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Coreceptor Choice and T Cell Depletion by R5, X4, and R5X4 HIV-1 Variants in CCR5-Deficient (CCR5Δ32) and Normal Human Lymphoid Tissue

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Coreceptor utilization by HIV-1 is an important determinant of pathogenesis. However, coreceptor selectivity is defined *in vitro*, while *in vivo* critical pathogenic events occur in lymphoid tissues. Using pharmacological inhibitors, we recently provided evidence that coreceptor selectivity by the R5X4 dual-tropic isolate 89.6 was more restricted in *ex vivo* infected lymphoid tissue than *in vitro* [S. Glushakova, Y. Yi, J. C. Grivel, A. Singh, D. Schols, E. De Clercq, R. G. Collman, and L. Margolis (1999). *J. Clin. Invest.* 104, R7–R11]. Here we extend those observations using CCR5-deficient (CCR5Δ32) lymphoid tissue as well as additional primary isolates. We definitively show that neither CCR5 nor secondary coreceptors used *in vitro* mediate 89.6 infection in lymphoid tissue. We also demonstrate that restricted coreceptor use in lymphoid tissue *ex vivo* compared with *in vitro* utilization occurs with other dual-tropic primary isolates and is not unique to 89.6. For all strains tested that are dual tropic *in vitro*, severe CD4 T cell depletion in lymphoid tissue correlated with preferential CXCR4 use in this *ex vivo* system.

Key Words: HIV; chemokine receptor; CCR5; CXCR4; T lymphocytes; pathogenesis; RANTES; AMD3100.

INTRODUCTION

HIV-1 isolates can be typed by their *in vitro* ability to enter and infect cells transfected with CD4 and specific coreceptors (reviewed in Berger *et al.,* 1999; Locati and Murphy, 1999). Viruses that transmit infection and dominate early stages of HIV disease use CCR5 [R5 variants (Berger *et al.,* 1998)]. HIV-1 variants isolated at late stages of HIV disease often use CXCR4 (reviewed in Berger *et al.,* 1999) [X4 variants (Berger *et al.,* 1998)]. Dual-tropic R5X4 isolates are also typical for late stages of disease (Bjorndal *et al.,* 1997; Connor *et al.,* 1997; Doranz *et al.,* 1996; Scarlatti *et al.,* 1997; Simmons *et al.,* 1996). The ability of viral isolates to use CXCR4 *in vitro*, exclusively or in addition to CCR5, is associated with massive loss of $CD4^+$ T cells *in vivo* and rapid progression to AIDS (Tersmette *et al.,* 1989; Zhu *et al.,* 1993). The coreceptors actually used by these viruses in lymphoid tissue, where critical events in HIV-1 disease occur, remain to be fully defined (Glushakova *et al.,* 1999; Zhang and Moore, 1999).

We previously reported that coreceptor use *in vitro* was not completely predictive of coreceptor use in lymphoid tissue *ex vivo* (Glushakova *et al.,* 1999). In particular, we found that one HIV-1 dual-tropic prototype and one of two genetically constructed dual-tropic variants preferentially used only one of the coreceptors in human lymphoid tissue *ex vivo*, even though they were all dual-tropic *in vitro* (Glushakova *et al.,* 1999). However, these results relied on the use of coreceptor ligands as blocking agents. For CXCR4, the bicyclam AMD3100 is a potent and effective inhibitor. In contrast, the CCR5 ligands including RANTES, used as a probe for CCR5 in those tissue-based studies, do not completely inhibit replication even of monotropic R5 HIV (Glushakova *et al.,* 1999; Margolis *et al.,* 1998). Whether that is because viruses use alternative epitopes of the same receptor or because RANTES did not saturate CCR5 in lymphoid tissue is unknown. Alternatively, since RANTES has agonist activity and can activate at least two other chemokine receptors expressed in lymphoid tissue, CCR3 and CCR1, the effects observed could be due to factors independent of or in addition to RANTES blockade of CCR5 (Murphy *et al.,* 2000).

Human lymphoid tissue derived from donors genetically deficient in specific coreceptors can provide a

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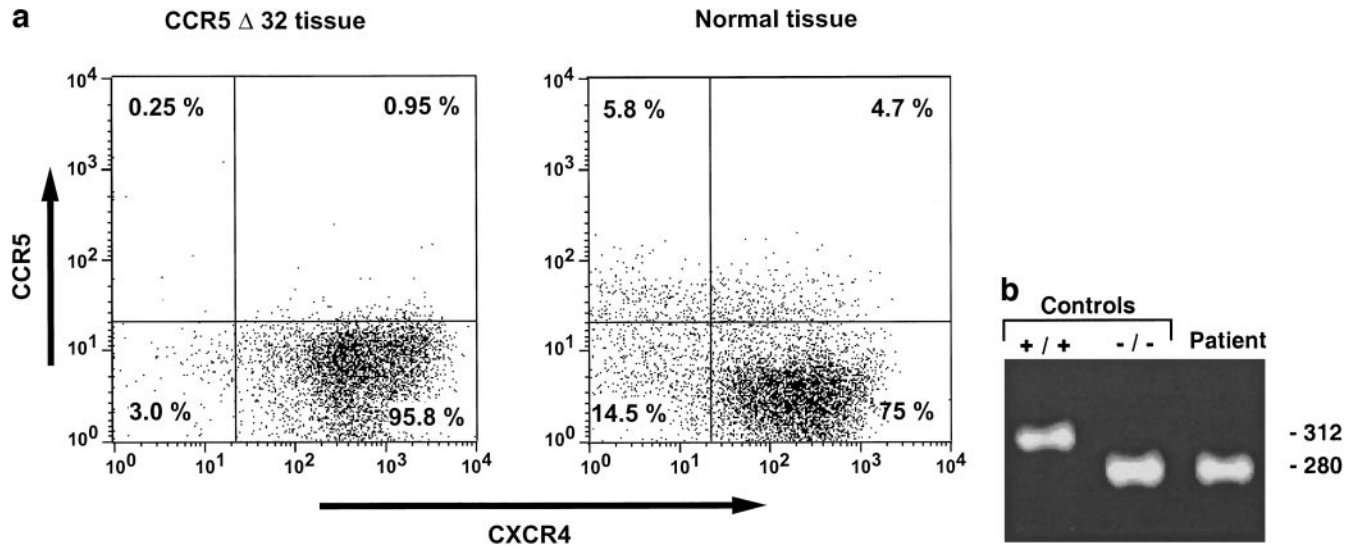

FIG. 1. Phenotypic and genotypic analysis of CCR5Δ32 lymphoid tissue. (a) Phenotypic analysis. Cells were mechanically isolated from tonsillar tissue and stained for CD3, CD4, CD8, CCR5, and CXCR4. The right panel shows a representative analysis of $CD4^+$ T cells isolated from normal donor tissue, and the left panel shows $CD4^+$ T cells from a tissue donor that lacks CCR5 expression. (b) Genotypic analysis. PCR amplification of the CCR5 gene was carried out on DNA obtained from tonsillar tissue from the donor shown in (a) who was found to lack CCR5 expression. Amplification was carried out in parallel using DNA from PBMC of two other donors of known genotype including CCR5 wild-type (+/+) and CCR5Δ32 homozygous (−/−). On the right are shown the sizes of the wild-type (312 bp) and Δ32 (280 bp) CCR5 amplicons.

novel and valuable opportunity to precisely define the coreceptor usage and pathogenic effects of HIV-1 variants in their native lymphoid microenvironment. Here we used tissue from a donor homozygous for the nonfunctional CCR5 Δ32 allele to definitively establish the coreceptor usage by a panel of R5, X4, and R5X4 HIV-1 variants. In addition, we used the pharmacological approach to define the coreceptors used in lymphoid tissue by two additional R5X4 primary isolates. These studies also enabled us to address additional questions: do R5X4 viruses all have similar cytopathic effects in lymphoid tissue, and is the cytopathicity of different R5X4 viruses determined by coreceptor preference? Is there evidence for use of alternative coreceptors in primary human lymphoid tissue? And does the Δ32 mutation in CCR5 affect the functional ability of CXCR4 to mediate HIV infection?

RESULTS

Human lymphoid tissue lacking CCR5 expression

As part of our standard analysis of tonsillar tissues obtained for experiments under approved protocols, lymphocytes are mechanically isolated from the tissues, stained for CD45, CD4, CD8, CCR5, and CXCR4, and analyzed by flow cytometry. By this analysis, we identified a donor tissue that did not express CCR5 (Fig. 1a). To test the donor's CCR5 genotype, we used a primer pair that spans the 32-bp deletion in the common CCR5Δ32 deletion allele and performed PCR amplification of genomic DNA isolated from whole tonsillar tissue. These amplification products were compared with those from two unrelated individuals of known CCR5 genotype (Fig. 1b). This genotypic analysis indicated that the donor was homozygous for the CCR5Δ32 mutation and confirms our phenotypic results since CCR5Δ32 homozygotes are known not to express detectable CCR5 on the surface of peripheral blood lymphocytes (Wu *et al.,* 1997). It also extends those previous observations by showing, as expected, that lymphocytes in lymphoid tissues from these individuals also do not express surface CCR5.

Replication of monotropic X4 and R5 HIV-1 variants in CCR5Δ32 lymphoid tissue

Since the lymphoid microenvironment is where critical pathogenic events take place *in vivo* and coreceptor use is a central determinant of pathogenesis, we utilized the CCR5-negative lymphoid tissue to definitively establish coreceptor selectivity profiles of a panel of viruses that we have previously studied using pharmacologic inhibitors. SF162 is classified as a monotropic R5 virus in transfected cell line assays. We inoculated CCR5Δ32 and control (normal) tissue with SF162 and evaluated its replication. In 11 experiments with normal tissue, the total production of p24 per tissue block ranged from 1 to 39 ng. In contrast, SF162 did not replicate at all in tissue lacking CCR5 (Fig. 2a), confirming this isolate's exclusive reliance on CCR5 in the lymphoid environment. We also tested whether in CCR5Δ32 tissue CXCR4 remains functional as a coreceptor for X4 HIV-1. We inoculated normal and CCR5Δ32 tissue with the X4 variant LAV.04 and evaluated its replication. In 18 experiments with normal

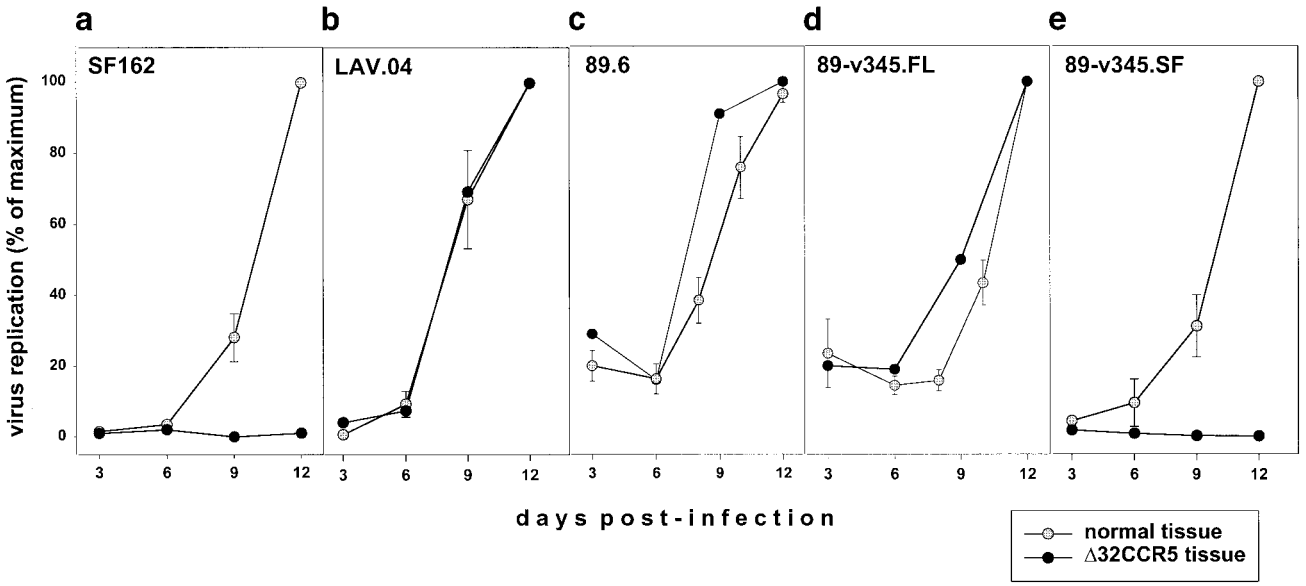

FIG. 2. Replication kinetics of HIV-1 in human lymphoid tissue from normal and CCR5-deficient donors. (a–e) Normal and CCR5Δ32 tissues were infected with different HIV-1 strains, and virus replication was assessed by p24 measurements. To normalize for variation in the absolute level of viral replication and to present average kinetics, data are presented as percentages of the maximum p24 production at the last day of experiments (means ± SD for tissues from 7 to 25 donors in experiments with normal tissues). 89-v345.SF and SF162 did not replicate in CCR5Δ32 tissues, and the amount harvested at each time point was less than at the previous time point. To demonstrate this in the same chart, we present the amount of p24 in CCR5Δ32 tissues infected with 89-v345.SF and SF162 as a percentage of what was produced by these two viruses in normal tissue on Day 12 p.i.

tissue, the total production of p24 per block for LAV.04 ranged from 20 to 48 ng/block. Neither the kinetics of LAV.04 replication nor the total amount of p24 produced by CCR5Δ32 tissue differed from those parameters in normal tissue (Fig. 2b), suggesting that CXCR4 coreceptor function is not affected by the absence of CCR5.

Inoculation of 89.6 and its isogenic chimeras in CCR5Δ32 human tonsillar tissue

To definitively establish whether HIV-1 variants that utilize multiple coreceptors *in vitro*, including both CCR5 and CXCR4, also do so in the context of human lymphoid tissue, we used the CCR5Δ32 tissue for infection with a panel of related R5X4 HIV-1 variants. 89.6 is a primary patient isolate, while 89-v345.FL and 89-v345.SF are chimeric constructs that are isogenic to 89.6 except for the V3-V5 region of gp120, which was replaced with corresponding sequences from SF162 and JR-FL, respectively. Each of these viruses efficiently uses both CCR5 and CXCR4 *in vitro* based on fusion with and/or infection of cell lines transfected with CD4 and the coreceptors (Glushakova *et al.,* 1999; Smyth *et al.,* 1998).

We compared these viruses' replication patterns in CCR5Δ32 and control human tonsillar tissue. In normal lymphoid tissue, each of the three viruses resulted in productive infection and similar kinetics of replication: an increase of p24 released into the media became evident between Days 6 and 9 p.i. and replication continued at least until Days 12–13 (Figs. 2c–2e). As we have described previously (Glushakova *et al.,* 1997, 1998), the absolute level of replication varies considerably in tissues from different normal donors. In seven experiments, the total production of p24 per tissue block ranged from 6 to 69 ng for 89.6, from 3 to 24 ng for 89-v345.SF, and from 4 to 15 ng for 89-v345.FL. However, there was no consistent difference among the three viruses in the absolute level of replication in individual tissues. In CCR5Δ32 and in normal lymphoid tissue, 89.6 and 89-v345.FL replicated with similar kinetics (Figs. 2c and 2d). The total production of p24 per block of CCR5Δ32 tissue infected with 89.6 or 89-v345.FL was 47 and 6 ng, respectively, which are within the range of production in normal tissues. In contrast, 89-v345.SF did not replicate in lymphoid tissue lacking CCR5 (Fig. 2e). Thus, 89-v345.SF relies exclusively on CCR5 for infection and so behaves as an R5 variant, while the two others variants are not restricted to CCR5, and so seem to use CXCR4 in addition to or instead of CCR5. Due to the scarcity of Δ32 donor tissue, for additional experiments, we used *ex vivo* HIV-1-infected tissues from normal donors treated with coreceptor ligands.

Sensitivity of dual-tropic HIV-1 variants to coreceptor ligands

To confirm which coreceptor was used in normal lymphoid tissue that expresses both CCR5 and CXCR4, tissues from three normal donors were pretreated for 3 h with or without the CXCR4-specific antagonist AMD3100,

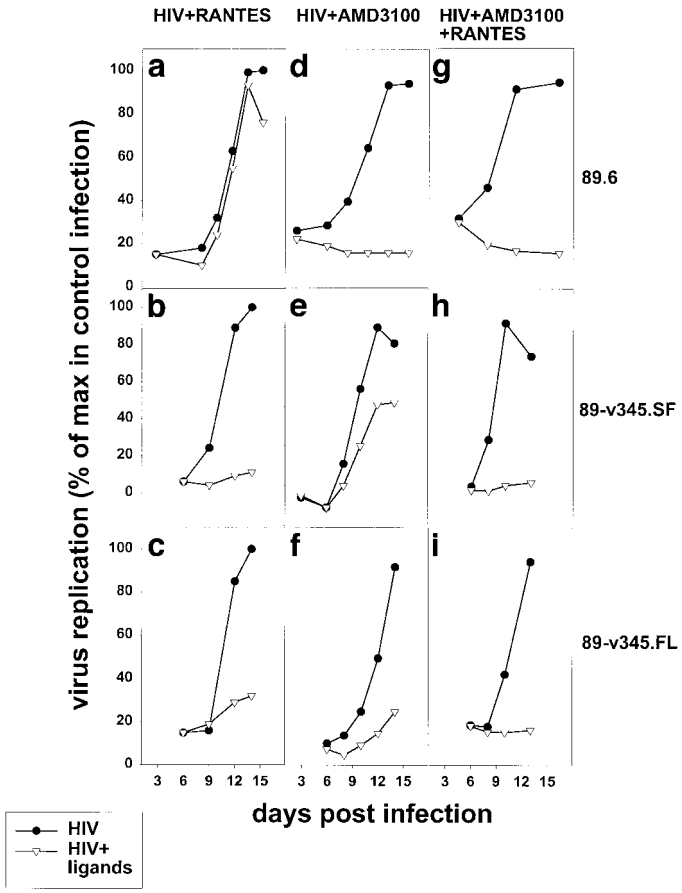

FIG. 3. Inhibition of replication of 89.6 and its chimeras by CXCR4 and CCR5 ligands. RANTES (100 nM; a–c), AMD3100 (1 μg/ml; d–f), or a mixture of both (g–i) was added to the culture medium 3 h prior to infection with 89.6 (a, d, and g), 89-v345.SF (b, e, and h), or 89-v345.FL (c, f, and i). Ligands were replenished with every medium change. To normalize for variation in the absolute level of viral replication and to present average kinetics, data are presented as percentages of the maximum p24 production at the last day of experiments. Typical replication curves from experiments with tissues from five to eight donors are shown.

the CCR5 ligand RANTES, or with a mixture of both, and then infected as described above. Agents were replaced as medium was changed every 3 days. To normalize for donor-to-donor variation in the absolute level of viral replication and to enable comparison of replication kinetics in experiments with tissues from different donors, p24 production at each time point was expressed as a percentage of maximum at the end of the experiments in control viral infection. In all experiments, inhibition of viral replication became evident at about Day 6 p.i. when the concentration of p24 in controls generally increased (Fig. 3).

The results of typical experiments are presented in Figs. 3a–3i. Consistent with our previous data (Glushakova *et al.,* 1999), replication of 89.6 was completely inhibited by AMD3100, with total p24 production in AMD3100-treated cultures reduced to 3 ± 1% of that in control untreated infected tissue (n = 7). In contrast, 89.6 replication was not sensitive to RANTES (83 ± 11% of control p24 production; n = 8). 89-v345.SF replication was reduced by RANTES to 10 ± 3% of control and by AMD3100 to 60 ± 7% of control (n = 5), whereas replication of 89-v345.FL was reduced by AMD3100 and RANTES to 34 ± 12 and 42 ± 14% of untreated infected tissue, respectively (n = 6). Consistent with preferential CCR5 use by 89-v345.SF and CXCR4 use by 89.6, RANTES reduced 89-v345.SF replication by approximately the same extent as it inhibited the CCR5-restricted strain SF162, whereas inhibition of 89.6 by AMD3100 was similar to that of the CXCR4-restricted strain LAV.04 (data not shown). We also treated tissues with a combination of AMD3100 and RANTES. Since AMD3100 alone and RANTES alone resulted in near-complete inhibition of 89.6 and 89-v345.SF, respectively, there was no additional inhibition seen with the addition of the second agent (Figs. 3g and 3h). For 89-v345.FL, however, neither agent alone completely blocked infection but the combination of both agents did (Fig. 3i). Thus, these studies support the notion that 89.6 and 89-v345.SF are predominantly restricted in lymphoid tissue to single coreceptors, CXCR4 and CCR5, respectively, whereas 89-v345.FL uses both coreceptors. These results also show, as we reported earlier, that AMD3100 is a potent inhibitor of X4 but not R5 HIV variants in *ex vivo* infected human lymphoid tissue (Glushakova *et al.,* 1999). In contrast, RANTES inhibits even completely CCR5-dependent R5 HIV-1 isolates by only 70–90%, but does not inhibit replication of X4 HIV variants (Glushakova *et al.,* 1999; Margolis *et al.,* 1998).

Coreceptor use in lymphoid tissue by other dual-tropic primary isolates

These studies demonstrate an important distinction between coreceptor use in transfected cell lines *in vitro* and in lymphoid tissue *ex vivo* for a set of viruses related to HIV-1 89.6. A critical question, however, is whether this discordance is unique to 89.6 or whether other primary isolates that use both coreceptors *in vitro* might also show distinctive patterns in lymphoid tissue. Therefore, to extend this analysis, we analyzed two additional primary isolates, 92US076 and 93US151, that exhibit R5X4 dual-tropic characteristics *in vitro* (the NIH AIDS Research and Reference Program). Because additional CCR5Δ32 lymphoid tissue was not available and since the above studies had confirmed that pharmacological inhibitors accurately identify coreceptor usage profiles in the lymphoid microenvironment, we used AMD3100 and RANTES to define the coreceptor usage profiles of 92US076 and 93US151 in lymphoid tissue *ex vivo*.

Replication by 93US151 was not inhibited by RANTES (total p24 production of 91 ± 9% of control; n = 3) but was almost completely blocked by AMD3100 (2 ± 2% of control, n = 3) or by AMD3100 plus RANTES (Fig. 4a). In

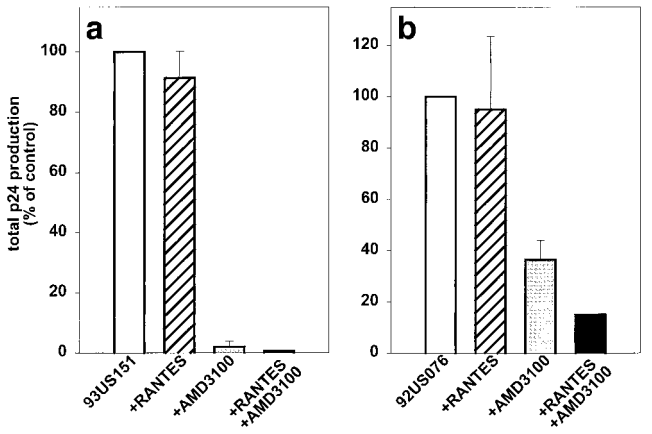

FIG. 4. Inhibition of HIV-1 primary isolates 92US076 (a) and 93US151 (b) by CXCR4 and CCR5 ligands. RANTES (100 nM), AMD3100 (1 μg/ml), or a mixture of both was added to the culture medium as described in the legend to Fig. 3. To normalize for variation in the absolute level of viral replication and inhibition, data are presented as percentages of the total p24 production in the course of the experiment relative to control infection (means ± SD for tissues from three donors in experiments with RANTES or AMD3100 and result of a single experiment with a mixture of RANTES and AMD3100).

contrast, 92US076 was only partially inhibited by AMD3100 to 37 ± 7% of control ($n = 3$). Although 92US076 was not significantly inhibited by RANTES alone (95 ± 28% of control; $n = 3$), the addition of RANTES to AMD3100 further inhibited 92US076 replication, reducing it approximately by 80% compared to control levels (Fig. 4b). The relatively poor 92US076 inhibition by RANTES and residual 20% replication despite blockade of both coreceptors likely reflect relative inefficient RANTES blocking of CCR5 rather than an alternative entry pathway other than CCR5 and CXCR4. Thus, the R5X4 primary isolate 93US151, like 89.6, appears to utilize CXCR4 exclusively for entry in lymphoid tissues, while 92US076, like 89-v345.FL, uses both coreceptors. In contrast, 89-v345.SF relies predominantly on CCR5.

Cytopathicity of dual-tropic HIV-1 variants in CCR5Δ32 and normal tissues

We next studied the correlation between coreceptor utilization and cytopathicity of the HIV-1 variants by evaluating the cumulative $CD4^+$ T cell depletion over the 12–13 days of infection in CCR5Δ32 and normal tissues. Since we have shown previously that $CD8^+$ T cells are not depleted by *ex vivo* HIV-1 infection (Glushakova *et al.,* 1997), $CD4^+$ T cell depletion in these experiments was evaluated as the decrease in $CD4^+/CD8^+$ ratio at Day 12–14 p.i. relative to matched uninfected control tissue from the same donor.

As shown in Fig. 5a, 89.6 severely depleted $CD4^+$ T cells in both normal and CCR5Δ32 lymphoid tissues. In both tissue types, only ~20% of $CD4^+$ T cells remained at Days 12–14 p.i. The monotropic X4 virus LAV.04 behaved similarly and also severely depleted $CD4^+$ T cells, with ~2% of $CD4^+$ T cells remaining in CCR5Δ32 tissue and 4 ± 2% remaining in LAV.04-infected normal tissue (Fig 5a). In contrast, depletion was milder in both CCR5Δ32 and normal tissues infected by 89-v345.FL, with about 60% of $CD4^+$ T cells remaining (Fig 5a). For dual-tropic 89.6, 89-v345.FL, and monotropic LAV.04 HIV-1 variants, there was no statistical difference between normal and CCR5Δ32 tissues in the level of $CD4^+$ T cell depletion ($P < 0.33$, $P < 0.15$, and $P < 0.16$, respectively). In contrast, there was significant difference between $CD4^+$ T cell depletion in CCR5Δ32 and normal tissues infected with 89-v345.SF or SF162 ($P < 0.0007$ and $P < 0.0002$, respectively). No depletion was detected when CCR5Δ32 tissue blocks were inoculated with 89-v345.SF or SF162 (Fig. 5a), as expected since neither of these viruses replicated in the tissue lacking CCR5 (Figs. 2d and 2e). In normal tissue, 89-v345.SF infection resulted in mild depletion with 75–90% of $CD4^+$ T cells remaining (Fig. 5a), similar to what was described for SF162 infection (Glushakova *et al.,* 1997, 1998). Tissues from four normal donors were infected with primary isolates 92US076 and 93US151. Both 92US076 and 93US151 severely depleted $CD4^+$ T cells in *ex vivo* infected tissues, resulting in

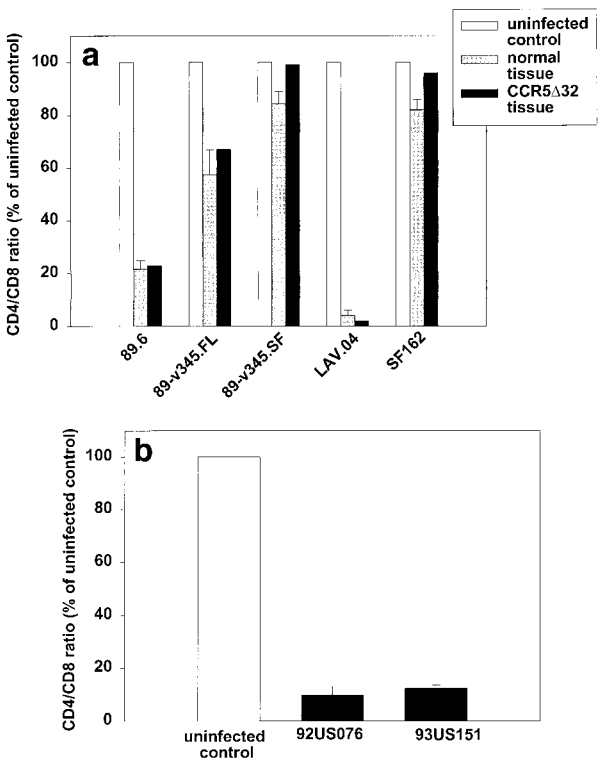

FIG. 5. $CD4^+$ T cell depletion in normal and CCR5Δ32 tissues infected *ex vivo* by HIV-1. Cells were mechanically isolated from uninfected and infected tissues, stained for CD45, CD3, CD4, and CD8, and analyzed with flow cytometry as described. Data are presented as $CD4^+/CD8^+$T cell ratio, relative to matched uninfected control tissue, on Days 12–13 after infection by dual-tropic HIV-1 variants 89.6 and its chimeras as well as by control mono-tropic X4 isolate LAV.04 or R5 isolate SF162 (a) or HIV-1 primary isolates 92US076 and 93US151 (b).

~10% of $CD4^+$ T cells remaining on 12–14 days after infection (Fig 5b). A mixture of AMD3100 and RANTES prevented $CD4^+$ T cell depletion in inoculated tissues, consistent with its inhibition of productive replication in lymphoid tissue (data not shown).

DISCUSSION

HIV-1 coreceptor utilization analysis is generally based on the ability of virus to infect and/or fuse with cell lines transfected with CD4 and coreceptor *in vitro*. So far more than a dozen coreceptors that support HIV or SIV fusion or infection *in vitro* have been identified (see Berger *et al.,* 1999). Only CCR5 and CXCR4 are unequivocally used *in vivo*, however (Bjorndal *et al.,* 1997; Connor *et al.,* 1997; Dean *et al.,* 1996; Huang *et al.,* 1996; Liu *et al.,* 1996; Samson *et al.,* 1996; Simmons *et al.,* 1997), indicating that coreceptor function *in vivo* is more restricted than studies *in vitro* would suggest. Dual-tropic R5X4 HIV-1 variants are defined as those that can enter cells *in vitro* expressing either CCR5 or CXCR4 (Berger *et al.,* 1998), and initially it was assumed that HIV-1 variants that use both major coreceptors *in vitro* would use the same spectrum of coreceptors in other systems. Here we asked what is the coreceptor specificity of the dual-tropic viruses in human lymphoid tissue.

In previous studies, we showed that the R5X4 primary isolate 89.6 is restricted mainly to CXCR4 in lymphoid tissue despite efficient use of both CCR5 and CXCR4 entry pathways *in vitro* (Glushakova *et al.,* 1999). We report here a similar pattern with another R5X4 primary isolate, 93US151. We also reported that a chimeric virus that was R5X4 *in vitro*, 89-v345.SF, preferentially used CCR5 in lymphoid tissue. Those conclusions were based on inhibition by chemokine receptor blockade, however, and are limited by the specificity and potency of such agents. AMD3100 blocks CXCR4 with high potency and specificity (Schols *et al.,* 1997), whereas the β-chemokines used to block CCR5 are considerably less efficient and specific (Margolis *et al.,* 1998). Furthermore, RANTES may potentially also influence viral replication through other mechanisms besides CCR5 blockade (Kinter *et al.,* 1998; Murphy *et al.,* 2000). The present study provides a definitive answer to the question of CCR5 usage in tissue by dual-tropic isolates by utilizing lymphoid tissue naturally lacking CCR5 expression. Surprisingly, only two of the five HIV-1 strains (and only one of the three primary isolates tested) defined as R5X4 dual-tropic in cell lines *in vitro* are truly dual-tropic in lymphoid tissue. Moreover, using this tissue, we answered whether the absence of CCR5 expression (or presence of the CCR5Δ32 mutant protein) affects the functional ability of CXCR4 to mediate HIV-1 infection. Analysis of kinetics of viral replication of LAV.04, 89.6, and 89v-345 FL revealed no difference between infection of CCR5Δ32 and normal lymphoid tissue.

Thus, we confirm and extend the observation that use of CCR5 and CXCR4 by primary isolates in lymphoid tissue *ex vivo* is more restricted than in cell lines *in vitro*. Recently, Zhang *et al.* (2000) reported that an SIV/HIV recombinant virus containing the 89.6 Env was restricted to CXCR4 for infection of PBMC *in vitro* suggesting that coreceptor choice in primary lymphocytes *in vitro* may also be restricted compared with transfected cells. They also found that AMD3100 completely blocked infection of CCR5-negative PBMC by several strains that could use a variety of secondary coreceptors such as STRL33, CX3CR1, CCR8, and Apj in transfected cells (Zhang and Moore, 1999), which argued that the secondary coreceptors were unlikely to be relevant pathways for infection *in vivo* or necessary targets for coreceptor-targeted antiviral strategies. Our results provide further support for that notion by extending it to the context of the natural lymphoid microenvironment. 89-v345.SF uses two coreceptors *in vitro* but fails to infect CCR5Δ32 tissue. Strain 89.6 uses a wide variety of secondary coreceptors *in vitro* such as CCR2, CCR3, CCR8, and Apj (Doranz *et al.,* 1996; Edinger *et al.,* 1998; Rucker *et al.,* 1997) but was completely blocked by AMD3100 in our system. This result indicates that the alternative coreceptors are not used by this strain for infection of lymphoid tissue *ex vivo*. We have not specifically examined expression of the other coreceptors in our system, so our data do not exclude their usage by HIV-1 strains in other compartments where they may be particularly highly expressed (He *et al.,* 1997; Lee *et al.,* 2000). For example, a recent study using human thymocytes suggested that entry into those cells could be mediated by an alternative coreceptor, possibly CCR8 (Lee *et al.,* 2000).

The mechanisms responsible for restricted CCR5 and CXCR4 utilization *ex vivo* are not clear. One possibility is that the expression of a particular coreceptor in tissue may be too low to mediate efficient entry, thus restricting an otherwise dual-tropic virus to only one coreceptor, while in transfected cell lines the level of expression may be higher. However, this would not explain why in samples of the same tissue some viruses are restricted to one coreceptor while other strains are restricted to the other. Alternatively, two coreceptors may be equally well expressed in lymphoid tissue but differently arranged relative to CD4 (Xiao *et al.,* 1999) or exist in distinct conformations (Lee *et al.,* 1999) that support entry by some but not other strains. Yet another possibility is that differences in the viral Env glycoprotein may be responsible for distinct coreceptor utilization in lymphoid tissue *ex vivo* versus cells *in vitro*. Since replication involves multiple rounds of virus production and re-infection in lymphoid tissue, each round of infection subsequent to the initial inoculum is supported by virions produced within the infected tissues. It is conceivable that the conformation or surface density of Env glycoprotein on virions produced by host cells in the native microenvi-

ronment may differ from Env on transfected cells or virions produced *in vitro*, leading to distinct coreceptor fusion capacities. Finally, distinct coreceptor-mediated infection by these strains may potentially result from differences in postfusion steps of infection. Env has been shown to interact with coreceptors to deliver signals to target cells (Davis *et al.,* 1997; Liu *et al.,* 2000; Weissman *et al.,* 1997), and in several systems Env–coreceptor interactions may influence postentry steps of infection (Chackerian *et al.,* 1997; Mori *et al.,* 1993). Unlike *in vitro* PBMC infection studies, these tissues are not subjected to exogenous activation and stimulation (Glushakova *et al.,* 1995, 1997, 1998). Although coreceptor use in stimulated PBMC may also differ from that in transfected cell lines (Zhang *et al.,* 2000; Zhang and Moore, 1999), it is possible that differences between viruses in signals transmitted to target cells through CCR5 and CXCR4 could determine whether permissive interactions occur at postentry stages of infection in lymphoid tissue. Whatever the mechanisms of preferential utilization of a particular coreceptor by a dual-tropic isolate, coreceptor choice apparently determines viral cytopathicity in *ex vivo* infected human lymphoid tissue.

Since the *ex vivo* lymphoid tissue model more closely recapitulates the natural viral reservoir *in vivo* than do transfected cells, our results suggests that major coreceptor usage is more restricted *in vivo* compared with what is suggested by *in vitro* studies. Like any experimental model, however, the *ex vivo* infected human lymphoid tissue has certain limitations, and results should be extrapolated to the situation *in vivo* with caution. Although it reflects the complex cytoarchitecture of lymphoid tissue where critical events in HIV infection occur *in vivo* more faithfully than PBMC cultures, the model does not simulate the immune response of an infected individual. Such an immune response could modulate CCR5 and CXCR4 expression and alter coreceptor distribution among $CD4^+$ T cells in lymphoid tissues of infected individuals compared to *ex vivo*. However, since both CCR5 and CXCR4 are expressed by $CD4^+$ T cells in the *ex vivo* tissues sufficiently for R5 and X4 HIV-1 infection (Grivel and Margolis, 1999), it is not the lack of the appropriate coreceptor that makes dual-tropic viruses behave as mono-tropic in lymphoid tissue *ex vivo*.

In summary, the experiments on *ex vivo* infection of normal and CCR5Δ32 human lymphoid tissue presented above prove genetically that dual tropism as defined *in vitro* using cell lines expressing either CCR5 or CXCR4 is not fully predictive of coreceptor usage in human lymphoid tissue *ex vivo* and confirm previous conclusions based on pharmacologic methods. The results indicate that the switch of coreceptor tropism *in vivo* during the course of HIV-1 disease may be more complex than that suggested by coreceptor use as reflected in *in vitro* assays. Moreover, our results indicate that CCR5Δ32 homozygosity does not affect CXCR4 coreceptor function in primary lymphoid tissue, suggesting that the absence of CCR5, and not other indirect pathways, is the key factor responsible for HIV resistance in individuals with this genotype. Furthermore, viruses that use CXCR4 exclusively or in addition to CCR5 are highly cytopathic, while those using exclusively CCR5 are mildly pathogenic, indicating that coreceptor selectivity in the lymphoid tissue may be the principal factor responsible for accelerated disease progression. The more restricted major coreceptor utilization and apparent lack of minor coreceptor utilization in lymphoid tissue *ex vivo* thus may offer cause for optimism regarding the ability to effectively inhibit HIV-1 replication *in vivo* through entry coreceptor blockade.

MATERIALS AND METHODS

Viruses

We used the R5X4 prototype dual-tropic isolate 89.6 (Collman *et al.,* 1992; Doranz *et al.,* 1996), two additional R5X4 primary isolates 92US076 and 93US151 (obtained from the National Institutes of Health AIDS Research and Reference Program), along with the monotropic X4 strain LAV.04 and R5 strain SF162. We also used two viral chimeras, 89-v345.FL and 89-v345.SF, in which the V3 through V5 env region of 89.6 was replaced with sequences from JR–FL or SF162, respectively, as previously described (Smyth *et al.,* 1998). Both chimeric viruses were R5X4 as evidenced by the ability to mediate fusion and infection of cell lines transfected with CD4 and either CCR5 or CXCR4 (Glushakova *et al.,* 1999; Smyth *et al.,* 1998).

Infection of human lymphoid tissue *ex vivo*

Human tonsils were obtained from patients undergoing tonsillectomy, dissected into ~2-mm blocks, and incubated on collagen gel at the air–liquid interface as described earlier (Glushakova *et al.,* 1997, 1998; Margolis *et al.,* 1998). Medium was changed every 2–3 days, and additional details of culture method were previously described (Glushakova *et al.,* 1997, 1998; Margolis *et al.,* 1998). Lymphoid tissue blocks were infected using 3–5 μl clarified virus-containing medium applied slowly on top of each tissue block. The dose of infection was chosen so that productive replication in tissue started between Days 3 and 6 p.i. and continued to increase in the course of the 12- to 14-day experiment (Glushakova *et al.,* 1997, 1998). The amount of viral p24 Gag antigen used to inoculate each tissue block was ~100 pg for LAV.04, 45 pg for SF162, between 50 and 400 pg for different stocks of 89.6, 600 pg and 1.1 ng in two different stocks of 89-v345.SF, and 180 pg and 1.1 ng for two different stocks of 89-v345.FL. For 92US076, the dose was 100 pg and for 93US151 was 300 pg. RANTES (PeproTech, Rocky Hill, NJ) and AMD3100 [obtained from

G. Henson, AnorMED, Langley, BC (Donzella *et al.,* 1998; Schols *et al.,* 1997) or as a generous gift from J. Moore] were added to the medium bathing tissue blocks 3 h prior to infection at concentrations of 100 nM and 1 μg/ml, respectively, and replenished with each medium change. The concentration of p24 gag antigen in the medium was measured by ELISA (Beckman Coulter, Miami, FL, or NCI, Frederick, MD).

Depletion of $CD4^+$ T cells

Cells were mechanically isolated from tissue blocks on Days 12 to 14 p.i. and stained with a mixture of anti-CD3-FITC, anti-CD4-APC, and anti-CD8-TC monoclonal antibodies (Caltag, Burlingame, CA) as described (Glushakova *et al.,* 1998, 1999; Grivel and Margolis, 1999). To analyze coreceptor expression on $CD4^+$ T lymphocytes, cells were stained with anti-CD3-FITC and CD4 TriColor (Caltag) as well as with anti-CXCR4-APC and anti-CCR5-PE (Pharmingen), and analyzed using a FACS calibur (Becton Dickinson, San Jose, CA). To normalize for differences in the size and cellularity of tissue blocks, depletion of total $CD4^+$ T cells and T cell subsets was expressed as a ratio of the number of these cells to the number of $CD8^+$ T cells, which are not affected by infection (Glushakova *et al.,* 1997).

Genotyping of the CCR5Δ32 allele

Genomic DNA was isolated from two individuals of known CCR5 genotype and from tonsillar tissue of CCR5-negative phenotype. Approximately 200 ng of DNA was amplified by PCR in a 50-μl reaction using 1.5 U Platinum *Taq* (Life Technologies, Rockville, MD), 2 mM $MgCl_2$, 175 μM of each dNTP, and 15 pM of each primer (5′-GTC TTC ATT ACA CCT GCA GCT CTC-3′ and 5′-GGT CCA ACC TGT TAG AGC TAC TGC-3′). Amplification was performed with 35 cycles of 1 min at 95, 65, and 72°C, preceded by 95°C for 3 min and followed by 72°C for 10 min. Amplification products were resolved by 2% agarose gel electrophoresis and stained with ethidium bromide.

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REFERENCES

Berger, E. A., Doms, R. W., Fenyo, E. M., Korber, B. T., Littman, D. R., Moore, J. P., Sattentau, Q. J., Schuitemaker, H., Sodroski, J., and Weiss, R. A. (1998). A new classification for HIV-1. *Nature* **391,** 240.

Berger, E., A., Murphy, P., M., and Farber, J. M. (1999). Chemokine receptors as HIV-1 coreceptors: Roles in viral entry, tropism and disease. *Annu. Rev. Immunol.* **17,** 657–700.

Bjorndal, A., Deng, H., Jansson, M., Fiore, J. R., Colognesi, C., Karlsson, A., Albert, J., Scarlatti, G., Littman, D. R., and Fenyo, E. M. (1997). Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. *J. Virol.* **71,** 7478–7487.

Chackerian, B., Long, E. M., Luciw, P. A., and Overbaugh, J. (1997). Human immunodeficiency virus type 1 coreceptors participate in postentry stages in the virus replication cycle and function in simian immunodeficiency virus infection. *J. Virol.* **71,** 3932–3939.

Collman, R., Balliet, J. W., Gregory, S. A., Friedman, H., Kolson, D. L., Nathanson, N., and Srinivasan, A. (1992). An infectious molecular clone of an unusual macrophage-tropic and highly cytopathic strain of human immunodeficiency virus type 1. *J. Virol.* **66,** 7517–7521.

Connor, R. I., Sheridan, K. E., Ceradini, D., Choe, S., and Landau, N. R. (1997). Change in coreceptor use coreceptor use correlates with disease progression in HIV-1–infected individuals. *J. Exp. Med.* **185,** 621–628.

Davis, C. B., Dikic, I., Unutmaz, D., Hill, C. M., Arthos, J., Siani, M. A., Thompson, D. A., Schlessinger, J., and Littman, D. R. (1997). Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. *J. Exp. Med.* **186,** 1793–1798.

Dean, M., Carrington, M., Winkler, C., Huttley, G. A., Smith, M. W., Allikmets, R., Goedert, J. J., Buchbinder, S. P., Vittinghoff, E., Gomperts, E., Donfield, S., Vlahov, D., Kaslow, R., Saah, A., Rinaldo, C., Detels, R., and SJ, O. B. (1996). Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. *Science* **273,** 1856–1862.

Donzella, G. A., Schols, D., Lin, S. W., Este, J. A., Nagashima, K. A., Maddon, P. J., Allaway, G. P., Sakmar, T. P., Henson, G., De Clercq, E., and Moore, J. P. (1998). AMD3100, a small molecule inhibitor of HIV-1 entry via the CXCR4 co- receptor. *Nat. Med.* **4,** 72–77.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., Parmentier, M., Collman, R. G., and Doms, R. W. (1996). A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **85,** 1149–1158.

Edinger, A. L., Hoffman, T. L., Sharron, M., Lee, B., Yi, Y., Choe, W., Kolson, D. L., Mitrovic, B., Zhou, Y., Faulds, D., Collman, R. G., Hesselgesser, J., Horuk, R., and Doms, R. W. (1998). An orphan seven-transmembrane domain receptor expressed widely in the brain functions as a coreceptor for human immunodeficiency virus type 1 and simian immunodeficiency virus. *J. Virol.* **72,** 7934–7940.

Glushakova, S., Baibakov, B., Margolis, L. B., and Zimmerberg, J. (1995). Infection of human tonsil histocultures: A model for HIV pathogenesis. *Nat. Med.* **1,** 1320–1322.

Glushakova, S., Baibakov, B., Zimmerberg, J., and Margolis, L. (1997). Experimental HIV infection of human lymphoid tissue: Correlation of $CD4^+$ T cell depletion and virus syncytium-inducing/non-syncytium-inducing phenotype in histoculture inoculated with laboratory strains and patient isolates of HIV type 1. *AIDS Res. Hum. Retroviruses* **13,** 461–471.

Glushakova, S., Grivel, J. C., Fitzgerald, W., Sylwester, A., Zimmerberg, J., and Margolis, L. B. (1998). Evidence for the HIV-1 phenotype switch as a causal factor in acquired immunodeficiency. *Nat. Med.* **4,** 346–349.

Glushakova, S., Yi, Y., Grivel, J. C., Singh, A., Schols, D., De Clercq, E., Collman, R. G., and Margolis, L. (1999). Preferential coreceptor utilization and cytopathicity by dual-tropic HIV-1 in human lymphoid tissue ex vivo. *J. Clin. Invest.* **104,** R7–R11.

Grivel, J. C., and Margolis, L. B. (1999). CCR5- and CXCR4-tropic HIV-1 are equally cytopathic for their T-cell targets in human lymphoid tissue. *Nat. Med.* **5,** 344–346.

He, J., Chen, Y., Farzan, M., Choe, H., Ohagen, A., Gartner, S., Busciglio, J., Yang, X., Hofmann, W., Newman, W., Mackay, C. R., Sodroski, J., and Gabuzda, D. (1997). CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **385,** 645–649.

Huang, Y., Paxton, W. A., Wolinsky, S. M., Neumann, A. U., Zhang, L., He, T., Kang, S., Ceradini, D., Jin, Z., Yazdanbakhsh, K., Kunstman, K.,

Erickson, D., Dragon, E., Landau, N. R., Phair, J., Ho, D. D., and Koup, R. A. (1996). The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. *Nat Med* **2,** 1240–1243.

Kinter, A., Catanzaro, A., Monaco, J., Ruiz, M., Justement, J., Moir, S., Arthos, J., Oliva, A., Ehler, L., Mizell, S., Jackson, R., Ostrowski, M., Hoxie, J., Offord, R., and Fauci, A. S. (1998). CC-chemokines enhance the replication of T-tropic strains of HIV-1 in CD4(+) T cells: Role of signal transduction. *Proc. Natl. Acad. Sci. USA* **95,** 11880–11885.

Lee, B., Sharron, M., Blanpain, C., Doranz, B. J., Vakili, J., Setoh, P., Berg, E., Liu, G., Guy, H. R., Durell, S. R., Parmentier, M., Chang, C. N., Price, K., Tsang, M., and Doms, R. W. (1999). Epitope mapping of CCR5 reveals multiple conformational states and distinct but overlapping structures involved in chemokine and coreceptor function. *J. Biol. Chem.* **274,** 9617–9626.

Lee, S., Tiffany, H. L., King, L., Murphy, P. M., Golding, H., and Zaitseva, M. B. (2000). CCR8 on human thymocytes functions as a human immunodeficiency virus type 1 coreceptor. *J. Virol.* **74,** 6946–6952.

Liu, Q. H., Williams, D. A., McManus, C., Baribaud, F., Doms, R. W., Schols, D., De Clercq, E., Kotlikoff, M., I, Collman, R. G., and Freedman, B. D. (2000). HIV-1 gp120 and chemokines activate ion channels in primary macrophages through CCR5 and CXCR4 stimulation. *Proc. Natl. Acad. Sci. USA* **97,** 4832–4837.

Liu, R., Paxton, W. A., Choe, S., Ceradini, D., Martin, S. R., Horuk, R., MacDonald, M. E., Stuhlmann, H., Koup, R. A., and Landau, N. R. (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86,** 367–377.

Locati, M., and Murphy, P. M. (1999). Chemokines and chemokine receptors: Biology and clinical relevance in inflammation and AIDS. *Annu. Rev. Med.* **50,** 425–40.

Margolis, L. B., Glushakova, S., Grivel, J. C., and Murphy, P. M. (1998). Blockade of CC chemokine receptor 5 (CCR5)-tropic human immunodeficiency virus-1 replication in human lymphoid tissue by CC chemokines. *J. Clin. Invest.* **101,** 1876–1880.

Mori, K., Ringler, D. J., and Desrosiers, R. C. (1993). Restricted replication of simian immunodeficiency virus strain 239 in macrophages is determined by env but is not due to restricted entry. *J. Virol.* **67,** 2807–2814.

Murphy, P. M., Baggiolini, M., Charo, I. F., Hebert, C. A., Horuk, R., Matsushima, K., Miller, L. H., Oppenheim, J. J., and Power, C. A. (2000). International union of pharmacology. XXII. Nomenclature for chemokine receptors. *Pharmacol. Rev.* **52,** 145–176.

Rucker, J., Edinger, A. L., Sharron, M., Samson, M., Lee, B., Berson, J. F., Yi, Y., Margulies, B., Collman, R. G., Doranz, B. J., Parmentier, M., and Doms, R. W. (1997). Utilization of chemokine receptors, orphan receptors, and herpesvirus- encoded receptors by diverse human and simian immunodeficiency viruses. *J. Virol.* **71,** 8999–9007.

Samson, M., Labbe, O., Mollereau, C., Vassart, G., and Parmentier, M. (1996). Molecular cloning and functional expression of a new human CC-chemokine receptor gene. *Biochemistry* **35,** 3362–3367.

Scarlatti, G., Tresoldi, E., Bjorndal, A., Fredriksson, R., Colognesi, C., Deng, H. K., Malnati, M. S., Plebani, A., Siccardi, A. G., Littman, D. R., Fenyo, E. M., and Lusso, P. (1997). In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. *Nat. Med.* **3,** 1259–1265.

Schols, D., Struyf, S., Van Damme, J., Este, J. A., Henson, G., and De Clercq, E. (1997). Inhibition of T-tropic HIV strains by selective antagonization of the chemokine receptor CXCR4. *J. Exp. Med.* **186,** 1383–1388.

Simmons, G., Clapham, P. R., Picard, L., Offord, R. E., Rosenkilde, M. M., Schwartz, T. W., Buser, R., Wells, T. N. C., and Proudfoot, A. E. (1997). Potent inhibition of HIV-1 infectivity in macrophages and lymphocytes by a novel CCR5 antagonist. *Science* **276,** 276–279.

Simmons, G., Wilkinson, D., Reeves, J. D., Dittmar, M. T., Beddows, S., Weber, J., Carnegie, G., Desselberger, U., Gray, P. W., Weiss, R. A., and Clapham, P. R. (1996). Primary, syncytium-inducing human immunodeficiency virus type 1 isolates are dual-tropic and most can use either Lestr or CCR5 as coreceptors for virus entry. *J. Virol.* **70,** 8355–8360.

Smyth, R. J., Yi, Y., Singh, A., and Collman, R. G. (1998). Determinants of entry cofactor utilization and tropism in a dualtropic human immunodeficiency virus type 1 primary isolate. *J. Virol.* **72,** 4478–4484.

Tersmette, M., Lange, J. M., de Goede, R. E., de Wolf, F., Eeftink-Schattenkerk, J. K., Schellekens, P. T., Coutinho, R. A., Huisman, J. G., Goudsmit, J., and Miedema, F. (1989). Association between biological properties of human immunodeficiency virus variants and risk for AIDS and AIDS mortality. *Lancet* **1,** 983–985.

Weissman, D., Rabin, R. L., Arthos, J., Rubbert, A., Dybul, M., Swofford, R., Venkatesan, S., Farber, J. M., and Fauci, A. S. (1997). Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. *Nature* **389,** 981–985.

Wu, L., Paxton, W. A., Kassam, N., Ruffing, N., Rottman, J. B., Sullivan, N., Choe, H., Sodroski, J., Newman, W., Koup, R. A., and Mackay, C. R. (1997). CCR5 levels and expression pattern correlate with infectability by macrophage-tropic HIV-1, in vitro. *J. Exp. Med.* **185,** 1681–1691.

Xiao, X., Wu, L., Stantchev, T. S., Feng, Y. R., Ugolini, S., Chen, H., Shen, Z., Riley, J. L., Broder, C. C., Sattentau, Q. J., and Dimitrov, D. S. (1999). Constitutive cell surface association between CD4 and CCR5. *Proc. Natl. Acad. Sci. USA* **96,** 7496–7501.

Zhang, Y., Lou, B., Lal, R. B., Gettie, A., Marx, P. A., and Moore, J. P. (2000). Use of inhibitors to evaluate coreceptor usage by simian and simian/human immunodeficiency viruses and human immunodeficiency virus type 2 in primary cells. *J. Virol* **74,** 6893–6910.

Zhang, Y., and Moore, J. P. (1999). Will multiple coreceptors need to be targeted by inhibitors of human immunodeficiency virus type 1 entry? *J. Virol* **73,** 3443–3448.

Zhu, T., Mo, H., Wang, N., Nam, D. S., Cao, Y., Koup, R. A., and Ho, D. D. (1993). Genotypic and phenotypic characterization of HIV-1 patients with primary infection. *Science* **261,** 1179–1181.