

Vaginal Langerhans Cells Nonproductively Transporting HIV-1 Mediate Infection of T Cells[∇]

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Although implied by other models, proof that Langerhans cells (LCs) in the human vagina participate in dissemination of infectious human immunodeficiency virus type 1 (HIV-1) has been lacking. Here, we show that LCs migrate from HIV-1-exposed vaginal epithelia and pass infectious virus to CD4⁺ T cells without being productively infected themselves, and we point to a pathway that might enable HIV-1 to avoid degradation in vaginal LCs. Transport by migratory LCs to local lymphatics in a nonproductive but infectious form may aid HIV-1 in evasion of topical microbicides that target its intracellular productive life cycle.

During recent years, substantial progress has been made in elucidating the events that occur between mucosal exposure to human immunodeficiency virus (HIV) and the systemic spread of infection (8, 11). Macaque models of simian immunodeficiency virus (SIV) infection and human explant models of HIV infection have independently identified mucosal CD4⁺ T lymphocytes as the prime targets for initial infection (6, 7, 12, 13, 15, 18, 23, 26). The details surrounding this critical event, including how HIV first reaches these cells, what factors in the mucosa or in semen inhibit or enhance infection, and how HIV travels from the mucosa to infect CD4⁺ T cells in draining

lymph nodes, are less clear. Answers to these questions may be of great importance for designing effective prevention strategies with microbicides or vaccines.

Langerhans cells (LCs) in the vaginal or foreskin epithelium, as well as other types of mucosal dendritic cells (DCs), are thought to play an important role in enhancing and spreading initial HIV infection (13). It has long been known that DCs can be invaded by HIV and, in some cases, productively infected (3, 25). We have shown that human vaginal CD1a⁺ LCs efficiently and rapidly endocytose HIV-1 virions (12). However, proof that these cells can pass infectious virions to CD4⁺ T

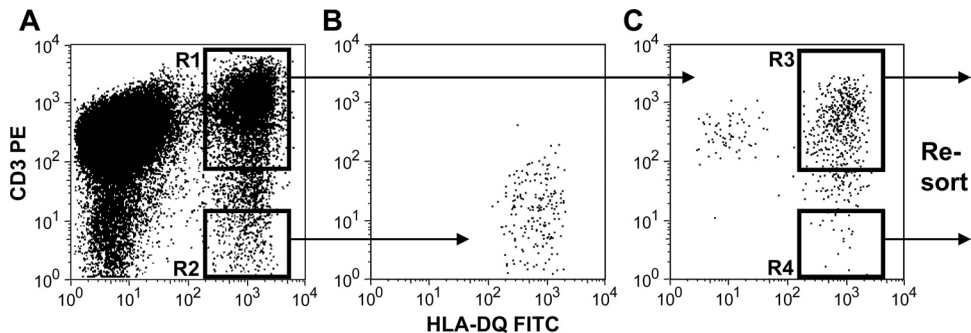


FIG. 1. Flow cytometric purification of discrete vaginal Langerhans cells (LCs) and LC-T-cell (LC-TC) conjugates isolated after emigration from HIV-1-exposed human vaginal epithelial sheets. Mucosal cells present in the media 48 h after emigration were stained with anti-CD45 allophycocyanin (APC), anti-CD3 phycoerythrin (PE), and anti-HLA-DQ fluorescein isothiocyanate (FITC) (BD Biosciences). CD45 positivity was used to identify leukocytes and exclude contaminating epithelial cells (data not shown). (A) Among cells gated for CD45, four populations could be distinguished: CD3⁺ HLA-DQ^{low} T cells, conjugates of CD3⁺ T cells with HLA-DQ^{high} LCs (R1), CD3⁻ HLA-DQ^{high} LCs (R2), and other leukocytes negative for both markers. To conservatively select only discrete LCs, R2 was set far from the LC-TC conjugates. (B) Purity control after sorting, using a fraction of the sorted cells and showing discrete LCs purified to >99%. (C) LC-TC conjugates after sorting, showing that a few conjugates separated into discrete T cells and LCs during sorting. To purify these additional discrete LCs, we resorted the LC-TC conjugates obtained after the initial sort, again selecting LC-TC conjugates (R3) and discrete LCs (R4). This procedure was repeated up to four times, and the discrete LCs from each round were pooled, thus markedly increasing their total yield.

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cells has been lacking, largely due to difficulties isolating and purifying sufficient quantities of viable LCs from HIV-1-exposed vaginal or foreskin mucosas. Moreover, whether productive infection of LCs is required for successful viral passage remains an important, unresolved question. The current generation of candidate topical microbicides most prominently includes antiviral drugs, such as tenofovir and dapivirine (1, 9, 22), which interfere with the intracellular productive life cycle of HIV-1. These drugs would be ineffective against infectious HIV-1 that is endocytosed and merely stored in LCs. If LCs, in their migratory capacity, transport such stored virions from the mucosa to local lymphatics, away from the local action of a topical microbicide, infection may spread unabated to lymphatic CD4⁺ T cells. Such a mechanism for HIV-1 to bypass the protective effect of a topical antiviral drug needs to be considered a potential reason for microbicide failure. We therefore sought to clarify the role of vaginal LCs in spreading HIV-1 to susceptible T cells.

To assess whether vaginal LCs are productively infected by HIV-1, we employed an *ex vivo* infection model of vaginal epithelial sheets as previously described (12, 20). Tissues were obtained from vaginal repair surgeries using a protocol approved by the Fred Hutchinson Cancer Research Center Institutional Review Board. Briefly, stroma-free epithelial sheets were obtained by EDTA-aided microdissection, spinoculated for 2 h with HIV-1_{BaL} (at 25 ng/ml Gag p24), washed thoroughly, and cultured for 2 days at 37°C under 5% CO₂. Cells which migrated into culture media were harvested, stained, and sorted on a FACSVantage fluorescence-activated cell sorter into discrete HLA-DQ⁺ LCs and HLA-DQ⁺/CD3⁺ LC-T-cell (LC-TC) conjugates (10). We tested these cell populations for proviral and chromosomally integrated HIV-1 DNA (20) or for passage of HIV-1 to phytohemagglutinin (PHA)-stimulated T cells. The presence or absence of chromosomally integrated HIV-1 DNA was interpreted as the ability or inability, respectively, to support a productive HIV-1 life cycle.

To overcome the limited yield of discrete LCs, we used multiple rounds of flow cytometric sorting (Fig. 1). After the first round of sorting into discrete LCs and LC-TC conjugates, discrete LCs were nearly pure (Fig. 1B), but some LC-TC conjugates apparently disassembled into discrete T cells and LCs (Fig. 1C). Since we could not separate true LC-TC conjugates *in vitro* using a combination of enzyme treatment and mechanical agitation (data not shown), this was likely due to instances where a discrete T cell and a discrete LC combined in one fluid droplet in the detection and sorting chamber and were thus erroneously interpreted as conjugates by the FACS machine. Resorting and combining LCs from each sort helped us to obtain enough discrete LCs from a number of tissue donors. From 11 tissue donors, we obtained an average of 7,405 discrete LCs (median, 2,776; range, 418 to 45,000) and 211,492 LC-TC conjugates (median, 219,735; range, 72,212 to 339,000).

We isolated DNA (QIAamp blood minikit; Qiagen) and performed an Alu-long-terminal-repeat (LTR)-based nested-PCR assay, which amplifies viral DNA only if it has integrated into the host cell genome (2, 20). Viral integration was detected in all four LC-TC conjugate samples tested (Fig. 2A) (37,664 mean copies per 10⁵ total cells; range, 6,335 to 68,924).

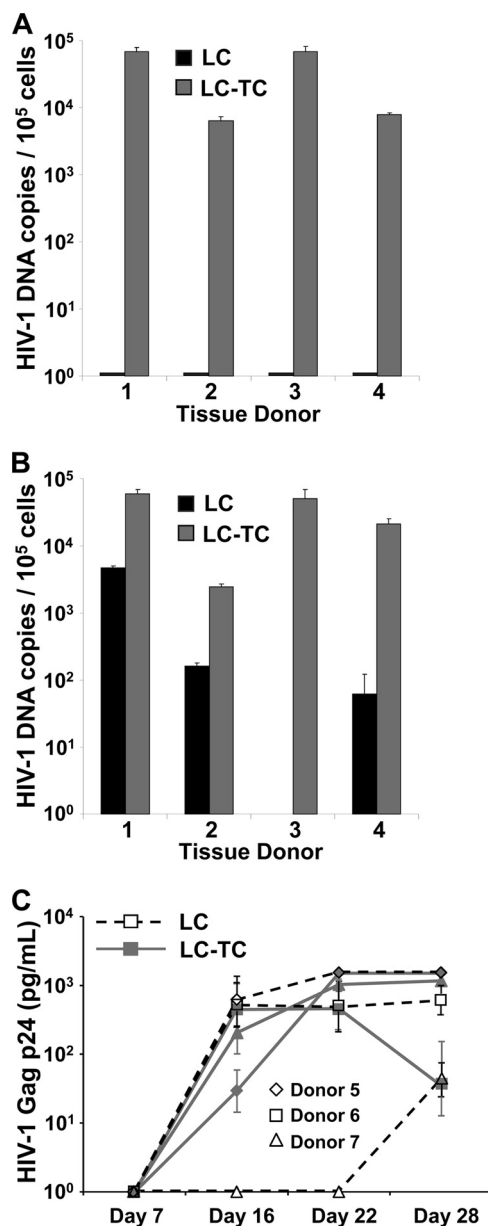


FIG. 2. HIV-1 genomic integration in vaginal LCs and passage to T cells. (A) Using an Alu-LTR-based nested-PCR assay, we detected viral integration in purified LC-TC conjugates from all four HIV-1-exposed vaginal epithelia tested but never in the discrete LCs from the same tissues. Error bars represent standard deviations of results of quadruplicate PCR assays. (B) Using DNA from the same cells, we also performed a nested-PCR assay for total viral DNA by replacing the two Alu primers with a second LTR primer (M667, with the sequence GGC TAA CTA GGG AAC CCA CTG) in the first PCR round. Total HIV-1 DNA was present in all four LC-TC conjugates and in three of four discrete LC samples. Error bars represent standard deviations of results of quadruplicate PCR assays. (C) HIV-1 Gag p24 production over time in cultures of PHA-activated peripheral blood mononuclear cells (PBMCs) mixed with discrete LCs (dashed lines) or LC-TC conjugates (solid lines) isolated from HIV-1-exposed vaginal epithelial sheets (3 donors), as measured by ELISA. Error bars represent standard deviations of results of duplicate or triplicate wells. Day 1 and day 4 p24 measurements are not shown and were consistently negative for all cocultures. Positive-control cultures of PHA-activated PBMCs directly inoculated with a titration of HIV-1_{BaL} were positive at all time points. The lower detection limit of the ELISA was 5 pg/ml p24, and the upper discrimination limit was reached at around 1,000 pg/ml p24.

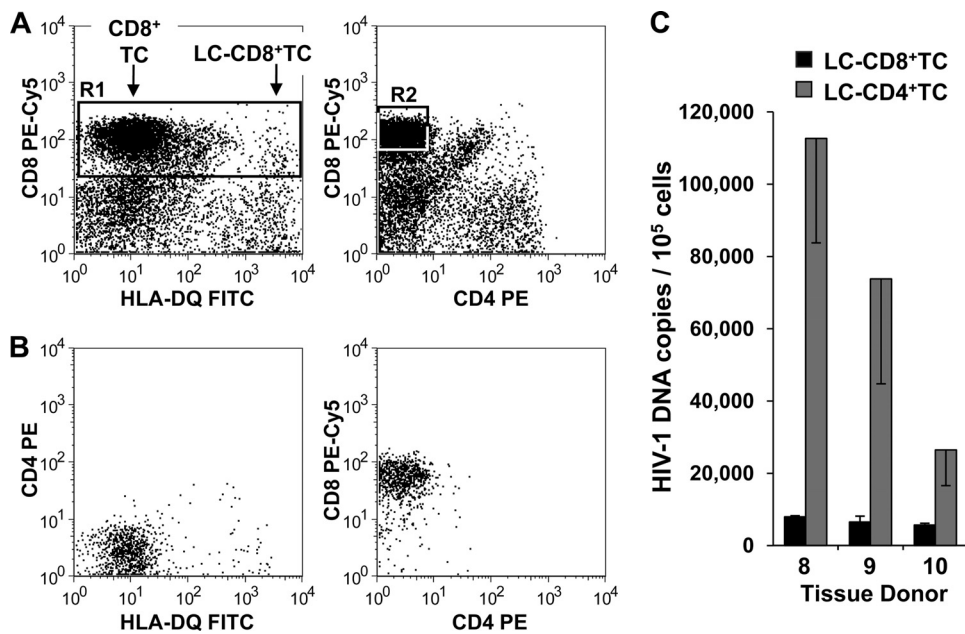


FIG. 3. HIV-1 genomic integration in vaginal LC-CD4⁺ TC versus LC-CD8⁺ TC conjugates. (A) Cells emigrating from vaginal epithelial sheets challenged *ex vivo* with HIV-1_{BaL} were stained with anti-CD4 PE, anti-CD8 PE-Cy5, and anti-HLA-DQ FITC (BD Biosciences) and sorted into a population containing LCs exclusively conjugated to CD8⁺ T cells (LC-CD8⁺ TC) (Boolean regions R1 and R2; discrete CD8⁺ TCs were included as filler cells) and all other cells, including LC-CD4⁺ TC conjugates and mixed conjugates. Region R2 was set very conservatively to exclude any contamination with CD4⁺ cells. (B) Control after sorting, demonstrating the lack of contaminating CD4⁺ cells in the population containing CD8⁺ TCs and LC-CD8⁺ TC conjugates. (C) The two sorted cell populations (LC-CD8⁺ TC and LC-CD4⁺ TC conjugates) were analyzed for integrated HIV-1 DNA in tissues from three donors. Error bars represent standard deviations of results of quadruplicate PCR assays. Total HIV-1 DNA was also measured and tested positive in all samples (not shown).

In contrast, we never detected integrated viral DNA in the discrete LCs from the same four donor tissues (Fig. 2A). While the total number of discrete LCs was always markedly lower than the number of LC-TC conjugates, we assayed equivalent amounts of total DNA, determined the overall sensitivity of the assay as ~5 copies of HIV-1 (20), and detected total viral DNA (i.e., unintegrated) in three of the four purified LC samples (Fig. 2B). This supports the reliability of our negative result for viral integration in purified vaginal LCs. Moreover, in prior studies using less-sensitive microscopic techniques for the detection of newly produced HIV-1 Gag or HIV-1 LTR-controlled green fluorescent protein, we also failed to detect productive infection of vaginal LCs (12). Thus, we conclude that the capacity of unconjugated vaginal LCs to support productive HIV-1 infection is minimal or absent.

To determine whether vaginal LCs, despite not being productively infected by HIV-1, can pass infectious virions to susceptible T cells, we cocultured purified, unconjugated LCs, derived from vaginal epithelial sheets challenged *ex vivo* with HIV-1_{BaL} as described above, with PHA-activated (2 days with 0.4 μ g/ml PHA [Remel]) peripheral blood-derived lymphoblasts. Lymphoblasts were seeded at 1×10^5 cells per well and discrete LCs were seeded at 1,200, 7,400, or 22,500 cells per well (depending on yield after sorting) in duplicate wells of 96-well plates. LC-TC conjugates were seeded in parallel in numbers matching the numbers of discrete LCs in each experiment. Cocultures were performed for 28 days with weekly replenishment of PHA-activated lymphoblasts and addition of 50 U/ml recombinant interleukin 2 (IL-2) (R&D Systems). We

harvested supernatant on days 1, 4, 7, 16, 22, and 28 and measured the Gag p24 concentration by a commercial enzyme-linked immunosorbent assay (ELISA) (PerkinElmer). Two of three cocultures with discrete LCs turned positive for p24 between days 7 and 16; the third turned positive between days 22 and 28 (Fig. 2C). All three cocultures with LC-TC conjugates turned positive for p24 between days 7 and 16 (Fig. 2C). This indicates that although they do not produce new HIV-1 progeny, discrete vaginal LCs can pass on infectious virions, preserved internally or membrane bound, for productive infection of CD4⁺ T cells.

The capacity to support HIV-1 replication is dramatically enhanced in DC-TC conjugates (3, 10, 21), in which viral budding from both cell types can be observed (10). We were therefore interested to test whether vaginal LCs conjugated to T cells remain resistant to HIV-1 integration. We sorted cells emigrating from vaginal epithelial sheets challenged *ex vivo* with HIV-1_{BaL} as described above into a population containing LCs conjugated exclusively to CD8⁺ T cells (Fig. 3A and B) and a population containing other types of LC-TC conjugates (LC-CD4⁺ TC and mixed conjugates) and analyzed them for integrated HIV-1 DNA. The LC-CD8⁺ TC conjugate population also contained discrete CD8⁺ T cells, but care was taken to exclude CD4-expressing cells. Vaginal LC-CD4⁺ TC conjugates from all three donors contained high numbers of integrated HIV-1 DNA copies (126,662, 73,804, and 26,463 copies per 10^5 total cells) (Fig. 3C). The population of discrete vaginal CD8⁺ TCs and LC-CD8⁺ TC conjugates also contained integrated HIV-1 DNA copies, although at lower copy numbers

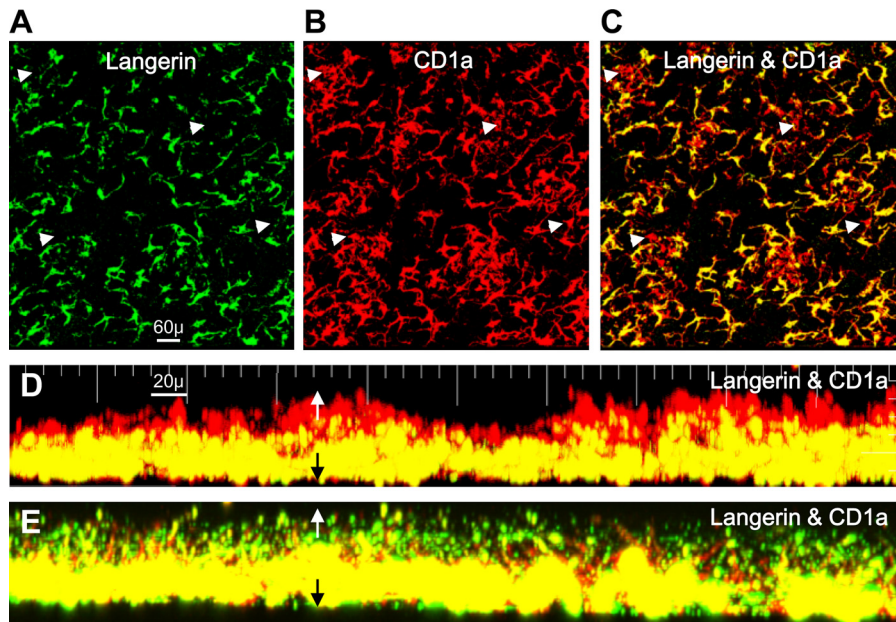


FIG. 4. *In situ* langerin and CD1a staining of LCs embedded within intact epithelial sheets. Epithelial sheets were permeabilized with 0.1% saponin (Sigma), stained with antilangerin (Immunotech) followed by goat anti-mouse Alexa Fluor 488, washed, stained with anti-CD1a Alexa Fluor 647 (all from Invitrogen), and fixed in 2% buffered paraformaldehyde. Stained sheets were embedded in Mowiol 40-88 containing 2.5% (wt/vol) Dabco (Sigma), the complete thickness of the epithelium was scanned using a Zeiss LSM 510 Meta confocal microscope, and acquired image stacks were visualized in three dimensions using Imaris software (Bitplane). (A to C) Horizontal, full-volume projections showing LCs embedded in all layers of the vaginal epithelium. (A) Langerin staining (green). (B) CD1a staining (red). (C) Overlay of langerin and CD1a stains (yellow signifies coexpression of both markers). (A to C) White arrowheads point to cells staining for CD1a but not for langerin. (D) Vertical full-volume projection of the same sheet demonstrating the vertical distribution of langerin and CD1a expression within the epithelium. The black arrow points to the basal side and the white arrow to the apical (vaginal) side of the squamous epithelium. In the lower layers of the epithelium, all CD1a⁺ LCs coexpress langerin (yellow). Toward the upper layer of the epithelium, a zone is reached where the CD1a⁺ LCs are not coexpressing langerin (red). Experiments with sheets from three donors showed similar results. (E) Identically processed skin epithelial sheet demonstrating langerin expression on all epidermal CD1a⁺ LCs.

(7,971, 8,538, and 5,704 copies per 10^5 cells) (Fig. 3C). These copy numbers were still far above the background level we have observed in previous experiments titrating antiretroviral compounds in our mucosal model (20). These results suggest that conjugation of vaginal LCs to CD8⁺ T cells provides an environment for measurable HIV-1 integration, and likely productive infection, in the LCs, CD8⁺ T cells, or both (5, 16, 17, 24). While we consequently cannot rule out the possibility that vaginal LCs lose resistance to HIV-1 integration when conjugated to T cells, this pathway of productive infection would likely be effectively inhibited by antiretroviral microbicides. It is thus less concerning than potential viral escape through nonproductive conservation and transportation of infectious virions, as demonstrated above using discrete LCs.

The ability of vaginal LCs to pass infectious HIV-1 virions on to CD4⁺ T cells stands in contrast to a report that in skin LCs, the C-type lectin langerin serves as a natural barrier to HIV-1 transmission by directing endocytosed HIV-1 to Birbeck granules for degradation (4). This discrepancy may be due to technical details of the assays; for instance, high viral dosages were reported to overcome the barrier effect (4). Alternately, vaginal LCs might exhibit more restricted langerin expression. Differences between skin and vaginal LCs have been identified in mice (14, 27), although in humans, this difference remains unexamined. We therefore evaluated langerin expression by intraepithelial CD1a⁺ vaginal LCs. By

confocal microscopy of three donor tissues, we consistently found that in the lower, more basal layers of the epithelium, all CD1a⁺ LCs coexpressed langerin, but the upper epithelial layers contained a zone of CD1a⁺ LCs lacking langerin expression (Fig. 4A to D). In contrast, langerin was uniformly expressed by skin LCs (Fig. 4E), demonstrating good penetration of all staining reagents into the saponin-permeabilized epithelial sheets. We conclude that a subset of vaginal LCs does not express langerin, and this may aid HIV-1 in bypassing a langerin-mediated degradation pathway.

In summary, we provide evidence that vaginal LCs encountering HIV-1 *in situ* are able to migrate from the epithelium and subsequently transmit virus to CD4⁺ T cells. Productive infection of LCs does not appear to be necessary for viral transmission. In fact, by not productively infecting LCs, HIV-1 may avoid an antiviral innate immune response in these cells (19). Moreover, nonproductive storage of infectious virions in vaginal LCs renders HIV-1 inaccessible to inhibition by reverse transcriptase inhibitors, such as tenofovir. Since HIV-1-harboring LCs may migrate away from the protective action of a topical antiviral drug in the mucosa, this potential mechanism of viral evasion needs to be considered when optimizing topical microbicide products for clinical use.

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