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R5 Macrophage-Tropic HIV-1 in the Male Genital Tract

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The entry tropism of HIV-1 Env proteins from virus isolated from the blood and genital tract of five men with compartmentalized lineages was determined. The Env proteins isolated from the genital tract of subject C018 were macrophage-tropic proteins, while the remaining cloned *env* genes encoded R5 T cell-tropic proteins. The detection of a macrophage-tropic lineage of HIV-1 within the male genital tract strongly suggests that evolution of macrophage-tropic viruses can occur in anatomically isolated sites outside the central nervous system.

Human immunodeficiency virus type 1 (HIV-1) develops considerable genetic diversity within an infected person. Typically, this diversity has been analyzed as that of the viral population in the blood; however, virus can also be detected in other bodily fluids (reviewed in reference 1). Most virus detected in the blood is likely produced in lymphoid tissues that are not themselves easily sampled; thus, our knowledge of HIV-1 diversity across tissues is limited (2). Furthermore, this limitation affects our ability to analyze the possible unequal distributions of viral diversity throughout the infected host, which could affect treatment and cure efforts. Virus in the blood typically requires high levels of CD4 and uses the CCR5 coreceptor (R5 T cell-tropic), with virus that can use the CXCR4 receptor evolving later in disease (X4 T cell-tropic) (3–5). Evolution resulting in the ability to use low levels of CD4 is associated with the ability to enter macrophages, and viruses with this phenotype ($CD4^{low}$/macrophage-tropic) are typically found late in disease in some subjects and within the central nervous system (CNS) (6). In this current study, we investigated viral tropism in 5 previously identified subjects with compartmentalized HIV-1 lineages in semen relative to blood (7). We show in one subject a clear example of a macrophage-tropic lineage of HIV-1 within the male genital tract. This observation demonstrates that macrophage-tropic viruses can occur in anatomical sites outside the CNS.

HIV-1 was analyzed from subjects who were recruited and who consented to participation and enrolled for a previous study (7, 8). $CD4^+$ T cell levels, viral loads, and GenBank accession numbers for the analyzed envelope genes are presented in Table 1. Previous analysis of viral RNA sequences in the seminal and blood plasma samples, performed first using a V3-specific heteroduplex tracking assay and then, more recently, using endpoint dilution PCR (i.e., single-genome amplification [SGA]) and sequence analysis, showed that a group of the seminal plasma-derived viral sequences from each of these five subjects formed a lineage genetically distinct from those of virus in the blood of the same subject (7, 9). Now we have performed phenotypic analysis of a subset of the full-length *env* genes amplified from each subject to determine the entry phenotype with respect to the ability to enter cells efficiently using low levels of CD4.

Representative *env* gene amplicons from the major viral population lineages in the blood and semen of all 5 subjects were selected for cloning and phenotypic analysis as pseudotyped virus. Briefly, the *env* gene was cloned into an expression vector, and each clone was cotransfected with the pNL4-3.LucR-E reporter backbone plasmid into 293T cells to generate an Env-pseudotyped luciferase reporter virus as described previously (6, 9, 10). Viral tropism was evaluated based on the ability to infect Affinofile cells expressing either high or low levels of CD4. Comparing infection levels of Affinofile cells expressing high versus low levels of surface CD4 provides a consistent assessment of the evolutionary step required to efficiently infect macrophages, i.e., the ability to infect cells with a low density of CD4 (6). When the level of residual infectivity of $CD4^{low}$ cells was 10% that of $CD4^{high}$ cells or greater, this was scored as a $CD4^{low}$/macrophage-tropic entry phenotype. Relative infectivity of $CD4^{low}$ cells of 2% or less indicated that the virus was T cell-tropic, and relative infectivity between those bounds constituted intermediate tropism. Viruses evolving the ability to infect cells with a low density of CD4 also become sensitive to neutralization by soluble CD4 (sCD4) early in the process (11–14). Thus, sensitivity to neutralization by sCD4 can be used to confirm macrophage tropism (15) by testing the sensitivity of infection to the presence of 5.0 μg/ml sCD4 when infecting TZM-bl cells. This amount of sCD4 efficiently inhibits the sensitive M-tropic viruses, while R5 T cell-tropic viruses are relatively resistant to this concentration.

The entry phenotype of the semen-compartmentalized viral population in all five subjects was initially established by assessing the tropism of one to three Env proteins from both the blood and seminal plasma samples (Fig. 1). This initial analysis

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TABLE 1 Patient cohort to analyze blood- and semen-compartmentalized Env tropism[a]

PID	Subtype	VL (copies/ml)[b]		CD4 count[c]	Amplicon ID	Analyzed *env* gene GenBank accession no.
		Blood	Semen			
701010380	B	5.1	3.6	129	0380_A7_S	HM638612
					0380_E4_S	HM638615
					0380_G3_B	HM638584
					0380_D9_B	HM638588
C018	C	6.2	6.4	116	C018_G11_S	HM638854
					C018_B2_B	HM638857
					C018_C6_B	HM638876
					C018_A7_B	New sequence
					C018_A6_B	New sequence
					C018_A2_B	New sequence
					C018_F3_B	New sequence
					C018_E10_S	HM638843
					C018_B11_S	HM638829
					C018_E8_S	New sequence
					C018_H5_S	New sequence
					C018_E5_S	HM638845
					C018_C9_S	HM638837
					C018_C11_S	HM638833
					C018_C10_S	HM638832
					C018_E8_S	HM638848
					C018_E6_S	HM638846
					C018_E9_S	HM638849
C047	C	5.1	5.3	291	C047_B11_S	HM638981
					C047_C7_S	HM638981
					C047_C6_B	HM638965
					C047_H7_B	HM639000
					C047_D3_B	HM639008
C083	C	4.8	5.4	423	C083_D12_S	HM639074
					C083_D9_B	HM639109
					C083_B11_B	HM639107
C113	C	4.6	4.6	279	C113_E4_S	HM639256
					C113_H10_S	HM639233
					C113_B8_B	HM639213
					C113_E11_B	HM639205

[a] PID, patient identifier; VL, viral load; Amplicon ID, amplicon identifier.
[b] Data represent $\log_{10}$ of the HIV-1 RNA load.
[c] Data represent numbers of $CD4^+$ T lymphocytes per cubic millimeter.

showed C018 to be the only subject with macrophage-tropic virus, which was found only in the semen. To confirm this finding, we reexamined multiple additional sequences from this subject, creating a more extensive sampling of viral proteins for entry phenotype analysis (Fig. 2). Clones were generated for 12 viral *env* gene amplicons from the seminal plasma and for 4 *env* gene amplicons from the subject's blood plasma. By this measure, clones from five *env* genes from the seminal plasma compartmentalized lineage-encoded Env proteins that were macrophage-tropic, with relative infectivity values ranging from 18% to 25% (Fig. 3A). One amplicon from the seminal plasma encoded a protein that displayed an intermediate tropism phenotype, with relative infectivity of 8%. The remaining *env* clones, six from seminal plasma and four from blood plasma (all grouping together in Fig. 2) encoded proteins that were T cell-tropic with a relative infectivity of less than 2% on $CD4^{low}$ cells (Fig. 3A). All of the pseudotyped viruses tested used CCR5 and not CXCR4 as a coreceptor for entry (data not shown).

Pseudotyped viruses from C018 were also evaluated for sensitivity to sCD4 (Fig. 3B). The presence of a low concentration of sCD4 significantly inhibited the infectivity of the pseudotyped viruses generated from the five macrophage-tropic viruses discussed above. In contrast, the 10 R5 T cell-tropic virus strains (6 from seminal plasma and 4 from blood plasma) were relatively resistant to sCD4 at the concentration tested. The clone with the intermediate entry phenotype showed intermediate sensitivity to sCD4 and represented a distinct lineage within the seminal plasma (Fig. 2).

HIV-1 from subject C018 constitutes the first clear demonstration of a population of macrophage-tropic virus outside the CNS in humans using the strict criteria of (i) *env* genes generated by endpoint dilution PCR, (ii) a genetically compartmentalized population, (iii) an entry phenotype determined using a well-controlled experimental approach where CD4 utilization can be assessed, and (iv) an entry phenotype confirmed by increased sensitivity to sCD4. The male genital tract has been shown to undergo depletion of $CD4^+$ T cells during chronic HIV-1 infection, and macrophages have been suggested to be an alternative host cell (7, 16), which could explain the emergence of a macrophage-tropic viral population in this subject, who had low levels of $CD4^+$ T cells in the blood at the time of sampling (116/μl; Table 1). Macrophage-tropic viruses, as determined by a capacity to infect human macrophages, have previously been identified in human lymphoid tissue from patients with HIV-associated dementia, but macrophage entry did not correlate with sCD4 sensitivity (17, 18). Significant infection of macrophages in peripheral tissues of macaques has

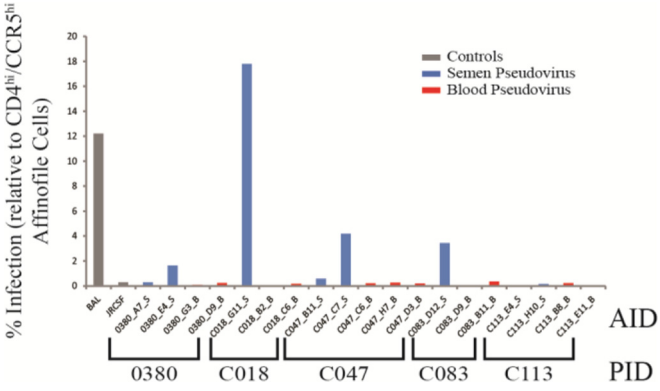
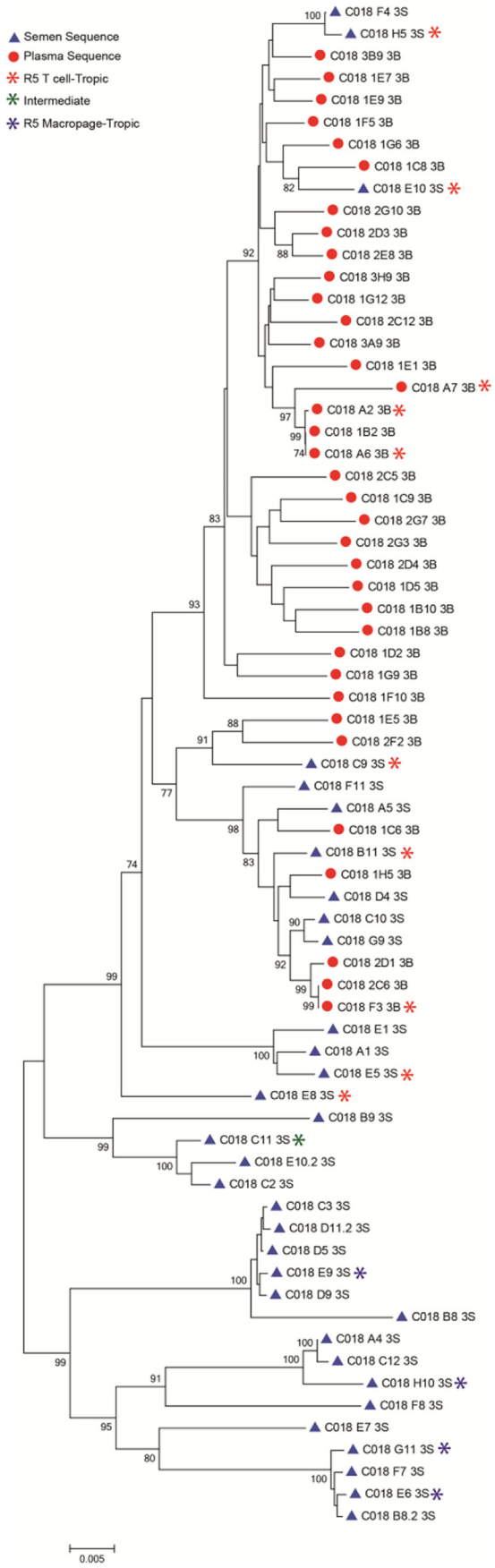


FIG 1 Tissue culture assays using pseudoviruses to define viral entry tropism phenotypes. Relative levels of infectivity of pseudoviruses (represented by blue bars for seminal Env and red bars for plasma Env) were determined by using Affinofile cells expressing low levels of CD4 compared to the level of pseudovirus infection of Affinofile cells induced to express high levels of CD4. Macrophage tropism is defined by relative infectivity of greater than ~10%, while T cell tropism is defined by relative infectivity of less than 2%. Relative infectivity from 2% to ~10% constitutes intermediate tropism. Results show that only 1 Env from the semen of C018 showed macrophage tropism and that C047 and C083 had 1 intermediate-tropism Env from the semen whereas all other Env showed T cell-tropic phenotypes from both the blood and semen. BAL, BaL viral Env protein; JRCSF, viral Env protein; AID, amplicon identifier; PID, patient identifier.



been described during late-stage infection with a highly pathogenic X4 simian-human immunodeficiency virus (SHIV) and early in infection with simian immunodeficiency virus (SIV) when the animals were depleted of $CD4^+$ T cells using an anti-CD4 antibody (2, 19). Collectively, these studies suggest that macrophage-tropic viruses evolve when $CD4^+$ T cells become limiting. In the case of subject C018, this limitation may have occurred in the genital tract tissue, in the absence of significant mixing of this local virus with the blood. Our study identified only one of five (20%) subjects who had compartmentalized virus in the male genital tract as having macrophage-tropic virus. We observed that two other subjects, C047 and C083, had *env* genes that encoded proteins with an intermediate entry phenotype, which could be evidence of the early steps of evolution toward macrophage tropism. There is likely to be interplay between $CD4^+$ T cell migration into tissue, the local immune response, and the level of local viral replication in the tissue that determines if and when macrophage-tropic viruses evolve, although this sampling of five subjects with compartmentalized virus is too small to define the individual contributions of these factors.

The correlation between the ability to efficiently infect Affinofile cells expressing low levels of CD4 and sensitivity to inhibition of infectivity by sCD4 identifies these viruses in the male genital tract of this subject as macrophage-tropic. This shows that macrophage-tropic viruses can be found in other tissues, outside the CNS. Additionally, these distinct evolutionary variants are present in the male genital tract and could be transmitted (although this has not been observed [20, 21]) or contribute to additional pathogenic mechanisms that would be distinct for each tissue where such viruses evolve. At present, we do not know if these viruses evolved in the genital tract to become macrophage-tropic or if this evolution took place first within the CNS and the virus migrated to another $CD4^+$ T cell-poor environment.

FIG 2 Phylogenetic analysis of *env* sequences reveals compartmentalization between the blood and seminal plasma. A neighbor-joining tree, created using MEGA, of the sequences produced using SGA is shown. Filled blue triangles represent *env* sequences from seminal plasma, and filled red circles represent *env* sequences from blood plasma. The clustering of seminal plasma *env* sequences into separate lineages and results of previously described compartmentalization tests show distinct viral evolution within the male genital tract. Cloned and tropism-analyzed *env* sequences are marked with an asterisk (*) beside each label as indicated in the key.

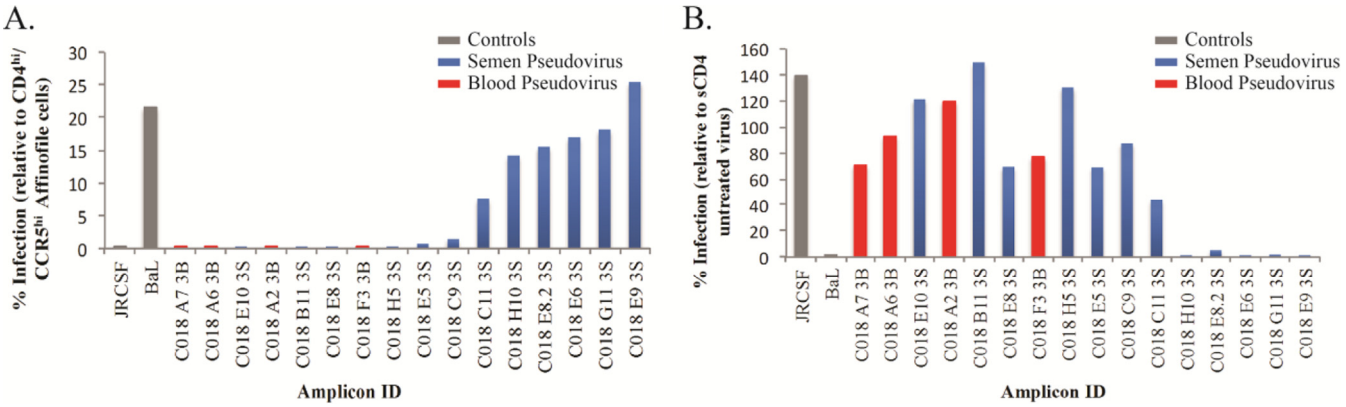


FIG 3 Tissue culture assays using pseudoviruses to define viral entry tropism phenotypes. (A) Relative levels of infectivity of pseudoviruses (represented in blue bars for seminal Env and in red bars for plasma Env) were determined by using Affinofile cells expressing low levels of CD4 compared to the level of pseudovirus infection of Affinofile cells induced to express high levels of CD4. Macrophage tropism is defined by relative infectivity greater than 10%, while T cell tropism is defined by relative infectivity less than 2%. Relative infectivity from 2% to 10% constitutes intermediate tropism. Results show five macrophage-tropic Env from the semen, one intermediate-tropic Env from the semen, six T cell-tropic Env from the semen, and four T cell-tropic Env from the blood. (B) Relative levels of infectivity of TZM-bl cells (which express high levels of CD4 and CCR5) were compared in the presence and absence of sCD4. Near-complete entry inhibition by sCD4 identified five macrophage-tropic Env from semen, and these Env corresponded to those identified as macrophage-tropic by the Affinofile cell assay. Intermediate entry inhibition by sCD4 confirmed the intermediate tropism of one semen Env, and a lack of entry inhibition by sCD4 confirmed the T cell tropism of six Env from semen and four Env from the blood.

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REFERENCES

1. **Swanstrom R, Coffin J.** 2012. HIV-1 pathogenesis: the virus. Cold Spring Harb Perspect Med **2:**a007443.
2. **Micci L, Alvarez X, Iriele RI, Ortiz AM, Ryan ES, McGary CS, Deleage C, McAtee BB, He T, Apetrei C, Easley K, Pahwa S, Collman RG, Derdeyn CA, Davenport MP, Estes JD, Silvestri G, Lackner AA, Paiardini M.** 2014. CD4 depletion in SIV-infected macaques results in macrophage and microglia infection with rapid turnover of infected cells. PLoS Pathog **10:**e1004467. http://dx.doi.org/10.1371/journal.ppat.1004467.
3. **Ping LH, Joseph SB, Anderson JA, Abrahams MR, Salazar-Gonzalez JF, Kincer LP, Treurnicht FK, Arney L, Ojeda S, Zhang M, Keys J, Potter EL, Chu H, Moore P, Salazar MG, Iyer S, Jabara C, Kirchherr J, Mapanje C, Ngandu N, Seoighe C, Hoffman I, Gao F, Tang Y, Labranche C, Lee B, Saville A, Vermeulen M, Fiscus S, Morris L, Karim SA, Haynes BF, Shaw GM, Korber BT, Hahn BH, Cohen MS, Montefiori D, Williamson C, Swanstrom R, CAPRISA Acute Infection Study and the Center for HIV-AIDS Vaccine Immunology Consortium.** 2013. Comparison of viral Env proteins from acute and chronic infections with subtype C human immunodeficiency virus type 1 identifies differences in glycosylation and CCR5 utilization and suggests a new strategy for immunogen design. J Virol **87:**7218–7233. http://dx.doi.org/10.1128/JVI.03577-12.
4. **Alexander M, Lynch R, Mulenga J, Allen S, Derdeyn CA, Hunter E.** 2010. Donor and recipient ENVs from heterosexual human immunodeficiency virus subtype C transmission pairs require high receptor levels for entry. J Virol **84:**4100–4104. http://dx.doi.org/10.1128/JVI.02068-09.
5. **Parrish NF, Wilen CB, Banks LB, Iyer SS, Pfaff JM, Salazar-Gonzalez JF, Salazar MG, Decker JM, Parrish EH, Berg A, Hopper J, Hora B, Kumar A, Mahlokozera T, Yuan S, Coleman C, Vermeulen M, Ding H, Ochsenbauer C, Tilton JC, Permar SR, Kappes JC, Betts MR, Busch MP, Gao F, Montefiori D, Haynes BF, Shaw GM, Hahn BH, Doms RW.** 2012. Transmitted/founder and chronic subtype C HIV-1 use CD4 and CCR5 receptors with equal efficiency and are not inhibited by blocking the integrin alpha4beta7. PLoS Pathog **8:**e1002686. http://dx.doi.org/10.1371/journal.ppat.1002686.
6. **Joseph SB, Arrildt KT, Swanstrom AE, Schnell G, Lee B, Hoxie JA, Swanstrom R.** 2014. Quantification of entry phenotypes of macrophage-tropic HIV-1 across a wide range of CD4 densities. J Virol **88:**1858–1869. http://dx.doi.org/10.1128/JVI.02477-13.
7. **Anderson JA, Ping LH, Dibben O, Jabara CB, Arney L, Kincer L, Tang Y, Hobbs M, Hoffman I, Kazembe P, Jones CD, Borrow P, Fiscus S, Cohen MS, Swanstrom R, Center for HIV/AIDS Vaccine Immunology.** 2010. HIV-1 populations in semen arise through multiple mechanisms. PLoS Pathog **6:**e1001053. http://dx.doi.org/10.1371/journal.ppat.1001053.
8. **Cohen MS, Hoffman IF, Royce RA, Kazembe P, Dyer JR, Daly CC, Zimba D, Vernazza PL, Maida M, Fiscus SA, Eron JJ, Jr.** 1997. Reduction of concentration of HIV-1 in semen after treatment of urethritis: implications for prevention of sexual transmission of HIV-1. AIDSCAP Malawi Research Group. Lancet **349:**1868–1873.
9. **Ping LH, Cohen MS, Hoffman I, Vernazza P, Seillier-Moiseiwitsch F, Chakraborty H, Kazembe P, Zimba D, Maida M, Fiscus SA, Eron JJ, Swanstrom R, Nelson JA.** 2000. Effects of genital tract inflammation on human immunodeficiency virus type 1 V3 populations in blood and semen. J Virol **74:**8946–8952. http://dx.doi.org/10.1128/JVI.74.19.8946-8952.2000.
10. **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun C, Grayson T, Wang S, Li H, Wei X, Jiang C, Kirchherr JL, Gao F, Anderson JA, Ping LH, Swanstrom R, Tomaras GD, Blattner WA, Goepfert PA, Kilby JM, Saag MS, Delwart EL, Busch MP, Cohen MS, Montefiori DC, Haynes BF, Gaschen B, Athreya GS, Lee HY, Wood N, Seoighe C, Perelson AS, Bhattacharya T, Korber BT, Hahn BH, Shaw GM.** 2008. Identification and characterization of transmitted and early founder virus envelopes in primary HIV-1 infection. Proc Natl Acad Sci U S A **105:**7552–7557. http://dx.doi.org/10.1073/pnas.0802203105.
11. **Traunecker A, Luke W, Karjalainen K.** 1988. Soluble CD4 molecules neutralize human immunodeficiency virus type 1. Nature **331:**84–86. http://dx.doi.org/10.1038/331084a0.
12. **Hussey RE, Richardson NE, Kowalski M, Brown NR, Chang HC, Siliciano RF, Dorfman T, Walker B, Sodroski J, Reinherz EL.** 1988. A soluble CD4 protein selectively inhibits HIV replication and syncytium formation. Nature **331:**78–81. http://dx.doi.org/10.1038/331078a0.
13. **Fisher RA, Bertonis JM, Meier W, Johnson VA, Costopoulos DS, Liu T, Tizard R, Walker BD, Hirsch MS, Schooley RT, Flavell RA.** 1988. HIV infection is blocked in vitro by recombinant soluble CD4. Nature **331:**76–78. http://dx.doi.org/10.1038/331076a0.
14. **Deen KC, McDougal JS, Inacker R, Folena-Wasserman G, Arthos J, Rosenberg J, Maddon PJ, Axel R, Sweet RW.** 1988. A soluble form of CD4 (T4) protein inhibits AIDS virus infection. Nature **331:**82–84. http://dx.doi.org/10.1038/331082a0.
15. **Montefiori DC.** 2005. Evaluating neutralizing antibodies against HIV,

SIV, and SHIV in luciferase reporter gene assays. Curr Protoc Immunol **2005:**Chapter 12:Unit 12.11. http://dx.doi.org/10.1002/0471142735.im1211s64.

16. **Politch JA, Mayer KH, Anderson DJ.** 2009. Depletion of CD4+ T cells in semen during HIV infection and their restoration following antiretroviral therapy. J Acquir Immune Defic Syndr **50:**283–289. http://dx.doi.org/10.1097/QAI.0b013e3181989870.
17. **Dunfee RL, Thomas ER, Gabuzda D.** 2009. Enhanced macrophage tropism of HIV in brain and lymphoid tissues is associated with sensitivity to the broadly neutralizing CD4 binding site antibody b12. Retrovirology **6:**69. http://dx.doi.org/10.1186/1742-4690-6-69.
18. **Peters PJ, Duenas-Decamp MJ, Sullivan WM, Clapham PR.** 2007. Variation of macrophage tropism among HIV-1 R5 envelopes in brain and other tissues. J Neuroimmune Pharmacol **2:**32–41. http://dx.doi.org/10.1007/s11481-006-9042-2.
19. **Igarashi T, Donau OK, Imamichi H, Dumaurier MJ, Sadjadpour R, Plishka RJ, Buckler-White A, Buckler C, Suffredini AF, Lane HC, Moore JP, Martin MA.** 2003. Macrophage-tropic simian/human immunodeficiency virus chimeras use CXCR4, not CCR5, for infections of rhesus macaque peripheral blood mononuclear cells and alveolar macrophages. J Virol **77:**13042–13052. http://dx.doi.org/10.1128/JVI.77.24.13042-13052.2003.
20. **Ochsenbauer C, Edmonds TG, Ding H, Keele BF, Decker J, Salazar MG, Salazar-Gonzalez JF, Shattock R, Haynes BF, Shaw GM, Hahn BH, Kappes JC.** 2012. Generation of transmitted/founder HIV-1 infectious molecular clones and characterization of their replication capacity in CD4 T lymphocytes and monocyte-derived macrophages. J Virol **86:**2715–2728. http://dx.doi.org/10.1128/JVI.06157-11.
21. **Chikere K, Webb NE, Chou T, Borm K, Sterjovski J, Gorry PR, Lee B.** 2014. Distinct HIV-1 entry phenotypes are associated with transmission, subtype specificity, and resistance to broadly neutralizing antibodies. Retrovirology **11:**48. http://dx.doi.org/10.1186/1742-4690-11-48.