Virus and host determinants of HIV-1 infection and AIDS pathogenesis
Blaak, H.

Citation for published version (APA):

General rights
It is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), other than for strictly personal, individual use, unless the work is under an open content license (like Creative Commons).

Disclaimer/Complaints regulations
If you believe that digital publication of certain material infringes any of your rights or (privacy) interests, please let the Library know, stating your reasons. In case of a legitimate complaint, the Library will make the material inaccessible and/or remove it from the website. Please Ask the Library: http://uba.uva.nl/en/contact, or a letter to: Library of the University of Amsterdam, Secretariat, Singel 425, 1012 WP Amsterdam, The Netherlands. You will be contacted as soon as possible.
Chapter 10

'Differential coreceptor expression within CD45RA* naive and CD45RO* memory CD4* T cells provide distinct cellular niches for independent NSI and SI HIV-1 evolution'


Submitted for publication (1999)

*Authors contributed equally to this study
Chapter 10

Differentiation correlated expression of CD155, CD166A, and CD265D in memory CD4+ T cells and gastrointestinal epithelial cells. Initial evidence for independent regulation of #1 and #2 HIV-1 replication.

#1 and #2 show high levels of expression. #1 and #2 are co-expressed in...
Differential coreceptor expression within CD45RA⁺ naive and CD45RO⁺ memory CD4⁺ T cells provide distinct cellular niches for independent non-syncytium-inducing and syncytium-inducing HIV-1 evolution

In HIV-1 infection, virus variants with syncytium-inducing (SI) capacity in the MT2 T cell line can evolve after a non-SI (NSI)-predominated phase. Recently, we showed that NSI variants can be isolated from CD45RO⁺ (memory) and SI variants can additionally be isolated from CD45RA⁺ (naive) CD4⁺ T cells, corresponding with the differential expression of the HIV-1 coreceptors, CCR5 and CXCR4, on these T cell subsets. Here, we longitudinally analyzed biological HIV-1 clones that were obtained from four patients during the switch from NSI to SI HIV-1, with respect to tropism for CD45RA⁺ and CD45RO⁺ CD4⁺ T cells, coreceptor usage, and phylogenetic relationships based on the envelope V3 region. NSI variants were generally isolated from the CD45RO⁺ CD4⁺ T cell population and were CCR5-restricted throughout infection. SI HIV-1 variants seemed to evolve and persist in the CD45RO⁺ CD4⁺ T cells. In three of four patients, the first MT2-tropic clones preferentially used CCR5 and were unable to infect PBMC from a CCR5 Δ32/Δ32 donor. These clones still had an NSI-like V3 loop and clustered with the NSI clones in phylogenetic analyses. SI clones that efficiently used CXCR4 showed several drastic changes in the V3 loop as compared to co-existing NSI variants and over time the NSI and SI populations in the patients diverged. With the ongoing evolution after SI conversion, SI clones lost the capacity to use CCR5. In concordance, increased SI HIV-1 infection of CD45RA⁺ CD4⁺ T cells was observed that ultimately equaled the SI HIV-1 infection of the CD45RO⁺ T cell population. Differential CCR5 and CXCR4 expression provides distinct cellular compartments even within the CD45RO⁺ CD4⁺ T cell population, thus contributing to the co-existence of NSI and SI HIV-1 and their independent evolutionary pathways.
The capacity to replicate and induce syncytia in T cell lines seems to be determined mainly by the second and third variable loop (V2 and V3) of gp120, as demonstrated by chimeric viruses carrying these envelope regions from SI variants in an NSI background. Furthermore, the presence of a positively charged amino acid at either one or both of two fixed positions of V3 (position 11 or 25) was highly associated with SI capacity in primary isolates. This suggests that these amino acids play a crucial role in the interaction of HIV-1 with CXCR4. Indeed, later studies confirmed the involvement of V1/V2 and or V3 in usage of CCR5, CCR3 and CXCR4 as coreceptors.

Through the acquisition of the capacity to infect CXCR4 expressing cells, SI conversion results in a greatly increased potential target cell population. Still, SI HIV-1 variants emerge in only 50% of HIV-1-infected individuals. The finding that SI conversion seems to occur only once during HIV-1 infection, the rarity of virus variants with intermediate genotypes, and the non-gradient character of evolution to SI HIV-1 might suggest that the virus evolves to the SI phenotype through several stages with reduced fitness. The relatively low frequency of SI conversion, despite the high mutation frequency of HIV-1, might then be explained by an incompetence of intermediate, less fit variants to compete with the well-established NSI population.

Once established, SI variants appear to be more replication competent than NSI variants, given the broader target cell range and the in general higher replication kinetics of SI HIV-1 in vitro. In this light, it seems paradoxical that NSI variants persist and may even expand in vivo after the emergence of SI HIV-1. A possible explanation would be that NSI and SI HIV-1 have their own cellular niche within an infected individual. Indeed, recently we showed that NSI variants are mainly isolated from memory, CD45RO CD4+ T cells whereas SI variants are isolated from naive, CD45RA CD4+ T cells as well.

Although the existence of distinct target cell populations could explain the co-existence of NSI and SI HIV-1 variants, it seems contradictory with the difficulty for emerging SI variants to compete with the established NSI population. To gain more insight into the development of NSI to SI HIV-1 and their potential to co-exist in vivo, we studied coreceptor usage in relation to tropism for CD45RA+ and CD45RO+ CD4+ T cells and the evolutionary relationships between the virus variants during NSI to SI evolution. In addition, expression patterns of the HIV-1 coreceptors were determined on these CD4+ T cell subsets.

**MATERIALS AND METHODS**

**Patients.** Four patients, ACH039, ACH171, ACH208, and ACH490, who developed SI HIV-1 variants during a progressive disease course were selected (Fig. 1). All patients were male homosexual participants of the Amsterdam Cohort studies on HIV-1 and AIDS. In this cohort, the presence of SI HIV-1 variants is prospectively determined at every visit by cocultivation of 10⁶ uncultured patient peripheral blood mononuclear cells (PBMC) with 10⁵ MT2 cells as previously described. SI conversion was calculated as the midpoint between the last MT2-negative and the first MT2-positive visit.

**Isolation of CD45RA+ and CD45RO+ CD4+ T cells from PBMC.** Cryopreserved patient PBMC were stained with monoclonal antibodies (mAbs) against CD45RA (PE-conjugated), CD45RO (FITC-conjugated) and CD4 (TC-conjugated; all antibodies were obtained from Caltag, Burlingham, CA) and FAC5-sorted on a FACStar (Becton Dickinson, San Jose, CA). Cells were separated in a CD45RO CD45RA+ and a CD45RO CD45RA+ CD4+ T cell population. Upon re-analyses of the purified cell populations, contamination by the reciprocal subset was on average 0.3%.

**Clonal isolation of virus.** Biological virus clones were obtained via cocultivation of 2× to 3-day phytohaemagglutinin-stimulated healthy donor PBMC (PHA-PBMC) with serial dilutions of patient PBMC, purified CD45RA+, or CD45RO+ CD4+ T cells in a 96-well plate under limiting-dilution conditions. Per well, 10,000 to 40,000 PBMC or 100 to 8,000 CD45RA+ or CD45RO+ CD4+ T cells were cocultivated with 10⁶ PHA-PBMC in a final volume of 200 µl of recombinant interleukin-2 (Proleukin, Chiron Benelux BV, Amsterdam, the Netherlands)-supplemented medium for 28 days. For each cell dilution, multiple cocultures (24 to 48 wells) were performed. At day 7, 14, and 21, one-third of the culture supernatants was harvested for analysis of p24 production by an in-house antigen-capture ELISA. Cells were resuspended and half of the cells was transferred to 96-well plates containing fresh healthy donor PHA-PBMC (10⁵ cells/well).
and further cultured in a final volume of 200 µl. To determine SI capacity, 25,000 MT2 cells per well were added to the remainder of the culture and scored by visual inspection for the presence of syncytia at days 3 to 7. From positive wells in the PBMC cultures, virus stocks were grown in 25 ml culture flasks, cell-free supernatants stored, and the infected PBMC used to confirm SI phenotype by co-cultivation with 10⁶ MT2 cells in a final volume of 3 ml for 10 days. In cases where MT2 infection did not correspond with the U87-CXCR4 infection, the MT2 assay was repeated and additionally p24 production was determined by an in-house p24 ELISA.

The frequency of productively infected cells was calculated with the formula for the Poisson distribution: F = -ln(1 - p), in which p is the fraction of negative cultures, and was expressed as tissue culture infectious dose (TCID) per 10⁶ cells. Viruses obtained via this procedure were considered to be clonal if less than one third of the wells were positive for p24, as can be estimated from the Poisson distribution.

Determination of coreceptor usage. Coreceptor usage was tested using human astroglia U87 cells that stably express CD4 and one of the HIV-1 coreceptors, CCR1, CCR2, CCR3, CCR5, CXCR4, BOB or BONZO. All coreceptor-expressing cell lines were maintained in Iscove’s medium supplemented with 10% FCS, 5 µg/ml polybrene, 100 µg/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml puromycin and were regularly selected for puromycin. U87-BOB and U87-BONZO were obtained via retroviral transfer. Briefly, a packaging cell line was transfected with either pBABE-BOB or pBABE-BONZO and the supernatants of each cell line and CCR1, CCR2, CCR3, CCR5, and CXCR4 expression on the corresponding U87 cell lines was confirmed by flow cytometry or RT-PCR (data not shown). An U87 cell line stably expressing CD4 but no coreceptor was included in all experiments as a control. This cell line was maintained in the same medium was tested using human astroglia U87 cells that stably express CD4 and one of the HIV-1 coreceptors, CCR1, CCR2, CCR3, CCR5, CXCR4, BOB or BONZO. All coreceptor-expressing cell lines were maintained in Iscove’s medium supplemented with 10% FCS, 5 µg/ml polybrene, 100 µg/ml penicillin, 100 µg/ml streptomycin and 1 µg/ml puromycin and were regularly selected for puromycin for p24 production was determined by an in-house p24 ELISA.

Sequencing. Viral gp120 V3 sequences were amplified by PCR as described. PCR products were purified and sequenced using the ABI prism BigDye Terminator sequencing kit (Perkin-Elmer) according to the instructions from the manufacturer using primers seq 5 and seq 6 (61). Sequences were analyzed on an ABI prism 377 DNA sequencer. A number of sequences were available from previous studies (36, 57).

Accession numbers for ACH039: AF022258; AF022262; AF022266; AF022267; AF022271; AF022273; AF022274; AF022277; AF022278; AF022280; AF022283; AF022287; AF022291; AF022293; AF022294; AF022299-AF022301; for ACH208: AF021477; AF021494; AF021499; AF021500; AF021502; AF021503; AF021505; AF021510; AF021514; AF021518; AF021523; AF021524; AF021532; AF021533; AF021536; AF021607; AF021608; AF021612-AF021614; AF021616-AF021618; AF021620; AF021622; AF021627-AF021630; AF021639; AF021647; AF021650; AF021651; AF021668-AF021670. Newly generated sequences are deposited in Genbank under accession numbers AF108098-AF108096 for ACH208, and AF108097-AF108076 for ACH39.

Phylogenetic analyses. Deduced amino acid sequences of the V3 region were obtained using TRANSLATE and aligned by PILE-UP (GGG-Sequence Analysis Package). Phylogenetic analyses were performed on the deduced amino acid sequences using the neighbor-joining method (86), as implemented in the PHYLIP package (87). Distance matrices were prepared in PROTDIST using the Kimura model for amino acid substitutions (98). For bootstrapping, SEQBOOT, PROTDIST and CONSENSE were used. DRAWTREE was used to produce the plots.

Expression of CCR5 and CXCR4 on CD4⁺ T cell subsets. Expression of CCR5 and CXCR4 on the CD4⁰ and CD4⁺ T cell subsets was determined by four-color flow cytometry. Cryopreserved PBMC from ACH171 and ACH490 were stained with mAbs directed against CCR5 (2D7-FITC; Pharmingen, San Diego, CA), CXCR4 (12G5-PE; Pharmingen), CD4 (-PERCP, Becton Dickinson) and CD4⁰ (-APC, Becton Dickinson) and analyzed on a FACScalibur (Becton Dickinson).
RESULTS

NSI to SI conversion during the course of HIV-1 infection. In order to analyze the sequential events in evolution of tropism and coreceptor usage during NSI to SI transition, 4 patients were selected from the Amsterdam Cohort Studies on HIV-1 infection and AIDS (ACS), who developed SI variants during follow-up. For each patient, the course of infection as characterized by changes in CD4\(^+\) T cell numbers and viral RNA load in serum is depicted in Figure 1. In the ACS, every participant is routinely monitored for the presence of SI variants by cocultivation of patient PBMC with MT2 cells as previously described\(^8\). The moment of SI conversion is calculated as the midpoint between the last time point at which SI variants were not detected and the first moment at which they were detected. Since the MT2 assay is generally performed every 3 months, the moment of SI conversion can be estimated with reasonable accuracy. It is important to note that SI conversion is by definition the moment of first detection in the MT2 assay performed on total patient PBMC, which is not necessarily the moment of first appearance of SI variants in vivo, because of detection limits of the assay. In order to isolate SI variants as early as possible after their first appearance, biological clones were isolated from several time points encompassing the estimated moment of SI conversion. In addition, time points were selected well before and after the moment of SI conversion (Fig. 1).

In two of the patients, SI variants were first detected within 1.5 years after seroconversion (16 and 14 months for ACH039 and ACH208, respectively). In ACH171 and ACH490, the transition to SI occurred 5 (ACH171) and 6 (ACH490) years after seroconversion.

Tropism for CD45RA\(^+\) and CD45RO\(^-\) CD4\(^+\) T cells. Recently, we demonstrated in a cross-sectional study, that while NSI variants are mainly isolated from CD45RO\(^+\) CD4\(^+\) T cells, SI variants are isolated from both CD45RA\(^+\) and CD45RO\(^-\) CD4\(^+\) T cells. To gain more insight into the evolution of tropism for CD45RO\(^+\) to tropism for CD45RA\(^+\) CD4\(^+\) T cells during transition from NSI to SI HIV-1, we performed clonal virus isolation on longitudinally obtained total PBMC and purified CD45RA\(^+\) and CD45RO\(^-\) CD4\(^+\) T cells from ACH171 and ACH490. For both patients, six PBMC samples were analyzed, obtained during a 40-month period spanning the moment of SI conversion.

The frequency of cells infected with either NSI or SI HIV-1 (hereafter: NSI- or SI-load) in the total, the CD45RA\(^+\) and the CD45RO\(^-\) CD4\(^+\) T cell population is depicted in Figure 2a. Throughout the course of infection, the NSI variants were predominantly isolated from CD45RO\(^+\) CD4\(^+\) T cells (NSI-load in the CD45RO\(^-\) T cells is about 100- and 40-times higher than NSI-load in the CD45RA\(^+\) T cells for ACH171 and ACH490, respectively; Fig. 2b). Still, NSI variants could be isolated from the CD45RA\(^+\) CD4\(^+\) T cells as well (Fig. 2a). Up to 20 months after SI conversion, SI variants were also predominantly isolated from the CD45RO\(^+\) CD4\(^+\) T cells (ratio SI-load in CD45RA\(^+\) cells to SI-load in CD45RO\(^-\) cells is <1 in both patients; Fig. 2b). However, after this point, the SI-load in CD45RA\(^+\) CD4\(^+\) T cells increased proportionally, and 2 years after SI conversion the SI-load in the CD45RA\(^+\) CD4\(^+\) T cells was even slightly higher than the SI-load in the CD45RO\(^+\) CD4\(^+\) T cells for both patients (~1.5 times higher for both patients; Fig. 2b). At this time point, NSI variants were no longer detected in the CD45RA\(^+\) CD4\(^+\) T cell subset (Fig. 2c). In ACH490, still 40% of the clones isolated from the CD45RO\(^-\) CD4\(^+\) T
cells had the SI phenotype. For ACH171 the percentage of SI variants in this subset was much lower. Interestingly in this patient the contribution of SI variants to the total load was slightly lower than that of the NSI variants (Fig. 2a). In general, NSI and SI HIV-1 contribute equally to the cellular infectious load in total CD4* T cells, as was already shown previously for ACH039 and ACH208 and can be seen here for ACH490.

Coreceptor usage during NSI to SI switch. Given the gradual evolution from preferential infection of CD45RO* CD4* T cells to preferential infection of CD45RA* CD4* T cells by SI variants, we next analyzed evolution of coreceptor usage during NSI to SI conversion. In addition to the clones obtained from the CD45RA* and CD45RO* CD4* T cell subsets from ACH171 and ACH490, we analyzed coreceptor usage of NSI and SI clones isolated in a previous study from total PBMC from ACH039 and ACH208.

Coreceptor usage of NSI and SI variants isolated from the different time points was analyzed by their capacity to infect U87 cell lines stably expressing CD4 in combination with either CCR3, CCR5, or CXCR4. None of the virus clones were able to infect the control U87-CD4 cell line. None of the tested virus clones from ACH039 and ACH208 were able to infect U87-CCR1, U87-CCR2, U87-BOB and U87-BONZO.

In agreement with their incapacity to infect the MT2 cell line, the majority of all NSI variants (108/111, 97%) were restricted to CCR5 usage throughout infection (Fig 3). Similarly, in line with their MT2-tropism, the majority of the SI variants (83/100, 83%) were capable of infecting the U87-CXCR4 cell line. CCR3 usage was observed among the SI variants in 3 of the patients (ACH39, 171, and 208). Interestingly, in 3 of the patients (ACH39, 171, and 208) early SI variants were dual-tropic for CCR5 and CXCR4 (R5X4 or R5X4).
Table 1. Infection of CCR5 Δ32/Δ32 PHA-PBMC by MT2 and U87 tropism

<table>
<thead>
<tr>
<th>Patient</th>
<th>MT2-</th>
<th>MT2+</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACH39</td>
<td>0(1)</td>
<td>100(5)</td>
</tr>
<tr>
<td>ACH171</td>
<td>0(43)</td>
<td>0(3)</td>
</tr>
<tr>
<td>ACH208</td>
<td>0(4)</td>
<td>0(2)</td>
</tr>
<tr>
<td>ACH490</td>
<td>0(32)</td>
<td>0(1)</td>
</tr>
</tbody>
</table>

Percentages of clones with the designated U87 and MT2 tropism that were able to infect CCR5 Δ32/Δ32 PHA-PBMC are indicated. Numbers in brackets indicate the number of clones analyzed.

Table 1. Infection of CCR5 Δ32/Δ32 PHA-PBMC by MT2 and U87 tropism

appeared to lose the capacity to use CCR5, whereas the capacity to use both CCR5 and CXCR4 seemed to be an early stage in the evolution of SI HIV-1.

For ACH171 and ACH490, we analyzed whether clones isolated from CD45RA* CD4* T cells and those isolated from CD45RO* CD4* T cells differed with respect to coreceptor usage. All types of SI variants could be isolated from both subsets in similar proportions, suggesting a dynamic exchange of SI HIV-1 variants between the CD45RA* and CD45RO* CD4* T cell compartments (Fig. 3b).

Unexpectedly, MT2-tropism did not correlate absolutely with the capacity to productively infect the U87-CXCR4 cell line. A minority of NSI variants appeared to be capable to infect the U87-CXCR4 cell line (3/111, 3%), while especially in ACH490, MT2-tropic variants were detected that were incapable to infect the U87-CXCR4 cell line (17/100, 17%). The discordance between the capacity of some variants to infect the MT2 and the U87-CXCR4 cell lines might indicate inefficient CXCR4 usage. To further analyze this, we determined the capacity of these variants to infect PHA-PBMC from an individual homozygous for the 32-bp deletion in the CCR5 gene (CCR5 Δ32/Δ32; Table 1). These cells do not express CCR5 on their cell surface and therefore replication must be due to entry via CXCR4 or possibly another HIV-1 coreceptor (e.g., CCR3).

None of the CCR5-restricted NSI variants tested (n = 80) and none of the variants with a discordant phenotype (i.e., the R5X4 NSI and R5 MT2-tropic variants, n = 20) were able to productively infect the CCR5 Δ32/Δ32 PBMC, supporting the idea that CXCR4 usage of these variants is highly inefficient. These variants were mainly isolated early after SI conversion, which suggests that they represent an early stage in the evolution
Fig. 4. Sequence alignment of the deduced amino acid sequences of the V3 region of virus clones from patients ACH-39 (a) and ACH-208 (b). Sequences were aligned with the consensus sequence of the first time point of each patient. SI capacity in MT2 cells, coreceptor usage, the number of positively charged amino acids within the V3 loop, and the ability to infect CCR5 A32/A32 PHA-PBMC are indicated. Positively charged amino acids at positions 11 and 25 (indicated in bold in the consensus sequence) are associated with SI capacity (reference 162).
toward efficient CXCR4 usage. The vast majority of the U87-CXCR4- and MT2-tropic SI variants (98%) were able to infect the CCR5 Δ32/Δ32 PBMC, confirming the results obtained in MT2 and U87 cell lines.

Sequence analysis of the third variable loop of gp120. The SI phenotype is strongly associated with positively charged amino acids at either one or both of two fixed positions of the V3 domain (residue 11 or 25)\(^\text{[102]}\). In order to study the relation between coreceptor usage and evolution of the V3 region, sequence and phylogenetic analyses were performed on virus clones from ACH039 and ACH208. Sequences from the majority of the virus clones were available from previous studies\(^\text{[105,107]}\): 387 and 189 nucleotides spanning the V3 region for ACH039 and ACH208, respectively. The deduced amino acid sequences of the V3 regions are depicted in Figure 4.

For ACH039 and ACH208, the first efficiently CXCR4-using SI clones already differed in the V3 loop by at least 7 and 4 amino acids, respectively, from the CCR5 using NSI clones isolated at the same time point. In both patients, all CXCR4-using SI variants had a positively charged amino acid at position 11 confirming previous observations. The association of a positive charge at this position with the SI phenotype indicates that the charge of the V3 loop might play an important role in the interaction with CXCR4. Indeed, one additional amino acid substitution between CXCR4-using SI clones and coexisting NSI clones resulted in the transition from a neutral to a positive charge (for ACH039 and ACH208) and one additional substitution resulted in the transition from a neutral to a negative charge (ACH039). Since the CXCR4-restricted SI clones (R3X4 and X4) did not have a higher positive charge than the clones that could use both CCR5 and CXCR4 (R3R5X4 and R5X4), a further increase in the charge of the V3 loop was not the determinant for the eventual loss of the ability to use CCR5.

The first MT2-tropic variants from ACH208 (-1.5 months) and ACH490 (-1.6 to 19.1 months) were very inefficient in their ability to use CXCR4, as characterized by their incapacity to productively infect CCR5 Δ32/Δ32 PHA-PBMC and the U87-CXCR4 cell line. Interestingly, these clones did not have a positively charged amino acid at positions 11 or 25 (ACH208: Fig. 4; ACH490; not shown). This may indicate that a region outside the V3 region may encode the first step towards SI capacity and CXCR4 usage and that efficient CXCR4 usage is not established until a positive charge at either or both of these V3 positions is acquired.

Phylogenetic relations of NSI and SI HIV-1. The neighbor-joining phylogenetic trees based on the amino acid sequences are depicted in Figure 5. The relatively large number of amino acid substitutions between CXCR4-using SI clones and coexisting NSI clones is reflected by the large phylogenetic distance. This divergence is stable in the bootstrap analyses, since it can be observed in 94 (ACH039) and 77 (ACH208) of 100 replicate analyses. After the acquisition of CXCR4 usage by SI variants, the NSI and SI HIV-1 populations continue to diverge as can be seen in the increasing distance between the NSI and SI clones obtained from the same time points. The virus clones that use both CCR5 and CXCR4 cluster between the CCR5-restricted clones and the CXCR4-restricted clones, which underlines their intermediate character in virus phenotype.
Fig. 6. Expression of CCR5 and CXCR4 on CD45RO⁺ and CD45RO⁻ CD4⁺ T cells. Cryopreserved PBMC from ACH171 (left) and ACH490 (right) obtained at 7 and 16 months after SI conversion, respectively, were stained with mAbs directed against CD4, CD45RO, CCR5, and CXCR4. The CD4⁺CD45RO⁺ (top) and CD4⁺CD45RO⁻ (bottom) cells were gated. Within these subsets, CCR5 and CXCR4 expression were determined. Numbers in the figures represent the percentages of gated cells in the corresponding quadrants.

Evolution. The early inefficiently CXCR4-using MT2-tropic clones from ACH208 (indicated by an asterisk in Fig. 5b) cluster with the CCR5-restricted NSI clones in the phylogenetic tree, which underlines their close relatedness to the NSI clones.

Expression of CCR5 and CXCR4 on CD45RO⁺ and CD45RO⁻ CD4⁺ T cells. The divergence of NSI and SI variants with respect to V3 sequence and coreceptor usage over time are compatible with the idea that both variants occupy different cellular niches within the CD4⁺ T cell population. Still, NSI and SI variants persistently co-existed in CD45RO⁺ CD4⁺ T cells. To analyze whether distinct NSI and SI target cell populations can be distinguished within the CD45RO⁺ CD4⁺ T cell population, we determined the expression of CCR5 and CXCR4 on CD45RO⁺ and CD45RO⁻ CD4⁺ T cells by four-color flow cytometry on cryopreserved PBMC from ACH171 and ACH490 obtained at 7 and 16 months after SI conversion, respectively.

Indeed, within the CD45RO⁺ CD4⁺ T cell subset, approximately 25% of the cells were CXCR4⁺ CCR5⁺ and 35% of the cells were CCR5⁺ CXCR4⁺ (Fig. 6). A minority of the CD45RO⁺ CD4⁺ T cells was CXCR4⁺ CCR5⁺ (12 and 7%) and 30 to 34% of the cells were negative for both CCR5 and CXCR4.

In agreement with previous results[518], the majority of the CD45RO⁺ CD4⁺ T cells were CXCR4⁺ CCR5⁺ (90 and 80% in ACH171 and ACH490, respectively). In this subset, only a small proportion of cells was positive for CCR5 (3 and 8%, respectively). Similar results were obtained from samples obtained well before SI conversion (25 and 10 months, for ACH171 and ACH490, respectively; data not shown).

Interestingly, these results were highly divergent from the expression levels observed in healthy donors. The majority of CD45RO⁺ CD4⁺ T cells were CXCR4⁺ CCR5⁻ (96%, average from 8 donors), as was described for the HIV-1-infected individuals. However, also in the CD45RO⁺ CD4⁺ T cell population the majority of the cells were CXCR4⁺ CCR5⁻ (68%), while only a minority of the cells was CCR5⁺ CXCR4⁻ (5%), and on average 14% were CCR5⁺ CXCR4⁺ (data not shown).

DISCUSSION

To gain more insight into the evolution from NSI to SI HIV-1 and to elucidate the basis of co-existence of NSI and SI virus populations within an infected individual, we longitudinally studied changes in HIV-1 coreceptor usage and viral tropism for CD45RA⁺ naive and CD45RO⁺ memory CD4⁺ T cells during NSI to SI conversion.

NSI HIV-1 variants were always predominantly isolated from the CD45RO⁺ T cell subset, independent of the presence of co-existing SI variants. Just after their emergence, SI variants were also mainly isolated from the CD45RO⁺ cells, but with time an equal distribution over CD45RO⁺ and CD45RA⁺ CD4⁺ T cell subsets seemed to be established, in agreement with previous observations from a cross-sectional study[518].

In agreement with their restricted tropism for CD45RO⁺ CD4⁺ T cells, the vast majority of NSI virus clones were unable to infect PBMC from a CCR5Δ32/Δ32 donor or U87 cell lines other than the one expressing CCR5. Early SI variants could use both CCR5 and CXCR4 and the early variants from ACH039 and ACH171 could additionally use CCR3. With increasing time after SI conversion, the capacity to use CCR5, but not CCR3, was lost. During the transition from the NSI to the SI phenotype, virus variants could be isolated that
Coreceptor expression and HIV-1 evolution

Fig. 7. Model of continuous evolution of SI HIV-1 population during and after the NSI to SI switch. NSI variants are CCR5 restricted and infect memory CD4+ T cells throughout infection. After the acquisition of a number of mutations, variants with a low capacity to use CXCR4 are established. Via several less fit stages, the capacity to efficiently use both CCR5 and CXCR4 evolves. These variants may infect CCR5 expressing memory cells, but have to compete with co-existing NSI variants. The presence of CXCR4/CCR5 naive and memory CD4+ T cells allow these SI variants to expand. Eventually, SI variants lose the ability to use CCR5, leading to a CXCR4-restricted SI and a CCR5-restricted NSI HIV-1 population with mutually exclusive target cell populations.

The variants were MT2-cell-line-tropic, but lacked the capacity to infect the U87-CXCR4 cell line and CCR5 Δ32/Δ32 PBMC, or were unable to infect the MT2 cell line and CCR5 Δ32/Δ32 PBMC but capable to infect the U87-CXCR4 cell line. These discordant phenotypes most likely reflect inefficient CXCR4 usage. In line with these observations, these variants with intermediate phenotypes had NSI-like V3 sequences (i.e., negatively or uncharged amino acids at positions 11 and 25) and clustered with NSI sequences in phylogenetic analyses. The capacity of these variants to replicate in MT2 cells, which completely lack CCR5 surface expression (data not shown), may thus implicate that the ability to use CXCR4, although inefficiently, is first determined by regions outside V3. In agreement with this, it was previously shown that the insertion of the V1/V2 region of an SI isolate in an NSI background (both viruses isolated during SI conversion) resulted in a low but significant SI capacity. The additional insertion of the V3 loop fully restored SI capacity, indicating that V3 is not the sole but major determinant of the phenotype.

A relatively large number of differences within the V3 loop and a large phylogenetic distance between the first efficiently CXCR4-using SI variants and the co-existing NSI variants and/or the CCR5 using MT2-tropic variants was observed. The inability to isolate variants with intermediate V3 sequences may indicate the existence of less fit variants that represent a minority of the total virus population but are important to establish an SI population with efficient CXCR4 usage. It is likely that after the acquisition of inefficient CXCR4 usage (as determined by regions outside the V3 loop), a specific combination of mutations, including mutations in V3, may be required for efficient interaction with CXCR4. Viruses that have not yet achieved the full combination of required mutations may be less replication competent and unable to compete with the co-existing NSI variants or may be more easily cleared from the body by a competent immune system.

After the acquisition of efficient CXCR4 usage by SI variants, the NSI and SI HIV-1 populations continued to diverge as can be seen in the increasing phylogenetic distance between the NSI and SI clones obtained from the same time points. The SI clones further evolve from the ability to use both CCR5 and CXCR4 to a CXCR4-restricted phenotype (either or not in combination with CCR3 usage).

The divergence of NSI and SI variants with respect to V3 sequence and coreceptor usage, and their co-existence throughout infection are compatible with the idea that they occupy different niches within the CD4+ T cell population. Otherwise, one would expect that a slight growth advantage would result in loss of the virus population with the lower replicative capacity. SI HIV-1 variants can be isolated from naive CD45RA+ CD4+ T cells, whereas NSI variants are isolated from this T cell subset only to low levels. Still the NSI and SI populations persistently co-exist in the memory CD45RO+ CD4+ T cells. Flow cytometric analysis showed that within the memory CD4+ T cells a large CCR5+ CXCR4+ population and a large CXCR4+ CCR5- population
exist in HIV-1-infected individuals (in contrast to observations in healthy donors in whom CCR5
CD4+ T cells in general also express CXCR4). These distinct populations within the CD45RO+ CD4+ T
cells may function as distinct cellular niches for NSI and SI HIV-1 variants.

Based on the results described in this study, we propose a model of continuous virus evolution
during and after the conversion to SI HIV-1 (Fig. 7). NSI clones are CCR5-restricted and therefore
capable of infecting the CCR5 expressing CD45RO+ memory CD4+ T cells throughout infection. NSI infection of CD45RA+ naive CD4+ T cells also occurs, though at low frequency probably due to the low number of CCR5-expressing CD45RA+ CD4+ T cells. Random mutations may occur, resulting in a low capacity to
use CXCR4. Still, these variants preferentially use CCR5 in vivo and therefore have to compete with
the co-existing and well-adapted NSI population for the same CCR5 expressing target cell
population. The acquisition of a combination of mutations, including a positively charged amino
acid at either position 11 or 25 in the V3 loop, eventually results in an increased efficiency to use
CXCR4, though initially at the expense of viral fitness. After the acquisition of efficient CXCR4
usage, the target cell population of the SI HIV-1 variants is extended to include the CD45RA+ and
the CXCR4+ CD45RO+ CD4+ T cells. Infection of these CXCR4+ CCR5+ CD4+ T cells offers the
opportunity to avoid competition with NSI variants, which may be very replication competent
especially late in infection\(^{[428]}\). The existence of this distinct cellular niche allows the SI variants
to expand in an environment where they are initially outnumbered by the NSI population and
eventually even reach equal levels as the NSI variants. The acquisition of increased affinity for
CXCR4 might exclude the capacity to use CCR5 because of structural constraints, explaining the
loss of the ability to use CCR5 by late stage SI variants. Eventually, two virus populations are
established with two largely mutually exclusive target cell populations and that have no
interaction with each other: an NSI virus population that is CCR5-restricted and within the T
cell pool preferentially infects CCR5+ memory CD4+ T cells, and an SI virus population that is
CXCR4-restricted and preferentially infects naive and CXCR4+ CCR5+ memory CD4+ T cells. The
occupation of the CXCR4+ cellular niche by this SI HIV-1 population may prevent the outgrowth of
possibly newly emerging SI variants by competition for CXCR4+ cells, thus explaining the
finding that SI variants seem to evolve only once during infection.

Acknowledgments

This study was performed as part of the Amsterdam Cohort Studies on HIV infection and AIDS, a
collaboration between the Municipal Health Service, the Academic Medical Centre, and the CLB, Amsterdam, the
Netherlands, and was financially supported by the Netherlands Ministry of Public Health and the
Netherlands Foundation for Preventive Medicine (grant no. 28-1547), within the Stimulation Program AIDS
Research of the Dutch Program Committee for AIDS Research (grant no. 1305).

We are greatly indebted to the cohort participants for their continuous participation. Dr. D. Liftman kindly
provided the U87 cell lines. Expression vectors pBABE-BOB and pBABE-BONZO were obtained from Dr. N.
Landau through the AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH.
The authors wish to thank Berend Hooibrink and Bert Hovenkamp for FACS sorting, Angelique van’t Wout for
helping with phylogenetic analyses, and Frank Miedema for critically reading the manuscript.