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Short Communication

Rescue of HIV-1 long-time archived X4 strains to escape maraviroc

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ABSTRACT

Entry of Human Immunodeficiency Virus type 1 (HIV-1) into target cells is mediated by the CD4 receptor and a coreceptor, CCR5 or CXCR4. Maraviroc interferes with HIV entry by binding the CCR5 coreceptor. Virological failure to maraviroc-containing regimens can occur through the emergence of resistance, or through tropism evolution and broadened coreceptor usage. In the latter case, the physiological relevance of minority strains is a major concern.

Here we report a retrospective analysis of coreceptor-usage and evolution based on 454-ultra-deep-sequencing of plasma and Peripheral Blood Mononuclear Cell (PBMC)-derived envelope V3-loops, accounting for coreceptor usage, from a patient who failed a maraviroc-containing regimen through the emergence of X4 strains. The X4 maraviroc-escape variant resulted from recombination between a long time archived proviral sequence from 2003 (5′-portion, including the V3-loop) and the dominant R5 strains circulating in plasma at the time of maraviroc-treatment initiation (3′-portion). Phylogenetic analyses and BEAST modeling highlighted that an early diverse viral quasispecies underwent a severe bottleneck following reinitiation of HAART and repeated IL-2 cycles between 1999 and 2001, leading to the transient outgrowth and archiving of one highly homogeneous X4 population from plasma, and to the expansion in plasma of one PBMC-derived R5 strain. Under maraviroc selective pressure, the early, no longer detectable X4 strains archived in PBMC were partially rescued to provide X4-determinants to the main circulating strain.

Entry of the Human Immunodeficiency Virus type 1 (HIV-1) into target cells requires the sequential binding of the viral envelope gp120 to the CD4 receptor and to a coreceptor (CCR5 or CXCR4). Maraviroc (MVC), the first HIV-1 entry inhibitor currently used in clinical practice, is an allosteric inhibitor of CCR5 that impedes recognition of MVC-bound CCR5 by gp120 (Tilton et al., 2010a). Because MVC specifically targets CCR5 (Dorr et al., 2005) and has no inhibitory effect against CXCR4-using (X4) viruses, the drug is recommended only for patients harboring exclusively strictly CCR5-dependent (R5) strains. Since R5, X4 and dual-tropic (R5X4) strains compete within the infected host, one of the major concerns is that MVC pressure could favor the selection of minority X4 variants, which are more cytopathic than R5 strains *in vitro* and are associated with increased viral loads and a more rapid decline of $CD4^+$ T-cell counts *in vivo*, resulting in a general deterioration of the patient's immune status (Brumme et al., 2005; Westby et al., 2006). Alternatively, MVC pressure could drive the evolution of viral strains from a strict CCR5-usage to broadened coreceptor usage (MacArthur and Novak, 2008; Tilton et al., 2010b).

We report the case of a patient infected with a subtype F1 virus who was diagnosed in 1992, began antiretroviral treatment in 1993 with dual-therapy, failed subsequent highly active antiretroviral therapy (HAART)-based regimens, which led to the selection of multidrug resistant strains. Between 1999 and 2001, the patient underwent 3 treatment interruptions and seven interleukin-2 (IL-2) injection cycles, at the end of which CD4 counts recovered from 50 cells/mm^3 to 490 cells/mm^3. In 2008, the patient was considered for receiving MVC as part of a salvage regimen also including raltegravir, emtricitabine + tenofovir, ritonavir-boosted-darunavir and etravirine. Strict R5 tropism was assessed by Trofile (Whitcomb et al., 2007) and by geno2pheno$_{[coreceptor]}$ (Sing et al., 2007) from plasma-derived sequences. At the time of MVC-treatment initiation, the patient's CD4 count was 250 cells/mm^3 and viral load (VL) was 4200 copies/ml. Within 2 months, VL became undetectable and CD4 counts increased to 410 cells/mm^3. However, 6 months later, VL rebounded (1755 copies/ml), CD4 counts

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Table 1
Patient clinical data, treatment and viral tropism.

Sample		VL	$CD4^+$ counts	Phenotype	Genotype		Median FPR [min, max]	Treatment
					%X4	%R5		
1998	Plasma	10,490	220	D/M	96.07	3.93	1.31 [1 31, 10.17]	d4T + IDV + NVP
	PBMC			nd	100	0	0.29 [0.29, 0.29]	
1999-I	Plasma	7804	60	D/M	47.7	52.3	10.17 [1.31, 10.17]	No treatment
	PBMC			D/M	43.7	56.3	10.17 [0.2907, 10.17]	
1999-II	Plasma	958	220	D/M	99.8	0.2	1.31 [1.31, 1.31]	d4T + 3TC + SQV + NFV + EFV
	PBMC			nd	0	100	10.17 [10.17, 10.17]	
2001	Plasma	595	610	X4	4.2	95.8	10.17 [10.17, 10.17]	ABC + ddI + LPV/r + EFV
	PBMC			X4	3.1	96.9	10.17 [10.17, 10.17]	
2003	Plasma	15,817	640	R5	3.04	96.95	22.67 [1.16, 22.67]	ABC + LPV/r + APV
	PBMC			X4	99.25	0.75	1.74 [1.163, 10.17]	
2005	Plasma	7594	310	R5	2.0	98.0	11.92 [11.92, 20.49]	ABC + FTC + NVP + LPV/r + SQV
	PBMC			X4	97.45	2.55	0.58 [0.5814, 99.56]	
2007	Plasma	1639	440	D/M	0	100	11.92 [11.92, 15.55]	FTC + TDF + ENF + ATV + DRV/r + ETR
	PBMC			D/M	100	0	1.89 [1.744, 1.890]	
2008-I	Plasma	4198	250	R5	0	100	11.92 [11.92, 11.92]	FTC + TDF + ATV + DRV/r + ETR
	PBMC			X4	96.4	3.6	1.31 [1.308, 10.17]	
2008-II	Plasma	1755	360	X4	100	0	1.16 [1.16, 1.16]	FTC + TDF + DRV/r + ETR + MVC + RAL
	PBMC			X4	100	0	1.74 [0.2907, 1.744]	

Patient CD4 counts, Viral Load (VL), tropism measured phenotypically (X4 or R5 or dual/mixed (D/M)), proportion of R5 and X4 454-pyrosequences expressed as Median FPR [min, max], and drug regimen at sampling time (ABC: abacavir; ddi: didanosine; FTC: emtricitabine; TDF: tenofovir disproxyl fumarate; NVP: nevirapine; EFV: efavirenz; ETR: etravirine; IDV: indinavir; NFV: nelfinavir; SQV: saquinavir; LPV/r: ritonavir-boosted lopinavir; APV: amprenavir; ATV: atazanavir; DRV/r: ritonavir-boosted darunavir; ENF: enfuvirtide; RAL: raltegravir).

declined (360 cells/mm^3) (Table 1) and the patient harbored strictly X4 viruses in plasma.

To gain insights into the mechanisms that led to treatment failure and to assess the role of minority and archived strains, the evolution and tropism of viral envelopes from longitudinal paired plasma- and Peripheral Blood Mononuclear Cell (PBMC)-derived samples was investigated.

As the main determinants of coreceptor usage have been mapped to the V3-loop (Hwang et al., 1991), 360 nucleotides spanning the V3-loop were amplified (in at least independent triplicates) from 1 ml of plasma or 5×10^5 PBMCs and pyrosequenced using the Roche FLX Titanium 454 platform with tagged patient-specific primers (forward primer: 5′-TACACATGGAATTAAACCAGT GG-3′ and reverse primer 5′-TAGGTCCCCTCCTGAGG-3′). A mean of 18,838 sequences (range 13,334–24,916) were generated per sample. Raw nucleotide sequences were compressed, filtered according to size (200 nucleotides < sequence < 500 nucleotides), aligned against HXB_2 and F1 reference strains, trimmed, then automatically processed to compensate for incorrect homopolymer counts (from $N \geqslant 3$) (Fischer et al., 2010) and carryover errors (within two bases) and finally further filtered to discard ambiguous reads and prematurely terminated sequences. A mean of 14,614 (9164–20,205) sequences were retained. The per-nucleotide error rate of the control sample (89% pNL4-3 (X4), 10% pYU-2 (R5) and 1% pAD8 (R5)) was 0.0099. Recombination due to reverse-transcription and PCR in the control sample was prominent in sequences <0.3%, therefore sequences representing less than 0.3% were excluded from further analysis. For genotypic analyses, tropism was predicted using $geno2pheno_{[coreceptor]}$ with a 10% false positive prediction rate. Tropism inferred by $geno2pheno_{[coreceptor]}$ was confirmed phenotypically using an in-house phenotypic Env-recombinant viral assay described previously (Baatz et al., 2011). Briefly, Env-recombinant viruses were produced trough homologous recombination between sample-derived *env* sequences and pNL4-3ΔEnv.luc, and used to infect U87.CD4 cells expressing either CXCR4 or CCR5. Genotypic and phenotypic results were concordant for all but one sample (2001) (Table 1), likely a consequence of PCR selection.

Prior to MVC treatment initiation, only R5 sequences were detected in plasma, while PBMCs harbored mainly X4 variants (Fig. 1 and Table 1). Phylogenetic analyses however indicated that the X4 MVC-escape sequences detected in plasma were unrelated to the pretreatment X4-proviruses, suggesting that the MVC-escape viruses did not emerge directly from the predominant contemporary PBMC reservoir. The major MVC-escape variant in both plasma and PBMCs was rather closely related to proviral sequences archived in earlier (2003 and 2007) PBMC samples (Fig. 1, 2003: pink, 2007: black, & Fig. S1), suggesting that MVC escape occurred through the emergence of minority archived X4 strains no longer detectable in the periphery, and further highlighting the importance of considering PBMC genotyping (Seclen et al., 2010). Although maraviroc escape has been previously described to occur mainly through the outgrowth of a minority X4 strains, most studies identified such minor variants in plasma or PBMCs drawn immediately prior to MVC-treatment onset (Archer et al., 2009; MacArthur and Novak, 2008; Westby et al., 2006).

Computational analysis of the plasma MVC-escape variant using the Recco software (Maydt and Lengauer, 2006), jpHMM (Schultz et al., 2009) and SimPlot v3.5.1 (Lole et al., 1999), revealed that this cluster was most likely ($p < 0.001$ according to Recco and jump probability set to 10^{-9} for jpHMM) a recombinant form including the V3-loop from a 2003-proviral sequence and the dominant sequence found in plasma prior to MVC-treatment onset (plasma 2007-2 and plasma sequences from 2008-I, 3′-portion of the sequence) (Table 2). Because the cross-over point of recombination lies near the 3′ end of the sequence, the plasma MVC-escape sequence appears to be related to the 2003-PBMC sample and very distant from the 2007/2008 strains circulating in plasma (Fig. 1). Sanger-sequencing of a longer Env fragment (HXB_2 nt 6601–7331) and SimPlot analysis further confirmed the mosaic pattern of the MVC-escape variant, consisting of the 2003-PBMC V3-loop inserted within the 2008-I dominant circulating strain sequence (data not shown). Here, recombination played a pivotal role as a source of diversity, representing a leap in genetic evolution leading to the inclusion of MVC-escape determinants (i.e. CXCR4-usage) within the viral genomic context most adapted to drug selective pressure, (Shi et al., 2010).

The 2003-PBMC sequence was itself phylogenetically related to X4 strains from a very heterogeneous, mixed (R5 + X4) quasispecies circulating in the earliest available plasma and PBMC samples

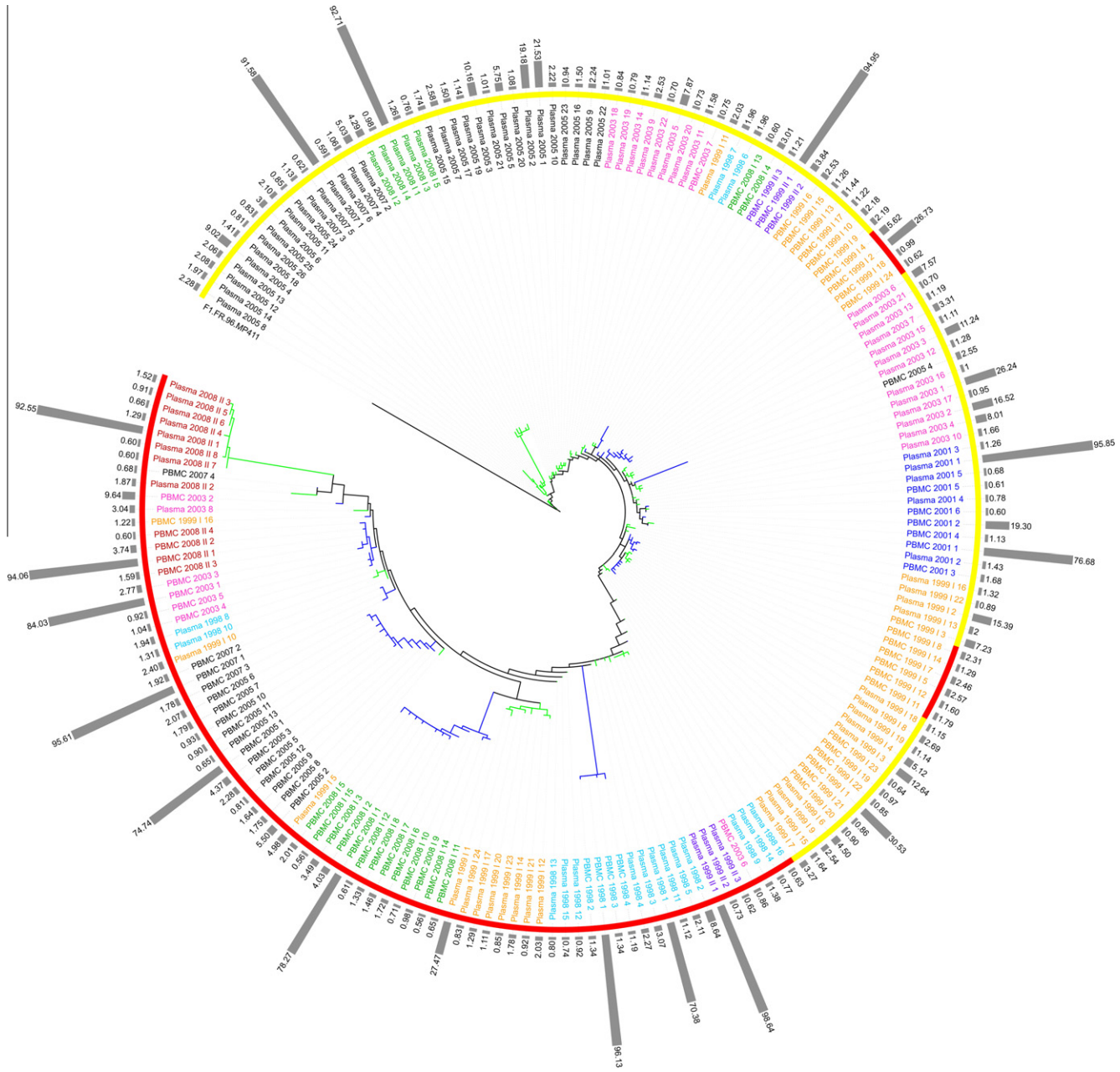

Fig. 1. Phylogenetic tree of patient plasma and PBMC samples from 1998 to MVC failure. The evolutionary model GTR + γ suggested to best fit the sequences by FindModel from the HIV database of the Los Alamos National Laboratory was used. The best fitted maximum likelihood phylogenetic tree, calculated with RAxML v7.2.8, was chosen among 500 distinct runs with complete random starting trees. The F1 reference strain F1.FR.96.MP411 was included as an outlier. Plasma samples are shown with green branches and PBMC samples with blue branches. The number after the year of sampling ranks the sequence. The percentage of detection of each sequence is shown as gray bars spiking from the tree, followed by its numerical value. Tropism, inferred by geno2pheno$_{[coreceptor]}$ with a FPR set at 10%, is shown as a colored ring circling the sample name: R5 strains are represented in yellow and X4 strains in red.

(1998 and 1999-I, light blue and orange in Fig. 1), but undetectable in plasma thereafter. These early diverse viral populations were scattered throughout the phylogenetic tree, without a net compartmentalization between both sources (Fig. 1). Following structured treatment interruptions and reinitiation of HAART concomitantly to IL-2 injections, these quasispecies underwent a severe bottleneck in both plasma and PBMCs (Fig. 1 & Fig. S1). The absence of viral rebound and the limited acquisition of resistance mutations within PR and RT during IL-2 therapy (not shown) suggest that resistance mutations and drug pressure alone unlikely accounted for such a drastic decimation of viral diversity. A variability model based on the pairwise maximum likelihood distances between unique/compressed sequences using RAxML confirmed that variability was drastically more constrained during IL-2 treatment than throughout regimen changes alone (not shown), suggesting that IL-2 might have contributed to quasispecies selection. Variability remained highly constrained throughout IL-2 treatment in both compartments and led to the transient outgrowth of one X4 population in plasma and of one discrete strictly R5 population from the PBMC-proviruses from one common ancestor (Fig. 1 1999-II sample, purple & Fig. S1). The X4 population boosted in plasma in 1999 led to an evolutionary dead-end (Fig. 1, purple) and was outcompeted by the R5 strains detected in both plasma and PBMCs drawn in 2001 (Fig. 1, blue), which then

Table 2
Sequence alignment of the PBMC 2003_2, of the pre-MVC (2008-I_1) and MVC-escape (2008-II_1) plasma samples.

Sample	Sequence
	10 20 30 40 50 60 70
Plasma 2008-II_1	AAACCAGTGG TATCAACTCA GTTGTTGTTA AATGGCAGCC TAGCAGAAGA AGATATAATA ATCAGATCTC
Plasma 2008-I_1	AAACCAGTGG TATCAACTCA GTTGTTGTTA AATGGCAGCC TAGCAGAAGA AGATATAGTA ATCAGATCTC
PBMC 2003_2	AAACCAGTGG TATCAACTCA GTTGTTGTTA AATGGCAGCC TAGCAGAAGA AGAGATAGTA ATCAGATCTC
	V3-loop start\
	80 90 100 110 120 130 140
Plasma 2008-II_1	AAAATATCTC AGATAATACA AAAACCATAA TAGTACACCT TAATGAATCT GTACAGATTA AT|TGCACAAG
Plasma 2008-I_1	AAAATATCTC AGATAATGCA AAAACAATAA TAGTACACCT TAATGAGTCT GTACAGATTA AT|TGTACAAG
PBMC 2003_2	AAAATATCTC AGATAATACA AAAACCATAA TAGTACACCT TAATGAATCT ATACAGATTA AT|TGTACAAG
	150 160 170 180 190 200 210
Plasma 2008-II_1	ACCCTACAGC AATACAATAA CAAAGATACC TGTAGGACCA GGACGAGCAT TTTATACCAC AGGAAAAATA
Plasma 2008-I_1	ACCTAACAAC AATACAAGAA AAAGTATAGC TTTAGGACCA GGACGAGCAT TTTATGCAAC AGGAGACATA
PBMC 2003_2	ACCCTACAGC AATTCAGTAA GAAAGATACC TGTAGGACCA GGACGAGCAT TTTATACCAC AGGAGAAATA
	/V3-loop end
	220 230 240 250 260 270 280
Plasma 2008-II_1	AATGGAAACA TCAGAAAGGC ATATTGC|AAC GTTAATGGAG CACAATGGAA TAAAACGTTA GAGAGGGTAA
Plasma 2008-I_1	ATAGGAGACA TCAGAAAGGC ACACTGT|AAC GTTAGTAGAA CACAATGGAA TAAAACGTTA GAAAGGGTAA
PBMC 2003_2	AACGGAAACA TCAGAAAGGC ACATTGC|AAC GTTAGTGGAA CACAATGGAA TAAAACGCTA GAACGGATAA
	290 300 310 320 330 340
Plasma 2008-II_1	GAACAAAGCTA AAGGAAAACC TAAATCTCTC TGATGCAAC AATAAAATTT AACTCATCCT CAGGAGG
Plasma 2008-I_1	GAGAAAAGCTA AAGGAAAAAC TAAATCTCCC TAATGCAAC AATAAACTTT AACTCATCCT CAGGAGG
PBMC 2003_2	AGGCAAAGTTA AAGTCTCCTT CTTATTTCCC TAATGCAAC AATAAAATTT AACTCATCCT CAGGAGG

The start and the end of the V3-loop are indicated. In the 2008-II_1 (MVC-escape) plasma sequence, nucleotides in black are conserved between all three sequences, nucleotides highlighted in red are identical to the PBMC 2003 sequence and nucleotides highlighted in green are identical to the pre-maraviroc sequence (Plasma 2008-I_1). Nucleotides highlighted in gray are different from both sequences, and are likely somatic mutations.

expanded and evolved in plasma from 2003 (Fig. 1, pink) on, until 2008, including the R5 pre-MVC sample subjected to the Trofile assay (2008-I, Fig. 1, green). BEAST analysis (Fig. S1) confirmed that the 2008-II X4 determinants of the MVC-escape variant were derived from the 2003-PBMCs. BEAST modeling also indicated that the X4 strain that was archived in the 2003-PBMC proviruses and persisted in PBMCs until the 2008-I sample "switched" from plasma to PBMCs early in 1999 and that the branch leading to nearly all viral strains detected in plasma after 1999 "switched" from PBMCs to plasma between mid-1999 and early 2000.

It is conceivable that IL-2 boosts initially induced both R5 and X4 strains, or that IL-2 activation might have rendered the low division CXCR4-expressing naïve $CD4^+$ T-cells more permissive to infection and more prone to support viral replication, providing X4 viruses with new target cells. X4 strains, which are more rapidly countered by HAART, might however have been counter-selected and outcompeted by R5 strains in plasma (Philpott et al., 2001). This is, to our knowledge, the first report of the involvement of IL-2 treatment on viral diversity and on shaping viral quasispecies and reservoirs.

Although no longer detectable in plasma, early pre-IL-2 treatment X4 strains remained archived in PBMCs (1998, light blue and 1999-I, orange), where they likely persisted throughout the years, in the periphery (detected in 2003, 2005, 2007) or in lymphoid organs. They were then rescued (at least partially) under MVC pressure to provide X4-determinants to an otherwise adapted circulating strain that was dominant prior to MVC-treatment onset. Although frequent in HIV infection, recombination requires infection of an actively replicating cell by two different strains. Accordingly, the V3 sequences detected in PBMCs after MVC escape (PBMC-2008-II, red) were also closely related to most 2003-PBMC sequences, strongly suggesting that this dormant or archived pool was activated during MVC treatment. As MVC has been suggested to increase circulating $CD4^+$ T-cell counts (Asmuth et al., 2010; Funderburg et al., 2010), MVC-based HAART may have contributed to the availability of long-time archived viral strains from some lymphoid organ, although, in this study we could not evaluate the origin of these archived PBMCs nor the direct contribution of strains archived in solid lymphoid tissues. Nonetheless, the inclusion of determinants from a long-time archived strain rather than from a contemporary minority strain underscores that the entire pool of archived viruses, even when they are no longer detectable in plasma or in circulating PBMCs, can be rescued by MVC and contribute determinants of resistance.

Ethical statement

This study was approved by the Luxembourg Comité National d'Ethique de Recherche and the patient provided informed consent to this study.

Conflicts of interest

All authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.antiviral.2011.10.003.

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