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Inhibition of Two Temporal Phases of HIV-1 Transfer from Primary Langerhans Cells to T Cells: The Role of Langerin

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Epidermal Langerhans cells (eLCs) uniquely express the C-type lectin receptor langerin in addition to the HIV entry receptors CD4 and CCR5. They are among the first target cells to encounter HIV in the anogenital stratified squamous mucosa during sexual transmission. Previous reports on the mechanism of HIV transfer to T cells and the role of langerin have been contradictory. In this study, we examined HIV replication and langerin-mediated viral transfer by authentic immature eLCs and model Mutz-3 LCs. eLCs were productively infected with HIV, whereas Mutz-3 LCs were not susceptible because of a lack of CCR5 expression. Two successive phases of HIV viral transfer to T cells via cave/vesicular trafficking and de novo replication were observed with eLCs as previously described in monocyte-derived or blood dendritic cells, but only first phase transfer was observed with Mutz-3 LCs. Langerin was expressed as trimers after cross-linking on the cell surface of Mutz-3 LCs and in this form preferentially bound HIV envelope protein gp140 and whole HIV particles via the carbohydrate recognition domain (CRD). Both phases of HIV transfer from eLCs to T cells were inhibited when eLCs were pretreated with a mAb to langerin CRD or when HIV was pretreated with a soluble langerin trimeric extracellular domain or by a CRD homolog. However, the langerin homolog did not inhibit direct HIV infection of T cells. These two novel soluble langerin inhibitors could be developed to prevent HIV uptake, infection, and subsequent transfer to T cells during early stages of infection.

H
uman immunodeficiency virus is transmitted sexually by crossing epithelial barriers in the anorectal and genital tracts (1). Langerhans cells (LC), a dendritic cell (DC) subtype residing in the stratified squamous epithelia of the skin or mucosae, efficiently capture Ags and are among the first cell types to encounter HIV within the female genital tract (2) and the inner foreskin (3, 4) in human explant cultures. Regarding the latter, circumcision has been shown to reduce female-to-male transmission by ∼60% in three clinical trials (5–7). Importantly, LCs efficiently transfer HIV to its primary target cells, CD4+ T cells, either in the submucosa or after migration to lymph nodes (2, 4, 8). In the SIV–macaque model, foci of infected T cells underlying intact mucosa of the vagina and ectocervix have been observed by 48 h postinfection (hpi) (9), and some evidence for LC uptake also has been shown (10–12). LCs can either encounter HIV as the virus diffuses between the tight junctions of keratinocytes in an intact mucosa (4) or where there is a breach in the mucosa caused by genital ulcerative diseases such as HSV or epithelial abrasions that occur during intercourse. In foreskin/skin tissue explants (13) or in emigrant LCs infected in situ (14–16), epidermal LCs (eLCs) were permissive to de novo infection by CCR5 using HIV-1 viral strains (R5 HIV-1) at >24–72 hpi.

LCs characteristically express CD1a (17) and the HIV entry receptors CD4 and CCR5 (18) in their immature state, rendering them susceptible to infection primarily by R5 HIV-1 (2, 4, 12). They also uniquely express the HIV binding C-type lectin receptor (CLR) langerin (19) but not DC-specific ICAM-3-grabbing non-integrin (DC-SIGN) or mannose receptor (MR). Langerin has an extracellular domain consisting of a single carbohydrate recognition domain (CRD), a neck domain involved in oligomerization, a short transmembrane domain, and a cytoplasmic tail, which mediates endocytosis, vesicular trafficking, and cytoskeletal movement (20–22). After pathogen or Ag acquisition, LCs usually undergo a process of maturation characterized by upregulation of molecules associated with T cell activation (CD80, CD86, CD83, and CD40), Ag presentation (MHC class I and II) and migration (CCR7), and downregulation of molecules associated with Ag uptake (langerin) and epithelial adhesion (E-cadherin) (23, 24).

Monocyte-derived DCs (MDDCs) are usually used as a cell model to study the interaction of HIV with DCs because tissue DCs are difficult to isolate, require enzymatic digestion resulting in their maturation, and often yield low numbers. Unlike LCs, MDDCs express the CLR s DC-SIGN and MR at high levels. Viral transfer from MDDCs (25, 26) and primary blood myeloid DCs (27) to CD4+ T cells has been reported to occur in two successive phases,
corresponding to the two main routes of HIV entry into DCs. After binding to the concentrating CLRs, the majority of HIV is either taken up into a neutral pH tetraspanin CD81+ compartment termed a virus-containing compartment (VCC) (28, 29) or into the endolysosomal system (30). The minority (<10%) is transferred from CLRs to the HIV entry receptors CD4 and CCR5 (31). Virions that enter the endosomal system are degraded by acid–proteolytic digestion within 6–24 h. If the DC contacts CD4+ T cells in the first few hours after encountering the virus (probably in the mucosa), HIV residing in the VCC can then be transferred to T cells across an “infectious synapse” representing what is termed “first-phase” transfer (32). HIV entering via CD4/CCR5 undergoes de novo replication within 48 h (26), and the virus is then transferred to T cells across a viral synapse (33). This “second phase” of viral transfer occurs at a later stage than the viral transfer from VCCs and increases with time.

Although DC-mediated infection and viral transfer to T cells is well documented using MDDCs and blood myeloid DCs, whether two temporal phases of HIV transfer from eLCs to T cells occurs is still controversial (4, 8, 14, 34–36). Specifically, differences in cell models used (35), cell isolation methods (36), potencies of anti-langerin inhibitory Abs, and titers of HIV inocula used (36) have led to contradictory reports on the relative roles of langerin and CD4/CCR5 in HIV binding, entry, and replication in LCs.

In this study, we have examined whether primary human immature eLCs and a model DC derived from the Mutz-3 cell line (Mutz-3 LCs) can transfer HIV to CD4+ T cells in two successive phases, corresponding to the two temporal phases of HIV trafficking in MDDCs and myeloid DCs. Because oligomerization of CLRs such as DC-SIGN (37–39) and MR (40, 41) has been reported to be essential for the binding of oligosaccharide ligands, we showed that native trimeric HIV envelope protein (HIVAD8 gp140) and HIVbul particles bound strongly to langerin trimers on Mutz-3 LCs, suggesting that soluble langerin homologs may need to be trimeric for maximal inhibition. Thus, we examined inhibition of HIV binding to langerin on eLCs and subsequent first- and second-phase viral transfer to T cells with a langerin-specific mAb and by langerin homologs consisting of soluble trimeric full-length extracellular domain (ECD) or the monomeric truncated carbohydrate recognition domain (CRD). These inhibitors could provide a useful strategy for blocking entry of HIV into anogenital LCs during sexual transmission, particularly if combined with others such as CCR5 inhibitors, perhaps as a novel microbicide.

**Materials and Methods**

**Ethics statement**

Written and informed consent approved by the Western Sydney Local Health District human research ethics committee (Westmead Hospital, Sydney, NSW, Australia) was obtained from all patients undergoing breast reduction or abdominoplasty.

**Isolation of immature skin LCs**

Skin was collected immediately after surgery and was cleared of s.c. fat using a dermatorome. Skin nets were generated using a skin graft mesher (Zimmer, Warsaw, IN) and were incubated at 4°C in RPMI 1640 medium (Sigma-Aldrich) supplemented with 25 µg/ml gentamicin (Sigma-Aldrich) and 2.5 mg/ml disperse II (Roche Biochemicals). After overnight incubation, the epidermis and dermis were separated using forceps. Epidermal sheets were washed twice in PBS and incubated for 60 min at 37°C in RPMI 1640 medium containing collagenase blend F (2.5 mg/ml) and DNase (50 µg/ml). Alternatively, trypsin (0.06%) was used for epidermal dissociation. Viability of eLCs was >90% in both collagenase and trypsin preparations. Isolated eLCs were stained for CD1a, CD45, CD3, and CD14. eLCs were sorted by gating on the CD3+CD14+CD1a+CD45+ population (1–3 × 10^5 LC) on a FACSVantage fluorescence-activated cell sorter to a purity of >97%. eLCs were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated human AB serum (RH10; Invitrogen) and 200 ng/ml GM-CSF (Invitrogen).

**Generation of Mutz-3 LCs, MDDCs, and isolation of CD4+ T cells**

Human CD34+ acute myeloid leukemia Mutz-3 cells provided by S.I.A.M. Santegoets (VU University Medical Center, Amsterdam, the Netherlands) were cultured and treated as previously reported by Santegoets et al. (42) to generate Mutz-3 LCs. MDDCs were generated from CD14+ monocytes using CD14 magnetic beads (Miltenyi Biotec) as described previously (26). CD4+ T cells were isolated from PBMCs using CD4 magnetic beads (Miltenyi Biotec). They were then activated with PHA (5 µg/ml; Sigma-Aldrich) for 48 h, washed, and cultured in 20 U/ml IL-2 (Roche Molecular Biochemicals, Indianapolis, IN).

**Transfer of HIVbul from MDDCs, eLCs, and Mutz-3 LCs to T cells and inhibition assays**

A total of 2.5 × 10^5 MDDCs, Mutz-3 LCs, or eLCs were mock treated or inoculated with either the laboratory adapted R5 using strain HIVR+AZT; the laboratory adapted four times using strain strain NL43 or the primary strain N82 (43) at multiplicities of infection (MOIs) ranging from 0.005 to 10 for 2 h at 37°C to assess their infectivity and whether viral transfer to T cells is MOI dependent. They were then washed three times in PBS and plated in 96-well plates. For the viral transfer inhibition binding proteins LCs and eLCs were treated with either neutralizing mAb to langerin (HIV+anti-lang; 1.4 µM) purchased from Dendritics (clone 817G7) or the reverse transcriptase inhibitor AZT, obtained from National Institutes of Health AIDS Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (HIV+AZT; 50 µM), for 30 min at 4°C or 37°C, respectively, before HIVbul inoculation (MOI of 0.5) for 2 h at 37°C. In other settings, HIVbulk was treated with soluble langerin ECD (aa 70–328) or CRD (aa 200–328) generated in a mammalian Chinese hamster ovary expression system (Apollo Life Sciences Pty, Sydney, Australia) for 1 h at 37°C before addition to the cells at the final concentrations of 0.1 µM HIV+ECD or HIV+CRD. Infected cells were washed three times in PBS and 1 × 10^5 PHA-activated CD4+ T cells or the CD4+ T cell line JLTR were added to mock, HIV-ECD, HIV-CRD, mAb to langerin, AZT, or HIVbul-treated cells at the indicated time points. Half the medium was replenished every 48 h. HIV infection was determined by either staining with an intracellular p24 mAb (KC57-RD1; Beckman Coulter, Fullerton, CA) or by measuring the GFP fluorescence intensity of JLTR cells. Samples were run on a FACSCan II flow cytometer (BD Biosciences) and FlowJo (Tree Star, Ashland, OR) was used for data analysis.

**Measurement of gene expression by quantitative PCR**

Total unamplified RNA was DNase I treated (Promega, Madison, WI) and reverse transcribed using oligo(dT) and superscript III (Invitrogen). The cDNA was subject to QPCR using CCR5 primers (Sigma-Aldrich), accesion number NM-000579 and SYBR Green (Invitrogen) as described previously (24).

**Affinity purification of langerin complexes and protein identification using mass spectrometry**

Chemical cross-linking of cell surface molecules using disuccinimidyl suberate (DSS) (Pierce, Rockford, IL) has been described previously (37). Affinity beads were generated by coupling 100 µg IgG, or anti-langerin (R&D Systems) to cyanogen bromide-activated Sepharose (Sigma-Aldrich), according to the manufacturer’s instructions. Nonreactive beads were removed by incubating the lysate from 2 × 10^7 DSS+ treated Mutz-3 LCs with the IgG; Sepharose beads for 2 h at 4°C. The lysate was then incubated with 200 µl anti-langerin Sepharose beads, and the beads were washed with 10 mM TBS containing 0.1% Triton X-100 (pH 8). Langerin complexes were eluted from the beads by boiling for 5 min in SDS buffer. Affinity purified proteins were identified by electrospray ionization mass spectrometry as described previously (40).

**HIVAD8 gp140 and whole HIVbul binding to Mutz-3 LCs and inhibition assay**

For HIVAD8 gp140 binding assays, mock or cross-linked cells were resuspended in binding buffer (RPMI 1640 medium, 1% BSA, and 10 mM HEPES [pH 7.5]) at 1 × 10^6 cells/50 µl. Three micrograms of biotinylated trimeric HIVAD8 gp140 was added to cells for 30 min at 4°C as described previously (40). To inhibit gp140 binding, 5 mg/ml mannan, 10 mM EGTA,
and 1.4 μM anti-langerin (12D5/DCGM4) were added to the cells for 30 min at 4°C prior to gp120 addition. Cells were then washed three times with PBS, and bound ligands were labeled by incubating the cells with 1 μg/ml streptavidin-PE for 30 min for detection by flow cytometry. For HIV\textsubscript{nat} binding assays, 3 × 10\textsuperscript{4} mock-treated and cross-linked Mutz-3 LCs were incubated with HIV\textsubscript{nat}, ranging from 0.27 to 4.25 μg/ml p24 Ag for 2 h at 4°C. Cells were washed three times with PBS and then lysed in lysis buffer (10 mM HEPES, 140 mM NaCl, 1% Triton X-100, and 10 mM DTT with protease inhibitors). Total protein content was measured using a DC Protein Assay Kit (Bio-Rad, Hercules, CA), and p24 Ag was quantified by ELISA (Beckman Coulter, Brea, CA).

**Statistical analysis**

Differences between treatments were analyzed by Student \( t \) test and modified for unequal variance. A \( p \) value < 0.05 was considered statistically significant.

**Results**

**HIV\textsubscript{nat} infection of eLCs and two phase transfer of HIV\textsubscript{nat} to T cells**

Ex vivo immature eLCs were isolated from epidermis by enzymatic collagenase digestion and flow sorting to collect the CD3\textsuperscript{-}CD14\textsuperscript{-}CD45\textsuperscript{-}CD1a\textsuperscript{+} population (Supplemental Fig. 1A). Because LCs are the only epidermal cells that coexpressed CD1a and langerin (Supplemental Fig. 1B), CD1a was used as a marker to select for eLCs, leaving the langerin epitope unbound and thus available to bind HIV immediately after sorting while eLCs are still in an immature state. To determine whether immature isolated eLCs can be infected with HIV, eLCs from five donors were inoculated with HIV\textsubscript{nat}, at different MOIs for 24 and 72 h. The proportion of HIV p24 Ag-positive (Ag\textsuperscript{+}) eLCs was 3.6 ± 1.2, 2 ± 0.6, 0.8 ± 0.5, and 0.3 ± 0.2% at MOI of 10, 5, 1, and 0.1, respectively, at 72 hpi (Fig. 1A, 1B). No intracellular p24 Ag\textsuperscript{+} cells were detected at 24 hpi. Extracellular p24 Ag was detected by ELISA in supernatants of infected cells at 72 but not at 24 hpi. In addition, no intracellular p24 Ag\textsuperscript{+} cells or extracellular virus were detected at 72 hpi when eLCs were pretreated with the antiretroviral reverse transcriptase inhibitor, zidovudine (AZT), for 1 h before HIV inoculation (Fig. 1B).

We next assessed the ability of eLCs to transfer virus to the JLTR CD4 T cell line. JLTR cells were used as a target for viral transfer from eLCs because they encode the GFP flanked by the HIV-1 promoter, and therefore, they fluoresce green when infected with HIV. eLCs were infected with HIV\textsubscript{nat} (MOI of 1 and 0.1) for 2, 24, 48, and 96 hpi and then cocultured with JLTR cells for 4 d. The amount of transferred virus declined progressively between 2 and 48 hpi as shown by the reduction in the number of GFP\textsuperscript{+} JLTR cells. This was followed by an increase in GFP\textsuperscript{+} JLTR cells when they were added at 96 hpi (Fig. 1C). This increase in transfer from 24/48 to 96 hpi correlated with the kinetics of HIV infection of LCs at 24 and 96 hpi (Fig. 1D). We also compared the kinetics of viral transfer of the laboratory adapted R5 using HIV\textsubscript{nat} to that of a primary R5 strain (NB2) and a laboratory adapted four times using strain (NL43). Both HIV\textsubscript{nat} and NB2 showed the same kinetics of viral transfer at 2, 24, 48, and 96 hpi. However, with NL43 there was transfer only at 2 and 24 hpi (Fig. 1E). In addition, treatment of eLCs with AZT prior to coculture with T cells (Fig. 1F) did not decrease the magnitude of HIV\textsubscript{nat} viral transfer at 2 hpi (first phase) but almost completely abrogated viral transfer at 96 hpi (second phase). Thus, the first phase of viral transfer from eLCs occurs independently of de novo HIV replication within the eLCs, whereas the second phase is dependent on the ability of eLCs to complete de novo viral production prior to transfer to T cells at 96 hpi.

Because trypsin has been reported to cleave CD4 (44) and has been used in other studies (36), we also compared transfer of HIV\textsubscript{nat} (MOI of 0.5) from eLCs isolated using collagenase or trypsin at either 2 or 96 hpi. Viral transfer was observed when JLTRs were added at 2 hpi from both trypsin- and collagenase-isolated eLCs as shown by the number of GFP\textsuperscript{+} JLTR cells. However, at 96 hpi, GFP\textsuperscript{+} cells were detected in collagenase- but not trypsin-isolated eLCs after 4 d of coculture (Fig. 1G). Also, there was a progressive increase in HIV spread in JLTR cells cocultured in collagenase-treated eLCs up to 8 d but still no detectable GFP\textsuperscript{+} cells in those cocultured with trypsin-treated eLCs (data not shown).

To exclude the fact that our findings were not influenced by fluctuation in viability and proliferation of JLTRs used as T cells in the viral transfer assay and which might affect viral replication, we assessed their proliferation using CFSE staining and their viability using DAPI at different time points after passage. There was no to little fluctuation in viability (90 ± 7% over 6 d postpassage (Supplemental Fig. 2A). Similarly, over this 6-d culture period, the cells continued to proliferate uniformly as shown by the gradual decrease in the intensity of CFSE stain (Supplemental Fig. 2B). Furthermore, the HIV\textsubscript{nat} infection of JLTRs showed a uniform increase in the proportion of GFP\textsuperscript{+} cells over time (Supplemental Fig. 2C).

**HIV\textsubscript{nat}-infected Mutz-3 LCs only mediate first-phase viral transfer**

Model Mutz-3 LCs were produced after 10 d of cytokine treatment of the parental Mutz-3 cells as reported previously (42). Mutz-3 LCs expressed CD1a, langerin, and CD4 but not DC-SIGN, MR, and CCR5 (Fig. 2A). Thus, they resembled authentic eLCs apart from the undetectable cell surface CCR5 expression. This lack of CCR5 surface expression on Mutz-3 LCs was investigated and compared with precursor Mutz-3 cells and also eLCs. Prior to cytokine treatment, Mutz-3 expressed low levels of cell surface CCR5 (7 ± 1.5%) and moderate levels intracellularly (20 ± 2.7%) (Fig. 2B). After cytokine treatment to convert them into Mutz-3 LCs, CCR5 was detected at high levels intracellularly (69 ± 5%) but was not expressed on the cell surface. In contrast, eLCs from four donors variably expressed cell surface CCR5 (12 ± 6.3%) (two donors shown in Fig. 2B). In addition, CCR5 mRNA levels were slightly higher in Mutz-3 LCs than Mutz-3 cells, but both were markedly and significantly lower when compared with eLCs from three different donors (Fig. 2C). The lack of CCR5 expression by Mutz-3 LCs is partly due to their maturation state as measured by the proportion of cells expressing costimulatory molecules CD80 (80 ± 4.3%), 86 (30 ± 3.2%), and especially CD83 (25 ± 2.7%) (Fig. 2D, panel 1). In addition, Mutz-3 LCs could be further matured by the addition of maturation mix for 24–48 h as the expression of CD83 increased from 25 to 58%, whereas CD86 increased from 30 to 90% (Fig. 2D, panel 2). However, when freshly isolated eLCs (Fig. 2D, panel 3, CD83 73%) matured (Fig. 2D, panel 4, CD83 64%), leading to a greater proportion of CD83 expression than Mutz-3-LCs (25%), CCR5 was still detectable on mature eLCs, indicating that the magnitude of CD83 expression is not an accurate predictor of CCR5 expression between cells. Thus, maturation of eLCs and Mutz-3 LCs downregulates CCR5, but the effect is greater on Mutz-3 LCs probably because of the lower expression levels on the parental Mutz-3 cell line.

When Mutz-3 LCs were inoculated with HIV\textsubscript{nat} at different MOIs (10, 4, 1, 0.5, and 0.005), they did not produce virus de novo as demonstrated by the fact that no intracellular p24 Ag was detected when compared with eLCs up to 96 hpi (Fig. 2E). In this LC model, HIV entry via fusion that usually leads to productive infection was impaired because Mutz-3 LCs did not express CCR5. However, Mutz-3 LCs infected at an MOI of 1 with the
G protein vesicular stomatitis virus (VSV-G) pseudotyped HIVBaL, which enters cells independently of CD4/CCR5 for fusion and entry, showed a significant population of p24+ cells (24.6 ± 5%) by 96 hpi (Fig. 2F). Overall, these results show that Mutz-3 LCs express langerin but not CCR5 and therefore could not be infected de novo with HIVBaL because of failure of entry.

In the absence of productive infection, the kinetics of HIVBaL transfer from Mutz-3 LCs to JLTR cells was assessed to investigate their suitability as a model to study eLC-mediated first-phase viral transfer to T cells. Mutz-3 LCs were exposed to HIVBaL at an MOI of 1 before JLTR cells were added to the culture at serial time points up to day 4. As shown in Fig. 3A, first-phase transfer was observed from Mutz-3 LCs to JLTR cells between 0 and 48 hpi, but the rate of viral transfer gradually decreased over time, probably as a result of endolysosomal degradation. In addition, when Mutz-3 LCs were inoculated with HIVBaL at different MOIs (4, 1, 0.5 and 0.005), the amount of transfered virus correlated with the MOI used (Fig. 3B). Varying the MOI did not affect transfer kinetics, because a relative decline in the amount of virus transferred over the 96-h period was observed, irrespective of the MOI used. Furthermore, treatment of Mutz-3 LCs with AZT prior to HIV exposure did not affect their ability to transfer virus to JLTR cells (Fig. 3C), demonstrating that productive infection of these cells was not required for first-phase transfer. In addition, when PHA-activated CD4+ T cells were cocultured at 2 hpi with HIV-exposed Mutz-3 LCs, the proportion of infected CD4+ T cells...
was higher than those infected with cell-free supernatants at a similar MOI (Fig. 3D), indicating that uninfected HIV-exposed Mutz-3 LCs are effective vehicles for HIV transfer to primary CD4+ T cells. In summary, this indicates that the degree of virus transfer from Mutz-3 LCs was MOI dependent and that they were only able to engage in first-phase vesicular trafficking and transfer.
experiments is shown. (A) 8-aminoguanidine was added to Mutz-3 LCs at 2, 4, 6, 10, 20, 24, and 48 hpi, and the percentage of GFP+ JLTR cells was determined 96 h postcoculture by flow cytometry. Data are representative of three experiments. The percentage of infected JLTRs is shown in the top right of each histogram. (B) Mutz-3 LCs were exposed to a different MOI of HIVBaL ranging from 4 to 0.005. HIV transfer was determined by assessing the percentage of GFP+ JLTR cells using flow cytometry 4 d postcoculture. (C) Mutz-3 LCs were either mock treated, infected with HIVBaL, or HIVBaL exposed Mutz-3 LCs. CD4 infectivity was determined by flow cytometry (Fig. 5D). Similarly, a 2-fold enhancement in gp140 binding was demonstrated on DSS+ cells compared with that in DSS$^-$ cells. The select binding of HIVBaL viral particles was demonstrated on DSS+ cells in all six clones. In the absence of cytotoxicity, DSS$^+$ cells displayed an enhanced gp140 binding level, with a 2-fold increase in gp140 binding compared with that in DSS$^-$ cells, as detected by flow cytometry (Fig. 5B). Similarly, a 2-fold enhancement in the binding of HIVBaL viral particles was demonstrated on DSS$^+$ cells by p24 ELISA over a range of input virus (Fig. 5C). The gp140–langerin interaction was further characterized by using various inhibitors to specifically block gp140 binding to langerin on DSS$^-$ and DSS$^+$ cells. The inhibitors used included the following: 1) yeast derived mannan, which like gp140, is highly glycosylated and expresses carbohydrate structures recognized by the CRD (clones 122D5/DCGM4 and 817G7). In DSS$^+$ cells, mannan, EGTA, and anti-langerin reduced gp140 binding to baseline levels (95% inhibition), suggesting that gp140 binds to langerin in both a carbohydrate and Ca$^{2+}$-dependent manner.

**Inhibition of HIV-1 Transfer from LCs**

To determine the predominant oligomerization state of langerin on the cell surface to guide the use of inhibitors, Mutz-3 LCs were cross-linked with increased concentrations of DSS. DSS maintains protein–protein interactions between langerin molecules, thus preserving its oligomeric state on the cell surface. In the absence of DSS, the oligomeric langerin dissociated and resolved to a monomeric molecular mass of 40 kDa (Fig. 5A). However, langerin resolved to a dimeric molecular mass of 80 kDa when the cells were exposed to low DSS concentrations (15–120 μM). Langerin dimers gradually increased in abundance with increasing DSS concentration, and this increase also was associated with both the appearance of a trimeric band and a concomitant decrease in the abundance of the monomeric band. Langerin trimers continued to increase in abundance and remained the predominant oligomerization species at saturating DSS concentrations (1–2 mM), whereas no monomeric langerin and little to no dimeric langerin were detected with DSS concentrations >1 mM (Fig. 5A). To investigate the possibility that proteins other than langerin may be participating in the formation of langerin complexes, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry analysis was performed on peptides isolated from trypsin-digested affinity-purified monomeric and trimeric langerin from DSS$^+$ Mutz-3 LCs. Table I lists the peptides identified from gel slices corresponding to monomeric and trimeric langerin. A total of eight langerin-derived peptides were identified in the monomeric band (expectation value = 5.7 × 10$^{-5}$) and six were identified in the trimeric band (expectation value = 0.023), confirming the presence of langerin in these bands. The absence of peptides from other potentially interacting proteins suggests that langerin mainly exists as homotrimers on cell membranes of Mutz-3 LCs.

**Kinetics of viral transfer in Mutz-3 LCs, MDDCs, and eLCs**

We next compared the kinetics of HIV transfer to T cells from langerin-expressing Mutz-3 LCs and eLCs with that of MDDCs (expressing MR and DC-SIGN) to T cells. Both MDDCs and eLCs showed two phases of viral transfer to JLTR cells, whereas Mutz-3 LCs exhibited only first-phase transfer (Fig. 4). When the kinetics of viral transfer were compared between MDDCs and eLCs, we observed that the first-phase transfer by eLCs was extended up to 48 hpi compared with <24 hpi in MDDCs, indicating that eLCs had slower kinetics of first-phase transfer compared with MDDCs.

FIGURE 3. HIVBaL transfer from Mutz-3 LCs to T cells. (A) JLTR cells were added to Mutz-3 LCs at 2, 4, 6, 10, 20, 24, and 48 hpi, and the percentage of GFP+ JLTR cells was determined 96 h postcoculture by flow cytometry. Data are representative of three experiments. The percentage of infected JLTRs is shown in the top right of each histogram. (B) Mutz-3 LCs were either mock treated, infected with HIVBaL, or HIVBaL-exposed Mutz-3 LCs. CD4 infectivity was determined by flow cytometry (Fig. 5D). Similarly, a 2-fold enhancement in gp140 binding was demonstrated on DSS+ cells compared with that in DSS$^-$ cells, as detected by flow cytometry (Fig. 5B). Similarly, a 2-fold enhancement in the binding of HIVBaL viral particles was demonstrated on DSS$^+$ cells by p24 ELISA over a range of input virus (Fig. 5C). The gp140–langerin interaction was further characterized by using various inhibitors to specifically block gp140 binding to langerin on DSS$^-$ and DSS$^+$ cells. The inhibitors used included the following: 1) yeast derived mannan, which like gp140, is highly glycosylated and expresses carbohydrate structures recognized by the CRD (clones 122D5/DCGM4 and 817G7). In DSS$^-$ cells, these inhibitors reduced total gp140 binding by ~50% (Fig. 5B). However, in DSS$^+$ cells, mannan, EGTA, and anti-langerin reduced gp140 binding to baseline levels (95% inhibition), suggesting that gp140 binds to langerin in both a carbohydrate and Ca$^{2+}$-dependent manner.
Second-phase transfer was inhibited by 30% and showed a 66% reduction in first-phase transfer and a 79% reduction in second-phase transfer when JLTR cells were added at 2, 24, 48, and 96 hpi. The mean data of GFP+ JLTR cells from three experiments is shown with SE (± SE) bars.

**Soluble langerin ECD, CRD, and neutralizing anti-langerin mAb inhibits HIV transfer from eLCs to JLTR cells**

We first checked the oligomeric status of ECD and CRD by native PAGE and showed that soluble langerin CRD exists solely as a monomer (17 kDa), whereas a mixture of monomeric (40 kDa) and trimeric (120 kDa) langerin was present in the soluble ECD (Fig. 6A). Next, the potential cytotoxicity of soluble langerin ECD and CRD was assessed on Mutz-3 LCs prior to the use of these compounds in the inhibitory assays. The ECD and CRD were not cytotoxic at concentrations ranging from 0.1 and 1 μM, whereas some cytotoxicity (20%) was observed at 2 μM (Fig. 6B). Because LCs and DCs are potent stimulators of T cells, we also assessed whether the ECD form of soluble langerin impaired the ability of DCs to stimulate allopurplication of T cells using a CFSE-based MLR assay. Treatment of Mutz-3 LCs with concentrations of ECD ranging from 0.1 to 1 μM did not affect their ability to stimulate T cell proliferation (Supplemental Fig. 3).

To determine whether soluble langerin can act as a potential inhibitor of HIV transfer, HIVBaL that was pretreated with either ECD or CRD was then added to eLCs or Mutz-3 LCs with final concentrations of 0.4 μM. The concentrations selected were based on surface plasmon resonance binding data of gp120 to CRD and ECD (data not shown), which is similar to recently published findings (46), and took into account our cytotoxicity studies and available amounts of reagent. eLCs or Mutz-3 LCs pretreated with AZT (50 μM) before HIV inoculation were used as positive controls for HIV transfer. HIV-ECD (0.1 μM) showed inhibitory activity against both first- and second-phase transfer (Fig. 6C), with a peak reduction of 55% in first-phase transfer and a 12% in first-phase transfer of virus to JLTR cells when the latter cells were added at 2 h after HIV inoculation of eLCs and a 66 ± 12.5% reduction in second-phase transfer when JLTR cells were added 96 h after eLC inoculation. HIV-CRD (0.1 μM) also effectively inhibited both phases of viral transfer, with a 46 ± 10% reduction in first-phase transfer and a 79 ± 7% reduction in second-phase transfer of virus to JLTR cells. Higher levels of inhibition of first-phase (56 ± 6%) and second-phase (85 ± 4%) transfer were observed at higher CRD concentrations (0.4 μM; data not shown). Also, we assessed whether the CRD-specific mAb (clone 817G7), which has been shown to strongly inhibit oligomeric langerin–gp140 interactions (Fig. 4B), can inhibit viral transfer from eLCs to T cells. eLCs pretreated with the mAb to langerin (1.4 μM) showed a 66 ± 5% reduction in first-phase viral transfer (Fig. 6C). Second-phase transfer was inhibited by 30 ± 10% when the mAb was added once to eLCs 30 min before and during the 2-h infection. However, when the mAb was also maintained postinfection and replenished every 48 h, there was an 82 ± 10% reduction in JLTR infection (Fig. 6C). Thus, both the langerin mAb and soluble langerin ECD and CRD inhibited both phases of HIVBaL transfer from immature eLCs to JLTR cells but sustained presence of the mAb in the medium was required to inhibit second-phase transfer.

Furthermore, to further verify the inhibitory effect of the ECD and anti-langerin on first-phase transfer, we treated Mutz-3 LCs with HIVBaL, that was preincubated with langerin ECD (0.1 μM) and showed that transfer from Mutz-3 LCs to PHA-activated CD4+ T cells was inhibited by 65 ± 7%. In addition, when Mutz-3 LCs were pretreated with either the anti-langerin mAb or AZT, there was 55 ± 8% reduction in first-phase transfer to CD4+ T cells (Fig. 6D), whereas AZT had no effect on such transfer. As a control, we examined the effects of langerin ECD on direct HIV
infection of JLTR cells and found that there was no difference in the proportion of infected JLTR cells whether they were inoculated with either HIV or HIV preincubated with langerin ECD (Fig. 6E).

**Discussion**

In this study, authentic immature primary eLCs were infected with CCR5 using laboratory (HIVBaL) and primary (NB2) strains at different MOIs ranging from 0.05 to 10. Two successive phases of HIV transfer from eLCs to T cells were observed at all MOIs: first-phase transfer mediated by the CLR langerin and a second phase of transfer followed de novo replication mediated through CCR5 and CD4 and facilitated by HIV binding to langerin. These two phases of viral transfer from eLCs to T cells were similar to those previously reported in MDDCs (25, 26) and as shown in Fig. 4. However, LCs showed slower kinetics of first-phase transfer and also a delayed onset of second-phase transfer, which was inhibited by AZT. Thus, the processes of viral transfer in MDDCs and eLCs differ only in their kinetics. As previously observed in MDDCs, the CXCRI4 using strain NL43 showed only first-phase but not second-phase transfer in eLCs. This is expected because CXCRI4 expression is very low on immature MDDCs and LCs, and these cells do not support productive replication of X4 strains (47).

These results clarify the apparent contradictions in reports of first and second phase transfer and the controversial role of langerin in HIV infection, for which there is no consensus. First, comparing these results to previous studies of first-phase vesicular uptake and transfer (26, 27), they are consistent with those using isolated CD34+ stem cells (35) but not with others (36). CD34+ stem cell–derived LCs express MR and langerin, and consequently, the role of langerin could not be distinguished from MR (35). Our study used authentic eLCs, which do not express MR and can only bind the virus via langerin (19). In addition, our study used cell-free virus and authentic isolated immature eLCs like others (36), but there were several clear differences, particularly in their low levels of HIV inoculum, the different clones of anti-langerin Ab used and LC dissociation by trypsin, which unlike collagenase used in this study, cleaves off the HIV binding domain of membrane-bound CD4 (44). With respect to viral inocula, we used a range of HIV MOIs from low (0.1) to high (10), similar to those used previously to demonstrate two-phase transfer in MDDCs and blood myeloid DCs (26, 27). Higher titers of HIV are required for de novo infection of DCs compared with macrophages and T cells because the majority of the HIV inoculum is destroyed via vesicular uptake, probably in an undefined endocytic pathway (30). For an optimal single cycle of HIV infection, we have found that >90% of DCs need to take up the virus, and >5% of cells within the cell sheet are then infected (26). This need for high inoculum appears to be a consistent property of all immature DCs when infected with HIV, partly because of constitutive SAMHD1 expression (48) and inducible restriction factors (49). If the MOI is too low, observations for over a week are necessary to detect infectious virus. Exposure of DCs to high titer HIV inocula is, however, physiologic as high levels of virus (burst size) are released from T cells, and 100–10,000 infectious particles/cell (50, 51) are potentially available to contact LCs, especially in an ulcerated anogenital epithelium. Comparison of eLCs isolated using trypsin rather than collagenase showed that eLCs isolated by trypsin were able to engage in first- but not second-phase transfer as shown by GFP detection when JLTR cells were added at 2 hpi but not 96 hpi of eLCs (Fig. 1F).

Furthermore, because HIV-gp120 binds to cell surface langerin (19, 52), we examined the oligomeric status of cell surface langerin on Mutz-3 LCs. We showed that langerin can be expressed as a homotrimer, similar to that of the soluble extracellular domain ECD (53) and that this enhances its ability to bind HIV, with no evidence of covalent langerin binding to CD4 or CCR5. These studies guided the choice of soluble langerin homologs to test as inhibitors of HIV binding to eLCs, including the trimeric ECD and the key-binding domain, the CRD. Both inhibited HIV binding and transfer, as did certain mAbs to langerin. The mAb to langerin CRD used in our transfer inhibition studies was carefully selected and based on our studies examining inhibition of oligomeric langerin–HIV gp140 interactions in view of previous reports that some of these Abs do not block either these interactions or viral transfer (31, 38). This was also the reason for using three types of inhibitors.

Thus, the role of langerin in mediating first-phase transfer is clearly shown by inhibition of such transfer with both an Ab and soluble langerin homologs. Two studies of LCs in genital epithelium in ex vivo explants also claimed to demonstrate first-phase transfer: Ballweber et al. (8) inoculated vaginal explants with cell-free virus and demonstrated that emigrating LCs showed no integrated HIV at 48 hpi but were still able to transfer virus to T cells. They interpreted this appropriately as first-phase transfer. As we show in this study, the kinetics of HIV transfer from LCs

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**Table I. Tryptic peptides identified by mass spectrometry in bands containing monomers and oligomers of langerin**

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<td>Langerin</td>
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<td>227–244</td>
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Data indicate whether the peptide was identified in a gel slice containing monomeric langerin or oligomeric langerin.

Data were calculated monoisotopic masses of uncharged peptides.

Δ indicates data were calculated as difference between the experimentally derived mass of the precursor and the calculated mass of the peptide.

M* indicates oxidized methionine. Amino acids flanking the peptide on both sides are shown separated from the peptide sequence by a full stop.

Given are the accession numbers for the SwissProt database.

Data indicate the position of the peptide in the unprocessed precursor obtained from the SwissProt database.
are slower compared with MDDCs, and no HIV was detected in supernatants until 72–96 hpi. Therefore, perhaps the presence of integrated HIV DNA should be assessed at later time points before concluding that vaginal LCs do not support de novo infection. The transfer that they observe on days 7–16 could still be “second phase.” Ganor et al. (4) infected inner foreskin explant cultures with either cell-free or cell-associated virus and showed rapid uptake of HIV by LCs and formation of LC–T cell conjugates within 1 h. However, transfer of HIV within these conjugates was assumed rather than demonstrated. Neither studies were able to assess the role of langerin. The exact mechanism for the longer duration of first-phase transfer in LCs needs to be investigated. Although first-phase viral transfer has been clearly shown to occur from VCCs in MDDCs, rapid degradation over 6 h also occurs (Fig. 4) (26), suggesting an interaction between the VCC and the endocytic pathway, which has not yet been defined. This may differ somewhat between the two cell types, resulting in slower degradation and longer preservation of infectious viruses in LCs.

For second-phase or de novo infection and transfer, our results are consistent with previous reports from the Blauvelt and Kawamura laboratories (34), except for the role of langerin. Using CCR5 inhibitors (PSC-RANTES) or eLCs from CCR5

FIGURE 6. Inhibiting HIV transfer from LCs to T cells using soluble langerin homologs and neutralizing mAb to langerin. (A) A representative native PAGE gel showing the oligomeric states of purified soluble langerin CRD and ECD used in blocking experiments. (B) Cytotoxicity of soluble langerin ECD and CRD. Mutz-3 LCs were seeded in 96-well plates at 50,000 cells/100 μl media, and both forms of soluble langerin were added individually at the final concentrations of 0.1, 0.2, 0.5, 1 and 2 μM. After 72 h, Mutz-3 LCs were counted using trypan blue to assess viability (n = 5). (C) eLCs were 1) HIV\textsubscript{Int} treated (HIV), 2) treated with HIV that was preincubated with either ECD (HIV+ECD) or CRD (HIV+CRD) for 1 h at 37°C, 3) treated with mAb to langerin CRD (clone 817G7) for 30 min at 4°C before HIV\textsubscript{Int} infection (HIV+anti-lang), or 4) treated with the reverse transcriptase inhibitor AZT (HIV+AZT) for 30 min before HIV\textsubscript{Int} infection. Cells were infected at an MOI of 0.5 for 2 h at 37°C. JLTR cells were added after eLCs were thoroughly washed three times in PBS either at 2 hpi (first-phase transfer) or at 96 hpi (second-phase transfer). Data shown represent the mean GFP\textsuperscript{+} JLTR ± SE from three separate experiments derived from different donors eLCs using flow cytometry after 96 h postcoculture (**p ≤ 0.02, ***p ≤ 0.005). (D) Mutz-3 LCs were similarly treated with inhibitors as described in (C), and CD4\textsuperscript{+} T cells were added after infected Mutz-3 LCs were washed three times in PBS. Intracellular p24 staining was assessed after 96 h postcoculture (n = 3, **p = 0.02 for both ECD and anti-langerin). (E) JLTR cells were inoculated with either HIV or HIV-ECD at an MOI of 0.2 or 1 for 2 h at 37°C, followed by three washes in PBS. Data shown represent the mean GFP\textsuperscript{+} JLTR ± SE (n = 3, *p = 0.04).
heterozygotes, they reported that CD4 and CCR5 are of key importance in productive HIV infection of LCs and in subsequent transfer to T cells. However, their conclusion that there was no role for langerin in concentrating or transferring virus to these receptors was based on a single application of mannan (14, 18, 34) and one report of anti-langerin Ab (14) (clone 122D5/DCG4M4) as inhibitors. These studies used LCs infected in situ in epidermal explants, which then emigrated 3 d. The apparent disparity with our results may be explained in Fig. 6. Blocking second-phase transfer required sustained presence of the langerin Ab in the culture, not a single dose prior and at the time of viral application. Presumably this is due to HIV persistence beyond the activity of the Ab both in vitro culture and within epidermal explants. Furthermore, other clones of anti-langerin CRD Ab are claimed to be more potent inhibitors (36). The results with the ECD and CRD inhibitors indicate that the role of langerin is similar to that of DC-SIGN in enhancing both phases of viral transfer. As a control, ECD langerin did not impair direct HIV infection of T cells, indicating the inhibition was specific and not just steric hindrance of HIV binding to CD4/CCR5.

Using transcriptomic analysis, we recently reported that Mutz-3 cells are different from authentic eLCs (54). However, these model cells are useful in examining HIV binding to surface langerin and in assessing the role of langerin, especially when it is impossible to obtain sufficient eLCs for biochemical studies. After cytokine manipulation, Mutz-3 LCs expressed high levels of CD4 and langerin but lacked the expression of DC-SIGN, MR, and cell surface CCR5. In addition, intracellular CCR5 RNA was much lower than in eLCs. Thus, as shown in Fig. 3, Mutz-3 LCs could only be productively infected with VSV-G pseudotyped and not with wild-type HIVBaL. This explains the absence of the second phase transfer by Mutz-3 LCs at any HIV concentration (MOIs of 4, 1, 0.5, and 0.05), in contrast to a previous report (55). HIVBaL was used in this study as it was previously shown a higher affinity for CCR5 than any other commonly used HIV strain (43).

The relative importance of first versus second phase transfer from LCs to T cells in vivo or ex vivo requires further study despite the recent suggestions from foreskin and vaginal explant studies that first phase is more important (4, 8), contrasting with previous studies suggesting they are T- rather than M-tropic (57), which might suggest first phase transfer to be more important. However, second-phase transfer also would result in passage through mucosal T cells, so the latter cells remain as the filter even after transmission from DCs (57).

In the absence of a HIV vaccine, the search for effective microbicides to protect against sexual transmission remains an important approach to prevent infection. Currently, the only microbicide reported to be clinically effective is the reverse transcriptase inhibitor, Tenofovir, which showed a 39% decrease in infection rate (58). It is therefore likely that combination therapy with microbicides will be required to improve efficacy, similar to systemic antiviral therapy and/or chemoprophylaxis. Such a strategy should include inhibitors of HIV entry into mucosal DCs. Prior to this study, HIV transmission by LCs to T cells has been reported to be either independent (2, 8, 59) or dependent on productive LC infection (15, 16, 59, 60), and the controversy surrounding the function of langerin during the early stages of infection has led to uncertainty in designing and testing langerin inhibitors as potential anti-HIV microbicides. Thus, these two temporal phases of HIV uptake and transfer have been shown sequentially in authentic eLCs and that both are at least partially mediated by langerin. Furthermore, we showed that soluble langerin ECD and CRD and also mAb to langerin were potent in inhibiting first- and second-phase viral transfer to T cells. Perhaps a combination of specific inhibitors may be required to block both the interaction between HIV envelope and trimeric langerin on the eLC surface and also the interaction between HIV envelope and CD4/CCR5, thereby optimally inhibiting both the viral transfer from VCC and productive infection. In addition, the equivalent inhibitory efficacy of the smaller CRD monomer to the trimeric ECD is promising for the development of synthetic inhibitors.

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Disclosures
The authors have no financial conflicts of interest.

References


