Impact of maraviroc-resistant and low-CCR5-adapted mutations induced by in vitro passage on sensitivity to anti-envelope neutralizing antibodies

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The aim of this study was to generate maraviroc (MVC)-resistant viruses in vitro using a human immunodeficiency virus type 1 subtype B clinical isolate (HIV-1 KP-5) to understand the mechanism(s) of resistance to MVC. To select HIV-1 variants resistant to MVC in vitro, we exposed high-chemokine (C-C motif) receptor 5 (CCR5)-expressing PM1/CCR5 cells to HIV-1 KP-5 followed by serial passage in the presence of MVC. We also passaged HIV-1 KP-5 in PM1 cells, which were low CCR5 expressing to determine low-CCR5-adapted substitutions and compared the Env sequences of the MVC-selected variants. Following 48 passages with MVC (10 μM), HIV-1 KP-5 acquired a resistant phenotype [maximal per cent inhibition (MPI) 24 %], whilst the low-CCR5-adapted variant had low sensitivity to MVC (IC50 ~200 nM), but not reduction of the MPI. The common substitutions observed in both the MVC-selected and low-CCR5-adapted variants were selected from the quasi-species, in V1, V3 and V5. After 14 passages, the MVC-selected variants harboured substitutions around the CCR5 N-terminal-binding site and V3 (V200I, T297I, K305R and M434I). The low-CCR5-adapted infectious clone became sensitive to anti-CD4bs and CD4i mAbs, but not to anti-V3 mAb and autologous plasma IgGs. Conversely, the MVC-selected clone became highly sensitive to the anti-envelope (Env) mAbs tested and the autologous plasma IgGs. These findings suggest that the four MVC-resistant mutations required for entry using MVC-bound CCR5 result in a conformational change of Env that is associated with a phenotype sensitive to anti-Env neutralizing antibodies.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) entry into target cells is triggered by the interaction of the viral envelope glycoproteins (Env) with its receptor CD4 and one or two major coreceptors, chemokine (C-C motif) receptor 5 (CCR5) or chemokine (C-X-C motif) receptor 4 (CXCR4), and culminates in fusion of the viral and cell membranes. Env is organized into trimers on virions, and consists of the gp120 surface and gp41 transmembrane subunits (Wyatt & Sodroski, 1998). The small-molecule CCR5 antagonist maraviroc (MVC) was the first CCR5 inhibitor licensed for clinical use (Gulick et al., 2008). Although MVC and another CCR5 inhibitor, vicriviroc (VCV), can efficiently suppress HIV-1 replication, resistant variants can arise both in vitro and in vivo, and these resistant viruses are adapted to use drug-bound CCR5 for entry (Berro et al., 2009; Kuhmann et al., 2004; Marozsan et al., 2005; Ogert et al., 2009, 2010; Ratcliffe et al., 2013; Roche et al., 2011b; Tilton et al., 2010; Tsibris et al., 2008; Westby et al., 2007; Yuan et al., 2011; Yusa et al., 2005). Current models of gp120 binding to a coreceptor suggest that the crown of the gp120 V3 loop interacts principally with the second extracellular loop region of the coreceptor, whilst the gp120 bridging sheet, which is formed after CD4 binding, and the stem of the V3 loop interact with the N terminus of the coreceptor (Brelot et al., 1999; Cormier & Dragic, 2002; Farzan et al., 1999; Huang et al., 2005). The development of resistance is an important issue for HIV treatment regimens incorporating MVC, as is the case for any antimicrobial agent.
HIV-1 can develop clinical resistance to CCR5 antagonists by two routes. The first pathway is through emergence of pre-existing CXCR4-using viruses (Fätkenheuer et al., 2008; Landovitz et al., 2008; Westby et al., 2006). CCR5 inhibitor evasion can also occur by the accumulation of multiple mutations in gp120 and/or gp41 without a switch in coreceptor usage (Dragic et al., 2000; Maeda et al., 2006, 2008a; Roche et al., 2011b; Tsamis et al., 2003). The resistant pathway is characterized not by shifts in IC50 (a competitive inhibition), but rather by reductions in the maximal per cent inhibition (MPI). Reductions in MPI are due to the resistant virus developing the ability to bind to the antagonist-modified form of CCR5 (Westby et al., 2007). However, one study reported that chimeric clones bearing the N425K mutation in C4 replicated at high MVC concentrations and displayed significant shifts in IC50s, characteristic of resistance to all other antiretroviral drugs, but not MVC (Ratcliff et al., 2013).

Escape mutants to the CCR5 inhibitor, AD101 (SCH-350581), have been found to be more sensitive than the parental isolate to a subset of neutralizing mAbs against V3 and a CD4-induced (CD4i) epitope (Pugach et al., 2007; Berro et al., 2009). To date, however, it is not clear which mutation(s) induced by MVC affect the accessibility of neutralizing mAbs to the epitopes in Env.

Therefore, to determine the resistance mechanisms to MVC, we passaged a primary CCR5-tropic (R5) subtype B isolate in the high-CCR5-expressing T-cell line PM1/CCR5 in the presence of MVC (Figs. S1, available in the online Supplementary Material) and compared the Env sequences of variants with those cultured in the low-CCR5-expressing parental PM1 cell line (Fig. S1). We also investigated the phenotypic change in the MVC-resistant clone against anti-Env antibodies, especially for anti-V3 neutralizing mAbs and autologous plasma IgGs, and compared the results with the low-CCR5-adapted clone to determine the key mutations for accessibility of neutralizing mAbs to the epitopes in Env.

### RESULTS

**Anti-HIV-1 activities of MVC toward laboratory strains and primary HIV-1 isolates**

Initially, we determined the MPI and the IC50 values of MVC against different laboratory-adapted and primary HIV-1 isolates, including both CXCR4-tropic (X4) and R5 viruses. MVC inhibited the laboratory-adapted HIV-1 R5 strains HIV-1Bal and HIV-1JR-FL with MPIs of 98 and 97 %, respectively, but did not inhibit the X4 virus HIV-1HXB or dual-tropic virus HIV-199.6 (MPI <20 %, Table 1). We also tested MVC against 14 R5 primary isolates, including subtypes B, C and G, and the circulating recombinant form CRF08_Bc. MVC effectively inhibited all of these primary isolates at concentrations of 1.2–26 nM (MPI 92–100 %), but did not inhibit three primary X4 isolates (two CRF01_AE and one subtype B) with MPI <20 % (Table 1).

<table>
<thead>
<tr>
<th>Virus</th>
<th>Subtype</th>
<th>IC50* (nM)</th>
<th>MPI (%)</th>
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<tr>
<td><strong>Laboratory adapted</strong></td>
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<tr>
<td>R5</td>
<td>HIV-1Bal</td>
<td>B</td>
<td>26</td>
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<td></td>
<td>HIV-1JR-FL</td>
<td>B</td>
<td>6.9</td>
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<tr>
<td><strong>Dual</strong></td>
<td>HIV-189.6</td>
<td>B</td>
<td>&gt;1000</td>
</tr>
<tr>
<td><strong>X4</strong></td>
<td>HIV-1HXB</td>
<td>B</td>
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<td><strong>Primary</strong></td>
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<tr>
<td>R5</td>
<td>HIV-1KP-5</td>
<td>B</td>
<td>26</td>
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<td>24</td>
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<td>HIV-1KP-6</td>
<td>G</td>
<td>20</td>
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<td>CRF01_AE</td>
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<td>HIV-1KP-20</td>
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*PM1/CCR5 cells (2 × 10⁴) were exposed to 100 TCID₅₀ of each virus and then cultured in the presence of various concentrations of MVC. The IC₅₀ values were determined by the WST-8 assay using a Cell Counting kit-8 on day 7 of culture. All assays were conducted in duplicate or triplicate.

**Selection of MVC-resistant variants**

To select MVC-resistant HIV-1 variants *in vitro*, we exposed PM1/CCR5 cells to HIV-1KP-5, which had the highest IC₅₀ value (26 nM) and lowest MPI (92 %) among the primary isolates tested, and serially passaged the viruses in the presence of increasing concentrations of MVC. As a control, HIV-1KP-5 was passaged under the same conditions without MVC in PM1/CCR5 cells (designated the passage control). Moreover, to compare the differences between the MVC-resistant variant and low-CCR5-expressing-cell-adapted variant, we passaged HIV-1KP-5 in low-CCR5-expressing parental PM1 cells (designated low-CCR5-adapted). The selected virus was initially propagated in the presence of 1 nM MVC and during the course of the selection procedure the concentration of MVC was increased to 10 µM over 48 passages (Fig. 1a).

Resistance to small-molecule CCR5 inhibitors is known to vary according to the cell type used (Anastassopoulou...
et al., 2009; Ogert et al., 2008; Pugach et al., 2007; Westby et al., 2007). To characterize the resistance profiles of the passaged variants, we tested the sensitivities of the three variants and HIV-1 BaL to MVC in phytohaemagglutinin (PHA)-activated PBMCs (Fig. 1b). The MPI of the MVC-resistant variant was lower than the MPIs of the passage control, low-CCR5-adapted variant and HIV-1 BaL (MPI 80.3 versus 92.3, 94.5 and 95.7%, respectively).

The MVC-selected variant became highly resistant to MVC (Fig. 2), with an MPI of 24% at 48 passages. However, the low-CCR5-adapted variant, which was passaged in PM1 cells, became low sensitive to MVC compared with the passage control (IC_{50} 279 versus 26.3 nM), but we did not find a reduction in the MPI.

We also determined the sequential MPIs and IC_{50} values of each passaged variant to MVC (Fig. 2). From passages

![Graph showing selection of MVC-resistant and low-CCR5-adapted virus variants.](image-url)

**Fig. 1.** Selection of MVC-resistant and low-CCR5-adapted virus variants. (a) The selection was carried out in PM1/CCR5 and PM1 cells as described in Methods. (b) Sensitivities of the MVC-selected (48 passages), low-CCR5-adapted (48 passages), passage control (48 passages) variants and HIV-1 BaL (BaL) to MVC as determined by p24 antigen measurement. PHA-activated PBMCs (1×10^6 cells ml^{-1}) were exposed to 100 TCID_{50} of each variant and cultured in the presence or absence of various concentrations of the drug in 96-well microculture plates. The amounts of p24 antigen produced by the cells were determined on day 7. All assays were performed in triplicate.
1 to 14, the MVC-selected and low-CCR5-adapted variants had almost equal IC_{50} values and the MPIs were high. After 16 passages, the IC_{50} values of the MVC-selected variants continued to increase to >10 μM, whilst the MPIs decreased to 24% at 48 passages, especially after 27 passages. The low-CCR5-adapted variants maintained an IC_{50} value of ~200 nM and high MPIs (90–100%) until the end of the experiment (passage 48). Conversely, the passage control variants did not show remarkable changes in their IC_{50} values and MPIs throughout the passages (IC_{50} values of ~20 nM, MPI 95–100%). The low-CCR5-adapted variant was also resistant to two other CCR5 inhibitors, APL and TAK-779 (data not shown).

These findings suggested that the phenotype of the MVC-selected variants under low concentrations of the drug corresponded with that of the low-CCR5-adapted variants until 14 passages; then, under high concentrations, the MVC-selected variants acquired additional mutations for high resistance to the CCR5 inhibitor.

Comparison of the Env region sequences of the MVC-selected and low-CCR5-adapted mutants

To determine the genetic basis of the resistance in the HIV-1_KP.5 variants and compare the substitutions between the MVC-selected and low-CCR5-adapted variants, the Env genes were sequenced (Figs 3, 4 and S2). At 17 passages, all substitutions in both the MVC-selected and low-CCR5-adapted variants were selected from the baseline viruses. Five of these substitutions in gp120, i.e. K8R, C11W (signal peptide), D141N (V1), E321D (V3) and I463T (V5), were observed in both passaged variants. Conversely, at positions 137 (K or E), 148 (Q or K) and 187 (G or D), the amino acids differed between the MVC-selected and low-CCR5-adapted variants. After 16 passages, the MVC-selected variants acquired four additional mutations, i.e. T297I (V3), M434I (C4), V200I (C2) and K305R (V3), which were not observed in the low-CCR5-adapted variants (Figs 2–4 and S2). After acquisition of M434I in C4 (21 passages), the MPI of the MVC-selected variants decreased gradually.

Fig. 2. Susceptibility of passaged variants to MVC. The sensitivity and MPI of each passaged variant to MVC was determined by a multi-round assay using the WST-8 assay as described in Methods. The x-axis shows the passage number, concentration of MVC (μM) and MPI values. The mutations observed in the highly MVC-resistant variants are shown above the graph.
The most important amino acid substitution for the reduction in the MPI might be K305R, because the MPI of the variant cultured without MVC after 48 passages increased by reverting from R to K at position 305 (data not shown). Three additional mutations, i.e. F317W (V3), V84I (C1) and A436T (C4), were observed in the low-CCR5-adapted variants at 17, 21 and 48 passages, respectively. These mutations might be compensatory for viral fitness following culture in the low-CCR5-expressing cells, because the MPIs of the variants with these three mutations did not differ from those of the variants prior to the acquisition of these mutations (>90%)

These findings suggest that under low concentrations of MVC, the variants were selected from the baseline viruses similar to the low-CCR5-adapted variants (IC50 shift and high MPI), whilst under high concentrations of the drug, the selected variants required additional mutations to use drug-bound coreceptors for entry into the target cells.

To compare the two mutation profiles obtained from the MVC-selected and low-CCR5-adapted variants at 48 passages, the crystal structure of gp120 was used (Figs 3 and 4). Comparison of the sequences of the two passaged variants based on the Protein Data Bank (PDB ID: 2B4C) crystal structure of gp120 showed that the MVC-selected variant harboured many substitutions within and around the V3 region, i.e. the CCR5 N-terminal-binding site, compared with the low-CCR5-adapted variant in the three-dimensional (3D) position. In a magnification of the CCR5 N-terminal-binding site (Fig. 4), three of four mutations, i.e. T297I, M434I and V200I, were concentrated around the V3 base and finally K305R appeared in the V3 stem region after 41 passages.

To determine the positions of MVC-selected mutations in the gp120 trimer form, we illustrated the sites of mutations on the structure of the BG505 SOSIP trimer obtained from the PDB (ID: 3J5M) (Lyumkins et al., 2013). Almost all of the MVC-selected mutations occurred at the upper and outer side of the trimer. Several MVC-selected mutations, i.e. V65K, V200I, K305R, M309I, F317L and M434I, lay relatively close to the neighbouring gp120. These findings demonstrated that these mutations may affect trimer formation and expose neutralizing antibody epitopes.

### Susceptibilities of the infectious clones with mutant Env to anti-Env mAbs

In a previous study, a CCR5 inhibitor (AD101)-resistant infectious clone was sensitive to neutralization via V3 and...
CD4i epitopes (Berro et al., 2009). To examine whether our three passaged variants became sensitive to anti-Env mAbs, we constructed three infectious clones with each 48-passaged Env (Fig. 5). The clone with the Env of the MVC-selected variant showed a low MPI (56%) under a high concentration of MVC, which was also seen with the passage control and low-CCR5-adapted clones (Fig. 5a). Using these infectious clones, we tested the susceptibilities to the anti-Env mAbs b12 [anti-CD4 binding site (anti-CD4bs)], 4E9C (anti-CD4i) and KD-247 (anti-V3). As shown in Fig. 5(b), the MVC-selected and low-CCR5-adapted clones showed higher sensitivity to b12 than the passage control clone, with IC50 values of 0.22, 0.31 and 0.86 µM, respectively. The MVC-selected and low-CCR5-adapted clones became highly sensitive to 4E9C compared with the passage control clone (IC50 values of 0.08, 0.41 and >5 µM, respectively) (Fig. 5c). Moreover, the clone with the MVC-selected Env was highly sensitive to anti-V3 mAb KD-247, while the low-CCR5-adapted and passage control clones were not (IC50 values of 0.04, >100 and >100 µM ml⁻¹, respectively) (Fig. 5d).

These findings indicated that the MVC-selected clone with its greater number of mutations might contribute to exposure of neutralizing epitopes for these three mAbs, whilst the low-CCR5-adapted mutations could change the conformation of Env to become sensitive to anti-CD4i and CD4bs mAbs, but not anti-V3 mAb.

**Susceptibilities of the infectious clones with mutant Env to autologous plasma IgGs**

We also examined whether the infectious clones with the passaged Env mutations were neutralized by autologous plasma IgGs. As shown in Fig. 6(a), none of the autologous plasma IgGs could neutralize the passage control clone at concentrations up to 100 µg ml⁻¹. In the low-CCR5-adapted clone, some of the plasma IgGs slightly inhibited the replication of the virus under high concentrations, but did not reach the 50% inhibition level (Fig. 6b). Conversely, all seven plasma IgGs were able to completely neutralize the clone with the MVC-selected Env (IC50 2.6–37 µg ml⁻¹, MPI 79–97%) (Fig. 6c).

These findings show that the MVC-selected clone with the greater number of mutations also might contribute to exposure of neutralizing epitopes for autologous plasma IgGs.
Fig. 5. Sensitivities of infectious clones with the passage control, low-CCR5-adapted and MVC-selected Env mutations to MVC and anti-Env mAbs. The sensitivities of the infectious clones with the passage control (filled symbols), low-CCR5-adapted (filled symbols and dotted lines) and MVC-selected (open symbols) Env mutations to (a) MVC, (b) b12, (c) 4E9C and (d) KD-247 are shown. The sensitivities of each infectious clone to MVC and mAbs were determined by the WST-8 assay as described in Methods. All assays were conducted in duplicate.

Fig. 6. Sensitivities of infectious clones with the passage control, low-CCR5-adapted and MVC-selected Env mutations to autologous plasma IgGs. The sensitivities of the infectious clones with the (a) passage control, (b) low-CCR5-adapted and (c) MVC-selected Env mutations to seven autologous plasma IgGs (KP-5-IgG-1 to KP-5-IgG-7; coloured symbols) and normal human plasma IgG (NH-IgG; black symbols) are shown. The sensitivity of each infectious clone to the plasma IgGs was determined by the WST-8 assay as described in Methods. All assays were conducted in duplicate.
The CCR5 inhibitors, MCV and VCV, are allosteric inhibitors of virus entry, hence resistance to these drugs is evidenced by a reduction in the plateau of virus inhibition curves rather than by increases in IC₅₀ (Dragic et al., 2000; Maeda et al., 2006, 2008a; Roche et al., 2011b; Tsamis et al., 2003). One study reported that resistant mechanisms contribute to the altered recognition of drug-bound CCR5 by an MVC-resistant HIV-1 strain. This study demonstrated very efficient usage of drug-bound CCR5, characterized by increased dependence on the CCR5 N terminus (Tilton et al., 2010). Another report demonstrated a similar yet distinct mechanism of escape from MVC by MVC-resistant Env, with comparatively less efficient usage of drug-bound CCR5 (Roche et al., 2011b). In the absence of the drug, MVC-resistant Env maintains a highly efficient interaction with CCR5, similar to that of MVC-sensitive Env, and displays a relatively modest increase in dependence on the CCR5 N terminus (Roche et al., 2011b). However, in the presence of the drug, MVC-resistant Env interacts much less efficiently with CCR5 and becomes critically dependent on the CCR5 N terminus. In the current study, we induced MVC-resistant HIV-1, which harboured many substitutions within and around the V3 region, i.e. the CCR5 N-terminal-binding site in vitro. In order to determine whether the resistant variant displayed an increased CCR5 N-terminal dependence, we determined the sensitivity of each variant to anti-CCR5 N-terminal mAb, CTC-5. All passaged variants were completely resistant to CTC-5; however, the MVC-selected variant became sensitive to CTC-5 when MVC (1 μM) was added in the assay, as reported previously (Berro et al., 2009). These results suggest that the four mutations associated with the CCR5-binding site in the MVC-selected variant might create an increased dependency on interaction with the CCR5 N terminus.

In this study, we attempted to determine the difference between the MVC-selected and low-CCR5-adapted variants in parallel using an in vitro passage system. Under low concentrations of MVC, the MPI reduction was not observed in either the MVC-selected variant or the low-CCR5-adapted variant, although both passaged variants had common substitutions in the V1, V3 and V5 regions from quasi-species. Compared with the baseline viruses, under high concentrations of MVC, the resistant variants harboured I317F, A322D and I323V substitutions in the V3 loop, whilst the other resistant clone (24-Res) had P308S and Ala inserted at the 313 position in the V3. In our MVC-resistant variant, we found some mutations at the same positions (305, 309, 317 and 321) in the V3 region as those of 17-Res and 24-Res clones (Roche et al., 2011a). It is still not clear whether such mutations around the V3 loop stem region contribute to increased reliance on sulfated tyrosine residues in the CCR5 N terminus without common gp120 resistance mutations. One resistant clone (17-Res) harboured I317F, A322D and I323V substitutions in the V3 loop, whilst the other resistant clone (24-Res) had P308S and Ala inserted at the 313 position in the V3.
not only anti-CD4bs and anti-CD4i mAbs, but also the anti-V3 mAb and autologous plasma IgGs. In preliminary data, we have confirmed the presence of such anti-CD4i and anti-V3 antibodies in plasma samples from the subject from whom HIV-1KP-5 was isolated (unpublished data). In vivo, where potent levels of Env neutralizing antibodies may be present, the MVC-selected variants may become neutralization-sensitive and not survive. For this reason, it is possible that CCR5 inhibitors, such as MVC, suppress HIV replication for long periods, especially in patients with high levels of circulating anti-Env neutralizing antibodies prior to treatment with MVC.

As some of the mutations in the MVC-selected variant are close to the epitope for KD-247, those mutations might influence the sensitivity and/or binding affinity to KD-247. Moreover, the mutations around and within the V3 loop may also affect the association with the V2 loop by opening of the trimer. Our study did not allow us to distinguish this possibility. Thus, further studies with single and combinations of mutations in Env to determine the binding affinity to the neutralizing antibodies by FACS and/or ELISA are ongoing.

Following CD4 binding, the CD4-binding site on gp120 becomes ordered and the bridging sheet subdomain forms, drawing the V1/V2 loops into a ‘down’ orientation and positioning them alongside CD4 (Guttman et al., 2012). The MVC-selected variant in our study became highly sensitive to anti-CD4i and V3 neutralizing mAbs compared with the passage control virus. Further analysis of the effect of the CCR5 inhibitor-resistant Env to neutralizing antibodies would be of interest because, as reported in our previous work (Yoshimura et al., 2006), the anti-V3 mAb KD-247-resistant variant became highly sensitive to CCR5 inhibitors.

**METHODS**

**Viruses.** Primary HIV-1 viruses were isolated from patients and passaged in PHA-activated PBMCs. Infected PBMCs were co-cultured for 5 days with PM1/CCR5 cells and the culture supernatants were stored at –150 °C until use (Yoshimura et al., 2010). HIV-1KP-5 was isolated from a subject prior to MVC therapy but who has subsequently been taking combination antiretroviral therapy containing MVC since September 2009. The HIV-1KP-5 was isolated before starting the combination antiretroviral therapy.

**Cells, culture conditions and reagents.** The CD4+ T-cell line PM1 (Lusso et al., 1995) was obtained through the AIDS Research and Reference Reagent Program (ARRRP). The PM1/CCR5 cell line was a kind gift from Dr Yousoke Maeda (Kumamoto University, Kumamoto, Japan) (Maeda et al., 2008b). The CCR5 inhibitor MVC was kindly provided by Pfizer (Groton, CT, USA).

**Flow cytometric analysis.** PM1 and PM1/CCR5 cells were analysed for surface expression of CCR5 and CXCR4. The cells (5 × 10^6) were incubated with phycoerythrin-labelled anti-CCR5 mAb 2D7, phycoerythrin-labelled anti-CXCR4 mAb 12G5 or isotype-matched control mAbs (BD Biosciences) and analysed using a FACS Calibur (Becton Dickinson).

**In vitro selection of HIV-1 variants using anti-HIV drugs.** HIV-1KP-5 was infected into PM1/CCR5 cells and treated with various concentrations of MVC to induce the production of MVC-resistant variants as described previously (Harada et al., 2013; Hatada et al., 2010; Yoshimura et al., 2006, 2010), with minor modifications. Briefly, PM1/CCR5 cells (4 × 10^5) were exposed to 500 TCID_{50} HIV-1KP-5 and cultured in the presence of MVC. The culture supernatant was harvested on day 7 and used to infect fresh PM1/CCR5 cells for the next round of culture in the presence of increasing concentrations of MVC. We also passaged the virus in the absence of MVC in PM1/CCR5 cells and the parental cell line PM1. Proviral DNA was extracted from lysates of infected cells at different passages and subjected to nucleotide sequencing.

**Amplification of proviral DNA and nucleotide sequencing.** Proviral DNA was subjected to PCR amplification using PrimeSTAR GXL DNA polymerase and Ex-Taq polymerase (Takara) as described previously (Harada et al., 2013; Hatada et al., 2010; Yoshimura et al., 2006, 2010). Primers 1B and H were used for the gp120 region (Harada et al., 2013; Hatada et al., 2010). The first-round PCR products were used directly in a second round of PCR using primers 2B and F for gp120 (Harada et al., 2013; Hatada et al., 2010). The second-round PCR products were purified and cloned into the pGEM-T Easy Vector (Promega), and the env region in each passaged virus was sequenced using a 3500xL Genetic Analyzer (Applied Biosystems).

**Susceptibility assay.** The sensitivities of the passaged viruses to various drugs were determined as described previously (Harada et al., 2013; Hatada et al., 2010; Yoshimura et al., 2006, 2010), with minor modifications. Briefly, PM1/CCR5 cells were plated in 96-well round-bottom plates (2 × 10^4 cells per well), exposed to 100 TCID_{50} of the viruses in the presence of various concentrations of drugs and incubated at 37 °C for 7 days. The IC_{50} values were then determined using a Cell Counting kit-8 (WST-8 assay; Dijindo Laboratories). All assays were performed in duplicate or triplicate.

**Construction of chimeric NL4-3/KP-5 env proviruses.** Chimeric proviruses were constructed from the pNL4-3 proviral plasmid (ARRRP) by overlapping PCR as described previously (Shibata et al., 2007), with minor modifications. Briefly, the gp160 coding sequences were amplified from the cloning vectors using the primers EnvFv (5’-AGCAGAAGACAGTGGCAATGAGAGCGAAG-3’) and EnvRv (5’-CTTCGCTCTCATTGCCACTGTCTTCTGCT-3’) by overlapping PCR as described previously (Shibata et al., 2007) with minor modifications. Primers 1B and H were used for the gp120 region (Harada et al., 2013; 2010). Primers 1B and H were used for the gp120 region (Harada et al., 2013; 2010). The second-round PCR products were used directly in a second round of PCR using primers 2B and F for gp120 (Harada et al., 2013; Hatada et al., 2010). The second-round PCR products were purified and cloned into the pGEM-T Easy Vector (Promega), and the env region in each passaged virus was sequenced using a 3500xL Genetic Analyzer (Applied Biosystems).
Dr Dennis Burton, Scripps Research Institute, La Jolla, CA (Kessler et al., 1997), 4E9C (anti-CD4i mAb) (Yoshimura et al., 2010) and autologous plasma IgGs were also determined by the WST-8 assay. Plasma samples were collected from the patient seven times from January 2010 to April 2011 and purified using Protein A Sepharose Fast Flow (GE Healthcare) (Kimmer, et al., 2002; Yoshimura et al., 2010). The purified plasma IgGs were designated KP-5-IgG-1 to KP-5-IgG-7.

**Crystal structure of gp120.** To compare the sequences of the MVC-selected and low-CR5-adapted variants in 3D space, the crystal structures of the gp120 monomer and trimer were obtained from the PDB (IDs: 2B4C and 3JSM). Figures were generated using ViewerLite version 5.0 (Accelrys).

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