Virus and host determinants of HIV-1 infection and AIDS pathogenesis
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'In vivo HIV-1 infection of CD45RA+CD4+ T cells is established primarily by SI variants and correlates with the rate of CD4+ T cell decline

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In vivo HIV-1 infection of CD45RA+CD4+ T cells is established primarily by syncytium-inducing variants and correlates with the rate of CD4+ T cell decline.

Switch from non-syncytium-inducing (NSI) to syncytium-inducing (SI) human immunodeficiency virus type 1 (HIV-1) is associated with accelerated CD4+ T cell depletion, which might partially be explained by higher virulence of SI variants compared to NSI variants. Since NSI and SI variants use different coreceptors for entry of target cells, altered tropism might offer an explanation for increased pathogenesis associated with SI HIV-1 infection. To investigate whether SI and NSI HIV-1 variants infect different CD4+ T cell subsets in vivo, the distribution of SI and NSI variants over CD4+ memory (CD45RA-RO) and naive (CD45RA+RO) cells was studied using limiting-dilution cultures. In contrast to NSI variants that were mainly present in CD45RO cells, SI variants were equally distributed over CD45RO* and CD45RA* cells. Infection of memory cells by both NSI and SI HIV-1 and infection of naive cells primarily by SI HIV-1 corresponded closely with the differential cell surface expression of CXCR4 and CCR5. The frequency of SI-infected CD45RA+ CD4+ T cells, but not the frequency of NSI- or SI-infected CD45RO+ CD4+ T cells, correlated with the rate of CD4+ T cell depletion. Infection of naive cells by SI HIV-1 may interfere with CD4+ T cell production and thus account for rapid CD4+ T cell depletion.

In 50% of HIV-1-infected individuals, a switch occurs from the non-syncytium-inducing (NSI) phenotype to the syncytium-inducing (SI) phenotype during the course of disease. The emergence of SI variants is associated with an accelerated CD4+ T cell loss and more rapid progression to AIDS. The enhanced pathogenesis of SI HIV-1 infection may be explained by the more virulent characteristics of SI variants observed in vitro, such as enhanced cytopathicity and higher replication kinetics.

Since SI and NSI variants use different coreceptors for entry of target cells, altered tropism could offer an alternative (or additional) explanation for the increased pathogenicity associated with the presence of SI variants. The vast majority of NSI variants are restricted to the use of CCR5, while SI variants use CXCR4 and may use CCR5 and/or other chemokine receptors in addition. CCR5 and CXCR4 are differentially expressed on different CD4+ T cell subsets: CXCR4 is mainly expressed on cells with a resting/naive phenotype and CCR5 is mainly expressed on activated/memory T cells. Moreover, we recently found that in HIV-1-infected individuals expression of CXCR4 and CCR5 is largely mutually exclusive within both the naive (CD45RO) and memory (CD45RO) CD4+ T cell subset. Given the existence of two major and distinct HIV-1 target cell populations within the CD4+ T lymphocyte compartment, one expressing the NSI-coreceptor and the other expressing the main SI-coreceptor, the total number of potential HIV-1 target cells expands considerably after the emergence of SI variants. This could explain the higher viral load observed in individuals with SI variants compared to individuals with only NSI variants at comparable CD4+ T cell counts, and hence the accelerated disease progression.

Next to the probable impact of an increase in the number of potential target cells, it can be envisaged that the extent to which HIV-1 infection affects the rate of CD4+ T cell decline depends on the type of cells that are infected. Recently it was shown that both naive and memory CD4+ T cells are productively infected with HIV-1 in vivo. Since naive cells contribute exponentially to the memory CD4+ T cell pool, HIV-1-induced loss of naive cells is likely to have a large impact on CD4+ T cell replenishment and would consequently contribute significantly to CD4+ T cell depletion. Here we tested the hypothesis that specifically the conversion to the SI phenotype enables HIV-1 to efficiently infect...
naive CD4* T cells, thereby contributing to the increased pathogenesis associated with the presence of SI HIV-1.

MATERIALS AND METHODS

Subjects. The study group consisted of 18 participants of the Amsterdam Cohort Studies on AIDS in homosexual men (ACS). For 6 of the individuals, a time point was analyzed at which only NSI variants were present (N1-N6), and for 10 individuals a time point was analyzed at which both SI and NSI variants were present (S1-S10). For 2 individuals both a peripheral blood mononuclear cell (PBMC) sample obtained before and one obtained after HIV-1 phenotype switch were analyzed. For the samples obtained before SI conversion, these 2 individuals were included in the NSI group (N7 and N8) and for the time point after phenotype switch, these individuals were included in the SI/NSI group (N7=N11, N8=N12). In the SI/NSI group, the moment of analysis was at least 6 months after SI conversion (median: 23 months; range, 6.7 to 57.1).

Isolation of CD45RA* and CD45RO* CD4* T cells from PBMC. PBMC were isolated from fresh blood and used immediately (N1, N3, N4, N6, S5, S7, S10) or after cryopreservation. PBMC (20 to 30 x 10^6) were stained with monoclonal antibodies (mAbs) directed against CD4 (conjugated with tri-color [TC]), CD45RA (conjugated with phycoerythrin [PE]) and CD45RO (conjugated with fluorescein isothiocyanate [FITC]) (all mAbs from Caltag, FL). CD4* CD45RA*RO* cells and CD4* CD45RA* cells were separated by sorting with a FACStar (Becton Dickinson, CA). CD4* CD45RA*RO* cells and CD4* CD45RA* cells were separated by sorting with a FACStar (Becton Dickinson, San Jose, CA). After cell sorting, contamination with the reciprocal CD4* T cell subset was on average 0.3% (CD4*CD45RO* in CD4*CD45RA*cell fraction) and 0.2% (CD4*CD45RA* in CD4*CD45RA*cell fraction).

CCR5 and CXCR4 expression on CD45RA* CD4* and CD45RO* CD4* cells. CCR5 and CXCR4 expression on the memory and naive CD4* T cell subsets was analyzed by three-color flow cytometry. PBMC samples were obtained from time points maximally 3 months before or after the moment at which the PBMC samples were used for virus isolation were obtained. PBMC (2.5 x 10^6) were stained with a combination of mAbs directed against 1) CD4 (TC; Caltag), CD45RA (2H4-RD1-PE; Coulter, Hialeah, FL) and CCR5 (5C7-FITC; Pharmingen, San Diego, CA); 2) CD4 (TC), CD45RO (UCHL-1-FITC; DAKO, Glostrup, Denmark) and CXCR4 (12G5-PE; Pharmingen); or 3) CD4 (TC), CD45RA (2H4-RD1-PE) and CD45RO (UCHL-1-FITC). The latter staining was performed to identify cell populations single positive for only one of the two CD4 isoforms, which was extrapolated to the CCR5/CD45RA staining and the CXCR4/CD45RO staining. Thus, CCR5 expression was analyzed on the CD45RA* (=CD45RO*hi) and CD45RA*RO* (=CD45RO*) cells and CXCR4 expression was analyzed on the CD45RO* (=CD45RA*hi) and CD45RO* (=CD45RA*) cells (Fig. 3a).

Biological cloning of HIV-1 and determination of virus load. CD45RA*, CD45RO* CD4* T cells, and total PBMC were independently cocultivated with freshly phytohaemagglutinin (PHA)-stimulated healthy donor peripheral blood lymphocytes under limiting diluting conditions, as described previously. Cultures were maintained for 4 weeks and every 7th day culture supernatant was analyzed for virus production in an in-house p24 antigen-capture ELISA. At weeks 2 to 4, SI phenotype of the biological virus clones was determined by analyzing the presence of syncytia after cocultivation with MT2 cells.

The frequency of productively infected cells was calculated with the formula for Poisson distribution: F(= -ln(Fo)), in which Fo is the fraction of negative cultures, and expressed as tissue culture infectious dose (TCID50) per 10^6 CD4*, CD45RA*, CD45RO*, and CD45RO* CD4* T cells. The detection limit of the assay depends on the number of patient cells available for coculture and was calculated in cases where no virus clones were obtained. The proportions of biological virus clones with NSI and SI phenotype were used to calculate the NSI-virus load and the SI-virus load.

CD4* T cell decline. Lymphocyte immunophenotyping for CD4* T cells was carried out using flow cytometry at 3-month intervals. The patients were matched for the moment of sampling at which virus load was determined (t=0) and the group mean CD4* T counts were calculated at each 3-monthly time point a year before and a year after t=0 (from t-12 to t=12). These mean CD4* T cell values were used for linear regression analysis. Individual CD4* T cell decline in this period was also analyzed by linear regression analysis.

Participants receiving anti-retroviral combination therapy at t=0 (S5, S7, S9 and S10) were excluded from analysis. From the remaining 8 NSI individuals and 8 SI/NSI individuals, CD4* T cell data obtained prior to seroconversion (time points -12 to -6, Fig. 4a, top) and CD4* T cell data obtained after initiation of anti-retroviral combination therapy (time points 6 to 12, Fig. 4a, top) were excluded from analysis.

Statistical analyses. The Mann-Whitney U Test was used to analyze differences between individuals with only NSI variants and individuals with SI and NSI variants with respect to the CD45RA* load to CD45RO* load ratio. Differences between CD45RA* cells and CD45RO* cells within the group of individuals with SI variants were analyzed with the Wilcoxon signed rank test (i.e., proportion of biological clones with SI phenotype, and SI-load in CD45RA* and CD45RO* cells). CCR5 and CXCR4 expression on CD45RA*RO* and CD45RA*RO* subsets was
analyzed with the paired Student's t Test. Correlations of CD4+ T cell decline with virus load were determined using the Spearman's correlation coefficient ($r_s$).

**RESULTS**

Individuals with SI variants have a relatively high frequency of infected CD45RA+ CD4+ T cells. The frequency of productively infected CD4+ T cells, CD45RA+ CD4+ T cells and CD45RO- CD4+ T cells (expressed per $10^6$ cells of the respective T cell subsets), was determined for 8 individuals at a time point at which only NSI variants were present (N1-N8), and for 12 individuals at a time point at which both SI and NSI variants were present (S1-S12) (Table 1). The total virus load had a broad range and did not differ significantly between both groups (2 to 269 and 5 to 361 TCID/10^6 CD4+ T cells, respectively; $P = 0.3$).

In individuals with only NSI variants, the frequency of infected CD45RA+ cells was 30- to 70-fold lower than the frequency of infected CD45RO- cells (Table 1). This is visualized by the low ratio of CD45RA+-load to CD45RO- load (median = 0.02; Fig. 1). The high ratio observed for individuals with both NSI and SI variants (median = 0.63) reflects a relatively high frequency of infected CD45RA+ cells (9-fold lower to 18-fold higher) compared to the frequency of infected CD45RO- cells (Table 1, Fig. 1). The CD45RA+-load/CD45RO- load ratio differed significantly between the individuals with and without SI variants ($P = 0.001$).

CD45RA+ CD4+ T cells are preferentially infected with SI variants. The relatively high frequencies of infected CD45RA+ cells in individuals with both NSI and SI variants compared to individuals with only NSI HIV-1 (Table 1, Fig. 1), suggested a preferential infection of CD45RA+ cells by SI variants. To verify this, we analyzed the distribution of co-existing NSI and SI HIV-1 in CD45RA+ and CD45RO- cells from the individuals carrying both variants. Within each of the three populations of biological virus clones, obtained by cultivation of total PBMC, CD45RO-, and CD45RA+ cells, the proportion of NSI and SI viruses was calculated (Fig. 2). The distribution patterns of NSI and SI variants in total PBMC and CD45RO- CD4+ T cells were similar, yet differed in the CD45RA+ CD4+ T cells (Fig. 2a-c). Paired
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analysis of CD45RA+ CD4+ T cells and CD45RO+ CD4+ T cells revealed a significantly higher proportion of SI variants in CD45RA+ cells (median 93.7 versus 40.0%; \( P = 0.01 \); Fig. 2d). Thus, while the proportions of SI and NSI variants were similar in CD45RO+ cells, CD45RA+ cells were almost exclusively infected with SI variants, explaining the higher CD45RA+ load in carriers of SI variants compared to individuals with only NSI HIV-1.

Since both CD4+ T cell subsets were infected with SI variants, we calculated the frequencies of CD45RA+ and CD45RO+ cells infected with SI HIV-1. SI variants were equally distributed over CD45RO+ cells and CD45RA+ cells (median SI-load: 60 and 47 TCID/10^6 cells, respectively; \( P = 0.17 \); not shown).

CD45RA+ CD4+ T cells mainly express CXCR4, CD45RO+ CD4+ T cells express CXCR4 and CCR5. To determine whether the differential distribution of HIV-1 variants correlated with the cell surface expression of CCR5 and CXCR4 in the individuals under study, we analyzed CCR5 and CXCR4 expression on CD45RA+ and CD45RO+CD4+ T cells (Fig. 3). In agreement with Bleul et al., CXCR4 was mainly present on CD45RA+ cells (median CXCR4 positive: 95.3%), while CCR5 was mainly present on CD45RA+RO+ cells (median CCR5 positive: 34.8%) (Fig. 3b). More importantly, the proportion of CXCR4-expressing cells was higher than the proportion of CCR5-expressing cells both in the naive (95.3 and 9.1%, respectively; \( P < 0.001 \)) and in the memory subset (44.4 and 34.8%, respectively; \( P = 0.04 \)). These expression patterns of CXCR4 and CCR5 on CD45RA+ and CD45RO+ cells were indeed compatible with the distribution patterns of NSI and SI variants in these T cell subsets.

Rate of CD4+ T cell decline correlates with the SI-load in CD45RA+ CD4+ T cells. To investigate the relation between the virus load in different T cell subsets and CD4+ T cell decline, we first
In patients with SI variants, the rate of CD4⁺ T cell decline in this 2-year period correlated with the SI-load in CD45RA⁺ CD4⁺ T cells \( (r_s = 0.79, P = 0.04) \) and with the ratio of CD45RA⁺-load to CD45RO⁺-load \( (r_s = 0.75, P = 0.05) \) analyzed at \( t=0 \) (Fig. 4b). In contrast, no correlation was observed between CD4⁺ T cell decline and the SI-load in CD45RO⁺ cells \( (r_s = 0.36, P = 0.4; \text{not shown}) \), or total PBMC \( (r_s = 0.18, P = 0.7; \text{not shown}) \). The CD4⁺ T cell decline in patients with only NSI variants did not correlate with the virus load in any of the cell types (data not shown).

**DISCUSSION**

Although depletion of CD4⁺ T cells is one of the main features of HIV-1 infection, the mechanisms underlying this depletion are still not fully understood. Destruction of cells might occur through virus-mediated killing of infected cells and activation-induced apoptosis of uninfected cells (reviewed in reference 501). Although recent studies have shown that in HIV-1-infected individuals CD4⁺ T cell turn-over is maximally 2-to 3-fold increased\(^{358,359}\), limited CD4⁺ T cell renewal capacity in adult individuals\(^{136,137}\) might further contribute to the depletion of CD4⁺ T cells. In HIV-1-infected individuals the effect of natural limitations on T cell production may further be enhanced by virus induced impairment of the development of progenitor cells\(^{389,390}\). Additionally, the decrease in CD4⁺ T cells measured in peripheral blood might not be completely explained by actual depletion, but partly by increased trapping of cells in the lymphnodes during HIV-1 infection\(^{370,371}\).

Until recently, the accelerated loss of CD4⁺ T cells associated with the presence of SI variants\(^{28,202,429}\) was attributed to the higher in vitro cytopathicity of SI variants compared to NSI variants\(^{90}\). However, recently it was shown that NSI variants can be equally cytopathic as SI variants and that enhanced pathogenicity of SI variants can alternatively be explained by different tropism resulting in an increased target cell population\(^{498}\).

In the present study we showed that SI HIV-1-infected-individuals carry both NSI and SI variants in their CD45RO⁺ (memory) CD4⁺ T cells.
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Fig. 4. CD4* T cell decline and virus load, (a) For the 2-year period spanning the moment of virus isolation and virus load determination (t=0), the average CD4* T cell counts at each 3-monthly interval were calculated for individuals with NSI variants only (△) and individuals with both SI and NSI variants (○). At the top of the figure the number of included patients is shown. The linear regression coefficients (r) are depicted. Bars indicate the standard errors of the means, (b) For the SI-carrying individuals, the individual CD4* T cell slopes in the same period of 2 years were calculated and correlated with the relative SI-virus load (left) and the absolute SI-load (right) in the CD45RA* cells. The Spearman's correlation coefficients (r_s) and the p-values are shown.

However, while the CD4* memory cells appeared to be the main T lymphocyte target cell population of NSI variants, SI variants were additionally detected in the CD45RA* (mainly naive) CD4* T cells. On average, the SI-virus load was similar in CD45RA* and CD45RO* CD4* T cells. The partly differential distribution of SI and NSI variants in the two CD4* T cell subsets agreed with the almost exclusive presence of CXCR4-expressing cells in the CD45RA* T cell subset, and the presence of both CCR5 and CXCR4-expressing cells in the CD45RO* T cell subset.

The frequency of CD45RA* cells, but not the frequency of CD45RO* cells, infected with SI HIV-1 correlated with the CD4* T cell decline measured over a 2-year period encompassing the moment of viral load analysis. This suggests that not merely the expansion of the total HIV-1 target cell population, but specifically SI infection of the (largely) naive CD45RA* CD4* T cell subset, contributes to the accelerated CD4* T cell decline in individuals harboring SI variants. Since naive cells contribute exponentially to the memory cell pool and given the intrinsically slow generation of naive cells in adults[397,502], killing of naive cells by SI HIV-1 may have a relatively large impact on CD4* T cell renewal. Interference with CD4* T cell renewal may in its turn explain the accelerated CD4* T cell loss associated with the presence of SI HIV-1.

Although especially in HIV-1-infected individuals not all CD45RA* CD4* T cells are naive cells[395], Ostrowski et al. recently demonstrated that truly naive (CD45RA*CD62L+) CD4* T cells are HIV-1-infected in vivo, especially in individuals harboring CXCR4-using variants[503]. Though in the present study we cannot exclude that non-naive cells are among the infected CD45RA* cells, the truly naive cells are more likely to represent the major HIV-1-infected cells in the CD45RA* population for several reasons. First, non-naive CD45RA* CD4* T cells are considered to belong to the memory compartment. In agreement we recently observed that the frequency of CCR5-expressing cells among the non-naive CD45RA* CD4* T cells was similar to that of resting CD45RO* CD4* T cells, and intermediate to that of truly naive CD4* T cells and activated CD45RO* CD4* T cells[503] (HB, unpublished observations).

The low frequency of NSI variants in the CD45RA* cell subset would be difficult to explain if, within the CD45RA* CD4* T cells, the non-naive cells represent the major population of HIV-1 carrying cells. Furthermore, in most individuals studied here, we observed a low frequency of CCR5 and a high frequency of CXCR4-expressing cells in the CD45RA* cell population. This expression pattern is consistent with the assumption that at the moment of analysis, the majority of the CD45RA* cells were naive in most patients.

At this moment it is not clear at what time point during their life cycle peripheral naive cells become infected. If infection occurred when the T cells were already mature and expressing CD45RA, HIV-1 may be present as an incomplete
and labile proviral DNA species\textsuperscript{504}, in analogy to observations in quiescent cells\textsuperscript{65,66,77}. On the other hand, the existence of proliferating cells within the naive cell population\textsuperscript{506} may support integration of viral DNA in part of the naive cells. In agreement, Ostrowski et al. demonstrated that integrated proviral DNA is detected in naive cells, yet at lower levels compared to memory cells\textsuperscript{500}. The capacity of SI variants to infect resting CD45RA\textsuperscript{*} cells has been previously observed in vitro\textsuperscript{504,506-508}. In one of these studies, infection of CD45RO\textsuperscript{*} cells was shown to be more efficient than infection of resting CD45RA\textsuperscript{*} cells, as measured by the presence of proviral DNA\textsuperscript{500}. In the other studies however, no differences in viral entry and production of full length reverse transcripts in CD45RA\textsuperscript{*} and CD45RO\textsuperscript{*} cells was observed\textsuperscript{507,508}. In agreement, similar levels of virus production could be induced with PHA-stimulation of infected resting CD45RA\textsuperscript{*} and CD45RO\textsuperscript{*} cells\textsuperscript{504,506-508}.

Alternatively, peripheral HIV-1-infected naive cells may derive from infected thymocytes. Thymocytes can be infected during different stages of maturation\textsuperscript{113,114}. Since mature thymocytes are activated during the process of positive selection\textsuperscript{509,510}, they could theoretically support proviral integration and productive infection. Indeed, mature infected thymocytes are capable of virus production in the presence of cytokines normally present in the thymus\textsuperscript{114}. The existence of stably infected thymocytes, surviving infection during subsequent differentiation processes, is suggested by the existence of productively infected CD3\textsuperscript{+}CD8\textsuperscript{+}CD4\textsuperscript{+}CD8\textsuperscript{+} thymocytes\textsuperscript{511,512}, expressing viral mRNA\textsuperscript{511}. Survival of thymocytes with integrated provirus could thus result in peripheral naive cells with integrated HIV-1 DNA. Thymocytes express CXCR4 during most stages of maturation and only low levels of CCR5\textsuperscript{112-115}, compatible with preferential infection by SI variants. Although conflicting results in this respect have been obtained in vitro\textsuperscript{113,114,512}, SI variants were shown to be more thymocyte-tropic in vivo in a SCID-hu mice model\textsuperscript{118}.

Even though especially in SI-infected individuals naive cells were shown to carry replication competent HIV-1, it is not known whether naive cells support HIV-1 replication in vivo. However, virus production may be supported by low levels of residual proliferation or induced upon antigen induced stimulation, either way resulting in an increased death of naive cells. Since increased killing of CD45RA\textsuperscript{*} cells may have a large impact, even when relatively low numbers of cells are infected, the capability of SI HIV-1 to infect CD45RA\textsuperscript{*} cells next to CD45RO\textsuperscript{*} cells may represent the crucial difference between SI and NSI variants with respect to their pathogenicity.

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