Title

Dependence on the CCR5 Coreceptor for Viral Replication Explains the Lack of Rebound of CXCR4-Predicted HIV Variants in the Berlin Patient

Authors

Symons, Jori
Vandekerckhove, Linos
Hütter, Gero
et al.

Dependence on the CCR5 Coreceptor for Viral Replication Explains the Lack of Rebound of CXCR4-Predicted HIV Variants in the Berlin Patient

Jori Symons,[1] Linos Vandekerckhove,[2] Gero Hütter,[3] Annemarie M. J. Wensing,[1] Petra M. van Ham,[1] Steven G. Deeks,[4] and Monique Nijhuis[1]

[1]Department of Medical Microbiology and Virology, University Medical Center, Utrecht, The Netherlands; [2]Department of Internal Medicine, Infectious Diseases and Psychosomatic Medicine, Ghent University Hospital, Belgium; [3]Institute of Transfusion Medicine and Immunology, Heidelberg, Germany; and [4]Department of Medicine, University of California, San Francisco

The "Berlin patient" is the first patient cured of HIV-1 infection after allogeneic transplantation with nonfunctional CCR5 coreceptor stem cells. We demonstrate that CXCR4-predicted minority viruses present prior to transplantation were unable to rebound after transplantation due to their dependence on CCR5 for replication and high genetic barrier toward CXCR4 usage.

Keywords.   HIV; cure; Berlin-Patient; coreceptor; stem cell transplantation.

The "Berlin patient" is the first individual in whom human immunodeficiency virus (HIV) type 1 (HIV-1) infection has been cured following allogeneic transplantation to treat acute myeloid leukemia [1, 2]. The stem cells used were homozygous for the Δ32 frameshift mutation (CCR5Δ32), resulting in absence of the CCR5 receptor at the cellular surface [3]. Heterozygosis for CCR5Δ32 is associated with slower disease progression, and individuals homozygous for CCR5Δ32 are naturally resistant to CCR5-tropic HIV [3]. Binding of the viral envelope glycoprotein gp120 to a coreceptor (CCR5 or CXCR4) is essential for HIV entry into CD4^{+} host cells. HIV coreceptor tropism is mainly determined by the third hypervariable loop of the viral envelope (gp120-V3) [4].

The Berlin patient had a plasma viral load of 6.9×10^6 copies/mL during an episode of treatment interruption prior to stem cell transplantation (SCT). The viral population was predicted to be CCR5-tropic based on standard genotypic tropism testing (Geno2Pheno[coreceptor] false-positive rate [FPR] 24.2%) [1]. Detailed analysis using ultradeep sequencing detected a 2.9% minority viral population predicted to be CXCR4-tropic (Geno2Pheno[coreceptor] FPR range, 2.7%–9.3%). Two months after SCT, successful reconstitution of CD4^{+} T cells with the homozygous CCR5Δ32 phenotype was observed [1]. These donor derived T cells displayed normal levels of CXCR4 coreceptor surface expression. Given the detection of CXCR4-predicted viral variants prior to SCT, one would expect these variants to appear after SCT, especially as antiretroviral therapy was discontinued during the transplant procedure. However, no viral rebound was observed, and the patient remained free of HIV infection for >5 years post-SCT [5].

Previously, we demonstrated that some viruses capable of using CXCR4 have a clear CCR5 coreceptor preference in vivo [6]. Based on these findings, we postulate that a rebound of the CXCR4-predicted variants in the Berlin patient did not occur due to dependence on the CCR5 coreceptor for viral replication.

METHODS

HIV coreceptor tropism was assessed in silico using 4 different prediction tools: Geno2Pheno[coreceptor] [7], position-specific scoring matrices Web PSSM$_{X4-R5}$ [8], the 11–25 rule [9], and net charge [10–12] (Supplementary Methods). The result of the Geno2Pheno[coreceptor] interpretation is given as a quantitative value, the FPR, which defines the probability of classifying a CCR5-predicted virus falsely as CXCR4-predicted variant. The Geno2Pheno[coreceptor] system is based on a statistical learning method called a support vector machine that is trained with a set of nucleotide sequences with corresponding exclusive CCR5- or CXCR4-capable phenotypes [11].

To investigate viral replicative capacity and coreceptor dependence, we generated recombinant viruses. Because full envelope sequences were not generated prior to SCT, we cloned patient-derived gp120-V3 loop sequences of the CXCR4-predicted viruses and the most dominant CCR5-predicted strain of the

DOI: 10.1093/cid/ciu284

patient in the background of a CXCR4-using laboratory reference strain (pHXB2-Δgp120-V3). As a control, we also introduced gp120-V3 of HXB2 or CCR5-tropic BaL in the background of pHXB2-Δgp120-V3 (Supplementary Methods, Table 1). Coreceptor preference of the recombinant viruses was assessed in T cells (U373-MAGI-CCR5E and U373-MAGI-CXCR4$_{CEM}$ cell lines [13] expressing CD4$^+$CCR5$^+$CXCR4$^-$ or CD4$^+$CCR5$^-$CXCR4$^+$) or in natural target cells (healthy donor peripheral blood mononuclear cells [PBMCs] or patient-derived PBMCs post-SCT) in the presence or absence of coreceptor inhibitors (Supplementary Methods).

Target cell selection assays were performed to investigate evolution toward CXCR4 usage. Recombinant CXCR4-predicted viral variants (X2bp and X3bp) and CCR5-predicted variant Rbp or the cBaL control virus were continuously cultured in an increasing percentage of U373-MAGI-CXCR4$_{CEM}$ cells (Supplementary Methods).

RESULTS

The 2.9% CXCR4-predicted minority of the viral population observed prior to SCT consisted of 7 unique variants with an FPR ranging from 2.7% to 9.3% (X1bp–X7bp). In addition to Geno2Pheno[$_{coreceptor}$] [11], we used 3 other prediction algorithms (position-specific scoring matrix [PSSM$_{X4-R5}$], net charge rule, and 11/25 rule) [12] to assess coreceptor tropism (Figure 1). Of the 7 variants predicted to be CXCR4-tropic by Geno2Pheno[$_{coreceptor}$], only 1 strain (X3bp) was predicted to use CXCR4 in all other prediction tools. This viral variant harbored the positively charged amino acid lysine at position 25 of the gp120-V3 loop, which is strongly associated with CXCR4 usage [14].

We investigated viral replicative capacity of these variants by cloning of the patient-derived gp120-V3 loop sequence in the backbone of the CXCR4-tropic HXB2 reference strain. The dominant CCR5-predicted and 5 of the 7 CXCR4-predicted strains among which the single viral variant predicted to be

CXCR4-tropic by all 4 algorithms (X3bp) were replication competent (Figure 2A).

Subsequently, coreceptor usage was assessed in MAGI cell lines expressing either CCR5$^+$ or CXCR4$^+$ as coreceptor [13]. The dominant CCR5-predicted strain from the patient (Rbp) and the CCR5-tropic control virus (cBaL) were able to infect the cells expressing CCR5 but not those expressing CXCR4 (Figure 2B). The CXCR4-tropic reference strain (cHXB2) was only able to infect CXCR4 expressing cells reflecting a full CXCR4 phenotype. Remarkably, all patient-derived CXCR4-predicted variants, including X3bp, replicated only in cells expressing CCR5, demonstrating CCR5 dependence (Figure 2B).

Because coreceptor preference may differ in cell lines vs natural target cells that express both coreceptors, we also assessed coreceptor usage in PBMCs [6]. We used maraviroc (MVC), a CCR5 antagonist, to mimic the CCR5Δ32/Δ32 phenotype. Likewise, we used AMD-3100 to block CXCR4 coreceptor binding. As expected, the dominant patient-derived CCR5-predicted strain and cBaL were fully inhibited by MVC and not by AMD-3100 (Figure 2C), reflecting a full CCR5 phenotype. The control virus cHXB2 was completely inhibited by AMD-3100, and no effect of MVC was observed (Figure 2C). Interestingly, the CXCR4-predicted variants were not inhibited by AMD-3100 but demonstrated CCR5 dependence in natural target cells, as shown by complete inhibition of replication by MVC (Figure 2C).

Finally, we investigated whether the viral constructs could replicate in posttransplant-derived CCR5-Δ32/Δ32 PBMCs of the Berlin patient. These experiments showed that the Berlin patient's new target cells can be infected by a CXCR4-tropic reference strain, but not with the pre-SCT patient-derived viruses (Figure 2D).

Given the presence of long-lived CCR5-positive macrophages [2] we explored the potential to evolve toward CXCR4 usage by performing cell selection experiments. The CCR5-tropic control virus cBaL and the patient-derived Rbp, X2bp, and X3bp were cultured in the presence of increasing percentages of CXCR4-expressing cells (0%–90%). After 10 weeks of selection,

Viral construct	1	2	3	4	5	6	7	8	9	10	11	12	13	14	14;1	14;2	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	g2p	PSSM	CHARGE	11/25
Rbp	C	T	R	P	N	N	N	T	R	K	G	I	H	I	-	-	G	P	G	R	A	F	Y	T	T	G	E	I	M	G	D	I	R	Q	A	H	C	24,2	-10,55	3,1	R5
X1bp	·	A	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	9,3	-10,35	3,1	R5
X2bp	·	·	·	·	·	·	K	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	2,7	-8,09	4,1	R5
X3bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	K	·	·	·	·	·	·	·	·	·	·	5,0	-6,27	5,1	X4
X4bp	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	R	·	·	·	·	6,8	-9,32	4,1	R5
X5bp	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	8,5	-9,88	3,1	R5
X6bp	·	·	·	·	·	·	·	D	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	7,8	-9,19	2,1	R5
X7bp	·	·	·	·	·	·	·	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	5,3	-8,45	3,1	R5
cBaL	·	·	·	·	·	·	·	·	·	·	S	·	·	·	·	·	·	·	·	·	·	L	·	·	·	·	·	·	I	·	·	·	·	·	·	·	·	51,8	-9,79	3,1	R5
cHXB2	·	·	·	·	·	·	·	·	·	·	·	·	R	·	Q	R	·	·	·	·	·	·	V	·	I	·	K	·	I	·	N	M	·	·	·	·	·	0,0	3,27	10,0	X4

Figure 1. Coreceptor tropism prediction of patient derived gp120-V3 loop sequences obtained prior to stem cell transplantation. Schematic representation of gp120-V3 loop amino acid sequences of the patient-derived dominant CCR5-predicted variant (Rbp) and CXCR4-predicted minority variants, X1bp–X7bp, and the control viruses cBaL and cHXB2. Genotypic tropism prediction by Geno2Pheno[$_{coreceptor}$] (10% false-positive rate) [7], PSSM$_{X4-R5}$, net charge rule, and 11/25 is given. CCR5 prediction is indicated in green; CXCR4 prediction in red.
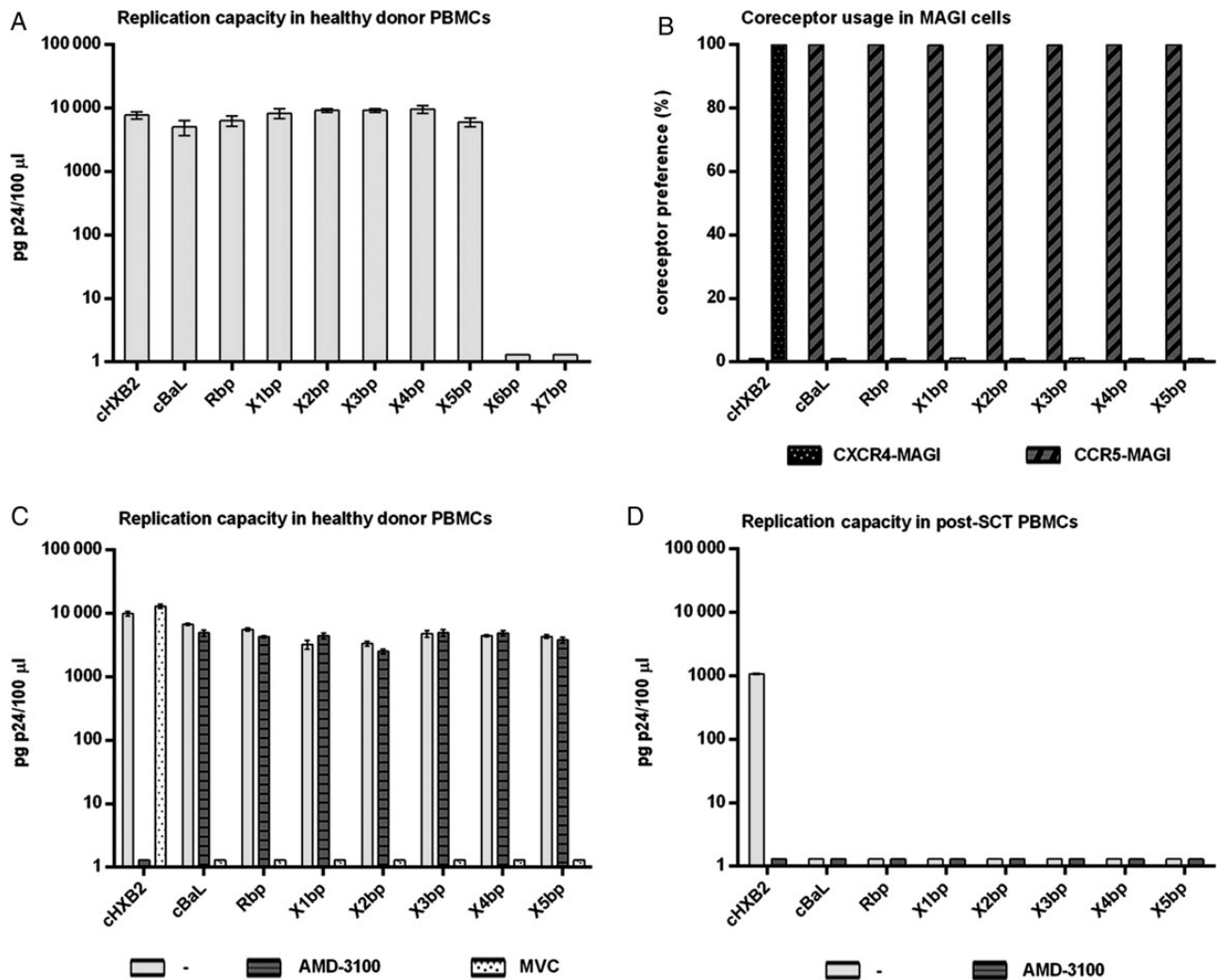
Figure 2. Replication capacity and phenotypic coreceptor usage. *A*, Replication capacity analysis in peripheral blood mononuclear cells (PBMCs) from healthy donors expressing both coreceptors CCR5 and CXCR4 (picogram [pg] p24/100 μL supernatant at day 7). *B*, Coreceptor usage was determined by expressing the percentage of viral entry in CXCR4 or CCR5 U373-MAGI cells. *C*, Replication capacity in PBMCs from healthy donors with 1 μM MVC (CCR5 antagonist) or 10 μM AMD-3100 (CXCR4 antagonist) or without inhibitor (pg p24/100 μL supernatant at day 7). *D*, Replication capacity in patient-derived PBMCs obtained after allogeneic homozygous CCR5-Δ32 stem cell transplantation (SCT) as tested with 10 μM AMD-3100 or without inhibitor (pg p24/100 μL supernatant at day 7). cHXB2 and cBal were used as control viruses. Rbp is the dominant CCR5-predicted viral construct and X1bp–X5bp are the viable Geno2Pheno$_{[coreceptor]}$ CXCR4-predicted viral constructs. Error bars are standard deviation of quadruplicate wells. All experiments were performed at least twice, and the figures are based on 1 representative experiment.

no coreceptor switch was observed, suggesting a relatively high genetic barrier toward CXCR4 usage.

DISCUSSION

The Berlin patient is the first patient with a cured HIV infection. Prior to SCT, the viral population was genotyped and a minority population was predicted to use the alternative CXCR4 coreceptor in a Web-based algorithm (Geno2Pheno$_{[coreceptor]}$) [1]. Considering the normal levels of CXCR4 coreceptor expression

on the donor-derived cells and susceptibility of these cells for CXCR4-tropic virus, it was remarkable that HIV did not rebound post-SCT in the absence of combination antiretroviral therapy (cART) [2]. Our study demonstrates that CXCR4-predicted minority viruses present prior to transplant were unable to rebound after transplant due to their dependence on CCR5 for replication and a high genetic barrier toward CXCR4 usage.

The main determinant of coreceptor usage is the gp120-V3 loop. Genotypic prediction algorithms, such as

Geno2Pheno[coreceptor], use this region to predict coreceptor tropism. The result of the Geno2Pheno[coreceptor] interpretation is given as a quantitative value, the FPR, which defines the probability of classifying a CCR5-predicted virus falsely as CXCR4-predicted variant. Varying the threshold value for FPR classification changes the sensitivity and specificity for CXCR4 tropism prediction. In clinical practice, conservative cutoff FPR values that show good correlations with virological outcome during CCR5 inhibitor–based therapy are generally applied to prevent underestimation of the presence of CXCR4-tropic viruses (FPR cutoffs 3.5%–10%) [11, 15]. The lowest FPR of the minority variants in the Berlin patient prior to SCT was 2.7%. Although this is below the lowest cutoff FPR suggested for tropism prediction based on deep sequencing (3.5%) and therefore genotypically predicted to be CXCR4-tropic, the variant was dependent on CCR5 for viral entry in phenotypic assays. This single case study shows that predicted genotypic coreceptor tropism may not always reflect biological behavior and suggests that larger studies are needed to explore the use a lower cutoff FPR for maraviroc eligibility in clinical practice. In case of a CCR5Δ32-SCT procedure, a more lenient FPR and/or phenotypic testing should be considered.

Envelope regions outside the gp120-V3 sequence can modulate coreceptor affinity [16], and commercial phenotypic coreceptor usage assays are based on (near) full-length envelope sequences representing the plasma population virus [11]. Unfortunately, prior to SCT, full envelope sequences were not generated from the Berlin patient, and no additional samples were stored to enable full envelope sequencing. Therefore, we were inherently limited in assessment of the coreceptor phenotype of the minority variants and were restricted to the V3 loop sequences generated by ultradeep sequencing prior to SCT. In absence of patient-derived full envelope sequences, we decided to clone the gp120-V3 loop sequences of the Berlin patient in the background of a CXCR4-tropic HIV-1 laboratory strain to limit bias for CCR5 usage.

In the Berlin patient, CCR5-tropic permissive cells could still be detected for at least 5.5 months in the colon, and proviral DNA was observed 2 months after SCT [1, 2]. More than 5 years post-SCT, extremely low levels of HIV DNA and RNA were intermittently detected using very sensitive assays [5]. Given the volatile combination of long-lived CCR5-expressing cells and a potential CCR5-tropic reservoir, HIV replication could potentially have continued. Residual replication of CCR5-tropic viruses in the setting of increasing numbers of CCR5⁻CXCR4⁺ cells may then result in evolution toward CXCR4 usage. We tested the evolution potential for the most CXCR4-predicted patient-derived viruses and did not observe viral evolution toward CXCR4 usage, suggesting a relatively high barrier for coreceptor switch. Moreover, it has also been hypothesized that the number of residual CCR5-expressing

CD4⁺ cells after SCT was too low to support replication of the CCR5-tropic variants and therefore evolution toward CXCR4 usage [17].

A recent study in which 2 HIV-infected heterozygote CCR5Δ32 patients with a small viral reservoir received a CCR5WT/WT transplant in the presence of cART unfortunately demonstrated viral rebound after interruption of cART [18, 19]. In the Berlin patient, aside from absence of CXCR4-tropic virus, the lack of a rebound of CCR5-tropic virus immediately post-SCT in the absence of cART indicates that transplantation of CCR5Δ32/Δ32 stem cells was pivotal to the apparent cure of HIV. These results provide a rationale for CCR5-based SCT and gene therapy studies in which in-depth analysis of HIV coreceptor usage is essential.

Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online (http://cid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

References

1. Hütter G, Nowak D, Mossner M, et al. Long-term control of HIV by CCR5 delta32/delta32 stem-cell transplantation. N Engl J Med 2009; 360:692–8.
2. Allers K, Hütter G, Hofmann J, et al. Evidence for the cure of HIV infection by CCR5Δ32/Δ32 stem cell transplantation. Blood 2001; 117:2791–9.
3. Samson M, Libert F, Doranz BJ, et al. Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature 1996; 382:722–5.
4. Cardozo T, Kimura T, Philpott S, Weiser B, Burger H, Zolla-Pazner S. Structural basis for coreceptor selectivity by the HIV type 1 V3 loop. AIDS Res Hum Retroviruses 2007; 23:415–26.
5. Yukl SA, Boritz E, Dahl V, et al. Challenges in detecting HIV persistence during potentially curative interventions: a study of the Berlin patient. PLoS Pathogens 2013; 9:e1003347.
6. Symons J, Lelyveld SFL, Hoepelman AIM, et al. Maraviroc is able to inhibit dual-R5 viruses in a dual/mixed HIV-1-infected patient. J Antimicrob Chemother 2011; 66:890–5.
7. Sing T, Low AJ, Beerenwinkel N, et al. Predicting HIV coreceptor usage on the basis of genetic and clinical covariates. Antiviral Ther 2007; 12:1097–106.

8. Jensen MA, Li FS, van 't Wout AB, et al. Improved coreceptor usage prediction and genotypic monitoring of R5-to-X4 transition by motif analysis of human immunodeficiency virus type 1 *env* V3 loop sequences. J Virol **2003**; 77:13376–88.

9. Isaka Y, Sato A, Miki S, et al. Small amino acid changes in the V3 loop of human immunodeficiency virus type 2 determines the coreceptor usage for CXCR4 and CCR5. Virol **1999**; 264:237–43.

10. Briggs DR, Tuttle DL, Slealsman JW, Goodenow MM. Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages). AIDS **2000**; 14:2937–9.

11. Vandekerckhove LPR, Wensing AMJ, Kaiser R, et al. European Consensus Group on Clinical Management of Tropism Testing. European guidelines on the clinical management of HIV-1 tropism testing. Lancet Infect Dis **2011**; 11:394–407.

12. Garrido C, Roulet V, Chueca N, et al. Evaluation of eight different bioinformatics tools to predict viral tropism in different human immunodeficiency virus type 1 subtypes. J Clin Microbiol **2008**; 46:887–91.

13. Vodicka MA, Goh WC, Wu LI, et al. Indicator cell lines for detection of primary strains of human and simian immunodeficiency viruses. Virology **1997**; 233:193–8.

14. Fouchier RA, Groenink M, Kootstra NA, et al. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J Virol **1992**; 66:3183–7.

15. Swenson LC, Mo T, Dong WWY, et al. Deep V3 sequencing for HIV type 1 tropism in treatment-naive patients: a reanalysis of the MERIT trial of MVC. Clin Infect Dis **2011**; 53:732–42.

16. Ogert RAM, Lee K, Ross W, Buckler-White A, Martin MA, Cho MW. N-linked glycosylation sites adjacent to and within the V1/V2 and the V3 loops of dualtropic human immunodeficiency virus type 1 isolate DH12 gp120 affect coreceptor usage and cellular tropism. J Virol **2001**; 75:5998–6006.

17. Deeks SG, McCUne JM. Can HIV be cured with stem cell therapy? Nat Biotech **2010**; 28:807–10.

18. Henrich TJ, Hu Z, Li JZ, et al. Long-term reduction in peripheral blood HIV type 1 reservoirs following reduced-intensity conditioning allogeneic stem cell transplantation. J Infect Dis **2013**; 207: 1694–702.

19. Henrich TJ. Challenges and strategies towards functional cure: how low do you need to go. Sixth International Workshop on HIV Persistence during Therapy, Miami, USA, **2013** [abstract 45].