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LACK OF IN VIVO COMPARTMENTALIZATION AMONG HIV-1 INFECTED NAIIVE AND MEMORY CD4+ T CELL SUBSETS

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ABSTRACT

Viral compartmentalization between naive and memory CD4+ T cell subsets has been described, but only for individuals who were receiving antiretroviral therapy (ART). We present here an extensive analysis of the viral quasispecies residing in the naïve, central and effector memory CD4+ T cell subsets in a number of therapy naïve individuals. We longitudinally analyzed subset-specific infection and evolution in a patient who switches coreceptor usage and one who does not. The central memory subset, being the predominantly infected subset, harbors a more diverse viral population compared with the others. Through sequence analysis of the env C2V3 region we demonstrate a lack of viral compartmentalization among all subsets. Upon coreceptor switch, we observe a more pronounced increase in naïve subset infection levels, as compared with a non-switching patient, which does not result in viral compartmentalization. Our findings emphasize the importance of all CD4+ T cell subsets to viral evolution.
INTRODUCTION

During human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus (SIV) infection the memory CD4+ T cell population in the gut-associated lymphoid tissue (GALT) is preferentially and rapidly depleted\(^1\)-\(^6\). This population must be replenished by naïve and/or central memory cells in order to maintain immune function at effector sites\(^7\),\(^8\). This replenishment also results in an increased presence of target cells in the tissues fueling virus replication and immune activation. Due to the continuous destruction of tissue effector memory cells, increasingly more naïve and central memory cells need to repopulate the effector memory subset\(^6\),\(^9\)-\(^10\). This phenomenon, in combination with many other events, may eventually lead to decreased replenishment\(^11\). A reduction in tissue delivery of the effector memory subset, in combination with destruction of this decreasing subset will ultimately lead to disease progression\(^12\).

HIV-1 populations isolated from various tissues have been shown to differ in cell tropism, diversity and drug resistance phenotype\(^13\)-\(^15\). At the cellular level compartmentalization has also been shown to exist among CD4+ T cells, CD8+ T cells and cells of the monocytic lineage\(^16\)-\(^18\). Within the variant CD4+ T cell populations, the naïve cells can be productively infected with HIV-1 despite low levels of CCR5 expression. The lymphoid tissue microenvironment and/or HIV-1 envelope signaling may therefore overcome low CCR5 expression levels and result in productive infection of this cell type\(^19\)-\(^22\). In addition, part of the SIV-infected memory CD4+ T cell pool has no detectable CCR5 expression, although this might also be explained by down-regulation of this coreceptor upon infection\(^6\). Different CD4+ T cell subsets express variant levels of CCR5 with the effector memory expressing higher levels than the central memory cells and the naïve expressing low to undetectable levels of CCR5\(^23\),\(^24\). Besides differences in coreceptor expression, variations in cell number, localization and proliferation stage will also presumably influence viral compartmentalization and contribute to the characteristics of the viral quasispecies\(^7\),\(^11\).

Various publications have studied the relationship between coreceptor expression and compartmentalization among CD4+ T cell subsets\(^25\). From in vitro experiments it is known that CCR5 (R5) HIV-1 variants preferentially infect effector memory CD4+ T cells, while CXCR4 (X4) variants are mainly found within the central memory and/or naïve subset\(^26\)-\(^28\). Among two in vivo studies under the influence of highly active antiretroviral therapy (HAART) treatment there is no consensus on the presence of viral compartmentalization between naïve and memory CD4+ T cells subsets, while comparable coreceptor usage was observed\(^16\),\(^29\). A detailed in vivo analysis of the viral genotypes residing in the naïve, central and effector memory CD4+ T cell subsets without the influence of antiretroviral therapy (ART) is still lacking.

Viral diversity within the gp120 part of the envelope gene (env) and specifically within the C2V3 region has been previously linked to disease progression\(^30\),\(^31\). The variable V3 region of the HIV-1 gp120 envelope, together with the V1V2 region, has been extensively linked to coreceptor usage\(^32\)-\(^35\),\(^61\). Changes in amino acid composition, length, V3 charge as well as glycosylation patterns of these regions have been shown to influence coreceptor usage and therefore likely influence the cell type of infection.
HIV-1 infection levels of CD4⁺ T cell subsets (including naïve, central memory and effector memory cells) has previously been monitored for a group of North-American individuals, presumably infected with HIV-1 subtype B³⁶. We followed up on these results to examine the viral quasispecies residing in each of these subsets through env C2V3 sequence analysis in 13 patients without the influence of ART. Additionally, we studied HIV-1 infection levels and analyzed subset-specific viral evolution. Despite large variation in subset infection levels, we do not detect HIV-1 compartmentalization among the various CD4⁺ T cell subsets and we observe equal nucleotide distances. Upon coreceptor switch, the naïve subset demonstrates a more pronounced increase in infection levels and decrease of cell number as compared to the memory subsets, which does not result in viral compartmentalization in this patient.

MATERIALS AND METHODS

Patient characteristics and viral characterization
Thirteen HIV-1 positive individuals were included in this study, of which eleven between 1989 and 1997 regularly visited the outpatient clinic of the Academic Medical Center (AMC) in Amsterdam, the Netherlands. Two well-characterized patients, H671 and H434, were selected from the Amsterdam Cohort Studies (ACS) on HIV-1 infection and AIDS for more extensive follow-up. The individuals demonstrate large variation in viral load values and CD4⁺ T cell counts and harbor viruses representing six different HIV-1 subtypes, being A, B, C, D, F and G (Table 1). HIV-1 genotyping was performed using phylogenetic analysis (Neighbor-Joining method) of the env C2V3 region with a set of reference sequences from the Los Alamos database (http://www.hiv.lanl.gov). Viral loads were determined using the branch DNA assay. All patients were in their chronic phase of HIV-1 infection. Informed written consent was obtained from all study individuals and was approved by the Medical Ethical Committee of the Academic Medical Center in Amsterdam (the Netherlands). The clinical profiles of H671 and H434 have been previously described³⁷,³⁸. Replication competent biological clones (RCBCs) were isolated from peripheral blood mononuclear cells (PBMC) isolated at different time-points during infection by limiting dilution³⁷-³⁹. The syncytium inducing (SI) phenotype was determined utilizing the MT2 cell-line assay. Characterization of HIV-1 coreceptor usage was performed on the U87 astrogliaoma cell line stably transfected with CD4 and either CCR1, CCR2b, CCR3, CXCR4, or CCR5 and as well as on CD4⁺-enriched PBMC isolated from Δ32 CCR5 homozygous individuals⁴⁰,⁵⁹.

Polychromatic leukocyte cell sorting
PBMC were thawed and washed with RPMI 1640 medium, supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin, 100 μg of streptomycin and 1.7 mM sodium glutamate. Cells were subsequently stained with the appropriate monoclonal antibodies for 30 min and fixed with 1% paraformaldehyde. Three CD4⁺ T cell subsets were sorted: naïve, CD57⁻ memory (or central memory) and CD57⁺ memory (or effector memory) CD4⁺ T cells, with CD57 being a marker for replicative senescence and terminal differentiation⁴¹,⁴². We measured and separated the CD4⁺ cells with αCD3-FITC, αCD4-Alexa 594, αCD8-Cy7-PE, αCD11a-APC, αCD14-Cy5-PE, αCD19-Cy5-PE, αCD27-PE, αCD45RO-Texas Red-PE, αCD56-Cy5-PE and αCD57-Cas (BD Pharmingen). All cell sorts were performed utilizing a modified FACS DIVA and the strategy for leukocyte population sorting is graphed in supplementary data I. Post-sort analysis showed over 98% purity of the sorted cell populations. Sorted fractions were stored at -80°C.
Quantification of cell-associated HIV-1 DNA for patients H671 and H434 was described previously. Cellular infection levels for the AMC outpatient clinic samples were quantified using a semi-nested real-time PCR assay allowing the amplification of all HIV-1 subtypes. This assay is based on amplifying the HIV-1 long terminal repeat (LTR) region and amplifies full-length genomes. Viral DNA was first subjected to 15 cycles of pre-amplification using AmpliTaq DNA polymerase. The forward primer was LTRPREGP (5'-TAACCCTCAGATGCTGCATAwAAGCAGCyGCT-3') and reverse primer L-GAGMNEW (5'-AGCAAGCCGAGTCCTGCGTC-3'). The cycling conditions were as follows: 5 min 95˚C followed by 15 cycles of (1 min 95˚C – 1 min 55˚C – 2 min 72˚C) and 10 min 72˚C. The product was used as template in the TaqMan quantification step, which was performed using 2x Platinum quantitative PCR Super mix UDG (Invitrogen), 2.5 mM MgCl₂, 0.5 mM RoX reference dye, 0.9 μM of each primer and 0.19 μM of the TaqMan dual-labeled fluorescent probe (PGPLTR 5'-GTADCTAGATCCCTGGA-3') and reverse primer L-GAGMNEW (5'-AGCAAGCCGAGTCCTGCGTC-3'). The cycling conditions were as follows: 2 min 50˚C, 10 min 95˚C, followed by 45 cycles of (15 sec 95˚C – 1 min 60˚C). Plasmid DNA corresponding to the region of interest was used as an external standard. To determine cellular input, a standard

### Table 1. Patient description

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<th>Patient</th>
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<th>Gender</th>
<th>Age at 1st time-point</th>
<th>env subtype</th>
<th>months after 1st visit</th>
<th>Viral load (copies/ml)</th>
<th>CD4 count (cells/μl)</th>
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<td>29</td>
<td>B</td>
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<td>G</td>
<td>4</td>
<td>14,180</td>
<td>300</td>
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</table>

*Dom. Rep.: Dominican Republic. b mpsc: months post seroconversion. c n.a.: not available.
d viral load data obtained on 15, 60 resp. 117 mpsc.
curve was constructed based on the β-actin gene, which was run in parallel. Reactions were performed in duplicate and samples where the difference between the duplicates exceeded 0.5 log were not included. Results were analyzed using the ABI Prism 7700 Software.

HIV-1 genome amplification
Viral DNA from the cellular subsets was isolated utilizing a silica-based method, which was also used for RNA isolation from serum. For patients H671 and H434 the env C1C4 region of the RNA was reverse transcribed using avian myeloblastosis virus reverse transcriptase (AMV-RT) as described previously with slight modifications. The obtained cDNA and sorted leukocyte lysate containing viral DNA were used in the Expand Long Template PCR system. The volume of the added cell lysate corresponded to 100 to 500 copies of gag as determined by quantitative PCR (qPCR) as previously published.

For the AMC outpatient clinic samples, the C2V3 region (HXB2 positions 7032-7301) of the HIV-1 envelope gene was amplified and AMV-RT was used for reverse transcription of the serum-derived RNA. For the first PCR the following primers were used: 5'-AATGTCAGCACAGTACAATG-3' and 3'-TCTCCCTCCTCAGGYCTGAA-5'. Subsequently, a nested PCR using the primers 5'-CCAGTGGTATCAAATCTCAA-3' and 3'-ATTTTCTATGGGTATATACTCAA-5' was performed (100 ng/μl).

Interpatient cross-contamination was ruled out using bootstrapped maximum likelihood phylogenetic analysis (data not shown).

HIV-1 sequencing and sequence analysis
Positive PCR products were cloned into the TOPO II vector and bacterial colonies were selected using ampicillin. Positive colonies were sequenced bi-directionally using the BigDye Terminator Cycle Sequencing kit and analyzed using an ABI 377 automated sequencer. Six to twenty-six clones for each subset were sequenced. Quality of the sequences was analyzed using CodonCode Aligner version 1.5.1, after which they were automatically aligned with BioEdit 7.0.1. Using Textpad 4.5.0 the sequences were adjusted manually in respect to the gp120 open reading frame according to reference sequences from the Los Alamos HIV sequence database (http://www.hiv.lanl.gov). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4. Statistical analyses were performed using the Wilcoxon signed rank test and correlation coefficients were computed by Graphpad Prism 5.01 using the nonparametric Spearman correlation. Sequences containing stop codons were not included in the analyses. The predicted coreceptor usage of subtype B and C virus sequences was determined using a web-based position-specific scoring matrix (PSSM), available at http://ubik.microbiol.washington.edu/computing/pssm.

Nucleotide sequence accession numbers
The sequences described have been submitted to GenBank and assigned accession numbers are GQ389219-GQ389231.

RESULTS
Large variation in CD4+ T cell subset infection levels
Here we analyzed HIV-1 compartmentalization among various CD4+ T cell subsets in peripheral blood of thirteen ART-naïve HIV-1 infected individuals. For eleven out of thirteen individuals PBMC from multiple time-points were available and studied. Table 1 shows the patient characteristics of the first time-point for all patients and all time-points for patients H434 and H671. We included these two well-characterized
Chapter 2

Subtitle B infected individuals for longitudinal analyses. Since infection levels might influence compartmentalization, we quantified HIV-1 infection in FACS-sorted naïve, CD57 and CD57+ memory CD4+ T cell subsets (supplementary data I). Figure 1 depicts the relative infection levels of eleven out of thirteen patients tested. Infection levels were highly variable among the cellular subsets. The CD57+ memory subset was being predominantly infected in seven out of eleven patients, in accordance with previous findings36. The naïve and CD57+ memory subsets were predominantly infected in two patients. For eight out of the eleven patients, where multiple time-points were available, infection levels were consistent over time, up to three years for patient M12259.

When absolute cellular infection levels were compared to markers of disease progression, the naïve and CD57 - memory subsets showed a significant inverse correlation with the CD4 count ($r_s = -0.65$ and $p<0.01$ for the naïve subset; $r_s = -0.57$ and $p<0.05$ for the CD57- memory subset; data not shown). In addition, the CD57- memory subset also significantly correlated with the viral load ($r_s = -0.60; p<0.05$; data not shown) indicating that infection of specific CD4+ T cell subsets can be linked to markers of disease progression. The infection levels of the CD57- memory subset also correlated with those of the naïve and the CD57+ memory subset ($r_s = 0.50$, $p<0.05$ and $r_s = 0.78$, $p<0.0001$ respectively; data not shown). This confirms previous data and indicates viral genome exchange among the subsets or differentiation of one subset into another36.

In summary, viral infection levels vary greatly among the different cellular subsets and naïve and CD57- memory infection levels show a significant inverse correlation with CD4+ T cell counts in the patients.

Lack of viral compartmentalization

To study whether differences in infection levels influenced compartmentalization among the various CD4+ T cell subsets, we amplified the env C2V3 region from each subset as well as from serum. Based on phylogenetic analysis, we identify no compartmentalization among the different cellular subsets or serum in twelve out of thirteen patients studied over time (Fig. 2 and supplementary data II). Figure 2 shows a...
Figure 2. Neighbor-joining phylogenetic trees of patient M11814 9, 24 and 31 months after primary diagnosis. *Env C2V3* sequences from the various cellular subsets and serum are represented by different symbols, as depicted in the legend. Reference strains are depicted by diamonds and are shown at the bottom of each tree. For these bootstrap trees of 1,000 replicates, only bootstrap values higher than 80% are shown and the Kimura-2 parameter was used to calculate nucleotide distances.
representative phylogenetic analysis of three different time-points for patient M11814 with intermingling sequences from the various subsets. For all patients, this image was consistent when longitudinal samples were analyzed (data not shown). In addition, in patients H671 and H434 we observed comparable evolutionary rates among all cellular subsets and serum (M. Geels, unpublished results). Analysis of the viral sequences from patient M12817 demonstrated compartmentalization among the different cellular subsets for some but not all time-points. Unfortunately, serum samples of this patient were unavailable for analysis.

To confirm the findings of our phylogenetic analyses, we next calculated genetic distances between all subsets (Fig. 3). We found no significant differences among any of these comparisons confirming the lack of compartmentalization. Median nucleotide distances varied between 4.3% for CM vs. EM and 4.9% for N vs. EM, N vs. S and EM vs. S. Synonymous nucleotide distances (dS) ranged from 2.2 to 2.8% and non-synonymous nucleotide distances (dN) from 3.7 to 4.7% with no significant differences between any comparison (data not shown).

We also determined the viral diversity within each subset (Table 2). When analyzing the synonymous nucleotide distances, all subsets harbored comparable levels of diversity with no significant differences, although the CD57+ memory subset contained the least diverse viral population (median value of 1.4 %). For the non-synonymous distances, the CD57- memory subset harbored a significantly more diverse collection of viral sequences over the other subsets (a median value of 3.8%; p<0.05). Besides nucleotide distances, viral sequences from the different subsets were also analyzed for possible differences in dN/dS ratios (for evolutionary pressure) and G to A hypermutation levels (a potential indication of APOBEC activity)\(^{60}\). For both parameters, highly variable levels among subsets were observed without clear differences between the subsets, again indicating lack of subset-specific evolutionary pressure.

In conclusion, despite the observation that the CD57- memory subset demonstrates the highest level of infection and diversity, we find no evidence of compartmentalization

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**Figure 3.*** Nucleotide distances among all subset comparisons. The horizontal bar depicts median values. **N** - Naïve; **CM** - CD57- memory; **EM** - CD57+ memory; **S** - Serum.
between the variant CD4+ cellular subsets analyzed and observe similar genetic distances of viral sequences among all subset comparisons.

**Subset-specific infection and evolution**

In two well characterized HIV-1 seroconverters, patients H671 and H434\(^{37,38}\), we longitudinally studied compartmentalization along with subset-specific infection and evolution. We had previously reported that patient H671 underwent a switch from a non-syncytium inducing (NSI) to a syncytium inducing (SI) phenotype between 57 and 69 months post seroconversion (mpsc), while the viral phenotype of patient H434 remained NSI throughout follow-up. Replication competent biological clones (RCBCs) were generated from PBMC and coreceptor usage was determined\(^{37,38}\). All generated biological clones from patient H434 utilized only the CCR5 coreceptor on U87.CD4 cells, whilst the later RCBCs from patient H671 at 85 and 89 mpsc could utilize both U87.CD4 cells expressing CCR5 and CXCR4 (Fig. 4). This was confirmed by infection of CD4+ lymphocytes isolated from individuals homozygous for the 32bp deletion in the CCR5 gene\(^{40,59}\).

We longitudinally determined cellular infection levels of the three CD4+ T cell subsets in the peripheral blood of H671 and H434. The CD57- memory CD4+ T cell subset was the predominantly infected subset during the respective R5 stages of infection in both patients (Fig. 5A and 5B). Interestingly, we detected early infection of the naive cells albeit at very low levels (3.9 vs. 14.4 copies per 10^5 sorted cells respectively; Fig. 5A and 5B). Infection levels of all subsets increased over time, except

<table>
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<th>Subset</th>
<th>synonymous diversity(^a)</th>
<th>non-synonymous diversity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naïve</td>
<td>2.3 (0.2 - 4.6)</td>
<td>3.4 (0.2 - 9.0)</td>
</tr>
<tr>
<td>CD57- Memory</td>
<td>2.4 (0.0 - 5.1)</td>
<td>3.8 (0.1 - 9.2)(^b)</td>
</tr>
<tr>
<td>CD57+ Memory</td>
<td>1.4 (0.0 - 6.3)</td>
<td>2.4 (0.1 - 10.1)</td>
</tr>
<tr>
<td>Serum</td>
<td>2.0 (0.2 - 5.0)</td>
<td>3.4 (0.1 - 8.9)</td>
</tr>
</tbody>
</table>

\(^a\) values are in percentage and represent median values of all time-points studied; no significant differences between the subsets. \(^b\) The CD57- memory subset was significantly more diverse than all other subsets (p<0.05).

Figure 4. Coreceptor usage of R5X4 clones from patient H671, 85 and 89 mpsc. U87.CD4 cells expressing either CCR5 or CXCR4 were infected with H671 R5X4 biological clones to determine coreceptor usage. Read-out was after seven days and is depicted in ng/ml.
for the CD57+ memory subset of H671. A dramatic increase in the naïve infection level of patient H671 was observed after RSX4 viruses were first detected at 69 mpsc. At 89 mpsc, the naïve subset was the predominantly infected cell population in this patient (Fig. 5B). Following an increase in naïve infection levels, a 38.9-fold reduction in naïve cell number was observed, resulting in seven cells per μl at 89 mpsc (Fig. 5D). Changes for the CD57- memory subset were more modest (Fig. 5C and 5D). Absolute cell numbers of the CD57+ CD4+ T cell subset remained fairly constant in both patients despite an increase in infection over time in H434. Surprisingly, phylogenetic analyses of both patients demonstrated lack of compartmentalization at all time-points, despite coreceptor switch of patient H671 (M. Geels, unpublished results).

These observations suggest that in these patients a different pattern of subset infection and evolution, partly due to coreceptor switch, did not result in viral compartmentalization.

**Distribution of HIV-1 genotypes among CD4+ T cell subsets**

To correlate CD4+ T cell subset infection levels with the viral genotype within each of the different subsets and relate this to the viral variants present in the sera, we analyzed the env V3 charge within the three CD4+ T cell subsets, serum and RCBCs of patients H671 and H434. A higher V3 charge has been heavily associated with a switch in coreceptor usage towards CXCR4 utilization\(^{33,35}\). All subsets show a comparable distribution of HIV-1 genotypes among CD4+ T cell subsets.

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**Figure 5. Longitudinal analysis of HIV-1 infection levels and size of CD4+ T cell subsets.** (A, B) Cellular infection levels of patients H434 and H671 for the naïve, CD57- memory and CD57+ memory CD4+ T cell subsets are depicted on the left y-axis in number of gag copies per 10^5 cells as determined by qPCR. Serum viral load values can be read out in the same graph on the right y-axis. Time-points after coreceptor switch are marked with an asterisk. (C, D) Longitudinal overview of absolute CD4+ subset cell numbers in peripheral blood for patients H434 and H671.
increase in V3 charge over time (Fig. 6). During the R5 stages of both patients, all CD4+ T cell compartments were infected viruses that largely possess an R5-signature. After the switch in coreceptor usage in patient H671 at 69 mpsc, the higher charged viruses preferentially infected the naïve and CD57− memory subsets, while the CD57+

Figure 6. Longitudinal overview of the net env V3 charge distribution of HIV-1 variants isolated from different CD4+ T cell compartments of patients H671 (A) and H434 (B). The relative quantity of the viruses within the naïve, CD57− memory, CD57+ memory and serum subsets and of the RCBCs is depicted on the y-axis and the net V3 charge is depicted on the x-axis. Time of sampling in months post seroconversion (mpsc) and the number of env variants analyzed are given above each panel component. The CD57+ memory subset of 0.2 and 89 mpsc of patient H671 contains V3 clones from hyper-mutated viral variants, as indicated by asterisks.
memory subset harbored the lower charged variants. This distribution did not lead to compartmentalization in our phylogenetic analyses (M. Geels, unpublished data). At 89 mpsc, the charge of the viral variants among the different subsets showed equal distribution again, with +5 charged viruses dominating every subset. Coreceptor usage predictions by PSSM confirmed these findings. Throughout the entire follow-up (except for 69 mpsc with patient H671), viruses possessing similar V3 charges were isolated from all the different cellular compartments. These results indicate that no compartmentalization was detected with respect to molecular determinants associated with different HIV-1 coreceptor usage patterns in either the switching or the non-switching patient.

**DISCUSSION**

In this study we examined HIV-1 infection in various CD4+ T cell subsets *in vivo*. Our phylogenetic analysis and the observation of equal nucleotide distances of the viral quasispecies among naïve, central and effector memory CD4+ T cells indicate the lack of compartmentalization. One explanation could be the short lifespan of HIV-1 infected cells. Since the half-life of productively infected cells is estimated to be 1.6 days, the majority of infected cells we analyze are recently infected although we cannot exclude the presence of minor quasispecies in these peripheral blood subsets47. The main difference with the study from Delobel et al is that there is ongoing viral replication in our patients, while their study contains three patients who were receiving seven years of HAART16. The predominant African origin of our study population might also explain comparable subset distribution of the viral quasispecies. Increased immune activation among Africans compared with Europeans might promote viral replication and diversity and thereby reducing viral compartmentalization48-50. The presence of compartmentalization at some time-points within patient M12817 might be explained by the patients’ altered immune response, since viral diversity has been shown to partly coincide with selective immune pressure51,52. The cellular infection levels of this patient are somewhat lower than those of most other patients.

We observed high variation in HIV-1 infection levels of the different CD4+ T cell populations. In lymphoid tissues, where a large part of viral replication is believed to occur, the CD57- memory subset is the most prevalent CD4+ T cell subset, which may help explain its preferential targeting53. We observed more variation in infection levels than previously described36. An explanation for the patients showing predominant infection of naïve or effector memory cells might be that they were in a later stage of disease or had heightened immune activation at that time-point. The average viral load value of our patient group was twice as high as compared with the group in the previous study, where nearly half of the patients were receiving HAART. Differences could also reflect the subtype differences and/or the African origin of our patients, since individuals from certain African populations have been shown to harbor increased immune activation in comparison with Europeans48,49.

Infection of the naïve CD4+ T cell subset was detected in all patients, even after 0.2 mpsc in the case of patient H671, and this patient was infected with CCR5 using
is isolates early in infection. Previous findings have shown that viruses with a lower V3 charge are likely to possess higher CCR5 affinity, facilitating infection of the naïve subset where CCR5 expression levels are low. However, we cannot rule out that other envelope modifications can compensate for such charge variations. The observation that naïve CD4+ T cell infection is not restricted to X4-using viruses has previously been shown. Surprisingly, patients M12228 and M12020 harbored a substantial naïve infection level with M12228 having predominant +4 charged V3 regions. Subset infection levels did not correlate with viral diversity, only if all subsets were grouped and compared to synonymous distances (r_s = 0.38; p<0.005). This is in line with the findings of Lemey and co-authors where synonymous substitution rates predict HIV-1 disease progression.

For patients H671 and H434 infection levels of all CD4+ T cell subsets increased comparably over time, except for the effector memory subset of H671, followed by a decrease in naïve and central memory cell numbers. The constant effector memory cell levels likely reflect the minor influence of viral infection on the lifespan of this already short-lived cell type. Upon viral coreceptor switch in patient H671 the naïve subset showed a 69-fold increase in infection levels, which corresponds with previous literature. Initially, naïve cell numbers were maintained, possibly due to homeostatic mechanisms or low pathogenicity of the dual-tropic viral variants. Disappearance of higher charged viruses and a reduction in predicted X4 variants within this subset between 69 and 89 mpsc supports this explanation (data not shown). At 89 mpsc, the more pronounced destruction of the naïve subset compared with the relatively more preserved central memory subset can partly be explained by high naïve infection levels (11,445 vs. 1,066 gag copies per 10^5 cells). Another explanation may be that dual-tropic viruses more likely enter the naïve subset using CXCR4 and thereby induce more cell death, while R5-mediated entry of the memory subsets do not readily kill these cells. Alternatively, homeostatic mechanisms may fail over time and/or there is increased differentiation into a more effector (memory) phenotype. To predict absolute levels of viral production by the various subsets, we combined infection levels with subset cell numbers. The central memory subset was found to be the major producing subset, even when H671 switched coreceptor usage. However, these numbers remain predictions, since we did not directly determine production levels, which likely differ between the subsets.

In figure 6 we observed comparable V3 charge distribution among all subsets, including serum, with no evidence of target cell selection, except for 69 mpsc of patient H671. Higher charged (and predicted X4 phenotype) viruses were infecting naïve and central memory cells, whereas lower charged variants were found within the effector memory subset. This distribution did not result in viral compartmentalization, since all subsets contained both R5 and X4 using variants (unpublished data). A different ratio of coreceptor expression may explain this temporal preferential attraction of higher charged variants. At 89 mpsc the viral quasispecies in the naïve and central memory subset reduced its V3 charge and we observed a comparable charge distribution among all subsets. The low number of X4-using clones (1/13 or 7.7%) and low number of higher charged variants in the serum at 69 mpsc indicate that these variants might not have been fit enough to persist.
In other words, patient H671 demonstrated the same viral evolution pattern upon coreceptor switch despite the preferential viral propagation in the naïve subset. This can be explained by our observation that the dual-tropic variants infect all CD4+ T cell subsets, including the memory subset. In addition, all subsets, including the naïve population, are still infected with R5 variants. These remain present upon coreceptor switch and have been described by some groups to be more capable of producing infectious virions compared with X4-using variants\textsuperscript{56-58}. Our data from patient H434 indicates that also in a non-switching patient naïve infection levels increase over time. We must keep in mind, however, that these conclusions are based upon findings within a single individual.

In summary, we demonstrate that HIV-1 evolution within naïve and memory CD4+ T cell subsets is comparable. Despite variation in infection levels, no compartmentalization was detected among naïve, central and effector memory CD4+ T cells. Despite the more pronounced changes of the naïve subset upon coreceptor switch, we observe comparable virus evolution among all studied subsets. These findings emphasize the importance of all CD4+ T cell subsets to viral evolution and disease pathogenesis.

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**REFERENCE LIST**


Supplementary data I. FACS sorting strategy. Progressive gate, flow cytometry sorting strategy for CD4+ T cells. CD4+ T cell subsets were defined based on expression of CD3 and CD4 without expression of CD8 and CD14, CD16, CD56 (dump). Naïve CD4+ T cells were defined based on expression of CD27 and dull expression of CD11a and no expression of CD45RO or CD57. Memory CD4+ T cells were defined based on expression of CD45RO with high expression of CD11a. Memory CD4+ T cells were then separated based on expression of CD57. CD57+ memory CD4+ T cells represent the central memory fraction, while the CD57+ cells are regarded as the effector memory subset.
Supplementary data II. Phylogenetic analyses of all patients except for M11814. Neighbor-joining phylogenetic trees, constructed using C2V3 env sequences from the different cellular subsets and serum, which are represented by different symbols, as depicted in the legend. Reference strains are represented by open diamonds and shown at the bottom of each tree. Trees consist of 1,000 replicates and only bootstrap values higher than 80% are shown; the Kimura-2 parameter was used to calculate nucleotide distances. For patients H671 and H434 the first time-point was depicted. a) M11306; b) M11781; c) M12003; d) M12020; e) M12092; f) M12228; g) H434; h) H671; i) M13408; j) M16394; k) M12817; l) M12259.