(as compared to Asp^{L27d} and Asn^{L27d}). Similarly, the even longer side chains of Arg^{L27d} and Lys^{L27d} are likely to negatively affect interactions with Arg^{315} because of steric constraints. The ability of the N27dD variant to neutralize clade B HIV-1 suggests that KD-247 may be substituted with Asp^{L27d} to retain activity against clade B HIV. This substitution would still be able to participate in the intricate network of hydrogen bond interactions proposed in our model for recognition of Arg^{315}. Although many of the scFv variants at position 92 of the light chain demonstrated proper folding (Supplemental Fig. 1*C*), Phe^{L92} demonstrated enhanced binding to the clade B V3 loop peptide while Ala^{L92} and Arg^{L92} had similar binding compared to the WT scFv (Fig. 3*B*), but only Phe^{L92} showed effective neutralization of clade B HIV-1 Env pseudotyped virus (Fig. 3*C*). These results establish that the aromatic ring at Tyr^{L92} is essential for clade B V3 loop recognition. Overall, these observations highlight the importance of Asn^{L27d}, Tyr^{L32}, and Tyr^{L92} in neutralizing clade B HIV-1 containing a GPGR V3 loop arch. Importantly, these results reveal that binding of scFv variants to clade B V3 loop peptides does not necessarily correlate with efficient HIV-1 clade B neutralization. It is possible that other factors may affect binding of the antibody in the context of the full Env glycoprotein that do not factor in to V3 peptide binding. The recent crystal structure of the native Env trimer by Julien *et al.* (71) revealed that the gp120 subunits are stabilized by β-hairpin interactions of the V3 loop and the V1/V2 strands B and C near the top of the trimer. The arch of the V3 loop is hidden by an *N*-acetyl glucosamine from the Asn^{197} glycan at the C-terminal region of V2 strand D from a neighboring protomer. This glycan blocks access to the V3 arch and may affect mAb binding.

Our findings are in agreement with previous work highlighting the dramatic flexibility of V3 loop and underscore the challenges in engineering antibodies that will be useful for treatment and vaccine design. Antibody engineering based on the interactions reported in our and other structures may lead to enhanced interactions with both Arg and Gln residues at position 315 of the V3 loop. For example, we expect that mutations of KD-247 residue Asn^{L27d} to longer polar residues (N27dQ, N27dK, and N27dR) should maintain the hydrogen bond interaction with clade B Arg^{315} and also be able to hydrogen bond with the shorter non–clade B Gln^{315}. Additionally, mutations of KD-247 residue Asp^{L28} to long polar residues (D28K, D28R, and D28E) should reach and interact with both clade B Arg^{315} and non–clade B Gln^{315}. Similarly, the Y32R mutant should maintain van der Waals interactions with clade B Arg^{315} and also make hydrogen bond interactions with non–clade B Gln^{315}. Combinations of the above mutations in the 27d, 28, 32, and 92 positions of the light chain of KD-247 may also help to maintain interactions with Arg^{315} and improve interactions with Gln^{315}. Additional approaches may include mutations of other KD-247 residues that do not interact directly with residue 315 of the V3 loop. Such interactions are observed in the structures of the 2557 and 3074 Fabs (22, 60) in complex with the V3 loop. Finally, extended CDR H3 loops may be designed to make nonspecific main chain interactions with the V3 loop, as is the case with 447-52D (61) and 537-10D (59), which form three-stranded and four-stranded antiparallel β-sheets with the V3 loop target.

Although the propensity of V3 loop to serve as an immunogen to elicit broadly neutralizing antibodies against HIV-1 of multiple clades has been challenging, the extensive studies performed by the Zolla-Pazner, Gorny, Wilson, and Kong groups suggest that this strategy may be achievable (22, 25, 55, 58–61, 69, 70, 72). FJ

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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 (IC_{50} ~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.

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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). CCR5 inhibitors work by allosterically altering the conformation of CCR5 at the cell surface, thereby disrupting its interaction with HIV gp120 (Berger *et al.*, 1999; Dorr *et al.*, 2005). 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; Ratcliff *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.

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The GenBank/EMBL/DDBJ accession numbers for the envelope sequences of KP-5 are AB742145–AB742157.

Three supplementary figures are available with the online version of this paper.

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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 IC_{50} (a competitive inhibitor), 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 IC_{50}s, 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 (Fig. 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 IC_{50} 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-1_{BaL} and HIV-1_{JR-FL} with MPIs of 98 and 97 %, respectively, but did not inhibit the X4 virus HIV-1_{IIIB} or dual-tropic virus HIV-$1_{89.6}$ (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 1. Inhibitory activities of MVC toward infection by laboratory-adapted and primary strains of HIV-1

Virus	Subtype	IC_{50}* (nM)	MPI (%)
Laboratory adapted			
R5			
HIV-1_{BaL}	B	26	98
HIV-1_{JR-FL}	B	6.9	97
Dual			
HIV-$1_{89.6}$	B	>1000	<20
X4			
HIV-1_{IIIB}	B	>1000	<20
Primary			
R5			
HIV-1_{KP-5}	B	26	92
HIV-1_{KP-2}	CRF08_BC	24	95
HIV-1_{KP-6}	G	20	95
HIV-1_{KP-7}	B	18	95
HIV-1_{KP-8}	B	14	97
HIV-1_{KP-9}	B	13	96
HIV-1_{KP-10}	B	9.2	98
HIV-1_{KP-11}	C	8.6	96
HIV-1_{KP-12}	B	5.1	98
HIV-1_{KP-13}	B	4.0	96
HIV-1_{KP-14}	B	3.0	95
HIV-1_{KP-15}	B	3.0	94
HIV-1_{KP-16}	B	2.2	100
HIV-1_{KP-17}	B	1.2	98
X4/mix			
HIV-1_{KP-18}	CRF01_AE	>1000	<20
HIV-1_{KP-19}	CRF01_AE	>1000	<20
HIV-1_{KP-20}	B	>1000	<20

*PM1/CCR5 cells (2×10^3) were exposed to 100 $TCID_{50}$ of each virus and then cultured in the presence of various concentrations of MVC. The IC_{50} 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-1_{KP-5}, which had the highest IC_{50} 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-1_{KP-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-1_{KP-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

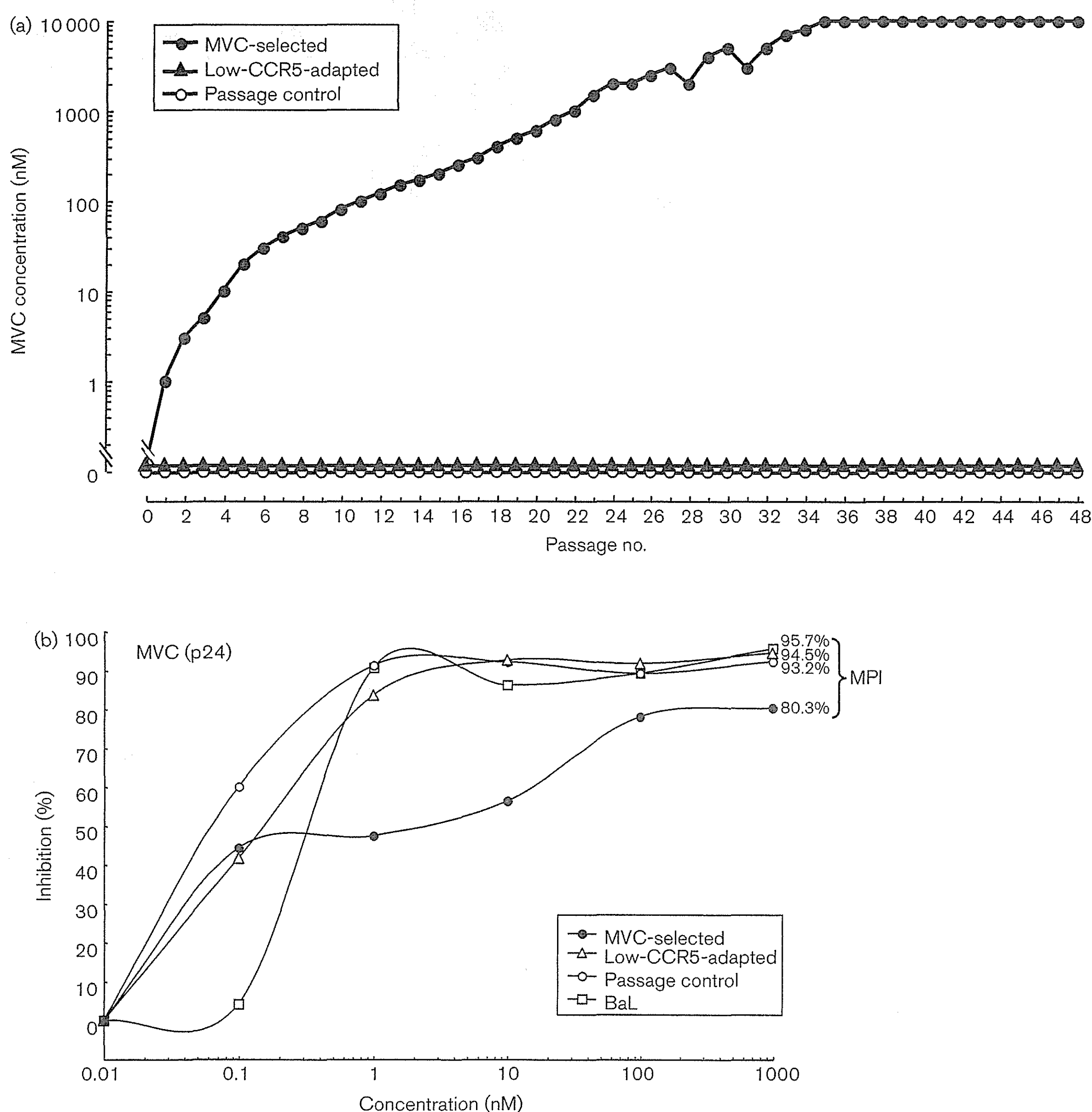

Fig. 1. Selection of MVC-resistant and low-CCR5-adapted virus variants. (a) The selection was carried out in PM1/CCR5 and PM1 cells as described in Methods. (b) Sensitivities of the MVC-selected (48 passages), low-CCR5-adapted (48 passages), passage control (48 passages) variants and HIV-1_{BaL} (BaL) 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.

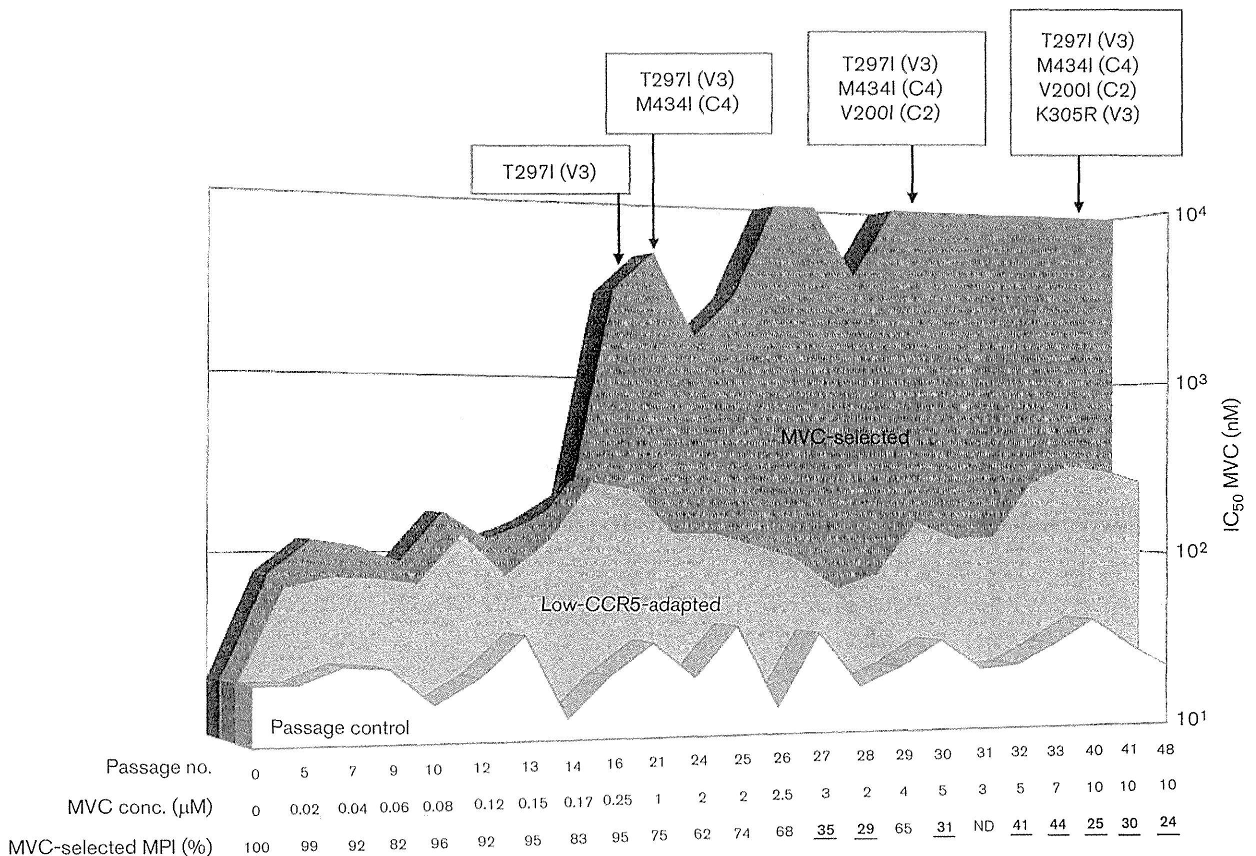

Fig. 2. Susceptibility of passaged variants to MVC. The sensitivity and MPI of each passaged variant to MVC was determined by a *multi-round* assay using the WST-8 assay as described in Methods. The *x*-axis shows the passage number, concentration of MVC (μM) and MPI values. The mutations observed in the highly MVC-resistant variants are shown above the graph.

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), at passages 17, 21, 34 and 41, respectively, 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

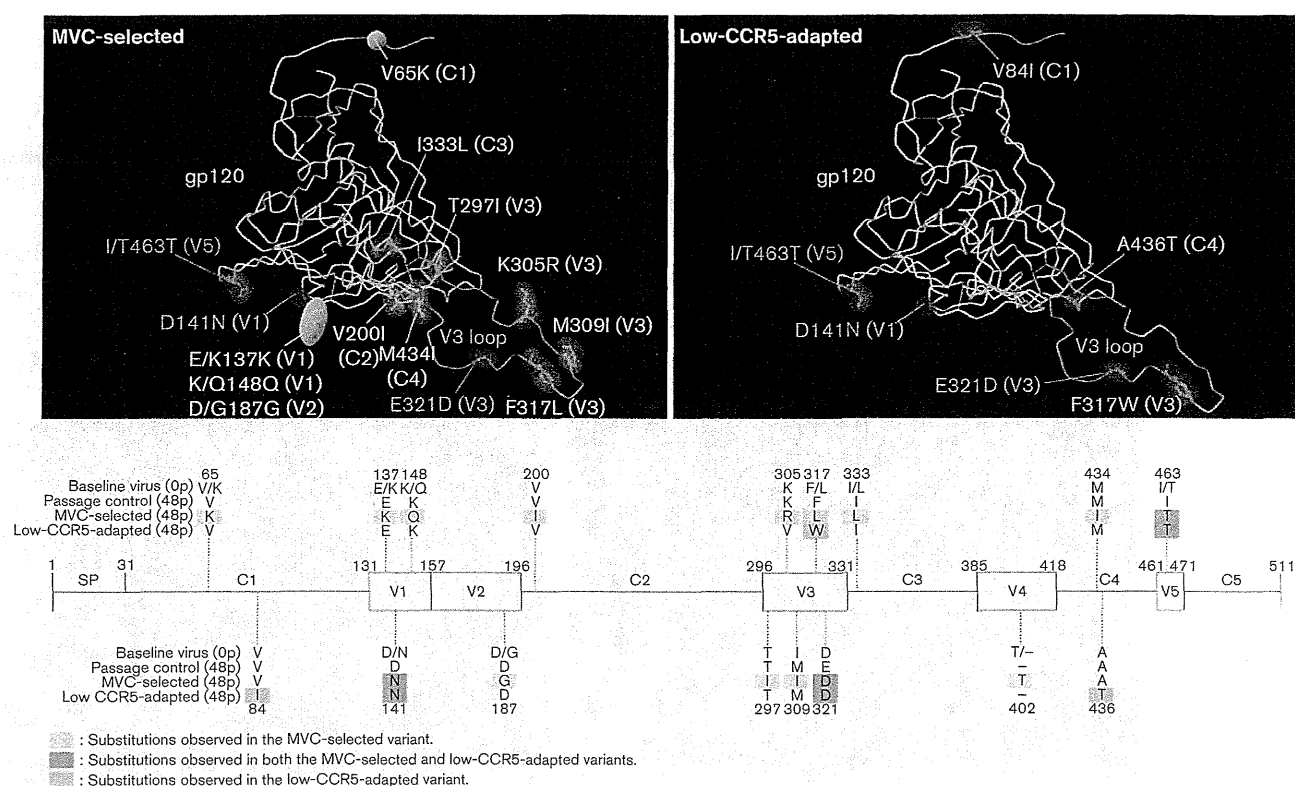

Fig. 3. Comparison of the locations of the mutations in the MVC-selected and low-CCR5-adapted gp120. The side chains of the mutated residues that appeared during the MVC selection (left) and low CCR5 adaptation (right) are shown in yellow (only MVC selection), pink (only low CCR5 adaptation) and green (both). A summary schema is also provided.

(from 74 to 24 %) (Fig. 2). 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 similarly to the low-CCR5-adapted variants (IC_{50} 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) (Fig. S3) (Lyumkis *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

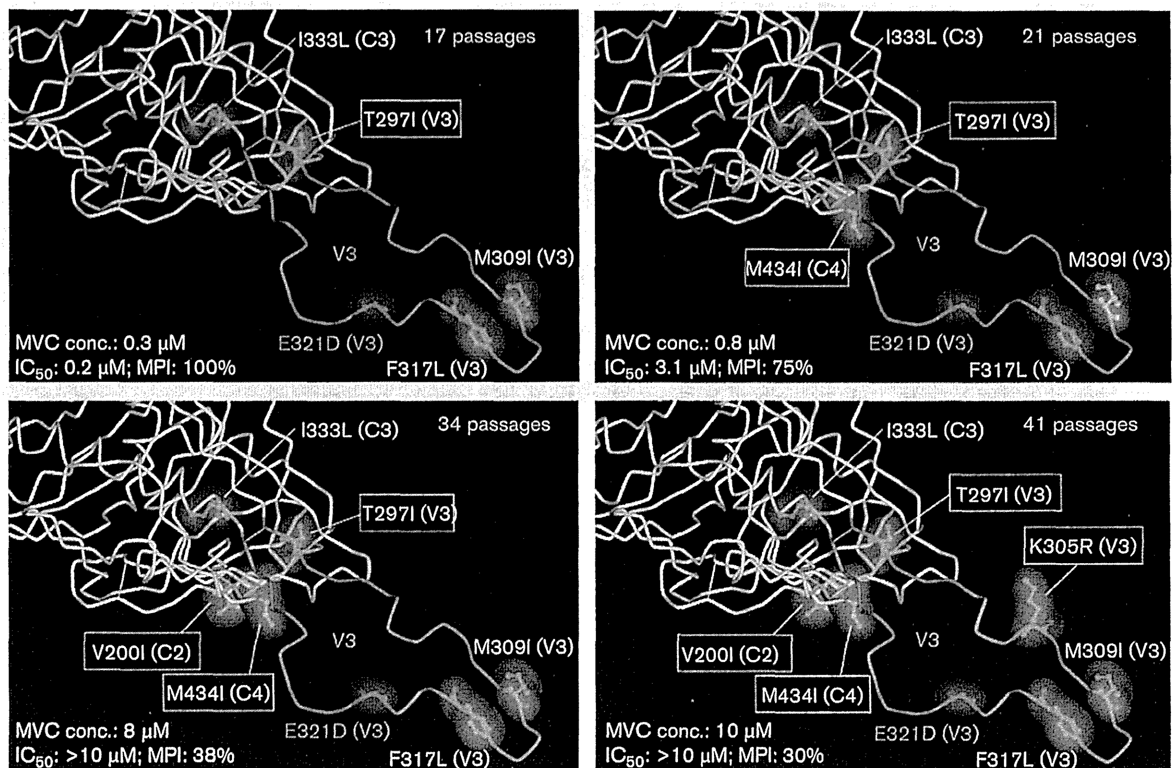

Fig. 4. Enlargement of the area of the CCR5 N-terminal-binding site and V3 loop in gp120. The side chains of the mutated residues that appeared during *in vitro* selection with MVC at 17 (upper left), 21 (upper right), 34 (lower left) and 41 passages (lower right) are shown. The crystal structure of gp120 was retrieved from the Protein Data Bank (PDB ID: 2B4C).

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 IC_{50} values of 0.22, 0.31 and 0.86 μg ml^{-1}, respectively. The MVC-selected and low-CCR5-adapted clones became highly sensitive to 4E9C compared with the passage control clone (IC_{50} values of 0.08, 0.41 and >5 μg ml^{-1}, 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 (IC_{50} values of 0.04, >100 and >100 μg 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 (IC_{50} 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.

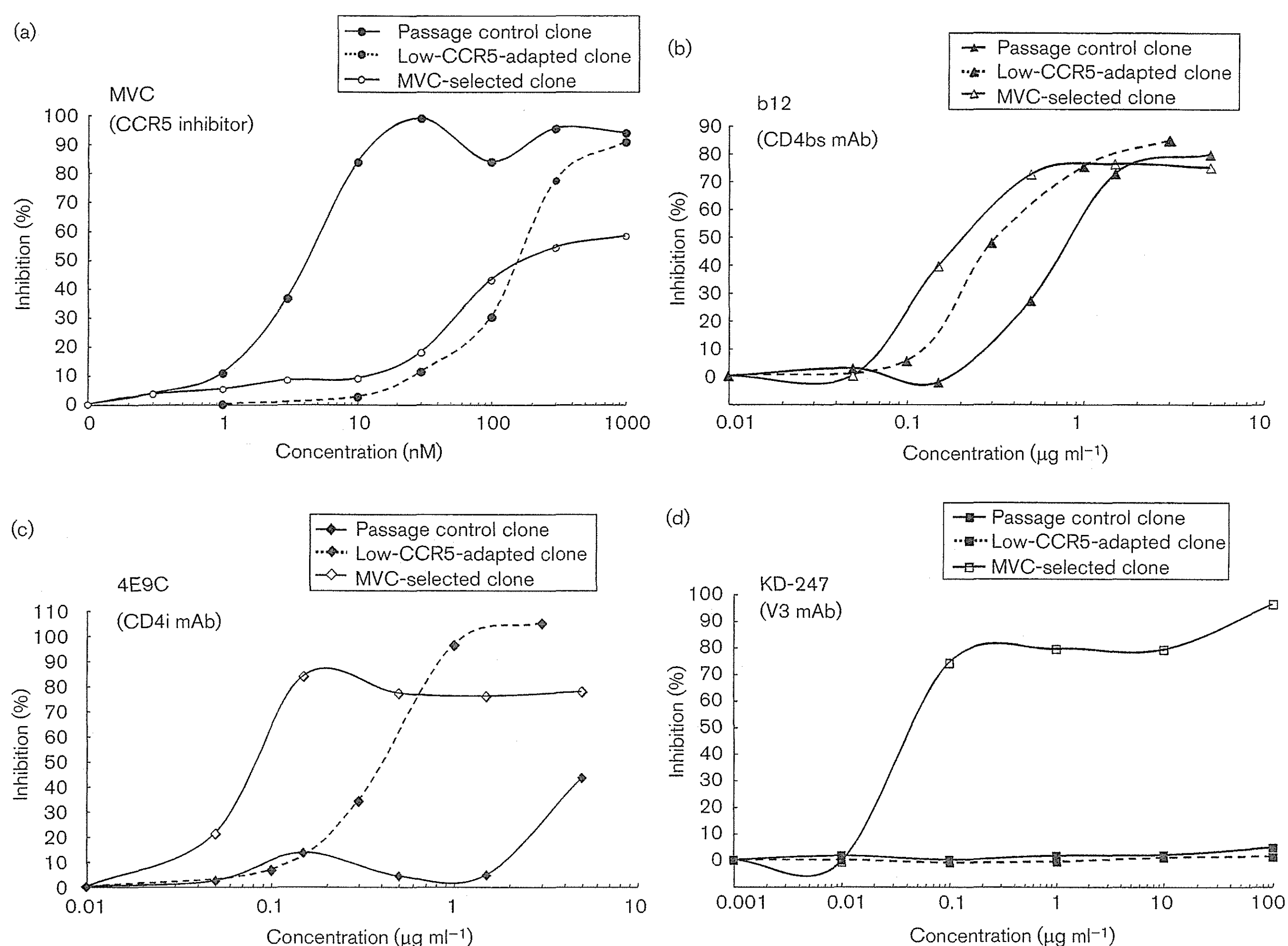

Fig. 5. Sensitivities of infectious clones with the passage control, low-CCR5-adapted and MVC-selected Env mutations to MVC and anti-Env mAbs. The sensitivities of the infectious clones with the passage control (filled symbols), low-CCR5-adapted (filled symbols and dotted lines) and MVC-selected (open 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.

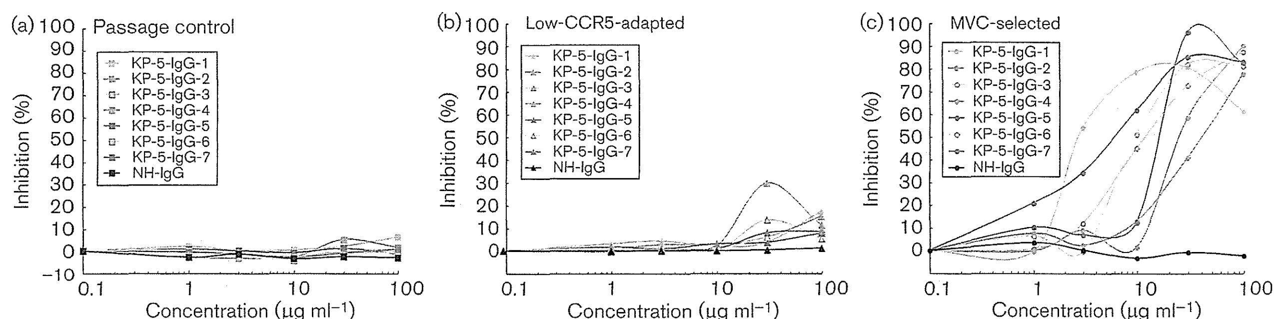

Fig. 6. Sensitivities of infectious clones with the passage control, low-CCR5-adapted and MVC-selected Env mutations to autologous plasma IgGs. The sensitivities of the infectious clones with the (a) passage control, (b) low-CCR5-adapted and (c) MVC-selected Env mutations to seven autologous plasma IgGs (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.

DISCUSSION

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 IC_{50} (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 acquired mutations within the area of the CCR5-binding site in gp120 by evolution and/or selection from minor subsets. Following acquisition of the latter mutations, the variants with mutant Env showed a considerably reduced MPI (24 %). These results indicate that mutants arising from passage in low-CCR5-expressing cells can influence the IC_{50} shift, but not a reduction in the MPI.

One previous study showed that although numerous changes were observed in V3 and other regions of gp160, genotypic analysis of the cloned *env* sequences revealed no specific mutational pattern associated with reduced susceptibility to VCV in a phase 2 clinical trial (Pantophlet & Burton, 2006). Using the Los Alamos Database, we found that the frequencies of the four mutations occurred in $<$10 % of 1501 subtype B viruses (V200I, 5.1 %; T297I, 5.9 %; K305R, 9.5 %; M434I, 2.5 %). Assays for these mutations might provide useful clinical markers for determining the sensitivity of HIV-1 to MVC. One limitation of the present study was that only one primary isolate was used and a single *in vitro* passage series was used for variant selection. Further studies using multiple isolates and multiple passage cultures are required to determine if this MVC-susceptibility model applies to other HIV subtypes and cell systems. Ogert *et al.* (2009) and Anastassopoulou *et al.* (2009) reported that multistep resistance mutations during *in vitro* selection that reduced the MPI values to VCV were driven by the K305R substitution and the H308P substitution was related to a reduction in the MPI plateau level to VCV resistance. Henrich *et al.* (2010) also reported such mutations *in vivo,* as S306P was not detected in the baseline virus population, but was necessary for maximal resistance when incorporated into V3 backbones that included pre-existing VCV resistance mutations. Our *in vitro* study also showed that the K305R mutation contributed to maximal resistance to MVC when incorporated into V3 and CCR5 N-terminal-binding site backbones that included pre-existing MVC resistance mutations. Moreover, our MVC-selected variant with MVC (1 μM) became sensitive to the CCR5 N-terminal mAb (data not shown). Conversely, Roche *et al.* (2011a) reported that the MVC-resistant variants 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. 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 the CCR5 N terminus, and further studies are needed to determine the relationship between each mutation and CCR5 N terminus dependency.

HIV Env evades antibody recognition of conserved epitopes by several means, including decoration with a dense glycan shield, hypervariable loops that mask conserved features and high intrinsic conformational dynamics that render it a poorly defined antigen (Pantophlet & Burton, 2006). In the present study, the infectious clone with the Env of the low-CCR5-adapted virus became sensitive to anti-CD4i mAb, but not anti-V3 mAb and autologous plasma IgGs. Conversely, the clone with the highly MVC-resistant Env was neutralized by the anti-V3 mAb at low concentrations ($<$0.1 μg ml^{-1}) and also by the autologous plasma IgGs. These findings suggest that the low-CCR5-adapted mutations are related to accessibility of the anti-CD4i mAb to its epitopes, whilst the greater number of mutations in the MVC-selected virus may provide access to the epitopes of

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-1_{KP-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-1_{KP-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-1_{KP-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 Yosuke 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 (5×10^5) 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 FACSCalibur (Becton Dickinson).

***In vitro* selection of HIV-1 variants using anti-HIV drugs.** HIV-1_{KP-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 (4×10^4) were exposed to 500 $TCID_{50}$ HIV-1_{KP-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 (2×10^3 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; Dojindo Laboratories). All assays were performed in duplicate or triplicate.

PHA-activated PBMCs (1×10^6 cells ml^{-1}) were exposed to 100 $TCID_{50}$ of each HIV-1 strain and cultured in the presence or absence of various concentrations of drugs in 96-well microculture plates. The concentration of p24 antigen produced by the cells was determined on day 7 using a Lumipulse F system (Fujirebio) (Maeda *et al.*, 2001). IC_{50} values were determined by comparison with the p24 production level in drug-free control cell cultures (Shirasaka *et al.*, 1995). All assays were performed in 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 EnvR (5′-TTTTGACCACTTGCCACCCATCTTATAGC-3′). A portion of the NL4-3 provirus spanning nt 5284–6232 was amplified with primers NL(5284)F (5′-GGTCAGGGAGTCTCCATAGAATGGAGG-3′) and NL(6232)Rv (5′-CTTCGCTCTCATTGCCACTGTCTTCTGCT-3′). This fragment encompasses the unique *Eco*RI restriction site in pNL4-3. Another fragment from the NL4-3 provirus spanning nt 8779–9045 was amplified using the primers NL(8779)F (5-GCTATAAGATGGGTGGCAAGTGGTCAAAA-3) and NL(9045)R (5-GATCTACAGCTGCCTTGTAAGTCATTGGTC-3). This fragment includes the unique *Xho*I restriction site in pNL4-3. Overlapping PCR was used to join the gp160 coding sequence from the desired clone to the fragment encompassing nt 8779–9045 that had been amplified from pNL4-3. The resulting fragment was then similarly joined to the amplified fragment encompassing nt 5284–6232 from pNL4-3.

The sensitivities of the three infectious clones to KD-247 (anti-V3 mAb) (Eda *et al.*, 2006), b12 (anti-CD4bs mAb; kindly provided by

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) (Kimura *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-CCR5-adapted variants in 3D space, the crystal structures of the gp120 monomer and trimer were obtained from the PDB (IDs: 2B4C and 3J5M). Figures were generated using ViewerLite version 5.0 (Accelrys).

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A Novel Tricyclic Ligand-Containing Nonpeptidic HIV-1 Protease Inhibitor, GRL-0739, Effectively Inhibits the Replication of Multidrug-Resistant HIV-1 Variants and Has a Desirable Central Nervous System Penetration Property In Vitro.

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Abstract

We report here that GRL-0739, a novel nonpeptidic HIV-1 protease inhibitor containing a tricycle (cyclohexyl-bis-tetrahydrofuranylurethane [THF]) and a sulfonamide isostere, is highly active against laboratory HIV-1 strains and primary clinical isolates (50% effective concentration [EC50], 0.0019 to 0.0036 μM), with minimal cytotoxicity (50% cytotoxic concentration [CC50], 21.0 μM). GRL-0739 blocked the infectivity and replication of HIV-1NL4-3 variants selected by concentrations of up to 5 μM ritonavir or atazanavir (EC50, 0.035 to 0.058 μM). GRL-0739 was also highly active against multidrug-resistant clinical HIV-1 variants isolated from patients who no longer responded to existing antiviral regimens after long-term antiretroviral therapy, as well as against the HIV-2ROD variant. The development of resistance against GRL-0739 was substantially delayed compared to that of amprenavir (APV). The effects of the nonspecific binding of human serum proteins on the anti-HIV-1 activity of GRL-0739 were insignificant. In addition, GRL-0739 showed a desirable central nervous system (CNS) penetration property, as assessed using a novel in vitro blood-brain barrier model. Molecular modeling demonstrated that the tricyclic ring and methoxybenzene of GRL-0739 have a larger surface and make greater van der Waals contacts with protease than in the case of darunavir. The present data demonstrate that GRL-0739 has desirable features as a compound with good CNS-penetrating capability for treating patients infected with wild-type and/or multidrug-resistant HIV-1 variants and that the newly generated cyclohexyl-bis-THF moiety with methoxybenzene confers highly desirable anti-HIV-1 potency in the design of novel protease inhibitors with greater CNS penetration profiles.

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Delayed emergence of HIV-1 variants resistant to 4'-ethynyl-2-fluoro-2'-deoxyadenosine: comparative sequential passage study with lamivudine, tenofovir, emtricitabine and BMS-986001.

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Abstract

BACKGROUND:

4'-Ethynyl-2-fluoro-2'-deoxyadenosine (EFdA) contains an ethynyl moiety and the 3'-hydroxyl and exerts highly potent activity against various HIV type-1 (HIV-1) strains including multi-drug-resistant variants.

METHODS:

Comparative selection passages against EFdA, lamivudine (3TC), tenofovir disoproxil fumarate (TDF), emtricitabine (FTC) or BMS-986001 (Ed4T) were conducted using a mixture of 11 highly multi-drug-resistant clinical HIV-1 isolates (HIV11MIX) as a starting virus population.

RESULTS:

Before selection, HIV11MIX was sensitive to EFdA with a 50% inhibitory concentration (IC50) of 0.032 μM, less susceptible to TDF and Ed4T with IC50s of 0.57 and 2.6 μM, respectively, and highly resistant to 3TC and FTC with IC50s>10 μM. IC50s of TDF against HIV11MIX exposed to EFdA and TDF for 17 (HIV11MIX(EFdA-P17)) and 14 (HIV11MIX(TDF-P14)) passages were 8 and >10 μM, respectively, while EFdA remained active against HIV11MIX(EFdA-P17) and HIV11MIX(TDF-P14) with IC50s of 0.15 and 0.1 μM, respectively. Both selected variants were highly resistant against zidovudine, 3TC, Ed4T and FTC (IC50 values >10 μM).

CONCLUSIONS:

The present data demonstrate that HIV11MIX developed resistance more rapidly against 3TC, FTC, TDF and Ed4T than against EFdA and that EFdA remained substantially active against TDF- and EFdA-selected variants. Thus, EFdA has a favourable resistance profile and represents a potentially promising new-generation nucleoside reverse transcriptase inhibitor.

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EFdA, a Reverse Transcriptase Inhibitor, Potently Blocks HIV-1 *Ex Vivo* Infection of Langerhans Cells within Epithelium

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TO THE EDITOR

Despite increasing access to antiretroviral drugs, sexual transmission of HIV-1 remains a significant public health threat. A recent clinical trial, CAPRISA 004, of a vaginally administered microbicide using a nucleoside reverse transcriptase inhibitor (NRTI), tenofovir (TDF), has demonstrated that 1% TDF gel reduced HIV-1 acquisition by an estimated 39% overall (Abdool Karim *et al.*, 2010), indicating a potential utility of NRTI-based microbicides. In the VOICE study, however, a once-daily dosing regimen with TDF gel failed to demonstrate protective effects in at-risk women. These studies demonstrate the need to develop additional more potent microbicide candidates to potentially increase the activity to protect women from HIV-1 transmission.

We previously reported that a series of 4′-substituted NRTIs have excellent antiviral properties (Ohrui, 2006), and through optimization of such 4′-substituted NRTIs, 4′-ethynyl-2-fluoro-2′-deoxyadenosine (EFdA) was found to exert extremely potent activity against a wide spectrum of HIV-1 strains including highly multidrug-resistant clinical HIV-1 isolates, with favorable *in vitro* cell toxicities (Nakata *et al.*, 2007; Ohrui *et al.*, 2007). EFdA inhibited HIV-1 replication in activated peripheral blood mononuclear cells with an EC_{50} of 0.05 nM, a potency several orders of magnitude greater than any of the current clinically available NRTIs (Michailidis *et al.*, 2009). As the prevalence of new infections with drug-resistant HIV-1 variants could increase in the coming years (Nichols *et al.*, 2011), EFdA may be useful as a topical microbicide.

Langerhans cells (LCs) are dendritic cells located, among other sites, within genital skin and mucosal epithelium (Lederman *et al.*, 2006). In female rhesus macaques exposed intravaginally to simian immunodeficiency virus, up to 90% of initially infected target cells were LCs (Hu *et al.*, 2000). *Ex vivo* experiments with human foreskin explants show that epidermal LCs in inner foreskin are primary target cells for HIV-1 infection, providing a plausible explanation for why circumcision greatly reduces the probability of acquiring HIV-1 (Ganor *et al.*, 2010; Zhou *et al.*, 2011). LCs also express CD4 and CCR5, but not CXCR4, and demonstrate the distinctive characteristics of emigrating from tissue to draining lymph nodes in order to interact with T cells following contact with pathogens (Lederman *et al.*, 2006). Indeed, epidermal LCs are readily infected *ex vivo* with R5-HIV-1, but not with X4-HIV-1, and initiate and promote high levels of infection upon interactions with cocultured $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013), consistent with previous epidemiologic observations that the majority of HIV-1 strains isolated from newly infected patients are R5-HIV-1 strains (Zhu *et al.*, 1993). Thus, LCs likely have an important role in disseminating HIV-1 soon after exposure to the virus.

To understand how HIV-1 traverses skin and genital mucosa, an *ex vivo* model was developed in which resident LCs within epithelial tissue explants obtained from suction blisters are exposed to HIV-1 and then allowed to emigrate from the tissue, thus mimicking conditions that occur following mucosal exposure to HIV (Kawamura *et al.*, 2000; Ogawa *et al.*, 2009, 2013). In this model, although relatively few productively infected LCs are identified, these cells induce high levels of HIV-1 infection when cocultured with resting autologous $CD4^+$ T cells (Kawamura *et al.*, 2000; Ogawa *et al.*, 2013). As expected, when epidermal tissue explants were pretreated with various concentrations of TDF, EFdA, and CCR5 inhibitor, maraviroc (MVC), prior to R5-tropic HIV-$1_{Ba\text{-}L}$ exposure, HIV-1 infection of resident LCs within epidermis as well as subsequent virus transmission from emigrated LCs to cocultured $CD4^+$ T cells was decreased in a dose-dependent manner (Figure 1a and c; for detailed methods, see Supplementary Material). The blocking was confirmed by repeated experiments using skin explants from three additional randomly selected individuals (Figure 1b and d). Strikingly, although the blocking efficiency of TDF or MVC even at 5,000 nM was partial, EFdA demonstrated complete blocking of R5-HIV-1 replication in LCs as well as subsequent virus transmission from emigrated LCs to $CD4^+$ T cells at doses of 100–5,000 nM (Figure 1a–d). Furthermore, EFdA blocked *ex vivo* virus infection of LCs as well as subsequent virus transmission when two strains of R5-HIV-1, HIV-$1_{JR\text{-}FL}$ and HIV-1_{AD8}, were utilized in experiments ($n=3$, Supplementary Figure S1 online).

Similar to the results in epidermal LCs, preincubation of monocyte-derived LCs (mLCs) with 100–5,000 nM of EFdA

Abbreviations: EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LC, Langerhans cell; mLC, monocyte-derived LC; MVC, maraviroc; NRTI, nucleoside reverse transcriptase inhibitor; TDF, tenofovir

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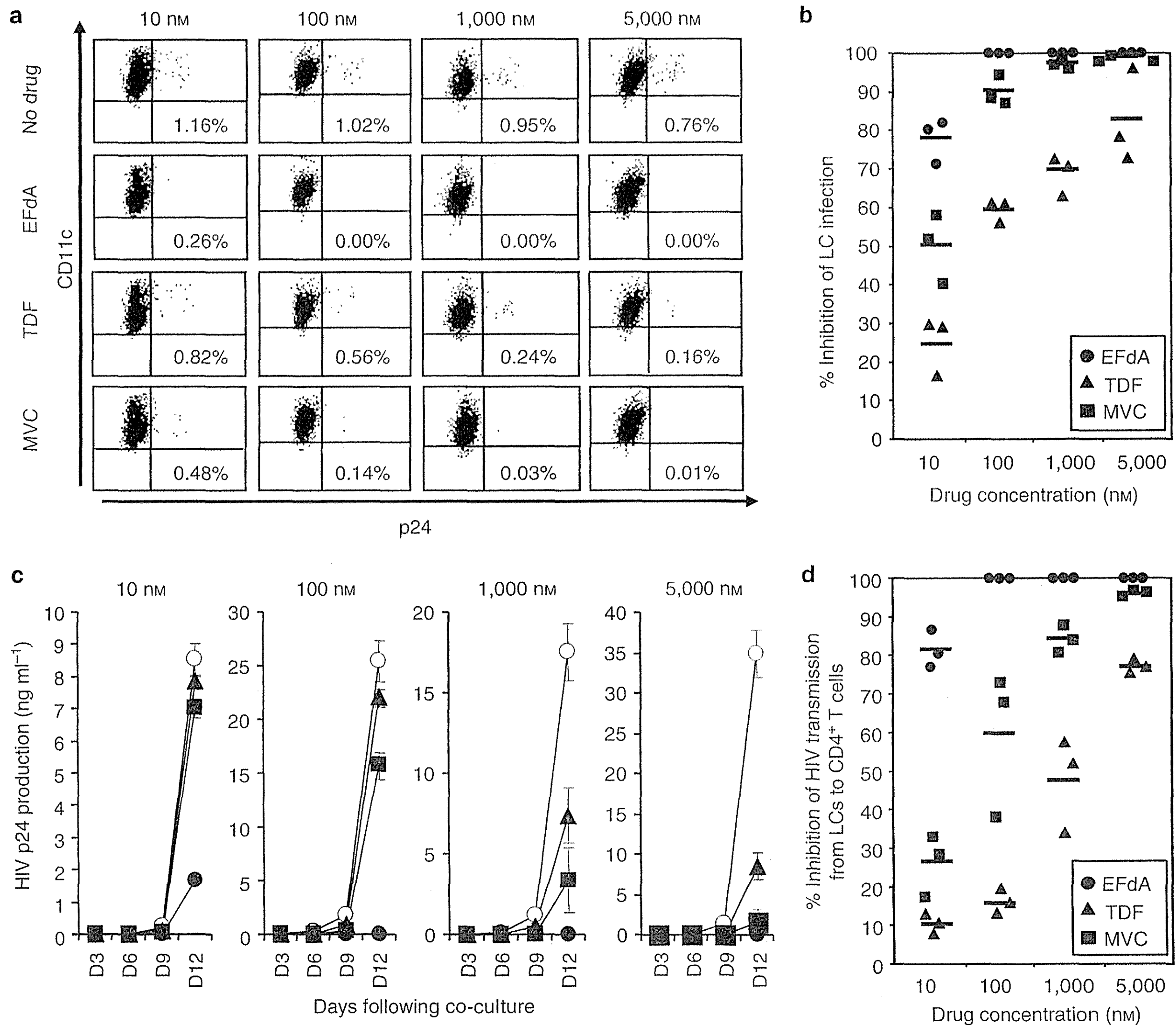

Figure 1. Preincubation of skin explants with EFdA blocks R5-HIV-1 infection in LCs and subsequent virus transmission to cocultured CD4$^+$ T cells. LCs within skin explants were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲), and MVC (■) for 30 minutes, exposed to HIV-$1_{Ba\text{-}L}$ for 2 hours, and then floated on culture medium to allow migration of LCs from the explants. Emigrating cells from the epidermal sheets were collected 3 days following HIV-1 exposure. HIV-1-infected LCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ LCs (**a**, **b**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**c**, **d**). Summary of percent inhibition of LC infection (**b**) and virus transmission to CD4$^+$ T cells (**d**) of 12 experiments using skin explants from 12 individuals with the indicated each concentration of EFdA (●), TDF (▲), and MVC (■) are shown. Mean values obtained from different donors are shown as horizontal marks (**b**, **d**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; MVC, maraviroc; TDF, tenofovir.

completely blocked HIV-1 replication in mLCs as well as subsequent virus transmission from mLCs to cocultured CD4$^+$ T cells, whereas both TDF and MVC at the same doses only partially inhibited the transmission (Figure 2a and b; for detailed methods, see Supplementary Material online). Intriguingly, even in 1–3 days following the removal of EFdA (1,000 nM), EFdA completely blocked HIV-1 infection of mLCs as well as subsequent virus transmission from mLCs to cocultured CD4$^+$ T cells, whereas TDF and MVC rapidly lost their anti-HIV-1 activity within days (Figure 2c–f). No cellular toxicity was noted for any of these drugs at the doses used in these experiments (Supplementary Figure S2 online). When similar experiments were conducted using peripheral blood mononuclear cell as target cells, virtually identical favorable persistency of EFdA in antiviral activity compared with that of TDF was observed (data not shown).

In the present work, we demonstrated that EFdA exerted extremely more potent anti-HIV-1 activity in LCs than did TDF and MVC, and the potent anti-HIV-1 activity of EFdA persisted for at least 3 days. Of note, the efficacy of TDF gel in CAPRISA 004 has been linked to its long intracellular half-life (Abdool Karim *et al.*, 2010; Rohan *et al.*, 2010). Our data strongly

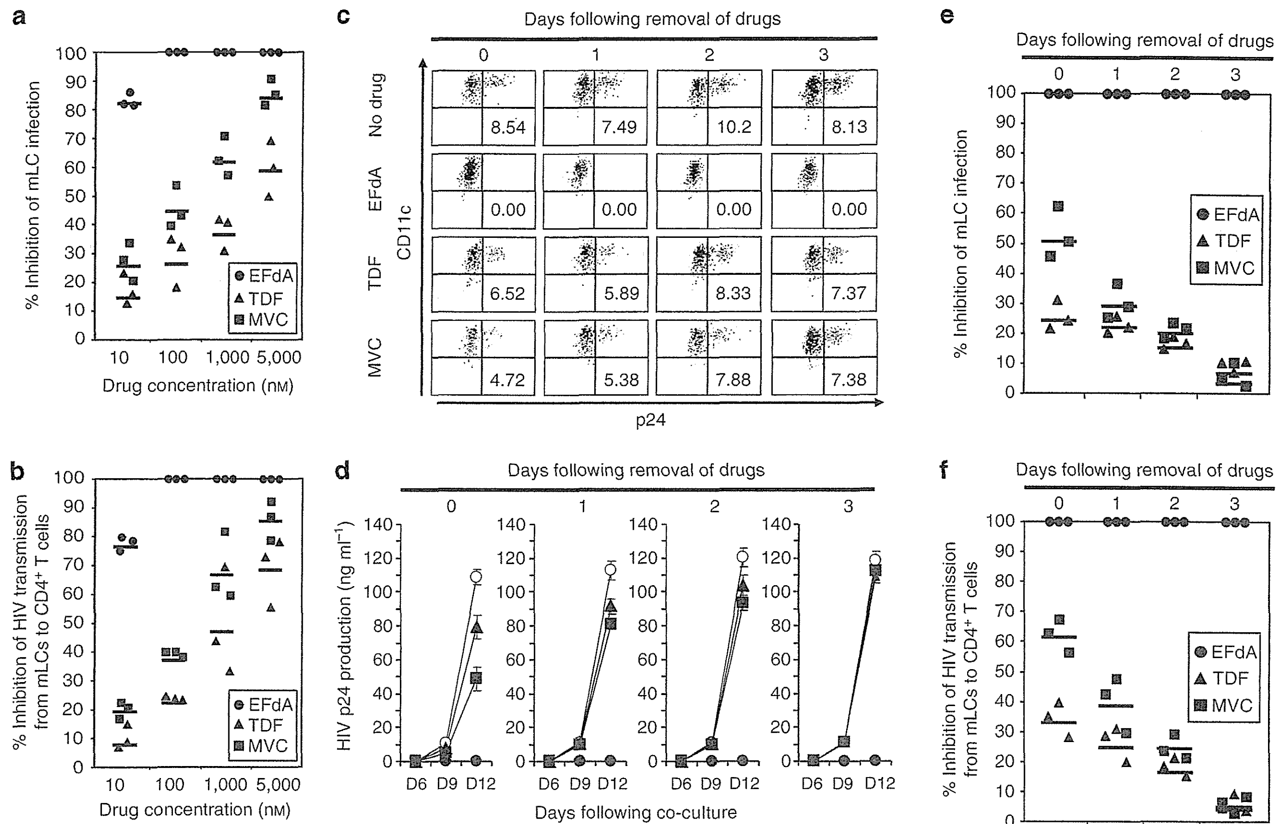

Figure 2. Preincubation of skin explants with EFdA blocks subsequent R5-HIV-1 infection in LC in a dose-dependent manner. mLCs were preincubated with no drug (○) or the indicated concentrations of EFdA (●), TDF (▲) and MVC (■) for 30 minutes, and then immediately exposed to HIV-1Ba-L for 2 hours (**a**, **b**), or thoroughly washed to remove the extracellular drug and further cultured for 1, 2, or 3 days prior to exposure to HIV-1Ba-L for 2 hours (**c**–**f**). After 7 days of HIV-1 exposure, HIV-1-infected mLCs were assessed by HIV-1 p24 intracellular staining in langerin$^+$ CD11c$^+$ mLCs (**a**, **c**, **e**), or further cocultured with autologous CD4$^+$ T cells and culture supernatants were assessed for p24 content by ELISA on the indicated days (**b**, **d**, **f**). Summary of percent inhibition of mLC infection (**a**, **e**) and virus transmission to CD4$^+$ T cells (**b**, **f**) of three independent experiments are shown. Mean values are shown as horizontal marks (**a**, **b**, **e**, **f**). EFdA, 4′-ethynyl-2-fluoro-2′-deoxyadenosine; LCs, Langerhans cells; mLCs, monocyte-derived LCs; MVC, maraviroc; TDF, tenofovir.

indicate that EFdA may serve as a promising microbicide to block sexual transmission of HIV-1 because of its potent anti-HIV-1 activity, low cytotoxicity, and superior persistence of antiviral activity against HIV-1 in LCs.

CONFLICT OF INTEREST

HM is among coinventors on a patent for EFdA; all rights, title, and interest to the patent have been assigned to Yamasa Corporation, Chiba, Japan. The other authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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