Influence of CD4+ cell types on HIV-1 infection
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VARIED SENSITIVITY TO THERAPY OF HIV-1 STRAINS IN CD4+ LYMPHOCYTE SUBPOPULATIONS UPON ART INITIATION

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**VARIED SENSITIVITY TO THERAPY OF HIV-1 STRAINS IN CD4+ LYMPHOCYTE SUBPOPULATIONS UPON ART INITIATION**

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**ABSTRACT**

Although antiretroviral therapy (ART) has proven its success against HIV-1, the long lifespan of infected cells and viral latency prevent eradication. In this study we analyzed the sensitivity to ART of the HIV-1 quasispecies in naïve, central and effector memory CD4+ lymphocyte subsets. From five patients, cellular HIV-1 infection levels were quantified before and after initiation of therapy (2-5 weeks). Through sequencing the C2V3 region of the HIV-1 gp120 envelope, we studied the effect of short-term therapy on virus variants derived from naïve, central and effector memory CD4+ lymphocyte subsets. HIV-1 infection levels declined in all lymphocyte subsets but not as much as RNA levels in serum. Virus diversity in the naïve and central memory lymphocyte populations remained unchanged, whilst diversity decreased in serum and the effector memory lymphocytes. ART differentially affected the virus populations co-circulating in one individual harboring a dual HIV-1 infection. Changes in V3 charge were found in all subjects after ART initiation with increases within the effector memory subset and decreases found in the naïve cell population. While certain cell populations can be targeted preferentially during early ART, some virus strains demonstrate varied sensitivity to therapy, as shown from studying two strains within a dual HIV-1 infected individual.
INTRODUCTION

Antiretroviral therapy (ART) has proven to be successful against human immunodeficiency virus type 1 (HIV-1) and results in undetectable plasma levels for many years. However, an increasing number of studies report on adverse events and toxicities\(^1,2\). Additional drawbacks to therapy are adherence and the considerable costs. In certain situations a more simplified antiretroviral regimen may be suitable, for instance as short-term use to prevent mother-to-child-transmission (MTCT), maintenance therapy after HAART or possibly as pre-exposure prophylaxis\(^3-7\). Despite the increased likelihood of viremia and emergence of resistance, prophylactic and/or short-term therapeutic use largely bypasses these disadvantages and more treatment options remain available.

The CD4\(^+\) lymphocyte is the main target cell for HIV-1 infection with the various subpopulations infected to a different extent\(^8,9\). Naïve and memory lymphocyte subsets differ in body distribution, proliferative capacity and in expression levels of the main coreceptor for HIV-1, CCR5\(^10-13\). Despite these differences, all cellular subsets are productively infected and display a lack of viral compartmentalization among circulating cells in peripheral blood\(^9,14,15\). Under the influence of long-term ART, most studies describe a lack of viral compartmentalization among HIV-1 infected CD4\(^+\) lymphocyte subsets\(^16-19\). Both central and transitional memory CD4\(^+\) lymphocytes are regarded as cellular reservoirs for HIV-1 under therapy\(^20\). Baldanti and colleagues show that naïve and memory cell numbers and HIV-1 infection levels do not differ greatly from each other under therapy\(^21\). These studies focus mainly on long-term ART and do not describe the influence on the cell subset-specific quasispecies during early therapy. Here we studied alterations to HIV-1 infection levels and viral diversity within specific cellular subsets after short-term ART.

MATERIALS AND METHODS

Five chronically HIV-1 infected individuals, who visited frequently the outpatient clinic of the Academic Medical Center (AMC), University of Amsterdam, the Netherlands, participated in this study. These patients received various antiviral regimens (Table 1) and their characteristics

### Table 1. Patient characteristics

<table>
<thead>
<tr>
<th>Patient</th>
<th>env subtype</th>
<th>therapy regimen</th>
<th># days on ART</th>
<th>viral load (copies/ml)</th>
<th>CD4 count (cells/μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M11306</td>
<td>C</td>
<td>amprenavir</td>
<td>14</td>
<td>52,436</td>
<td>3,160</td>
</tr>
<tr>
<td>M12020</td>
<td>D</td>
<td>zidovudine</td>
<td>18</td>
<td>5,352</td>
<td>304</td>
</tr>
<tr>
<td>M12259</td>
<td>F</td>
<td>zidovudine</td>
<td>33</td>
<td>246,572</td>
<td>25,588</td>
</tr>
<tr>
<td>M13408</td>
<td>A</td>
<td>d4t, 3tc, rtv(^a)</td>
<td>28</td>
<td>65,262</td>
<td>247</td>
</tr>
<tr>
<td>M16394</td>
<td>C</td>
<td>zidovudine</td>
<td>28</td>
<td>1,026</td>
<td>607</td>
</tr>
</tbody>
</table>

\(^a\)d4t - stavudine, 3tc - lamivudine, rtv - ritonavir
have been previously described. Serum and peripheral blood mononuclear cells (PBMC) were obtained and frozen according to standard protocols. Viral loads were determined with the Versant HIV-1 RNA Assay (bDNA; Bayer Diagnostics, Leverkusen, Germany). Determination of HIV-1 subtype was performed by phylogenetic analyses and by blasting the sequences using the Los Alamos database. This study was approved by the Medical Ethical Committee of the AMC and informed consent was provided by all participants.

PBMC were thawed and FACS-sorted as previously published. Cells were stained with various antibodies and three CD4+ lymphocyte subsets were sorted: naïve, CD57- memory (or central memory) and CD57+ memory (or effector memory) CD4+ lymphocytes. All cell sorts were performed utilizing a modified FACS DIVA. Viral DNA from the cell subsets was isolated utilizing a silica-based method, which was also used for RNA isolation from serum. Cellular HIV-1 infection levels were quantified using a semi-nested real-time PCR assay.

The LTR segment of the virus genome where the second strand transfer takes place and quantifies only fully reverse transcribed HIV-1 genomic DNA and has high specificity for all major HIV-1 subtypes. AMV-RT (Madison, WI, USA) was used for reverse transcription of the serum-derived RNA. The C2V3 region (HXB2 nucleotide positions 7032-7301) of the HIV-1 envelope gene was amplified using AmpliTaq DNA polymerase (PE Applied Biosystem, Foster City, CA, USA). The primers (100 ng/μl) for the first-round PCR were 5’-AATGTCAGCACAGTACAATG-3’ and 3’-TCTCCTCCTCCAGGYCTGAA-5’ and for the nested PCR 5’-CCAGTGGTATCAACTCAA-3’ and 3’-ATTITGAAGTCCCTCCTGA-5’. PCR products were clonally sequenced using the TOPO II cloning system (Invitrogen, Paisley, UK). Eleven to twenty-three clones from each subset were sequenced bi-directionally using the BigDye Terminator Cycle Sequencing kit and analyzed with the ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA). Quality of the sequences was analyzed using CodonCode Aligner version 1.5.1, after which the sequences were aligned with BioEdit and adjusted manually with respect to the gp120 open reading frame and according to reference sequences from the Los Alamos HIV sequence database. Molecular evolutionary analyses were conducted using MEGA version 4. Tamura-Nei was used as distance parameter and interpatient cross-contamination was ruled out. Statistical analyses were performed using the Mann-Whitney test.

**Sequence data**

The sequences described here were allocated the following Genbank nucleotide accession numbers: GQ389219, GQ389220, GQ389225, GQ389227 and GQ389228.

**RESULTS**

**Patient description and HIV-1 quantification in CD4+ lymphocyte subsets**

We studied the effect of antiretroviral therapy on HIV-1 infection levels of naïve, central and effector memory CD4+ lymphocyte subsets and on the viral quasispecies present in these subsets, two to five weeks after initiation of ART. The five patients studied, harbored various HIV-1 subtypes (A, C, D and F) and demonstrated a wide range of viral load values and CD4 counts (Table 1). Three out of five study subjects received an RT inhibitor (AZT), one a protease inhibitor (APV) and one received a three drug regimen (d4T/3TC/RTV). Plasma viral load declined in four individuals by 1 to 2.4 log and one subject (M16394) experienced only a small plasma load decline (Fig. 1A). This patient already had a low viral load prior to therapy (1026 copies/ml). Additionally,
this patient had a high CD4 count at time of therapy initiation (800 cells/μl), which
did not rise following therapy. In three of the four patients with complete data sets,
intracellular HIV-1 infection levels decayed by comparable levels for all cell subsets
studied, up to 1.1 log (Fig. 1B). One exception was the effector memory population
of subject M13408, the individual receiving the triple regimen, where infection levels
significantly increased 6.5-fold.

Influence of therapy on HIV-1 quasispecies in CD4+ lymphocyte subsets

Our goal was to determine how therapy affected the virus variants within naïve, central
memory and effector memory CD4+ T cell subsets during the initial phase of therapy.
Before therapy initiation, phylogenetic analysis of the C2V3 region of HIV-1 gp120
envelope did not demonstrate compartmentalization of the virus quasispecies within
serum or CD4+ T cell subsets (Fig. 2A). Only effector memory-derived sequences from
M12020 clustered. After therapy start, loss of diversity was observed predominantly
in serum, but also within the effector memory subset (Fig. 2B). Naïve- and central
memory-derived virus showed modest changes in diversity. The loss of diversity was
highly significant in serum (p=0.02 for subject M12259 and p<0.0001 for all other
patients; Fig. 3). No diversity loss was observed in the naïve or central memory
compartments.

To measure genetic evolution of the viral quasispecies, pairwise distances were
calculated between the virus populations before and after start of therapy. In serum,
divergence of the viral quasispecies was observed in three patients (indicated by an
asterisk; Fig. 3). This indicated selection of serum variants due to introduction of

Figure 1. Viral load and cellular infection levels before and after initiation of ART. (A) Viral load
values were calculated before (-) and after (+) initiation of ART and are plotted on logarithmic scale.
The median decline in copy number is inserted within the graph. (B) The number of HIV-1 gag copies per
10^5 cells of the respective cell subset is depicted on the y-axis in logarithmic scale. An occasional subset
was not included due to a large difference between the duplicate measurements.
Figure 2. Neighbor-joining phylogenetic analysis of the gp120 virus sequences. The Kimura-2 parameter and 100 replicates were used to calculate nucleotide distances and sequences from the Los Alamos HIV-1 database were used as reference strains. Circles indicate sequences from serum, diamonds from naïve CD4+ T cells, triangle from central memory and squares from effector memory cells. (A) Phylogeny of the strains isolated before initiation of therapy (B) Phylogeny of the strains isolated after therapy initiation. The black curved lines indicate strains from the effector memory population and the white curved lines indicate strains from serum. The dotted line indicates the two virus strains co-circulating in subject M12020.
Figure 3. Diversity and divergence of the viral quasispecies. (A) From each patient pairwise nucleotide distances before (-) and after (+) initiation of therapy were calculated for each cell subset and serum. Nucleotide distance is presented as percentage and the red bar represents the median value. Pairwise distances between both time-points were calculated (d) and when this value was higher than the diversity of either time-point it was identified as viral divergence, indicated by an asterisk. Statistical significance was calculated for the difference in diversity before and after therapy start; *** = p<0.0001. Data from the effector memory subset of M16394 was not available.
therapy. Viral divergence was absent in all cell subsets, with the exception of the effector memory subset in subject M12020. The absence of changes in viral diversity and divergence within naïve and central memory subsets as opposed to effector memory cells and serum, indicates that during early therapy the plasma and effector memory cell compartments are more susceptible to the effect of the drugs.

To investigate the relatedness of virus strains among the cellular fractions, the genetic distances between HIV-1 sequences derived from the various cellular fractions were calculated. Four out of five individuals demonstrated comparable distances before and after start of therapy ranging from 2.4% to 7.2% (Fig. 4). After therapy initiation no change in distances were observed and these were similar to virus diversity within each of the cell subsets. Subject M12020 was the exception to the above since intersubset distances before therapy were not only higher than those from any other individual, but as well higher than the values after therapy (Fig. 4). This person was found to be infected with two different subtype D virus strains, strain I and II, as shown by phylogenetic analysis (Fig. 2A). In addition, the analysis of virus sequences with DNAsp software indicated that up to 11 possible recombination breakpoints could be detected suggesting that these two virus strains were co-circulating within this individual for some time (data not shown). Before therapy, strain I was dominant in the effector cell population, while the other cell subsets harbored strain II. Both strains were present in serum. After therapy start, strain I disappeared from the effector memory subset but remained in some central memory cells (Fig. 2B). The replenishment of this cell subset by a different virus strain correlated with viral divergence (Fig. 3). Intersubset virus distances approached values observed for the other patients harboring mono-infections, demonstrating that although some cell populations may be more sensitive to the effects of antiretroviral therapy, differences in sensitivity amongst virus strains also exists. These data indicate that the occurrence of dual HIV-1 infection could be an additional hurdle for therapy to succeed.
Chapter 3

Influence of therapy on V3 charge

Previous observations by our group and others have shown that V3 charge influences coreceptor usage. Since CD4+ lymphocyte subpopulations differ in coreceptor expression levels, it was analyzed whether therapy initiation affected the V3 charge of the virus quasispecies in serum and lymphocyte subsets due to the variant expression profile. We therefore compared the V3 charge from all sequences found in the cell subsets before and after start of therapy. Sequences from all five patients were grouped together and we observed a clear increase in V3 charge within the effector memory subset in three out of four subjects (Fig. 5; p<0.0001). Within the central memory subset the V3 charge did not change whilst alterations in serum varied per patient (Fig. 5; no significance). Within the naïve subset the V3 charge decreased systematically in all patients (p=0.05), indicating that characteristics like coreceptor usage may be involved in viral selection after therapy initiation.

Discussion

In our study we observed comparable viral decay within all CD4+ lymphocyte subpopulations in the peripheral blood, except for one effector memory subset, confirming our previous observation that all CD4+ lymphocyte subsets are productively infected with HIV-1. The results also confirm findings from other studies demonstrating comparable decay of productively infected cells in peripheral blood. A report on preferential HIV-1 inhibition during AZT treatment in activated cells over slowly dividing cells in vitro, may indicate that the vast majority of virus in the circulation comes from activated cells. Although naïve and central memory lymphocyte subsets contain more long-lived resting cells than the effector memory subset and outnumber this subset, no difference in viral decay was observed. Two to five weeks after initiation of ART represents the start of the second phase of viral decay, which comprises loss of long-lived infected cells. Two to five weeks following

Figure 5. Change in V3 charge after initiation of ART. From all cellular subsets and serum the net V3 charge of each viral clone was calculated. The net V3 charges of all patients were grouped per time-point before (-) and after (+) initiation of ART. The graph depicts the mean value with standard deviation. *** = p<0.0001 and ns = not significant.
therapy start may be too early to detect a differential decay of these cells due to their slower decay rate. M13408 was the only patient who received a triple regimen and surprisingly increased in effector memory infection levels. Perhaps these cells possess high P-glycoprotein efflux activity decreasing intracellular antiviral drug concentrations\textsuperscript{30}. Although blood CD4\textsuperscript{+} lymphocyte levels only represent a minor fraction of the total body lymphocyte population, memory subsets in blood versus gut and lymphoid tissue counterparts are infected to the same extent\textsuperscript{20,31}, thereby indicating that studying HIV-1 infection in blood is a good representation of events that occur in other tissues.

The more pronounced changes in diversity of cell-free over cell-associated virus can be explained by the difference in half-life severely reducing serum copy numbers\textsuperscript{29}. Although virus diversity in serum decreased after therapy start, the pairwise distances between time-points before and during therapy increased indicating different genetic characteristics of the virus after therapy start. Virus may be produced by other cell types or stems from compartments less accessible to antiretroviral drugs\textsuperscript{19,32-34}. This is in agreement with studies where rebounding virus is distinct from variants before initiation of therapy\textsuperscript{35,36}. The absence of divergence in the cell subsets, apart from M12020, is explained by a moderate drop in infection levels and smaller changes in diversity. In M12020, the compartmentalization of effector memory-derived virus pre-therapy indicates that in case of dual HIV-1 infection, one strain can preferentially infect a specific CD4\textsuperscript{+} lymphocyte subset. We have previously observed in dual HIV-1 infection that one strain replicates preferentially within different cell types when compared with another strain indicating that the host cell environment influences viral replication\textsuperscript{9,37}. The shift in balance between strains I and II is likely caused by therapy, although differences in host immune pressure, virus fitness as well as high turnover of this cell subset may also play a role. The complete and specific infection of effector memory cells by strain I and fast replenishment with a different virus strain indicate that this cell subset quickly facilitates infection by different variants. Although strain I was not detected in serum during therapy, its presence in long-lived central memory cells at that time-point ensures persistence of both variants. This increases the chances of recombination and therapy resistance raising questions as to the efficacy of antiretroviral therapy in dual infected individuals\textsuperscript{36}. This is in line with the more resistant phenotype of HIV-2 over HIV-1 in dual-infected persons\textsuperscript{39}.

The pronounced increase in the gp120 V3 charge in effector memory cells from three out of four patients reflects increased sensitivity to therapy of virus within this cell subset. It has been speculated that such changes can influence coreceptor usage including a possible switch towards CXCXR4 usage\textsuperscript{25,26,40,41}. Four weeks of therapy restores CCR5 expression levels, which are increased during HIV-1 infection, while CXCXR4 expression levels demonstrate a modest change\textsuperscript{42}.

In conclusion, antiretroviral therapy resulted in a comparable decay of HIV-1 infection levels in naïve and central memory subsets with minor to no changes in the viral quasispecies. HIV-1 copy numbers in the effector memory subset not always decreased and the virus in this cell subset and in serum appeared to be more sensitive to therapy. We also observed variant sensitivity among virus strains in a dual-infected individual.
individual. These results give insight in the viral dynamics within CD4⁺ lymphocyte subsets during early therapy.

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REFERENCE LIST


27. Ince, W.L., Harrington, P.R., Schnell, G.L., Patel-Chhabra, M., Burch, C.L., Menezes, P., Price, R.W., Eron Jr, J.J. and Swanstrom, R.I. (2009). Major coexisting human immunodeficiency virus type 1 env gene subpopulations in the peripheral blood are produced by cells with...


