Invitrogen) according to the manufacturers' instructions.

**Dynamic immunofluorescent cytometric bead assay for HIV-1 p24<sub>gag</sub> quantification.** We evaluated productive HIV-1 infection from measurements of HIV-1 antigen p24<sub>gag</sub> accumulated in tissue culture medium, using a dynamic immunofluorescent cytometric bead assay. The assay was performed as described previously.<sup>43</sup>

**Real-time PCR for HIV-1 complete reverse transcription products.** DNA was extracted from tissue blocks with a DNeasy tissue kit from Qiagen (Valencia, CA). We performed real-time PCR to quantify complete reverse transcripts using the primer sets MH531 and MH532 as described previously.<sup>22</sup> We performed amplification and detection on an Applied Biosystems Prism 7500 sequence detection system (Applied Biosystems) using a Taqman universal master mix (Invitrogen), 300 nm forward and reverse primers, 100 nm probe, and 100 ng of template DNA in a 25-μl reaction volume. We created a reference standard curve using 8E51 cells serially diluted. The amount of cellular DNA was assessed by use of *erv-3* as a reference gene.<sup>44</sup>

**Statistical analysis.** Each datum point is the result of analysis of sets of 16 tissue blocks derived from each of *n* donors, where *n* is indicated in text. The statistical significance of differences between data from various experimental groups was evaluated with the paired or unpaired Student's *t*-test. As the distribution of the numbers of HIV-1 complete reverse transcription products failed the Kolmogorov-Smirnov normality test, we used distribution-free nonparametric methods (Wilcoxon's match-pairs signed-ranks test and Mann-Whitney *U*-test) to evaluate the significance of the differences between various experimental groups. However, when these data were log<sub>10</sub>-transformed, normality was achieved and we applied parametric methods (paired or unpaired Student's *t*-test). The statistical significance of differences between data from various experimental groups evaluated after transformation was similar to that evaluated with nonparametric methods applied to nontransformed results. Depending on the type of statistical analysis, the pooled data are presented either as means±s.e.m. or as medians and IQR. All hypothesis tests were two-tailed, and a *P*-value of ≤0.05 defined statistical significance.

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## DISCLOSURE

The authors declared no conflict of interest.

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