Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: Implications for HIV primary infection

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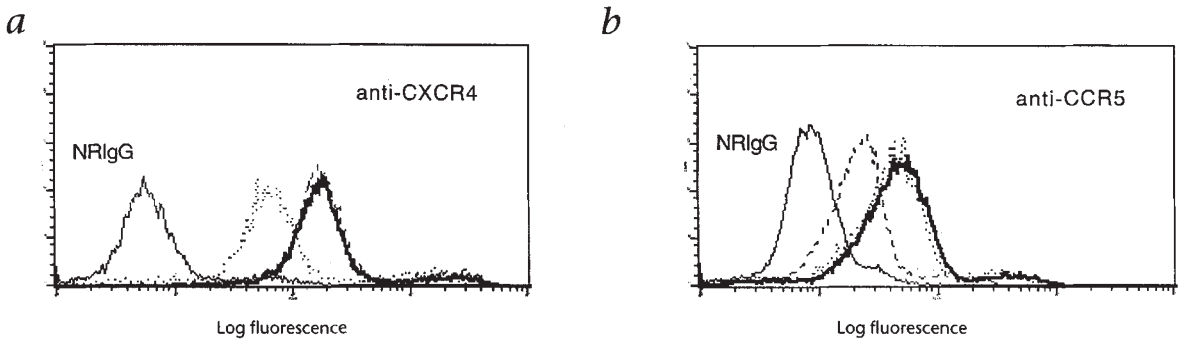
Transmission of HIV-1 is predominantly restricted to macrophage (MΦ)-tropic strains. Langerhans cells (LCs) in mucosal epithelium, as well as macrophages located in the submucosal tissues, may be initial targets for HIV-1. This study was designed to determine whether restricted transmission of HIV-1 correlates with expression and function of HIV-1 co-receptors on LCs and macrophages. Using polyclonal rabbit IgGs specific for the HIV co-receptors cytokines CXCR4 and CCR5, we found that freshly isolated epidermal LCs (resembling resident mucosal LCs) expressed CCR5, but not CXCR, on their surfaces. In concordance with surface expression, fresh LCs fused with MΦ-tropic but not with T-tropic HIV-1 envelopes. However, fresh LCs did contain intracellular CXCR4 protein that was transported to the surface during *in vitro* culture. Macrophages expressed high levels of both co-receptors on their surfaces, but only CCR5 was functional in a fusion assay. These data provide several possible explanations for the selective transmission of MΦ-tropic HIV variants and for the resistance to infection conferred by the CCR5 deletion.

Several studies have demonstrated that sexually transmitted HIV-1, isolated from newly infected individuals, represents minor variants of the swarm of viruses found in the transmitters' blood, and most often exhibits macrophage (MΦ)-tropism *in vitro*[1–5]. Since most new infections occur via mucosal surfaces, it is important to determine the distribution of the HIV-1 co-receptors, CXCR4 and CCR5, on target cells present at these surfaces and in underlying tissues. Langerhans cells (LCs) are $CD4^+$ $CD1a^+$ antigen-presenting cells in the dendritic cell (DC) family that are present within the epidermis and mucosal epithelium and that are believed to be the initial targets for HIV infection[6–8]. Indeed, after intravaginal inoculation of rhesus macaques with simian immunodeficiency virus (SIV), submucosal DCs were the first infected cells identified[9,10]. It has recently been shown that blood-derived DCs (ref. 11, 12) and activated skin-derived DCs (ref. 11) express CXCR4 and CCR5 mRNA (ref. 11) and protein[12], and that these cells could be infected with different HIV strains in a co-receptor-dependent manner[11,12]. However, these and other studies[13–15] have failed to show a correlation between the restricted sexual transmission of MΦ-tropic HIV-1 and specific target cell–HIV interactions.

Populations of LCs and other DCs (isolated from tissues and derived from blood precursors by using cytokines *in vitro*) demonstrate marked phenotypic and functional heterogeneity[16–19]. These features are generally dependent on the maturation state and particular tissue localization of DCs. For example, *in situ* epidermal LCs represent prototypic nonlymphoid DCs. They express relatively low levels of surface major histocompatibility complex (MHC) and costimulatory molecules; contain characteristic ultrastructural features, that is, Birbeck granules; and are functionally proficient at micropinocytosis, endocytosis and processing of foreign antigens[16,18]. However, following antigen uptake *in vivo*, LCs greatly upregulate MHC and costimulatory cell-surface molecules, migrate from epithelial surfaces to regional lymph nodes, and potently stimulate naive and memory T cells in an MHC-restricted antigen-specific manner[21,22]. Freshly isolated LCs resemble LCs *in situ*, whereas LCs placed into culture for 1–3 days, as well as LCs that spontaneously migrate from skin explants *in vitro*, phenotypically and functionally resemble LCs that have migrated to lymph nodes[13,14,16–18]. In the current study, expression of CXCR4 and CCR5 in freshly isolated epidermal LCs (as a model for mucosal LCs *in situ*[23–26]) was examined. Co-receptor expression in these cells was compared to that of cultured epidermal LCs, cytokine-derived blood DCs, and monocyte-derived macrophages. We show that freshly isolated LCs express functional CCR5, but not CXCR4, on their cell surface. Unlike freshly isolated LCs, cultured LCs and blood-derived DCs expressed low surface levels of both co-receptors. Macrophages expressed high levels of both HIV co-receptors; however, these cells were only able to fuse with cells expressing MΦ-tropic (and not T-tropic) envelopes. Our findings suggest that restricted sexual transmission of MΦ-tropic HIV-1 may in part be explained by the differential pattern of HIV-1 co-receptor surface expression and their function on initial target cells, that is, mucosal resident LCs and submucosal tissue macrophages, respectively.

Specificity of anti-CXCR4 and anti-CCR5 antiserum

Polyclonal immune serum specific for the extracellular amino termini of CXCR4 (ref. 27) and CCR5 was generated in rabbits,



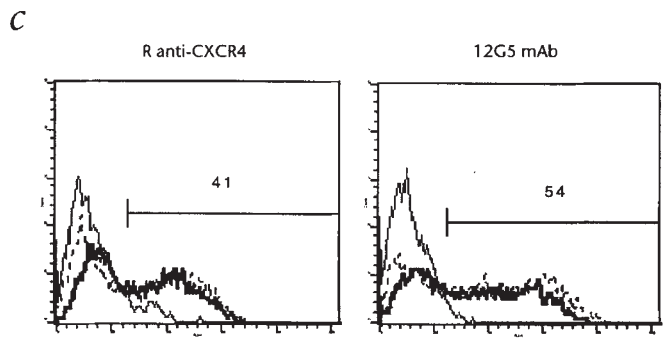


Fig. 1 Specific staining of elutriated monocytes with anti-CXCR4 and anti-CCR5 rabbit IgGs. Elutriated monocytes were incubated with human IgG (2 μg/ml) for 30 min to saturate FcR and were then incubated with normal rabbit preimmune IgG (NRIgG) (thin line), rabbit anti-CXCR4 (*a*) or rabbit anti-CCR5 (*b*) IgGs. The immune IgGs were preadsorbed with control glycine-Sepharose beads (heavy line) or with Sepharose beads conjugated to CCR5 peptide-KLH (dashed line) or CXCR4 peptide-KLH (dotted line). *c*, NIH.3T3.CD4.CXCR4 cells were treated with PBS (heavy line) or 0.25% trypsin solution (dotted line) for 30 min at 37 °C and washed several times with 10% FCS-containing medium. Cells were stained with rabbit anti-CXCR4 (as above) or biotin-conjugated 12G5 followed by TC-streptavidin. Negative controls were stained with NRIgG or biotin-conjugated anti-FasL mAb (PharMingen), respectively (thin line).

and the IgG fractions were isolated as described below. The peptides used for immunization showed no significant homology to the N termini of other known α- and β-chemokine receptors. The specificity of the immune IgGs was determined initially in a fusion inhibition assay using PM1 cells, a human T-cell line previously shown to fuse with both T-tropic and MΦ-tropic HIV-1 envelopes[28] (Table 1). Rabbit anti-CXCR4 IgG (but not preimmune IgG) blocked (by 54%) syncytium formation between PM1 and 12E1-vPE16 (T-tropic, IIIB envelope). This blocking was reversed by prior adsorption of antiserum with CXCR4-peptide-KLH, but not with CCR5-peptide-KLH, conjugated to Sepharose beads. Conversely, fusion of PM1 with 12E1-vCB28 (MΦ-tropic, JR-FL envelope) was blocked (51%) by anti-CCR5 rabbit IgG, and blocking was reversed by prior adsorption of antiserum with CCR5-peptide-KLH, but not with CXCR4-peptide-KLH conjugated to beads. These polyclonal IgGs were also shown to stain cell-surface chemokine receptors on murine fibroblast transfectants (obtained from NIH AIDS Reagent Repository), on human cell lines (data not shown), and on primary human cells. In the last case, a sensitive three-step staining protocol was developed (see the Methods section). When we followed this protocol, both antisera reacted strongly with elutriated human monocytes, and specific staining could be reduced by 60% when the reagents were preadsorbed with relevant (but not irrelevant) peptides (Fig. 1).

Because isolation of fresh LCs requires trypsinization of epidermis, it was important to determine whether CXCR4 and CCR5 are sensitive to trypsin degradation (as is the case with CD4, data not shown). Treatment of NIH.3T3.CD4.CXCR4 and NIH.3T3.CD4.CCR5 stable transfectants (kindly provided by Dan Littman) or monocytes with 0.25% trypsin did not reduce their surface CXCR4 and CCR5 expression (Fig. 1*c* and data not shown). Similar results were obtained by using either our rabbit anti-CXCR4 IgG or the 12G5 (ref. 29) murine monoclonal antibody (Fig. 1*c*).

Expression of CXCR4 and CCR5 on epidermal LCs

To determine which co-receptors are expressed on resident LCs, freshly isolated epidermal cell suspensions (consisting primarily of keratinocytes) were prepared by limited trypsinization of suction blister roofs as described[30]. LCs, which constitute only 2–3% in these cell suspensions, were easily identified by their characteristic expression of CD1a (Fig. 2*a*) and HLA-DR. Their "immature" phenotype was further confirmed by the presence of large numbers of

Table 1 Specific blocking of syncytium formation by anti-CXCR4 and anti-CCR5 rabbit IgGs

Target	Effector	Rabbit IgG[a]	Adsorbed[b]	No. of syncytia[c]	Percent blocking
PM1	12E1-vPE16	–	–	359 ± 39	0
	(IIIB env)	NRIgG[d]	–	353 ± 26	1
		RαCXCR4	–	167 ± 9	54
			CXCR4 pept-KLH	328 + 49	9
			CCR5 pept-KLH	156 ± 12	57
		RαCCR5	–	357 ± 42	0
	12E1-vCB28	–	–	507 ± 16	0
	(JR-FL env)	NRIgG	–	524 ± 29	0
		RαCXCR4	–	505 ± 11	1
		RαCCR5	–	250 ± 29	51
			CXCR4 pept-KLH	230 ± 9	55
			CCR5 pept-KLH	508 ± 15	0

[a]All polyclonal IgG preparations were used at 10 μg/ml (final concentration).
[b]Rabbit anti-CXCR4 and anti-CCR5 IgGs were adsorbed overnight with Sepharose beads conjugated to CXCR4-peptide-KLH, CCR5-peptide-KLH or glycine (control). No reduction in blocking activity was seen after adsorption with control beads.
[c]Syncytium formation was measured 3–4 h after coculture (1:1 ratio, 1×10^5 cells each) of PM1 target cells with CD4⁻ 12E1 effector cells infected with the indicated vaccinia recombinants at 10 PFU/cell. Numbers represent mean ± s.d. of triplicate cultures.
[d]NRIgG, preimmune normal rabbit IgG.

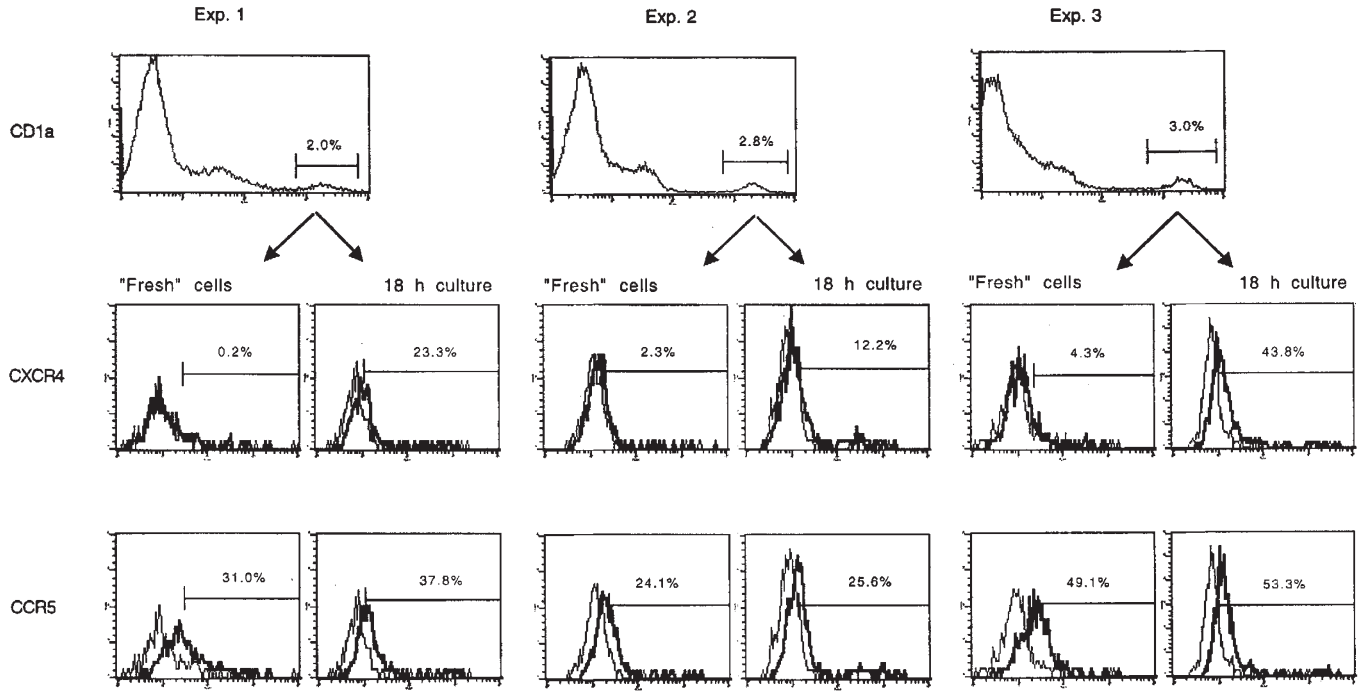


Fig. 2 Freshly isolated epidermal LCs express surface CCR5, but not CXCR4. Two-color flow cytometry was carried out by using FITC-conjugated anti-CD1a mAb and rabbit anti-CXCR4, anti-CCR5 IgG (heavy line) or NRIgG (thin line), as described in Fig 1. Epidermal cells were stained fresh or were first cultured for 18 h in complete RPMI medium supplemented with rhGM-CSF (200 U/ml). $CD1a^{hi}$ LCs (2–4% of total epidermal cells) were gated, and analyzed for co-receptor expression by using the FL-3 channel. Numbers in histograms represent percent positive cells after subtraction of background (staining with NRIgG).

Birbeck granules after ultrastructural analyses (J. Orenstein and A.B., data not shown). Two-color flow cytometry was performed on epidermal cells with fluorescein isothiocyanate (FITC)-conjugated anti-CD1a monoclonal antibody and rabbit anti-CXCR4, anti-CCR5 or preimmune IgG, followed by successive incubations with biotinylated goat anti-rabbit antibody and Tri-color (TC)-conjugated streptavidin. Freshly isolated $CD1a^+$ LCs expressed low levels of surface CCR5 (24–49% $CCR5^+$ cells in ten separate experiments), but not surface CXCR4. CXCR4 mRNA was expressed in freshly isolated highly purified (>99%) LCs, and intracellular CXCR4 protein was detected when cells were permeabilized before immunolabeling and flow cytometry (Fig. 3, *a* and *b*). This surprising finding was in contrast to the selective cell-surface expression of CCR5 by these cells (Fig. 2). No significant intracellular staining of other LC surface proteins, for example, CD1a and CD4, was found (data not shown). To confirm and extend these findings, we determined the kinetics of CXCR4 appearance on LCs over a period of 18 hours, by using our polyclonal rabbit anti-CXCR4 IgG and the 12G5 monoclonal antibody. As shown in Fig. 3*c*, no surface CXCR4 expression was seen on freshly isolated LCs or on LCs cultured for 2–4 hours. However, after overnight culture, 22–26% of LCs were $CXCR4^+$ as determined by both reagents, in agreement with earlier experiments (Fig. 2). In summary, cell-surface expression of the HIV co-receptors CXCR4 and CCR5 is differentially regulated on LCs, possibly by both transcriptional and post translational transport mechanism(s).

On the basis of these data, *in situ* LCs are predicted to be susceptible to infection with only MΦ-tropic strains of HIV-1. To correlate surface co-receptor expression with fusion potential, we isolated highly purified $CD1a^+$ LCs by cell sorting, and incubated the sorted cells with HIV envelope-expressing 12E1 cells (as in Table 1). Initial experiments failed to show syncytium formation, which was attributed to loss of surface CD4 during the trypsinization procedure. Subsequently, CD4 was re-introduced into sorted LCs by using a recombinant vaccinia-CD4 virus (vCB7)[31] (before the fusion assays). Three hours after infection, LCs were mixed with 12E1 effector cells expressing either T-tropic (IIIB) or MΦ-tropic envelopes (JR-FL, ADA, Ba-L) (Fig. 3*d* and data not shown). The kinetics of syncytium formation was followed for 18 hours and compared with that of PM1 cells as positive controls. No syncytia were formed between vaccinia-CD4 infected $CD1a^-$ keratinocytes and any of the HIV envelope-expressing cells, consistent with the lack of CXCR4 or CCR5 expression on these cells. The PM1 cells formed syncytia rapidly (within 3 hours), reaching plateau numbers within 6 hours (Fig. 3*d*). It was noteworthy that the sorted $CD1a^+$ vCB7-infected LCs started to fuse with the MΦ-tropic Env-expressing cells within 3 hours and that the numbers increased during the overnight incubation. In contrast, no syncytia were formed between LCs and T-tropic Env-expressing cells for at least 6 hours. Syncytia were first detected after 9 hours, and their numbers increased overnight, although they never reached the numbers of syncytia observed with the MΦ-tropic envelopes. The involvement of surface CXCR4 and CCR5 in these fusion events was confirmed by the specific blocking of fusion by the appropriate rabbit anti-CXCR4 and anti-CCR5 IgGs. Thus, a clear correlation was found between the kinetics of CXCR4 and CCR5 surface expression on LCs (Fig. 3*c*) and the kinetics of syncytium formation in the fusion assays (Fig. 3*d*). Together, these data demonstrate that freshly isolated epidermal LCs, which closely resemble resident

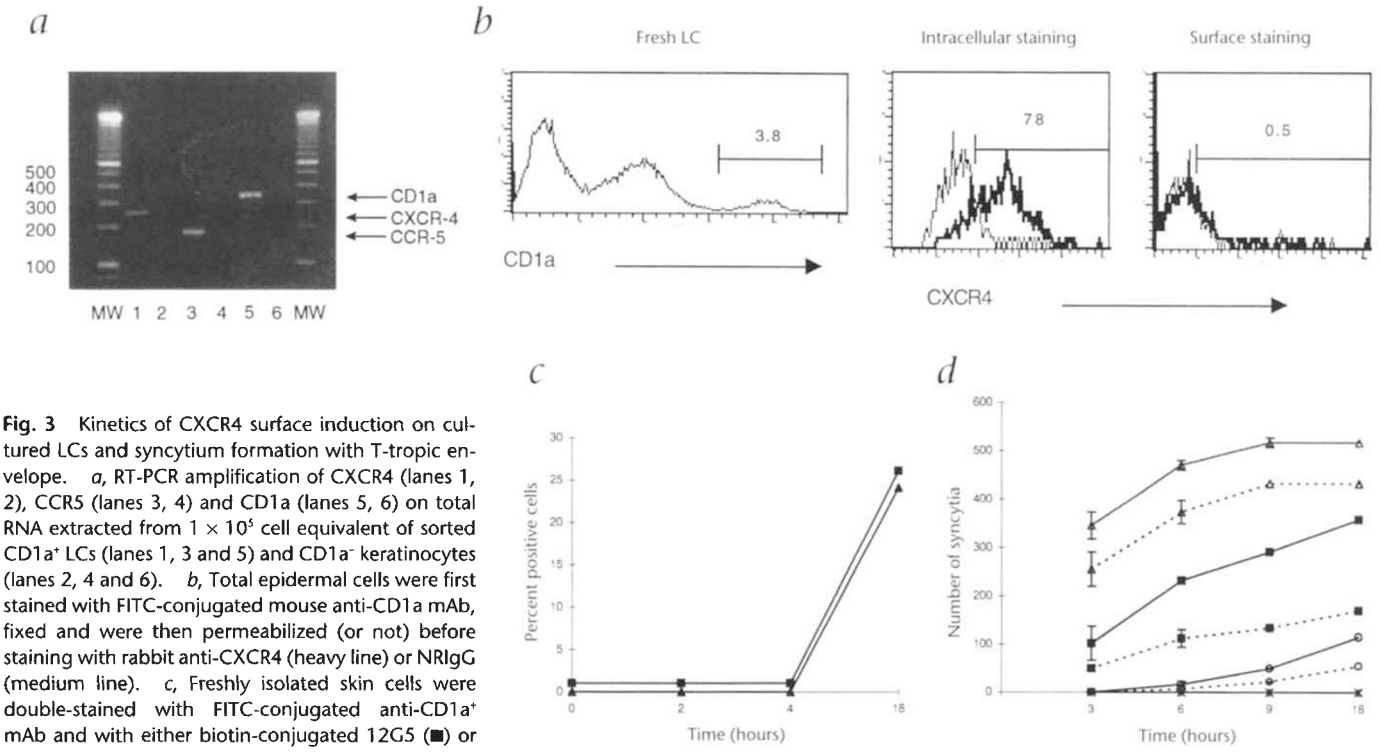


Fig. 3 Kinetics of CXCR4 surface induction on cultured LCs and syncytium formation with T-tropic envelope. *a*, RT-PCR amplification of CXCR4 (lanes 1, 2), CCR5 (lanes 3, 4) and CD1a (lanes 5, 6) on total RNA extracted from 1×10^5 cell equivalent of sorted $CD1a^+$ LCs (lanes 1, 3 and 5) and $CD1a^-$ keratinocytes (lanes 2, 4 and 6). *b*, Total epidermal cells were first stained with FITC-conjugated mouse anti-CD1a mAb, fixed and were then permeabilized (or not) before staining with rabbit anti-CXCR4 (heavy line) or NRIgG (medium line). *c*, Freshly isolated skin cells were double-stained with FITC-conjugated anti-$CD1a^+$ mAb and with either biotin-conjugated 12G5 (■) or rabbit anti-CXCR4 followed by biotin-conjugated goat anti-rabbit antibody (▲). Cells were stained immediately after isolation or after 2, 4 and 18 h in culture. *d*, Sorted $CD1a^-$ keratinocytes and $CD1a^+$ LCs were infected with recombinant vaccinia-CD4 (vCB7) at 2 PFU/cells for 3 h, after which they were washed and mixed with 12E1 cells infected for 10 h with: vPE16 (IIIB) in the absence (o—o) or presence (o----o) of rabbit anti-CXCR4 IgG (10 μg/ml), or vCB28 (JR-FL) in the absence (■—■) or presence (■----■) rabbit anti-CCR5 IgG (10 μg/ml). In parallel, PM1 cells were mixed with 12E1 expressing IIIB (Δ—Δ) or JR-FL (Δ----Δ) envelopes. Syncytia were scored at 3, 6, 9 and 18 h. Numbers represent the means ± s.d. of triplicate cocultures. The experiment was repeated twice.

LCs located within mucosal epithelium, express surface CCR5, but not CXCR4, and that this expression pattern correlates with fusion potential of LCs.

Expression of CXCR4 and CCR5 on LC-like DCs

The biology of DCs has been studied with cells derived from bone marrow, cord blood and peripheral blood DC precursors and monocytes [propagated in culture medium containing recombinant human granulocyte/macrophage colony-stimulating factor (rhGM-CSF) and recombinant human interleukin-4 (rhIL-4) (ref. 19, 20)]. These cytokine-derived DCs have proven to be useful experimentally, because relatively large numbers of purified cells can be readily obtained. The blood-derived DCs we have generated potently stimulated allogeneic and antigen-specific autologous T cells and exhibited a phenotype similar (but not identical) to that of freshly isolated LCs (Fig. 4*a* and ref. 12). In contrast to staining of freshly isolated LCs, staining of blood-derived DCs with our anti-CXCR4 and anti-CCR5 IgGs revealed a low but consistent surface expression of both co-receptors (15–30% positive cells in eight experiments) (Fig. 4*b*). More recently, identical results were obtained with rabbit IgGs specific for the third extracellular loops of CCR5 and CXCR4 (data not shown). In the fusion assays, DCs formed syncytia with effector cells expressing either T-tropic (IIIB) or MΦ-tropic (JR-FL, Ba-L) envelopes. It is important to note that syncytia were specifically blocked with the anti-HIV-1 co-receptor IgGs (Table 2). Thus, our data demonstrate that CXCR4 and CCR5 are expressed on LC-like blood-derived DCs at low levels, and that this expression is sufficient to support fusion with T-tropic and MΦ-tropic HIV-1 envelopes, respectively. However, the expression pattern on cytokine-derived DCs contrasts with the pattern observed on freshly isolated tissue DCs (that is, LCs). This suggests that cytokine-derived DCs may not be an optimal model to study HIV-1–LC interactions that may occur *in vivo*.

Macrophages only fuse with MΦ-tropic envelopes

Previous studies provided evidence that HIV-1 can be found within macrophages present in the upper and lower human female reproductive tract[32,33]. Normally, macrophages may be found in lamina propria adjacent to the mucosal epithelium, and it is possible that they could have direct contact with HIV in cases of genital ulceration or tears in mucosal epithelium. Staining of macrophages ($CD14^+$, $CD1a^-$) derived from elutriated peripheral blood monocytes revealed high surface levels of both CCR5 and CXCR4 (Fig. 4, *a* and *b*). These findings were also supported by polymerase chain reaction with reverse transcription (RT-PCR) analyses demonstrating specific mRNA for both co-receptors in pure macrophages (S.L., data not shown). However, in contrast to LCs and blood-derived DCs (Table 2), differentiated macrophages fused only with MΦ-tropic, but not with T-cell line-tropic envelopes (Table 3). This block of fusion is not lineage specific, since elutriated monocytes fused with both types of envelopes (Table 3). Experiments are under way to elucidate the differentiation-induced changes in the function of CXCR4. However, our data suggest that in spite of the presence of both co-receptors on cell surfaces of macrophages, this cell type is likely to be susceptible only to MΦ-tropic strains *in vivo*, and could contribute to the re-

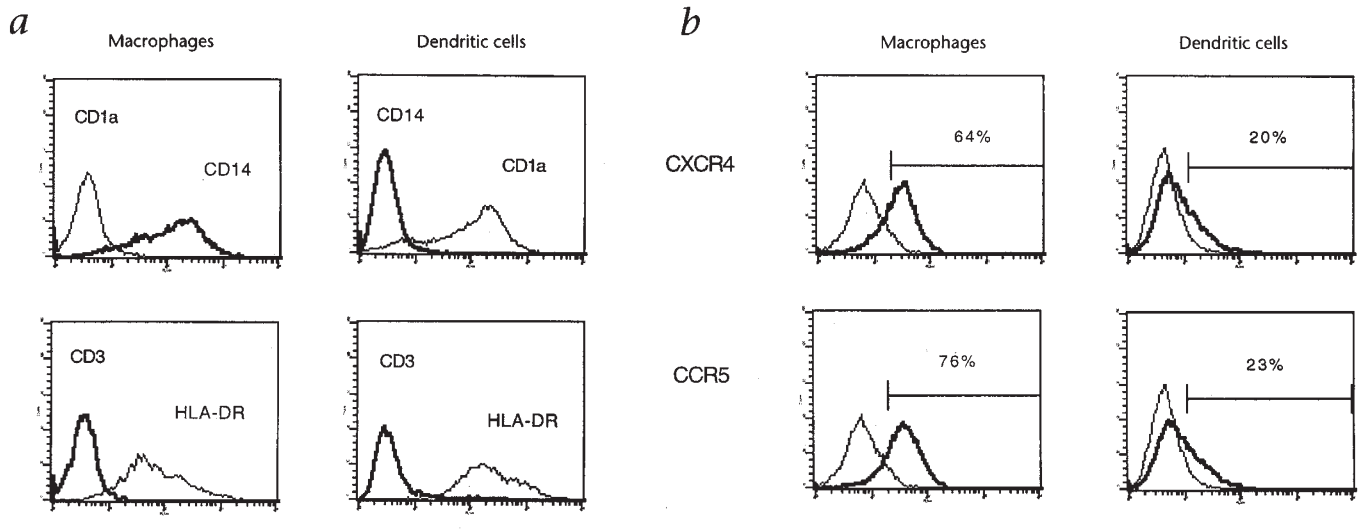


Fig. 4 Expression of CXCR4 and CCR5 by blood-derived DCs and macrophages. Macrophages and DCs were generated as described in Methods. *a*, Macrophages and DCs were stained either with FITC-conjugated mAbs against CD1a, CD14, CD3 HLA-DR or CD4 (not shown), or with FITC-conjugated isotype control mAb. *b*, Cells were stained with NRIgG (thin line), rabbit anti-CXCR4 or rabbit anti-CCR5 IgGs (heavy line), as described in Fig. 1. Data shown are representative of eight experiments with each cell population.

stricted transmission of MΦ-tropic strains of HIV-1 during mucosal exposure to virus.

Discussion

In studies on the role of LCs and other DCs in the immunopathogenesis of HIV disease, Steinman and colleagues, as well as other investigators, have used blood-derived DCs and activated skin-derived DCs to model initial events that may occur following mucosal exposure to virus[11–15,34–37]. However, these studies did not address HIV-1 co-receptor expression on freshly isolated nonactivated LCs, which closely resemble LCs *in situ*, and did not provide a possible explanation for the selective transmission of MΦ-tropic HIV-1. Our data clearly demonstrate expression of surface CCR5, but not CXCR4, on freshly isolated LCs and the induction of surface CXCR4 on cultured LCs. Indeed, the kinetics of induction of CXCR4 on LCs upon culturing correlated with the kinetics of fusion with T-tropic envelope-expressing cells. Thus, our findings stress the importance of examining tissue DCs in a manner that most closely resembles DC phenotype and function *in situ*. Although fusion assays do not always predict productive infection of HIV-1 in a given cell population, they are usually predictive of viral entry. Subsequent steps required for productive infection, including complete reverse transcription, integration and viral transcription, may require additional factors that depend on cellular activation and/or exogenous signals provided by other cell types. In this regard, Granelli-Piperno *et al.*[11] demonstrated that DCs derived from blood and skin explants could be infected by both T-tropic and MΦ-tropic strains, and limited reverse transcription was observed. However, T cells were required for productive infection to ensue. Thus, our data suggest that "*in situ*" LCs located on the mucosal surfaces allow preferential entry of MΦ-tropic HIV strains. Subsequent interactions with T cells following migration of the infected LCs to the submucosal areas and the regional lymph nodes will support productive viral replication and transmission. Similarly, monocytes may allow viral entry[38], whereas viral replication may depend on subsequent cellular interactions in specific tissue sites, further differentiation, or possibly the presence of other opportunistic infections[39]. Differentiated macrophages represent a unique case of discordance between surface expression and functionality of the CXCR4 co-receptor. These cells have been shown to fuse with and to support viral replication only of MΦ-tropic strains of HIV-1, in spite of high levels of CXCR4 expression (demonstrated in our study). Either differential processing of the CXCR4 molecules in monocytes versus macrophages, or the activity of other cellular molecules, might affect the ability of CXCR4 to function as an HIV co-receptor. H. Moriuchi *et al.* presented preliminary data suggesting that the CXCR4 molecules in macrophages are altered on the way to the surface, resulting in inefficient binding of T-tropic HIV strains (4th Conf. on Human Retroviruses and Related Infections, 22–26 Jan. 1997, Washington, DC). Their data, however, do not explain the differences between monocytes and macrophages observed in our study.

Our study provides several possible explanations for the preferential transmission of MΦ-tropic HIV-1 following mucosal exposure to HIV. It does not provide an explanation for the restricted transmission (although not absolute) of MΦ-tropic strains via other routes (that is, maternal–fetal, contaminated needles). However, it is conceivable that blood and tissue DCs

Table 2 Blood-derived DCs fuse with T-tropic and MΦ-tropic envelopes in a co-receptor-dependent manner

Target	Effector	Rabbit IgG[a]	No. of syncytia[b]	Percent blocking
Dendritic cells[c]	12E1-vPE16(IIIB)	NRIgG	60 ± 1	0
		RαCXCR4	25 ± 2	60
		RαCCR5	65 ± 7	0
	12E1-vCB28(JR-FL)	NRIgG	90 ± 23	0
		RαCXCR4	97 ± 15	0
		RαCCR5	28 ± 5	70
	12E1-vCB43(Ba-L)	NRIgG	166 + 20	0
		RαCXCR4	160 ± 15	1
		RαCCR5	43 ± 4	75

[a]All polyclonal IgG preparations were used at 10 μg/ml (final concentration).
[b]Syncytium formation was measured 3–4 h after coculture (1:1 ratio, 1×10^5 cells each) of PM1 target cells with $CD4^-$ 12E1 effector cells infected with the indicated vaccinia recombinants at 10 PFU/cell. Numbers represent mean ± s.d. of triplicate cultures.
[c]Dendritic cells were generated from plastic-adherent PBMCs as described in Methods.

Table 3 Elutriated monocytes, but not differentiated macrophages, fuse with cells expressing T-tropic envelopes

Cells[a]	No. of syncytia[b]				
	IIIB	MN	RF	JR-FL	Ba-L
Monocytes	132 ± 6	210 ± 20	195 ± 15	173 ± 8	125 ± 17
Macrophages	0	6 ± 3	4 ± 2	192 ± 45	155 ± 11

[a]Elutriated monocytes and differentiated macrophages were all <1% $CD3^+$, >85% $CD14^+$ and >95% $HLA\text{-}DR^+$ as determined by flow cytometry (see Fig. 3). Macrophages were derived from elutriated monocytes in 7-day cultures in medium supplemented with rhGM-CSF.
[b]Syncytium formation was measured by using effector 12E1 cells infected with recombinant vaccinia virus expressing T-tropic envelope (vPE16, IIIB) or MΦ-tropic envelopes (vCB28, JR-FL; vCB43, Ba-L) or with H9 cells chronically infected with IIIB, MN and RF T-tropic viruses (obtained from the NIH AIDS Research and Reference Reagent Program). Syncytia were scored after 4–5 h and after overnight cocultures.

play a role in initial infection via these routes as well (especially by cell-associated virus). Of interest are the findings of Cameron *et al.*[40] who showed that DCs isolated from tonsils and thymus permit viral entry by MΦ-tropic (Ba-L) but not T-tropic (IIIB) strains.

In addition, we show for the first time that surface expression of CXCR4 may be regulated by a post translational mechanism or mechanisms. A similar phenomenon was described for a subset of human memory T cells, which contain preformed intracellular CD40 ligand that is rapidly transported to the surface upon cell activation. These cells were found in the outer zone of the germinal centers where they function in cognate activation of memory B cells[43]. It is conceivable that the rapid transport of preformed CXCR4 to the surface of LCs contributes to LC activation and emigration from the epithelium after antigen uptake. This emigration process is associated with other phenotypic and functional changes in LCs as well, with the end result being a potent antigen-presenting cell capable of stimulating both naive and memory T cells within regional lymph nodes[16,18].

Our data also emphasize the importance of using multiple assays to determine chemokine receptor expression and function in primary cells. It suggests that immunostaining of fixed (permeabilized) tissue may demonstrate the presence of intracellular co-receptors proteins in certain cells, which may not always predict cell-surface expression. In addition, our data emphasize the point that cell-surface expression may not always correlate with chemokine receptor function. As a prototype for this situation, we show here that macrophages express high levels of CXCR4, yet are essentially unable to fuse with T-cell line-tropic envelopes in our functional assay. The reasons for the disparity between surface staining and function of CXCR4 on macrophages are currently under investigation, but this disparity may be due to altered receptor conformation and/or post-binding interference with fusion.

In summary, we have tested both surface expression and function of the HIV-1 co-receptors CXCR4 and CCR5 on possible initial targets for HIV-1 infection. Freshly isolated epidermal LCs, which are models for mucosal LCs in situ, express surface CCR5, but not CXCR4. Monocyte-derived macrophages, which we used to model submucosal tissue macrophages, expressed high levels of both HIV co-receptors, yet were only able to fuse with MΦ-tropic HIV-1 envelopes. Thus, by either restricted surface expression (LCs) or restricted function (MΦ), these data provide several possible explanations for the selective sexual transmission of MΦ-tropic HIV variants and for the resistance to infection conferred by the homozygous CCR5 deletion[41,42].

Methods

Generation of anti-CXCR4 and anti-CCR5 rabbit polyclonal IgGs. Two synthetic peptides corresponding to the predicted extracellular N termini of CXCR4 (MEGISIYTSDNYTEEMGSGDYDSMKEPCFREENANFNK) and CCR5 (MDYQVSSPIYDINYYTSEPCQKINVK-QIAAR) were synthesized, purified and characterized as previously described[27]. The linear peptides were conjugated to keyhole limpet hemocyanin (KLH) through the N-terminal cysteine residue[27]. New Zealand White rabbits were immunized with the KLH-conjugated peptides at 1 mg protein/dose emulsified in complete Freund's adjuvant followed by two or three boosts (3 weeks apart) in incomplete Freund's adjuvant. Preimmune and immune IgG fractions were prepared from the serum by two rounds of saturated ammonium sulfate precipitations. After primary titrations, all rabbit IgGs were used at a 10 μg/ml final concentration in both staining and fusion inhibition assays. When indicated, anti-CXCR4 and anti-CCR5 rabbit IgGs were adsorbed overnight (4 °C) with Sepharose 4B beads (Sigma, St. Louis, MO) coupled to KLH-conjugated peptides (CXCR4 or CCR5) or to glycine.

Isolation of epidermal cells and propagation of blood-derived DCs and macrophages. Epidermal cells were obtained from suction blister roofs on the anterior thighs of healthy volunteers, and epidermal cell suspensions were prepared by limited trypsinization of blister roofs as previously described[30], except that the concentration of trypsin was reduced to 0.25% (previously determined not to affect CXCR4 and CCR5 surface expression). DCs were propagated from plastic-adherent PBMCs in complete RPMI medium supplemented with rhGM-CSF (1000 U/ml, Immunex Corp., Seattle, WA) and rhIL-4 (1000 U/ml, R&D Systems, Minneapolis, MN) as previously described[12]. After 7 days in culture, DCs were purified by negative selection by using a cocktail containing mouse anti-human CD3, CD14, CD16 and CD19 monoclonal antibodies (mAbs; all from PharMingen, San Diego, CA) followed by incubation with sheep anti-mouse IgG-coated magnetic beads (Dynal, Great Neck, NY) to deplete contaminating cells. Macrophages were propagated from elutriated monocytes in DMEM (4.5 g/l glucose, 1 mM sodium pyruvate, 2 mM L-glutamine, 10% human serum) supplemented with rhGM-CSF (1000 U/ml, Immunex Corp.). Cells were harvested by scraping the surfaces of plastic culture dishes.

Flow cytometric analyses. All cell types were incubated with the various rabbit IgG reagents at 10 μg/ml for 1 h (4 °C). After washing cells were incubated with biotinylated goat anti-rabbit antibody [$F(ab')_2$] followed by TC-conjugated streptavidin (both from Caltag Laboratories, South San Francisco, CA, at 1:50 final dilution), and were analyzed on a FACScan instrument (Becton Dickinson, Mountain View, CA) by using a 650-nm long pass filter (FL-3 channel). Alternatively, cells were stained with biotin-conjugated anti-CXCR4 murine 12G5 mAb followed by PE-conjugated streptavidin (both from PharMingen). For some experiments, cells were double-stained with the rabbit polyclonal sera (or 12G5) and FITC-conjugated anti-CD1a mAbs (Ortho Diagnostic Systems, Raritan, NJ, at 10 μg/ml) and the $CD1a^+$ cells (that is, LCs) were gated and assessed for HIV co-receptor expression. For intracellular staining, total epidermal cells were first stained with FITC-conjugated anti-CD1a mAb for 1 h at 4 °C (surface staining) and then fixed in 1% paraformaldehyde solution for 15 min on ice, washed with PBS, and permeabilized with Fix and Perm kit (Caltag Labs), according to manufacturer's instructions. After permeabilization, cells were stained with preimmune rabbit IgG or with anti-CXCR4 and anti-CCR5 rabbit IgGs (as described above), or with FITC-conjugated anti-CD1a or FITC-conjugated anti-CD4 (Leu3a) antibodies.

Reverse transcriptase-PCR analyses. Freshly isolated epidermal cells were stained with FITC-conjugated anti-CD1a mAb and were sorted into $CD1a^+$ LCs (>99% pure) and $CD1a^-$ keratinocytes by using dual laser FACStar^{PLUS} (Becton Dickinson). Total RNA was extracted from 4 x 10^5 cells by using RNAzol (Teltest Inc., Friendswood, TX). Contaminating DNA was eliminated with RNase-free DNase 1 (Boehringer Mannheim, Indianapolis, IN). Oligo-dT primed cDNA was prepared by using Moloney murine leukemia virus RT (Gibco-BRL, Gaithersburg, MD) (control reactions contained no RT), and resuspended in 80 μl TE (10 mM Tris pH 8.0, 1 mM EDTA). Aliquots of 20 μl were amplified by using *Taq* polymerase (Perkin-Elmer

Cetus, Norwalk, CT) and primers specific for CXCR4, CCR5 or CD1a. Upstream and downstream oligonucleotide primers and conditions were as follows: CXCR-4 (34 cycles: denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, extension at 72 °C for 1min). 5'–TCA TCT ACA CAG TCA ACC TCT ACA; 3'– GAA CAC AAC CAC CCA CAA GTC ATT. CCR5 (40 cycles: denaturation at 94 °C for 1min, annealing at 57 °C for 1 min, extension at 72 °C for 1 min). 5'–TTC ATT ACA CCT GCA GCT CTC ATT T; 3'– TCA CAG CCC TGT GCC TCT TCT. CD1a (35 cycles: denaturation at 94 °C for 1 min, annealing at 63 °C for 1.5 min, extension at 72 °C for 2 min). 5'–TGA GAG ACC AGC AGC CCA AG; 3'–AAT GTG GCG GGA GTT CAG ACT. All amplifications were RNA specific, as no bands were seen in the control (no RT) samples (data not shown). The reaction products were separated on 2% agarose gels in the presence of ethidium bromide.

Fusion assays. Syncytium formation was measured at multiple time points after coculture of target cells (that is, LCs, blood-derived DCs, macrophages) with HIV envelope-expressing effector cells at a 1:1 ratio (2×10^5 cells/well, in triplicate). PM1 cells, which express both CXCR4 and CCR5 (data not shown) served as positive controls. As effector cells we used $CD4^-$ 12E1 cells infected with vaccinia recombinants expressing HIV-1 IIIB (T-tropic) or JR-FL and Ba-L (MΦ-tropic) envelopes at 10 plaque-forming units (PFU)/cell. In some experiments, chronically infected H9 cells (IIIB, MN, RF T-tropic strains) were used. When indicated, rabbit anti-CXCR4, anti-CCR5 or preimmune IgG were added to target cells for 1 h at 37° C before the addition of envelope-expressing 12E1 cells.

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