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# Replicative fitness of CCR5-using and CXCR4-using human immunodeficiency virus type 1 biological clones

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**Abstract**

CCR5-tropic viruses cause the vast majority of new HIV-1 infections while about half of the individuals infected with HIV-1 manifest a co-receptor switch (CCR5 (R5) to CXCR4 (X4)) prior to accelerated disease progression. The underlying biological mechanisms of X4 outgrowth in AIDS patients are still poorly understood. Although X4 viruses have been associated with increased "virulence" in vivo, in vitro replication and cytopathicity studies of X4 and R5 viruses have led to conflicting conclusions. We studied the replicative fitness of HIV-1 biological clones with different co-receptor tropism, isolated from four AIDS patients. On average, R5 and X4 clones replicated equally well in mitogen-activated T cells. In contrast, X4 variants were transferred more efficiently from dendritic cells to autologous $CD4^+$ T cells. These observations suggest that interaction between X4 viruses, DC and T cells might contribute to the preferential outgrowth of X4 viruses in AIDS patients.


*Keywords:* HIV-1; fitness; Replication capacity; CXCR4; CCR5; Dendritic cell; $CD4^+$ T cell; Transmission; AIDS

## Introduction

Infection of target cells by HIV-1 is mediated through high affinity binding of the viral envelope to the human CD4 receptor and an additional co-receptor of the human chemokine receptor family (CCR5 or CXCR4). Co-receptor use is correlated with the ability of an isolate to induce syncytia in the MT2 T-cell line (Asjo et al., 1986). Non-syncytium-inducing (NSI) viruses use CCR5 (R5) and syncytium-inducing (SI) variants use CXCR4 (X4) as entry co-receptor, while dual tropic isolates can use both CCR5 and CXCR4 (R5X4) (Koning et al., 2002; Philpott, 2003). Differential co-receptor usage (NSI/R5 and SI/X4) has been described for all different HIV-1 clades (Zhang et al., 1996), although the occurrence of SI/X4 variants in clade C viruses is rare (Abebe et al., 1999; Peeters et al., 1999). It is understood that these phenotypic properties are determined mainly by mutations in the V2–V3 region of the HIV-1 *env* gene (Koning et al., 2002).

In vivo studies indicate that the vast majority of transmitted HIV-1 is macrophage tropic, CCR5-using and non-syncytium inducing (NSI/R5). (Asjo et al., 1986; van 't Wout et al., 1994; Zhu et al., 1993). Subjects with the CCR5Δ32 deletion lack a functional CCR5 receptor and are resistant to HIV infection, although in rare cases infection with SI/X4 virus has been observed (Berger et al., 1999). Over the course of infection, the co-receptor preference of HIV-1 changes from CCR5 to CXCR4 in approximately 50% of infected individuals and usually precedes an accelerated $CD4^+$ T cell decline and consequently disease progression (Tersmette et al., 1989; Koot et al., 1993). Because only a limited number of mutations are required for this phenotypic switch (Shimizu et al., 1999; Verrier et al., 1999), it could be expected that X4 variants evolve on multiple occasions throughout infection. HIV-1 biological or phenotypic properties such as co-receptor usage, cell tropism, cytopathicity, and replication kinetics have been extensively studied in vitro (Schuitemaker et al., 1991, 1992; van 't Wout et al., 1998; Grivel and Margolis, 1999; van Rij et al., 2000; Kreisberg et al., 2001). However, the utilization of diverse cellular systems and assays to measure phenotypic

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properties of R5 and X4 HIV-1 has led to substantial confusion in current literature and the true biological mechanisms underlying the "phenotypic switch" during HIV-1 infection are still poorly understood.

In recent years, studies on HIV-1 replicative fitness have led to new insights in the field of HIV/AIDS research (Quinones-Mateu and Arts, 2001). Thus far, most research on HIV-1 fitness has established that primary drug resistance mutations reduce replication capacity (Harrigan et al., 1998; Martinez-Picado et al., 1999), while secondary or compensatory mutations could increase fitness (Nijhuis et al., 1999). More recent work has studied the impact of HIV-1 replicative fitness on disease progression (Blaak et al., 1998; Quinones-Mateu et al., 2000; Troyer et al., 2005, Ariën et al., 2005a), on the evolution of the pandemic (Ball et al., 2003; Ariën et al., 2005a, 2005b), and has also shown that entry/fusion is an important determinant of HIV-1 fitness (Ball et al., 2003; Rangel et al., 2003; Marozsan et al., 2005). We have recently defined two parameters of ex vivo fitness: (1) 'pathogenic fitness' which measures the relative HIV-1 replicative fitness in primary human T-cells, i.e., the major target cells during chronic HIV-1 infection, and (2) 'transmission fitness' which measures the efficiency of viral transfer from dendritic cells (i.e., crucial targets during sexual HIV-1 transmission) to T-cells (Blauvelt et al., 1997; Vanham et al., 2000a, 2000b; Ball et al., 2003; Sivard et al., 2004; Ariën et al., 2005a, 2005b).

In this study, we have investigated the replicative fitness of SI/X4 and NSI/R5 HIV-1 biological clones, isolated from AIDS patients. In addition, we also determined the replication capacity of SI/X4 and NSI/R5 variants from the same viral quasispecies, i.e., within a single patient. We performed viral competition experiments employing human activated T-cells and autologous co-cultures of dendritic cells and $CD4^+$ T-cells.

## Results

### *Characterization of SI/X4 and NSI/R5 HIV-1 biological clones*

HIV-1 biological clones with distinct co-receptor tropism were generated by limiting dilution (as described in detail by Zhong et al., 1995) from each of four AIDS patients ($CD4^+$ cell count typically below 200 cells/μl blood; Fig. 1) and subjected to head-to-head competitions in PHA/IL-2 activated PBMC (i.e., activated T cells) and in autologous co-cultures of dendritic cells and $CD4^+$ T-cells (Fig. 2). Since antiretroviral drug resistance mutations can influence HIV-1 replicative fitness (Harrigan et al., 1998; Nijhuis et al., 1999), we sequenced and analyzed the *Pro-RT* coding regions in the Stanford University HIV drug resistance database. None of the clones displayed any drug resistance mutations (data not shown). In addition, sequences of the *envelope* gp120 coding region were used for subtyping and determination of V3-loop amino acid charge (Fig. 1B). Viruses VI 968 and VI 943 clustered with subtype B reference strains and VI 761 was classified as subtype D. VI 820 clustered with subtype A and CRF02_AG references (Fig. 1A). V3-loop amino acid charges were higher for X4 clones as compared to R5 clones for each isolate, with differences in charge among clones with different tropism varying from 3+ for VI 820 and VI 761, to 3+ and 2+ for VI 943, and to 2+ and 1+ for isolate VI 968 (Fig. 1B).

### *SI/X4 and NSI/R5 HIV-1 have comparable replicative fitness in activated PBMC*

SI/X4 and NSI/R5 HIV-1 clones from four AIDS patients were competed against each other in mitogen-activated T-cells (Fig. 2). When considering the replicative fitness between clones from the same isolate, i.e., intra-isolate fitness (Fig. 3; black bars), it was clear that among VI 943 clones, the VI 943-1 (X4) clone was most fit (winning 6/6 competitions), followed by clone VI 943-4 (R5) (winning 4/6), clone VI 943-2 (X4) (winning 2/6) and finally clone VI 943-3 (R5) (losing all 6 competitions). Among the VI 968 clones, clone VI 968-1 (X4) was the most fit (winning 6/6 competitions), followed by clones VI 968-4 (X4) and VI 968-2 (R5) (both winning 2/6 competitions) and VI 968-3 (R5) was least fit (losing all 6 competitions). In the case of VI 761 and VI 820 clones, there was only a single intra-isolate competition. The R5 clone of VI 761-1 out competed the X4 clone VI 761-4, while we obtained no intra-isolate results on VI 820 clones, because we failed to distinguish the R5 and X4 clones of this isolate in the heteroduplex tracking assay.

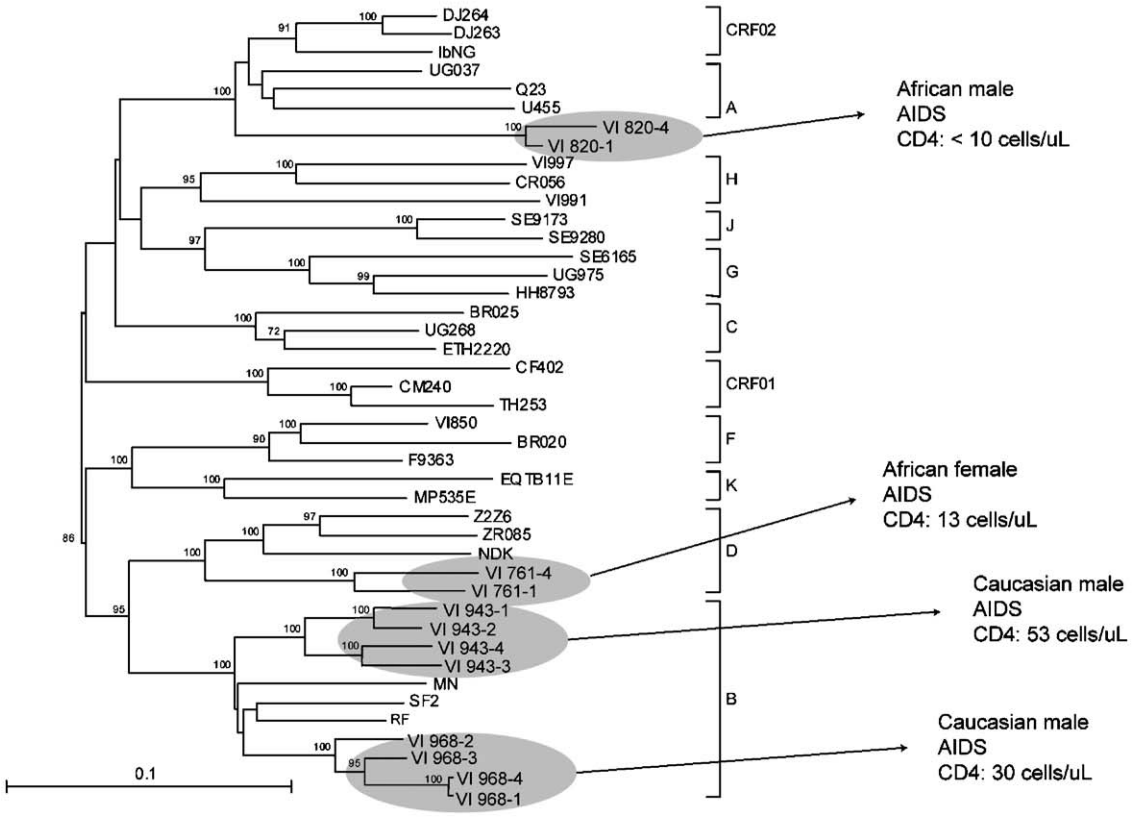
A different picture was obtained in inter-isolate competitions. All VI 943 clones were on average more fit than the R5 and X4 clones from the 3 other isolates in mitogen-activated human T cells (winning 26/29 competitions), despite the clear fitness differences amongst the VI 943 clones in intra-isolate competitions (Fig. 3; gray bars). VI 968 and VI 761 isolates harbored clones of intermediate replicative fitness, while both clones of VI 820 were completely out competed by all clones from other isolates (Fig. 3; gray bars). Clearly, in those cases where biological clones were very fit or very unfit (VI 943 and VI 820, respectively), the outcome of the competition was independent of viral co-receptor use or syncytium-inducing capacity.

### *SI/X4 HIV-1 clones are more efficiently transferred from dendritic cells to T-cells*

As a measure of viral transfer efficiency, we exposed monocyte-derived dendritic cells to mixtures of equal infectious doses of NSI/R5 and SI/X4 biological clones, washed away excess virus and subsequently added autologous $CD4^+$ T cells, which were not pre-activated with PHA. When competing clones derived from the same patient isolate (i.e., intra-isolate) (Fig. 4; black bars), the X4-clones appeared more fit than the R5 clones from VI 943, VI 761 and VI 820. In contrast, X4-clones VI 968-1 and VI 968-4 replicated less efficiently in $CD4^+$ T-cells after transfer from DCs (winning only 2/6 competitions), compared to the R5-clones VI 968-2 and VI 968-3 (winning 4/6 competitions).

When competing isolates from different AIDS patients against each other (i.e., inter-isolate) (Fig. 4; gray bars), all X4

**A.** Phylogenetic tree (*Env*) and virus characteristics




**B.** Amino acid sequence of V3 loop region of HIV-1 biological clones

| Clone N° | Subtype | V3 loop Sequence 10 | 20 | 30 | Phenotype | Charge |
|---|---|---|---|---|---|---|
| VI820-1 | CRF02 | C I R P G N N T R Q **S** V R I G P G Q A F F A T G **D** I I G D I R K A F C | | | NSI/R5 | 3+ |
| VI820-4 | CRF02 | C I R P G I N T R Q **G** V R I G P G R A F F I R N **N** I I G D I R K A F C | | | SI/X4 | 6+ |
| VI943-3 | B | C T R P S N N T R K **G** I H I G P G R A F Y A T G **E** I I G D I R Q A H C | | | NSI/R5 | 5+ |
| VI943-4 | B | C T R P S N N T R K **G** I H I G P G R A F Y A T E **E** I T G D I R Q A H C | | | NSI/R5 | 4+ |
| VI943-1 | B | C T R P N N Y T R K **G** I R I G P G R A V Y A A E **K** I V G N I R Q A H C | | | SI/X4 | 7+ |
| VI943-2 | B | C T R P N N Y T R K **G** I R I G P G R A V Y A A E **K** I V G N I R Q A H C | | | SI/X4 | 7+ |
| VI968-2 | B | C T R P N N N T R R **S** I H I G P G R A F Y T R - **D** I I G N I R Q A H C | | | NSI/R5 | 7+ |
| VI968-3 | B | C I R P N N N T R R **S** I H I G P G R A F Y T T - **D** I I G N I R Q A H C | | | NSI/R5 | 6+ |
| VI968-1 | B | C T R P H N T I K R **R** I H I G P G R A F Y T T K **G** I Q G D L R Q A H C | | | SI/X4 | 8+ |
| VI968-4 | B | C T R P H N T I K R **R** I H I G P G R A F Y T T K **G** I Q G D L R Q A H C | | | SI/X4 | 8+ |
| VI761-1 | D | C T R A S N N T R Q **S** V R I G P G Q A Y F T T - **E** I I G N I R R A H C | | | NSI/R5 | 5+ |
| VI761-4 | D | C I R P F Y K T R K **S** V R V G L G R A Y L T E - **R** I R G D I R Q A H C | | | SI/X4 | 8+ |

Fig. 1. Virus characteristics. Twelve HIV-1 biological clones with distinct bio-phenotype, from four AIDS patients, were selected. VI 761 and VI 820 originate from African patients. *Env* gp120 (partial) (Acc. No.: AM156911–AM156922) and *Pro-RT* were sequenced for subtyping and drug resistance analysis. (A) Phylogenetic tree. Sequences determined in this study are in bold. A total of 1000 bootstrap samples were analyzed. Bootstrap values are given in percentages at the internodes if they exceed the 70% level. The distance between two sequences is obtained by summing the lengths of the connecting branches by using the scale provided. The tree is rooted arbitrarily. (B) V3-loop amino acid alignment. Positive V3-loop charge of amino acids in positions 11 and 25 (gray boxes) correlate with SI/X4 phenotype for VI 943, VI 968, and VI 761. Viruses able to use the CXCR4 co-receptor have higher net V3-loop charges as compared to viruses that use CCR5. Arginine (R), lysine (K), and histidine (H) are positively charged, while aspartic acid (D) and glutamic acid (E) are negatively charged.

variants appeared to be more fit than the R5 clones (Figs. 4 and 5). Nevertheless, VI 968, VI 761 and VI 820 R5-clones were able to compete to some extent with the X4 clones, while that was much less the case for VI 943. The R5-clones VI 943-3 and VI 943-4 lost all competitions against the other clones, both X4 and R5, from AIDS patients (Fig. 4; gray bars).

In summary, we found that HIV-1 biological clones of distinct bio-phenotype and from different AIDS patients do not display significant differences in replicative fitness in direct competitions in activated PBMC (Fig. 5A, $P > 0.05$, $t$ test). In contrast, we showed that DCs can transfer both R5- and X4-using HIV-1 variants to T-cells but that X4 HIV-1 variants

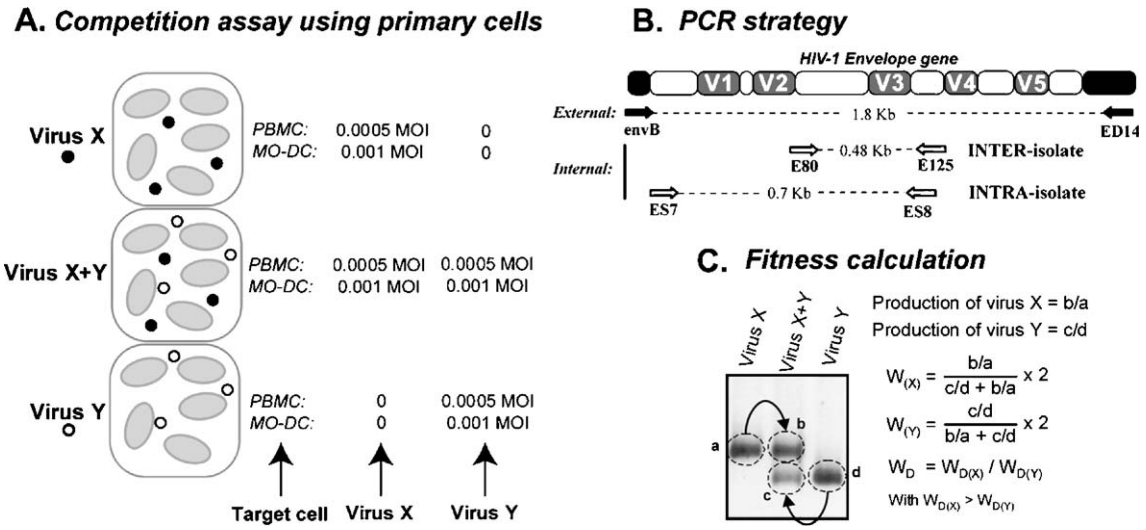



Fig. 2. Graphic representation of the experimental set up. (A) Dual infection/competition assay to measure relative viral fitness in diverse human cell types. Primary human cell cultures are infected with two viruses at equal multiplicity of infection (MOI), and monoinfections correspond to positive controls. Excess virus is washed away, culture medium is replaced every 3 days, and virus production is measured by p24 antigen detection. (B) Cells are harvested at peak p24 production, DNA is extracted and part of the HIV-1 *env*gene is PCR amplified. Primers to PCR amplify competitions between clones from different patients (inter-competitions) are spanning the C2V3-region, while a slightly larger region was amplified for competitions between clones of the same patients (intra-competitions). (C) Finally, heteroduplex tracking assays (HTAs) employing radiolabeled probes were performed to quantify the amount of each virus after competition. Relative fitness values ($W$) and a fitness ratio ($W_D$) were derived as shown.

seem to be more efficiently transferred from DCs to $CD4^+$ T-cells compared to R5 clones from the same or different HIV-1 quasispecies (Fig. 5B, $P < 0.05$, $t$ test).

*Entry as a determinant of HIV-1 fitness: impact of V3-loop charge?*

Previous studies have reported that HIV-1 replicative fitness is primarily determined by the efficiency of primary virus isolates to fuse to and enter host target cells (Ball et al., 2003; Rangel et al., 2003; Marozsan et al., 2005). We found that the V3-loop amino acid charge of X4 clones of VI 820, VI 761, and VI 943 is on average 2.75+ higher then that of R5 clones. In contrast, the difference in V3-loop charge of VI 968 R5 and X4 clones is only 1.5+ on average (Fig. 1B). The R5 clones of VI 968 showed slightly higher V3-loop amino acid charges (VI 968-2; 7+ and VI 968-3; 6+) compared to the other R5 clones (VI 820-1; 3+, VI 761-1; 5+, VI 943-3; 5+, VI 943-4; 4+), and were in the range of V3-loop charges observed for X4 HIV-1 clones (VI 820-4; 6+, VI 761-4; 8+, VI 943-1; 7+, VI 943-2; 7+, VI 968-1; 8+, VI 968-4; 8+) (Fig. 1B). Interestingly, VI 968 R5 clones also appeared to replicate more efficiently in DC/T cell co-cultures compared to R5 variants of other HIV-1 isolates. We speculate that the increased V3-loop amino acid charge of VI 968 R5 viruses may contribute to this fitness-advantage.

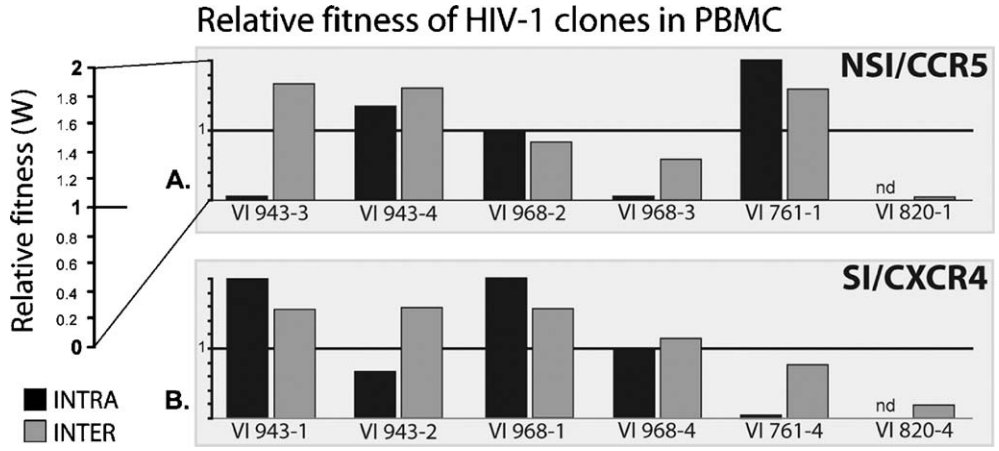



Fig. 3. Relative fitness of HIV-1 clones in human peripheral blood mononuclear cells (PBMC). Panel A shows the mean relative fitness of NSI/R5 clones of four AIDS patients (VI 943, VI 968, VI 761, and VI 820), while panel B shows the mean relative fitness of SI/X4 clones. Black bars represent the mean relative fitness of one virus clone against all other clones from the same HIV-1 isolate (i.e., intra-isolate). Gray bars represent the mean relative fitness of one clone against all clones from the other HIV-1 isolates (i.e., inter-isolate).

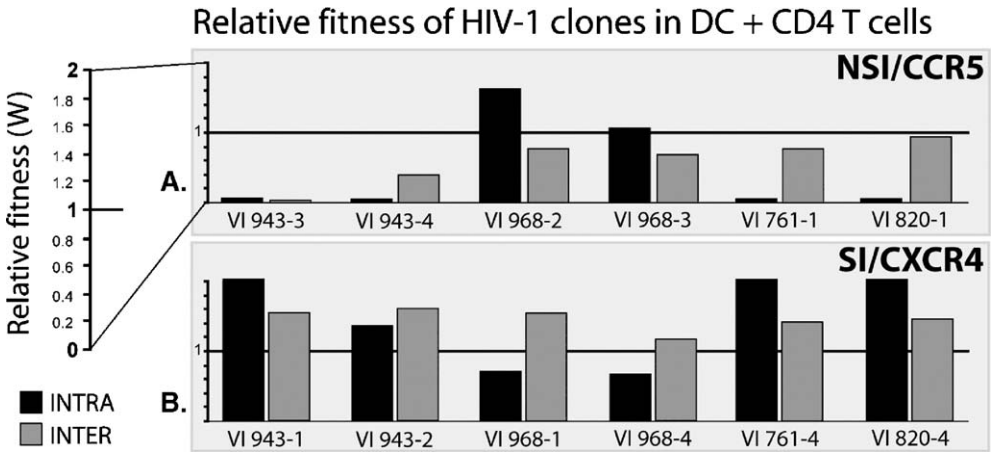



Fig. 4. Relative fitness of HIV-1 clones in co-cultures of human dendritic cells and autologous CD4$^+$ T cells. The mean relative fitness of NSI/R5 and SI/X4 clones from AIDS patients is shown in panels A and B, respectively. Black bars represent the mean relative fitness of one virus clone against all other clones from the same HIV-1 isolate (i.e., intra-isolate). Gray bars represent the mean relative fitness of one clone against all clones from the other HIV-1 isolates (i.e., inter-isolate).

*Differential co-receptor expression on target cells may skew to SI/X4 dominance*

We have analyzed the expression of CCR5 and CXCR4 co-receptors on gated CD4$^+$ T cells (before and after PHA/IL-2 stimulation of PBMC), dendritic cells, and purified CD4$^+$ T cells (before and after 3 days of co-cultivation with DC) from three different HIV seronegative blood donors, by indirect immunofluorescent staining and flow cytometry (Fig. 6). We found that CXCR4 expression is higher than that of CCR5 both on non-stimulated and PHA/IL-2 activated PBMC (Fig. 6). PHA/IL-2 stimulation of PBMC results in up regulation of CCR5 and down regulation of CXCR4, although the relative expression of CXCR4 remains higher than that of CCR5 even after activation with PHA and IL-2. As reported previously (Vanham et al., 2000a, 2000b), the relative expression of CCR5 (Mean fluorescence intensity (MFI) = 81.3) on DC is higher than that of CXCR4 (MFI = 16.5), while the opposite is seen for purified CD4$^+$ T cells (Fig. 6B). Interestingly, we found that physiologic activation of CD4$^+$ T cells by co-cultivation with autologous DC, significantly up regulates the expression of CXCR4 (MFI from 67.0 to 1008.1) and down regulates that of CCR5 (MFI from 10.3 to 5.6) on the CD4$^+$ T cell population (Fig. 6B). Clearly, the ratio of relative CXCR4/CCR5 expression is much higher in DC-activated CD4$^+$ T cells (i.e., 180.0 in donor 3) as compared to PHA-activated CD4$^+$ T cells (5.4 in donor 1 and 11.2 in donor 2). In conclusion, mitogen-activated PBMC and especially physiologically (i.e., by DC) activated CD4$^+$ T cells have higher CXCR4 expression relative to CCR5, and may therefore be more permissive for SI/X4 HIV-1 variants.

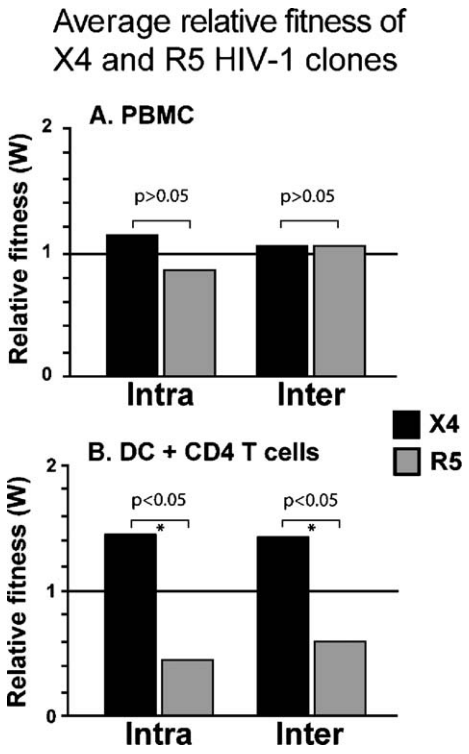
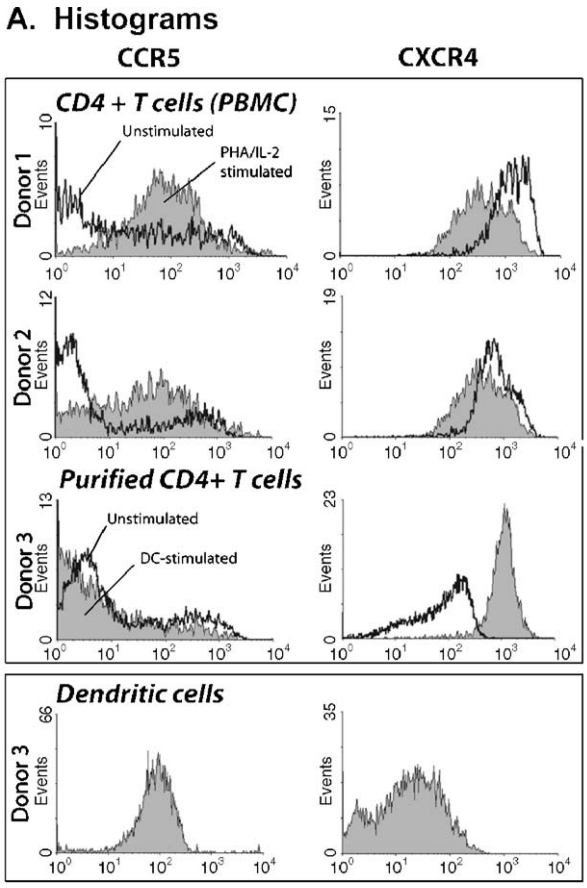



Fig. 5. Average relative fitness of X4 and R5 HIV-1 biological clones. Panel A depicts results from virus competitions in PBMC, while panel B shows results in DC and CD4$^+$ T cells. Direct competitions between diverse HIV-1 isolates in PBMC show that the average fitness is close to 1 (i.e., no difference), irrespective of the co-receptor use. SI/X4 clones appear to be slightly more fit than NSI/CCR5 clones from the same isolate, although not significantly ($P > 0.05$, $t$ test). In co-cultures of DC and T cells, the SI/X4 variants appear to be more fit then NSI/R5 clones from the same or another isolate ($P < 0.05$, $t$ test).

## Discussion

In this study we show that AIDS patients may harbor HIV-1 viruses with different replicative fitness in human activated T cells, regardless the viral co-receptor preference. When the efficiency of virus transfer between DC and naïve CD4$^+$ T cells was assessed, we found that X4 clones are more efficiently transferred to T cells than R5 variants. This latter observation is rather counterintuitive, because it is known that CCR5-using viruses are causing the majority of new HIV-1 infections and dominate during the early stages of infection. Immunofluorescent staining of various HIV-1 target cells (i.e., PBMC, DC, CD4$^+$ T cells) revealed that CCR5 and CXCR4 are differentially expressed on the cell surface and may have




B. Mean fluorescence intensity (MFI)

| | Donor 1 | | Donor 2 | |
|---|---|---|---|---|
| | *CCR5* | *CXCR4* | *CCR5* | *CXCR4* |
| CD4+ T cells (PBMC) | 51.4 | 1205.4 | 6.08 | 679.4 |
| PHA/IL-2 CD4+ T cells | 72.3 | 388.1 | 35.3 | 394.5 |

| | Donor 3 | |
|---|---|---|
| | *CCR5* | *CXCR4* |
| Purified CD4+ T cells | 10.3 | 67.0 |
| DC-stimulated CD4+ T cells | 5.6 | 1008.1 |
| DC alone | 81.3 | 16.5 |

Fig. 6. CCR5 and CXCR4 co-receptor expression on the cell surface of PBMC, DC and $CD4^+$ T cells. Flow cytometry histograms (A) showing CCR5 and CXCR4 expression on gated $CD4^+$ T cells within PBMC (before and after stimulation with PHA and IL-2), DC and $CD4^+$ T cells (before and after physiologic stimulation by DC). Panel B summarizes mean fluorescence intensity (MFI) values for CCR5 and CXCR4 surface expression.

significant impact on the preferential outgrowth of SI/X4 variants.

It was generally accepted that macrophages and dendritic cells are almost exclusively permissive for NSI/R5 strains and there is substantial debate on whether macrophages and DCs can sustain productive viral replication (Gendelman et al., 1988; Schuitemaker et al., 1991; Schmidtmayerova et al., 1998, Nobile et al., 2005). Tissue macrophages and also dendritic cells in the mucosa of the reproductive organs are regarded as selective portals of entry and constitute long-term viral reservoirs (Orenstein et al., 1997). However, it was recently reported that macrophages in human tonsil blocks (Jayakumar et al., 2005) and mucosal dendritic cells (Hlakdik et al., 1999; Prakash et al., 2004, Nobile et al., 2005) could be productively infected with CXCR4-using HIV-1 strains ex vivo. Although we previously showed that productive infection of MO-DC is largely restricted to R5 strains and clones (Vanham et al., 2000a, 2000b), our present observations clearly demonstrate that both R5 and X4 clones can be transferred from DCs to autologous $CD4^+$ T-cells, and that the transfer of X4 variants appears to be even more efficient than that of R5 viruses. It has been shown that DC-SIGN, a C-type lectin expressed on MO-DC (and their physiological counterparts in the mucosa) can capture and transfer both R5 and X4 viruses to $CD4^+$ T cells. During this process DC are not necessarily productively infected, whereas the $CD4^+$ T cells clearly are (Geijtenbeek et al., 2000; Hlakdik et al., 1999).

If X4 viruses can be successfully transmitted/transferred, why do they only appear in approximately 50% of HIV-1 infected individuals and usually only at later stages during disease progression? Mucosal epithelial cells are known to naturally secrete high levels of SDF-1α, the natural ligand of CXCR4, and as a result, CXCR4 expression on mucosal T-cells may be down regulated, rendering these cells less permissive for X4 strains (Agace et al., 2000). However, R5 strains also predominate in the early phases of parenteral infection (e.g., intravenous drug use), suggesting that other X4-restricting barriers or R5-enhancing factors must exist (Cornelissen et al., 1995; Harouse et al., 1999).

It is likely that R5 and X4 variants target different cell populations upon infection (van Rij et al., 2000). Previous studies reported that X4 strains preferentially target 'memory' $CD4^+$ T cells, while R5 viruses may infect a subset of activated mucosal T cells (Berger et al., 1999; van Rij et al., 2000, Mattapallil et al., 2005; Li et al., 2005). Other studies using human cervical tissue and monkey models have shown that both activated and resting $CD4^+$ T cells and not DC or LC are the first cells infected by HIV-1 during transmission (Zhang et al., 1999; Gupta et al., 2002). Our findings suggest that CXCR4 is significantly up regulated by direct physiologic stimulation from DC, as such advantaging the transfer of X4-tropic HIV-1 variants. Interestingly, this observation suggests that DC may play a less prominent role in transmission than generally thought, and that local mucosal T cells (CCR5 rich) could constitute the selective portal of entry.

In addition, different anatomical compartments have different expression levels of CXCR4 and CCR5. There is increasing evidence for a central role of the gut-associated lymphoid tissue (GALT) in the replication of HIV-1 in vivo (Veazey et al., 2001; Moore et al., 2004; Mattapallil et al., 2005; Li et al., 2005). The GALT contains over half of the human body's total T-lymphocytes, and these cells tend to be more activated than T cells in the peripheral blood. The majority of GALT $CD4^+$ T cells expresses CCR5 and has inherently low CXCR4 expression (Veazey et al., 2000; Poles et al., 2001). Another important site of viral replication is the thymus, the major organ of naïve T cell production, where 60–70% of the T cells express CXCR4 while CCR5 expression is marginally low (<5% of T cells) (Zamarchi et

al., 2002). The selective destruction of GALT 'memory' $CD4^+$ T cells during primary HIV and SIV infection is a continual but dynamic event, with the body making a valiant effort to replace these cells (Mattapallil et al., 2005; Li et al., 2005). It could be hypothesized that X4 variants may start to dominate when the number of susceptible GALT-lymphocytes has dropped below a critical level necessary for sustained R5 HIV-1 replication. Dendritic cells residing in the mucosa are known to continuously traffic from the peripheral tissues to the lymph nodes where they extensively interact with T cells, which than re-circulate to blood and tissues. This physiological process may play an important role in the distribution of the R5-variants, but equally well in the outgrowth of late-stage X4-variants.

In conclusion, we have shown that HIV-1 clones of both bio-phenotypes are efficiently transferred from DCs to $CD4^+$ T cells, with X4 clones being remarkably superior in this respect. Furthermore, our data suggest that the differential CCR5 and CXCR4 co-receptor expression on HIV target cells may be an important factor to favor X4 selection. Although our in vitro observations do not provide a final answer to the reason for the early dominance of R5 and late occurrence of an R5 to X4 'switch' observed in only a proportion of the patients with disease progression, they may have elucidated one of the mechanisms involved in this important aspect of AIDS pathogenesis.

## Materials and methods

### *Viruses*

Four HIV-1 primary isolates were selected from our patient cohort (ITM, Antwerp, Belgium) and twelve HIV-1 biological clones (6 SI/CXCR4 (X4) and 6 NSI/CCR5 (R5)) were generated by limiting dilution, as described in detail elsewhere (Zhong et al., 1995). All virus isolates were obtained from patients in advanced stage of disease (i.e., $CD4^+$ cells <200 cells/μl, AIDS) (Fig. 1A). Co-receptor tropism and syncytium-inducing capacity of all biological clones were determined on U87.CD4 cells expressing either CCR5 or CXCR4 and on MT2 T-cell lines, respectively. Virus stocks were generated by short-term propagation of each biological clone in HIV-seronegative donor PBMC. Afterwards, co-receptor tropism was checked again using the U87-assay, to ensure that biological clones had not evolved to dual receptor tropism during the propagation process. Subsequently, tissue culture dose for 50% infectivity ($TCID_{50}$) was calculated using the Reed and Muench method (Reed and Muench, 1938). Briefly, each stock of HIV isolates was serially diluted and then plated in 6-fold with $10^5$ PBMC in a 96-well plate. Infectivity in each well was tested using an in-house p24 antigen capture ELISA (Beirnaert et al., 1998).

### *Cells*

Peripheral blood mononuclear cells (PBMC) were obtained from a HIV-seronegative buffy coat by Ficoll-Hypaque density gradient centrifugation. PBMC were stimulated with 2 μg/ml of phytohemagglutinin (PHA) for 3 days and further maintained in RPMI 1640—2 mM L-glutamine medium supplemented with 10% fetal bovine serum (FBS), 10 mM HEPES, 1 ng/ml interleukin-2 (IL-2), 100 U/ml penicillin and 100 μg/ml streptomycin. As described in detail previously (Vanham et al., 2000a, 2000b), monocyte- and lymphocyte enriched fractions were generated by counterflow elutriation of $6 \times 10^8$ PBMC. Monocytes were further purified by sheep erythrocyte rosetting, yielding >95% $CD3^-$ $CD4^+$ monocytes and <0.5% T cells. Addition of IL-4 (20 ng/ml) and GM-CSF (20 ng/ml) to RPMI containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin differentiates monocytes into interstitial-type dendritic cells (MO-DC). Cell cultures were fed every 3 days and immunophenotyped ($CD3^+$/$CD4^+$, $CD1a^+$, $CD13^+$/$CD14^-$, DC-$SIGN^+$) before use in infection experiments on day 7. Frozen lymphocyte fractions were thawed on the day of infection and used to isolate autologous $CD4^+$ T cells.

### *Immunofluorescent staining and flow cytometry*

To assess the expression of CD4, CCR5 and CXCR4 on $CD4^+$ T-cells (before and after PHA/IL-2 stimulation of PBMC), DC and purified $CD4^+$ T cells (before and after co-culture with DC), indirect immunofluorescent staining and flow cytometry were used. Cells were washed once with Dulbecco's phosphate buffered saline (DPBS) (BioWhittaker Cambex BioScience, Belgium), and resuspended in 50 μl DPBS containing 5% human serum (PBS 5% HuS) and, 1 μg purified monoclonal antibody (mAb); anti-CD4, anti-human CCR5 or CXCR4. 1 μg IgG1 and $IgG_{2a}$ were used as isotype matched control mAbs. After 20 min at 4 °C, cells were washed with PBS containing 1% bovine serum albumin (PBS/BSA/azide) and resuspended in 50 μl PBS 5% HuS and 1 μg biotin-labeled goat anti-mouse. After incubating for 20 min at 4 °C, cells were washed once with PBS/BSA/azide and resuspended in 50 μl 5% HuS and 1 μg streptavidin-phycoerythrin (PE). After 20 min at 4 °C, cells were washed with PBS/BSA/azide and resuspended in 10 μl mouse serum. After 10 min at 4 °C, FITC-conjugated mAb to CD4 was added to the reaction. Subsequently, cells were washed with PBS/BSA/azide and fixed with paraformaldehyde, after 20 min at 4 °C. The cells were analyzed on a FACScan (BD, Belgium) to determine the level of surface CD4, CXCR4 and CCR5 expression. The data were analyzed using WinMDI 2.8 software (http://facs.scripps.edu/software.html). mAbs to CD4, CCR5 and CXCR4, biotinylated goat anti-mouse, FITC-conjugated mAb to CD4, and streptavidin-PE conjugate were obtained from BD PharMingen (San Jose, CA). Isotype matched control Abs IgG1 and IgG2a were obtained from Sigma-Aldrich (Saint Louis, Missouri USA).

### *Growth competition assays*

Dual infections/competitions (Quinones-Mateu et al., 2000; Ball et al., 2003; Troyer et al., 2005; Ariën et al., 2005a,

2005b) were performed with twelve biological clones of distinct viral phenotype derived from four HIV-1 primary patient isolates (Fig. 2). All dual infection/competition experiments were performed with PHA/IL-2 activated PBMC from one donor (same donor as for $TCID_{50}$ determination and same blood draw) on 24-well cell culture plates and in duplicate. A sub-set of competitions was repeated in PBMC from one additional donor to exclude host effects. Finally, the same isolates were used in dual infections with MO-DC and autologous 'resting' $CD4^+$ T-cells. Cells were infected with two viruses at equal multiplicity of infection (0.0005 MOI for PBMC and 0.001 MOI for MO-DC). Uninfected cultures were used as HIV-negative controls and mono-infected cell cultures of each virus correspond to positive controls. Virus mixtures were incubated with $2 \times 10^5$ PBMC or $10^5$ MO-DC at 37 °C in 5% $CO_2$ and washed three times with 1× phosphate-buffered saline (PBS) 24 h post infection and then resuspended in complete medium. Subsequently, $3 \times 10^5$ autologous $CD4^+$ T-cells were added to the MO-DC and cultures were washed and fed with complete medium twice a week. Cell-free supernatant was assayed for p24 antigen detection 4 and 7 days post infection. Two aliquots of supernatants and cells were harvested at day 10 after infection and stored at −80 °C for subsequent analysis.

*PCR strategy*

For all dual infected and mono infected cultures, proviral DNA was extracted from lysed PBMC using the QIAamp DNA Blood kit (Qiagen). Isolated viral DNA was PCR amplified using a set of external primers (envB: 5′-AGAAAGAGCA-GAAGACAGTGGCAATGA-3′ and ED14: 5′-TCTTGCCTG-GAGCTGTTTGATGCCCCAGAC-3′). Subsequently, nested PCR amplicons (C2V3-region) were obtained using internal primers E80 (5′-CCAATTCCCATACATTATTGTG-3′) and E125 (5′-CAATTTCTGGGTCCCCTCCTGAGG-3′) for the inter-isolate competitions, while a slightly larger, and in turn more diverse, fragment (V3V5-region) was PCR amplified for the intra-isolate competitions using internal primers ES7 (5′-TGTAAAACGACGGCCAGTCTGTTAAATGGCAGTC-TAGC-3′) and ES8 (5′-CAGGAAACAGCTATGACCCACTT-CTCCAATTGTCCCTCA-3′). Both the external and nested PCR reaction was carried out in a 100-μl reaction mixture under defined cycling conditions (Fig. 2) (as described previously by Quinones-Mateu et al., 2000; Ball et al., 2003; Troyer et al., 2005; Ariën et al., 2005a, 2005b).

*Heteroduplex tracking assay*

Nested PCR products in *env* (C2-V3) were analyzed by heteroduplex tracking assays (HTA) to determine the amount of virus production in the dual infection/competition experiments. Radiolabeled DNA probes were PCR amplified from regions of *env* using the same primer sets described above. For this amplification, one of the nested primers was radiolabeled using T4 polynucleotide kinase and 2 μCi of [γ-$^{32}$P] ATP. Subsequently, radiolabeled PCR-amplified probes were separated on 1% agarose gels and purified using the QIAquick gel extraction kit (Qiagen). The HTA reaction mixtures contained DNA annealing buffer (100 mM NaCl, 10 mM Tris–HCl [pH 7.8], 2 mM EDTA), 10 μl of amplified DNA from the competition culture, and 0.1 pmol of radioactive probe DNA were denatured at 95 °C for 3 min followed by incubation at 37 °C for 5 min and rapid transfer on wet ice to allow re-annealing. The DNA heteroduplexes were resolved on 5% TBE non-denaturing polyacrylamide gels (Bio-Rad) for 1h15 at 200 V. Afterwards, gels were dried for 45 min at 80 °C, exposed on a phosphorimaging screen and scanned with a phosphor imager (Cyclone, PerkinElmer) followed by analysis using the OptiQuant software package from PerkinElmer (Fig. 2) (described in detail elsewhere by Quinones-Mateu et al., 2000; Ball et al., 2003; Troyer et al., 2005; Ariën et al., 2005a, 2005b).

*Estimation of viral fitness*

In our dual infection/competition experiments, the final ratio of two viruses produced in a dual infection was estimated by heteroduplex tracking analysis and compared to the production in monoinfections. Production of individual HIV isolates in a dual infection ($f_o$) was divided by the initial proportion in the inoculum ($i_o$). This is referred to as relative fitness ($W = f_o/i_o$), while the ratio of the relative fitness values of each HIV variant in the competition is a measure of the fitness difference ($W_D$) or ratio between two HIV strains ($W_D = W_M/W_L$), with $W_M$ and $W_L$ corresponding to the relative fitness of the more and less fit virus, respectively (Fig. 2) (Quinones-Mateu et al., 2000).

*Subtyping and sequence analyses*

*Pol* fragments (nt 2280–3518) corresponding to protease amino acid 10 through RT amino acid 323 were PCR amplified using external primers PS1: 5′-TTTTTTAGG-GAAAATTTGGCCTTC-3′ and RTA9: 5′-TAAATTTAG-GAGTCTTTCCCCATA-3′ (1633 nt) and internal primers PS2: 5′-TCCCTCAAATCACTCTTTGGCAAC-3′ and RTA6-subB: 5′-CCATTGGCCTTGCCCCTGCTTCTG-3′ (1310 nt). Nucleotide sequencing of *pol* (nt 2280–3518) and *env* fragments (nt 688–1377) (Acc. No.: AM156911–AM156922) was performed using an ABI 3730 DNA sequencer and ABI PRISM BigDye Terminator cycle sequencing kits by the Genetic Service Facility of the Flanders Interuniversity Institute for Biotechnology (GSF-VIB). For phylogenetic subtyping analyses, nucleotide sequences were aligned using ClustalX and then manually edited for codon alignment. Alignments included a representative set of known-subtype sequences recommended by the Los Alamos National Laboratory HIV Sequence Database. Distance calculation, tree construction, and bootstrap analysis were realized with the software package TREECON (Van de Peer and De Wachter, 1994). Drug resistance analysis was performed using of the algorithms available in the Stanford University Drug Resistance Database (http://hivdb.stanford.edu/).

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