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Quantification of Entry Phenotypes of Macrophage-Tropic HIV-1 across a Wide Range of CD4 Densities

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ABSTRACT

Defining a macrophage-tropic phenotype for HIV-1 to assess a role in pathogenesis is complicated by the fact that HIV-1 isolates vary continuously in their ability to enter monocyte-derived macrophages (MDMs) *in vitro*, and MDMs vary in their ability to support HIV-1 entry. To overcome these limitations, we identified consistent differences in entry phenotypes between five paired blood-derived, T cell-tropic HIV-1 *env* genes, four of which are CCR5-using (R5) and one of which is CXCR4-using (X4), and cerebrospinal fluid (CSF)-derived, R5 macrophage-tropic *env* genes. We performed entry assays using the CD4- and CCR5-inducible Affinofile cell line, expressing a range of CD4 levels that approximates the range from MDMs to $CD4^+$ T cells. The macrophage-tropic viruses were significantly better at infecting cells expressing low levels of CD4 than the T cell-tropic viruses from the same subjects, with the titration of CD4 providing a distinctive and quantitative phenotype. This difference in CD4 utilization was not due to macrophage-tropic viruses being CD4 independent. Furthermore, macrophage-tropic viruses did not differ from paired T cell-tropic viruses in their ability to use low levels of CCR5 ($t_{paired} = -1.39$; $P = 0.24$) or their use of an alternative conformation of CCR5. We also infected MDMs with a panel of viruses and observed that infectivity of each virus differed across four donors and between three preparations from a single donor. We concluded that the evolutionary transition from replication in T cells to that in macrophages involves a phenotypic transition to acquire the ability to infect cells expressing low levels of CD4 and that this phenotype is more reliably measured in Affinofile cells than in macrophages.

IMPORTANCE

HIV-1 typically infects memory T cells by using CD4 and CCR5 to enter cells. The virus evolves to infect new cell types by changing the coreceptor from CCR5 to CXCR4 to infect naive T cells or adapting to the use of low levels of CD4 to infect macrophages. However, defining the phenotype of macrophage tropism has been difficult due to inherent variability in the use of macrophages generated in culture to support entry of HIV-1. We describe the use of Affinofile cells with inducible and variable levels of CD4 to identify a signature phenotype for macrophage-tropic HIV-1. The ability to define HIV-1 variants that have evolved an entry phenotype that allows more efficient entry into cells with low levels of CD4 sets the stage for a clearer placement of these variants in HIV-associated pathogenesis.

The HIV-1 Env protein determines the entry phenotype of the virus, typically using CD4 as the receptor and CCR5 as the coreceptor. The ability of HIV-1 to replicate in a novel cell type likely requires adaptation of the viral envelope protein to efficiently utilize the receptor and coreceptor present on that cell type. The emergence of CXCR4-using virus late in infection has long been thought to represent adaptation to infect a novel host cell (reviewed in reference 1), most likely $CD4^+$ naive T cells, which are known to express high levels of CXCR4 and very little CCR5 (2). This is consistent with a recent *in vitro* study showing that receptor-mediated entry of $CD4^+$ naive T cells requires use of the CXCR4 coreceptor (3).

Historically, viruses capable of growing in transformed T cell lines were called T cell-tropic viruses. Due to the fact that most T cell lines express CXCR4 but not CCR5, the early isolates capable of growth on these cell lines were predominantly CXCR4-using viruses. In order to distinguish these CXCR4-using "T cell-tropic" viruses, the remaining CCR5-using (R5) isolates were collectively called "macrophage-tropic" (M-tropic) viruses, based on the observation that at least some of these isolates could enter and, in some cases, replicate in macrophages. Thus, the early analyses of sexually and vertically transmitted HIV-1 suggested that transmitted/founder viruses are predominantly macrophage tropic (4, 5). These findings were supported by early studies suggesting that macrophages are the initial target cell for sexual transmission (6) and by observations that cervical explants could be infected by the macrophage-tropic virus Ba-L and not by two T cell-tropic strains (7). More recent studies, however, have contradicted this viewpoint by showing that infectious molecular clones (8–10) and *env* gene clones (11) generated from transmitted/founder viruses are predominantly CCR5-using viruses and infect monocyte-derived

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TABLE 1 CD4 sensitivities and fusogenicities of *env* clones derived from subjects with slow viral decay after initiation of therapy[b]

Patient	% Infectivity at lowest CD4 density			CD4 usage curve data						Ability to fuse to cells expressing CD4 and CCR5 (RLU)		
				CD4 ED_{50} (ABS/μm^2)			Hill slope					
	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference[a]	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference[a]	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference[a]
4013	0.4	15.9	−15.5	19.9	15.7	4.2	3.1	2.6	0.5	83.4	80.9	2.5
4051	1.5	19.7	−18.3	20.9	15.3	5.6	3.3	3.9	−0.6	86.7	234.3	−147.6
4059	0.9	22.8	−22.0	17.0	13.6	3.4	3.1	3.1	0.0	43.1	177.9	−134.8
5002	1.0	21.4	−20.4	18.2	17.9	0.3	2.9	2.3	0.6	24.4	141.9	−117.5
7115	0.1	23.5	−23.4	24.6	18.6	6.0	3.8	3.2	0.6	14.5	188.9	−174.4

[a] Significant difference between paired CSF and plasma clones (paired *t* test; $P < 0.02$).
[b] See Fig. 1A.

macrophages (MDMs) at levels well below those of prototypic macrophage-tropic viruses.

Uncertainty about the nature of most R5 viruses also comes from the study of macrophage-tropic HIV-1. A virus capable of replicating in MDMs was originally isolated from primary lung cultures taken from an infant who died of AIDS (12). This virus, $HIV\text{-}1_{Ba\text{-}L}$, was subsequently passaged on MDMs, where it likely adapted to replication in MDMs in culture. This phenotype has been linked to the ability to infect cells with low levels of CD4 (13–18), and these types of viruses have most often been found in brain tissue of subjects who died with neurologic involvement (15, 17, 19–21). However, there are also reports of these viruses being found in the blood (22, 23). A common observation in studies using MDMs is that they vary in the capacity to support HIV-1 entry, and this variability is usually dealt with by including several donors in a study. The lack of a quantifiable phenotype to measure viruses that enter macrophages with various efficiencies has left the concept of "macrophage-tropic" viruses vague and inconsistently applied to a wide variety of isolates, thus obscuring the role of these variants in transmission and pathogenesis.

In this study, we addressed this uncertainty by identifying phenotypes that differentiate viruses that have evolved *in vivo* to replicate in macrophages from those that replicate in T cells. We accomplished this by examining the entry phenotypes of well-characterized pairs of macrophage- and T cell-tropic viruses isolated from five subjects, as represented by cloned *env* genes. The macrophage-tropic viruses were all derived from cerebrospinal fluid (CSF), where the virus was previously shown to decay very slowly after initiation of antiretroviral therapy (24), indicating that the virus was being produced by long-lived cells, presumably either perivascular macrophages or microglia (25). In contrast, the T cell-tropic viruses were blood-derived viruses from the same subjects and decayed rapidly after the initiation of therapy, indicating that they were being produced by short-lived cells, presumably $CD4^+$ T cells. By carefully examining the entry phenotypes of these viruses, we were able to show that the evolutionary transition from replication in $CD4^+$ T cells to replication in macrophages selects for an increased ability to infect cells expressing low levels of CD4 but does not alter the ability to infect cells expressing low levels of CCR5. We observed distinctive macrophage-tropic and T cell-tropic phenotypes by using a CD4 titration curve, which is possible with the CD4- and CCR5-inducible Affinofile cell line (26). We also show that infection of MDMs *in vitro* is an inconsistent assay for defining a macrophage-tropic phenotype. As a result, T cell-tropic viruses may appear to be macrophage tropic when infecting MDMs from some donors/preparations and T cell tropic on others, and some macrophage-tropic viruses may show the reverse. Collectively, these studies provide a more quantitative definition of HIV-1 macrophage tropism, which will allow a more accurate identification of these variants that will lead to an improved understanding of their role in viral pathogenesis.

MATERIALS AND METHODS

Study subjects. In this study, we examined the entry phenotypes of previously generated *env* gene clones amplified from the blood and CSF of subjects infected with HIV-1 subtype B (23). These subjects participated in a study of HIV-associated dementia (23) at the University of California at San Francisco. Procedures for sample collection (27), viral decay assays (24), and cloning (23) (see below) have been described previously. All samples were collected with written informed consent, and all protocols were approved by institutional review boards at the collection sites.

Five of these subjects were diagnosed with neurological disease (stages 1 to 3) (Table 1) (23), and we consider the viruses in their CSF to be M-tropic based on measurements made both *in vivo* and *in vitro*. For four of the subjects (subjects 4013, 4059, 5002, and 7115), the CSF viral load decayed slowly upon initiation of antiretroviral therapy (24), and the *env* clones derived from the CSF mediated the infection of MDMs very efficiently (23), thus indicating that these clones are replicating in long-lived cells *in vivo* and are well adapted to entry into macrophages (using viruses pseudotyped with these Env proteins). Conversely, the blood viral loads of these subjects declined rapidly after initiation of therapy, indicating production from short-lived cells (24), and blood-derived clones from these subjects mediated the infection of macrophages very poorly, although this varied by the donor source for the MDMs (23). We consider these clones to be T cell-tropic viruses and indicate them as R5 (for four of the subjects) or X4 (for subject 5002) viruses, based on their coreceptor specificity. It is important to note this distinction of R5 T cell-tropic viruses, which are typically not accounted for in the literature; however, for simplicity, we will refer to these five rapid-decay viruses as T cell-tropic without specifying their coreceptor usage. We also included a subject (4051) who had a mixture of T cell-tropic and M-tropic viruses in the CSF, and we observed that the M-tropic lineage in the CSF of this subject increased in abundance relative to the T cell-tropic lineage with the initiation of therapy (unpublished data), consistent with its production from long-lived cells. Thus, we have a well-validated set of five CSF-derived, M-tropic *env* clones and paired blood-derived *env* clones that represent viruses that were growing in T cells and the majority of which are CCR5-using. We used these pairs of clones to develop a quantitative description of CD4 dependence for entry for two types of viruses: M-tropic and T cell-tropic viruses (see below).

We also examined *env* clones from five of these subjects with R5 T cell-tropic HIV-1 subtype B in the blood and CSF (Table 2). Their blood and CSF viral loads declined rapidly after the initiation of therapy, and *env* clones isolated from both compartments were unable to efficiently infect macrophages (23). The remaining two subjects were infected with R5 T cell-tropic HIV-1 subtype C. HIV *env* clones generated from the blood of these subjects were unable to infect Affinofile cells expressing low levels of CD4 (28).

TABLE 2 CD4 sensitivities of *env* clones derived from subjects with rapid viral decay after initiation of therapy[a]

Patient	% Infectivity at lowest CD4 concn			$CD4\ ED_{50}$ (ABS/μm^2)			Hill slope		
	Plasma clone (T-tropic)	CSF clone (T-tropic)	Difference	Plasma clone (T-tropic)	CSF clone (T-tropic)	Difference	Plasma clone (T-tropic)	CSF clone (T-tropic)	Difference
4012	1.0	0.6	0.4	24.9	21.0	3.9	3.5	3.0	0.5
4030	0.5	3.1	−2.6	21.1	16.9	4.2	3.2	2.9	0.3
4033	1.0	2.3	−1.3	16.5	16.8	−0.3	3.6	2.5	1.1
5003	1.0	0.5	0.5	17.8	21.5	−3.7	3.5	2.9	0.6
7036	0.2	0.8	−0.6	19.9	18.8	1.1	6.8	3.5	3.3

[a] See Fig. 1B.

Cloning of *env* genes. Two *env* clones—Ba-L and JRCSF—were obtained from the Division of AIDS, NIAID, through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. The remaining subtype B *env* clones were generated in a previous study (23). Here we briefly review the single-genome amplification (SGA) and cloning procedures. An oligo(dT) primer was used to generate cDNA from HIV-1 RNA isolated from blood plasma or CSF. SGA (29–31) was then used to amplify full-length *env* sequences through the 3′ U3 region. After one round of SGA, amplicons were reamplified, gel purified using a QIAquick gel extraction kit (Qiagen), and cloned into the pcDNA 3.1D/V5-His-TOPO expression vector (Invitrogen) by use of a pcDNA 3.1 directional TOPO expression kit (Invitrogen) and Max Efficiency Stbl2 competent cells (Invitrogen). An additional set of *env* clones representing the transmitted virus in the context of heterosexual transmission of subtype C HIV-1 (28) was also included in the study of infection of MDMs.

Cells. 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) with 4.5 g/liter glucose (Cellgro) supplemented with 10% fetal bovine serum (FBS), 50 U/ml of penicillin, and 50 μg/ml streptomycin. Affinofile cells (26) were maintained in DMEM with 4.5 g/liter glucose (Cellgro) supplemented with 10% dialyzed FBS (12 to 14 kDa; Atlanta Biologicals) and 50 mg/ml blasticidin (Invitrogen).

At three time points, monocytes were isolated from healthy donors. Approximately 40 ml of blood was collected from each donor into heparin-treated tubes. Buffy coats were purified using Ficoll-Paque Plus (GE Healthcare) following the manufacturer's directions. We then used negative selection to isolate monocytes (EasySep human monocyte enrichment kit without CD16 depletion; StemCell Technologies). Monocytes were resuspended and cultured in RPMI 1640 supplemented with 10% FBS, 100 mg/ml of penicillin and streptomycin, and 10 ng/ml recombinant human macrophage colony-stimulating factor (M-CSF; Gibco). Monocytes were then plated at a density of 1.1×10^6 cells per 60- by 15-mm dish (6 ml medium per dish) for flow cytometry and at 5×10^4 cells per well of a 48-well plate (0.5 ml medium per well) for infection. After 5 days, a partial medium change was performed. After an additional 2 days (for a total of 7 days), cells were either infected or processed for flow cytometry.

Flow cytometry. CD4 and CCR5 expression levels on Affinofile cells, monocytes, $CD4^+$ T cells, and MDMs were quantified using flow cytometry. In order to avoid disrupting surface receptors, Affinofile cells and MDMs were removed from their culture dishes by nonenzymatic methods. Affinofile cells were removed using cold phosphate-buffered saline (PBS; CellGro), and MDMs were removed using cell dissociation buffer (Gibco). Affinofile cells and MDMs were stained with Fixable Aqua dead cell stain (Invitrogen) and saturating concentrations of either phycoerythrin (PE)-conjugated anti-human CD4 antibody (clone RPA-T4; BD Biosciences) or PE-conjugated mouse anti-human CCR5 antibody (clone 2D7; BD Biosciences). CD4 expression and CCR5 expression on primary cells were quantified by staining peripheral blood mononuclear cells (PBMCs) with Brilliant Violet 421-conjugated anti-human CD3 antibody (clone UCHT1; BD Biosciences), allophycocyanin (APC)-Cy7-conjugated anti-human CD14 antibody (clone MφP9; BD Biosciences), and saturating concentrations of either PE-conjugated anti-human CD4 antibody (clone RPA-T4; BD Biosciences) or PE-conjugated mouse anti-human CCR5 antibody (clone 2D7; BD Biosciences). QuantiBRITE beads (BD Biosciences) were then used to translate the mean fluorescence per cell to the number of CD4 or CCR5 antibody binding sites (ABS) per cell. Six bead standards of known size (flow cytometry size calibration kit; Invitrogen) were used to translate measurements of forward scatter into estimates of cell diameter. All flow cytometry assays were performed using a Cyan flow cytometer (Beckman Coulter) and analyzed using FlowJo software (version 9.3.1).

Env-pseudotyped virus stocks. Env-pseudotyped luciferase reporter viruses were generated by cotransfecting 100- by 20-mm dishes of 293T cells with 5 μg of an HIV-1 *env* clone, 5 μg of pNL4-3.LucR-E− plasmid (obtained from the Division of AIDS, NIAID, through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH), and 30 μl of the FuGENE 6 transfection reagent (Promega). At 5 h posttransfection, the medium was changed and the cells were incubated at 37°C for an additional 43 h. Viral supernatants were then harvested, filtered through a 0.45-μm filter (Millipore), and stored at −80°C. Virus stocks were not subjected to multiple freeze-thaw cycles.

Affinofile cell assays. Affinofile cells were plated at a concentration of 1.8×10^4 cells/well in black poly-L-lysine (Sigma)-treated 96-well plates. After 24 h in culture, ponasterone A (Pon A; Invitrogen) and doxycycline (Doxy; Sigma) were added at various concentrations to the medium to induce CD4 and CCR5 expression. Twenty hours later, the medium was replaced with medium lacking Pon A and Doxy, and virus was added to the plates. Cells were spinoculated at 2,000 rpm for 2 h at 37°C and then incubated at 37°C. After 48 h, the cells were washed twice with PBS and lysed with 50 μl of 1× reporter lysis buffer (Promega), and the lysate was stored at −80°C. Virus entry was then assessed by thawing the lysates and quantifying luciferase expression by using a luciferase assay system (Promega).

CD4 usage was examined by infecting Affinofile cells expressing 10 levels of CD4 and a single, high level of CCR5. Affinofile expression of CD4 ranged from uninduced ([Doxy] = 0 ng/ml) to maximally induced ([Doxy] = 6 ng/ml), with eight levels in between (0.07, 0.1, 0.2, 0.3, 0.5, 0.7, 1.0, and 1.5 ng/ml Doxy). CCR5 expression was maximally induced ([Pon A] = 5 μM).

CCR5 usage was examined by infecting maraviroc-treated Affinofile cells expressing a single, high level of CD4 ([Doxy] = 6 ng/ml) and either a high ([Pon A] = 5 μM) or low ([Pon A] = 0 μM) level of CCR5. One hour prior to spinoculation, maraviroc was serially diluted and added to cells at 10 concentrations (0, 1.7, 8.2, 41.2, 123.5, 370.4, 1,111.1, 3,333.3, 10,000, and 50,000 nM maraviroc). After spinoculation, the maraviroc-containing medium was removed from each well and replaced with fresh medium.

Titration of virus stocks. Virus stocks were titrated by infecting Affinofile cells expressing the maximum levels of CD4 ([Doxy] = 6 ng/ml) and CCR5 ([Pon A] = 5 μM). We then calculated the volume of each virus stock that generated 800,000 relative light units (RLU) of luciferase expression when infecting maximally induced Affinofile cells. This quantity of virus, which was in the linear range for measuring infectivity, was used in all subsequent experiments infecting either MDMs or Affinofile cells.

Cell-cell fusion assay. A luciferase-based gene reporter assay was used to assess the ability of Env proteins to mediate cell-cell fusion (32, 33). Briefly, quail QT6 cells were transfected with individual HIV-1 *env* ex-

pression vectors by using $CaPO_4$ and then infected with a vaccinia virus expressing T7 RNA polymerase for 18 h. A second population of QT6 cells was transfected to transiently express human CCR5 and/or human CD4, or neither; all cells in the second population of QT6 cells were also transfected with the luciferase gene under the control of a T7 promoter and with a green fluorescent protein (GFP)-expressing vector to monitor transfection efficiency. The Env-expressing cells were then mixed with the receptor/coreceptor-expressing cells. Eight hours later, the cells were lysed, and luciferase expression was measured (represented in figures as mean RLU) with a luminometer. In this experimental design, luciferase activity is recorded as a function of the cells expressing the T7 RNA polymerase fusing with the cells expressing the luciferase gene under the control of the T7 RNA polymerase promoter.

Statistical analyses. All statistical analyses were performed using R statistical software (version 2.14.1). Model fitting of infection data was performed using the R statistical package (drc) designed to analyze dose-response models (drm). These analyses fit a four-parameter, log-logistic model to the data.

RESULTS

Affinofile cells readily distinguish M-tropic and R5 T cell-tropic viruses based on differences in sensitivity to surface CD4 levels. Affinofile cells have been engineered to have inducible and titratable levels of CD4 and CCR5, and they express CXCR4 constitutively. In our previous study, we showed that the ability to maintain a moderate level of infectivity on Affinofile cells with the uninduced level of CD4 on the surface was related to efficient infection of macrophages and the slow decay of viral load in the CSF (23). Here we explored the phenotype of CD4 dependency across the entire range of CD4 concentrations available using Affinofile cells. The sensitivity to CD4 level of viruses pseudotyped with different Env proteins was assessed by measuring their ability to infect Affinofile cells induced to express 10 CD4 levels (ranging from 1,425 to 81,649 CD4 molecules per cell, inferred as antibody binding sites per cell). The shape of the infectivity curve as a function of CD4 density (four-parameter, log-logistic curve) differed for comparing the M-tropic viruses to the R5 (and one X4) T cell-tropic viruses isolated from the same subject (Fig. 1A; Table 1).

There are three features of these titration curves that distinguish the two groups of viruses: (i) the M-tropic viruses retained the ability to infect cells at the lowest level of CD4 expression (1,425 CD4 molecules per cell), at 16 to 24% of their infectivity on high-CD4-expressing cells (81,649 CD4 molecules per cell), while the T cell-tropic viruses infected Affinofile cells with low levels of CD4 at only 0 to 2% of their infectivity on high-CD4-expressing cells (t_{paired} = 14.29; degrees of freedom [df] = 4; *P* value = 0.00014); (ii) the CD4 level that increased infectivity by 50% (CD4 ED_{50}) was significantly lower for the M-tropic viruses than for the paired T cell-tropic viruses (t_{paired} = 3.84; df = 4; *P* value = 0.018); and (iii) in most cases, the M-tropic virus approached a plateau level of infectivity at a lower CD4 density than that with the paired T cell-tropic virus. We observed a similar pattern of CD4 utilization for the prototypic M-tropic virus Ba-L (data not shown).

We repeated the above-described assays using T cell-tropic *env* clones isolated from the blood and CSF of five subjects who lacked M-tropic HIV-1 in the CSF (Fig. 1B; Table 2). We observed that pseudotyped viruses generated using these CSF- and blood-derived *env* genes did not differ in their minimal ability to infect Affinofile cells expressing the lowest CD4 level (t_{paired} = −1.25; df = 4; *P* value = 0.28) or in the CD4 level that increased infectivity by 50% (t_{paired} = 0.71; df = 4; *P* value = 0.52). Also, there was no pattern of difference in the approach to a plateau of infectivity at the highest CD4 levels. Thus, the ability to infect cells expressing low levels of CD4 (and the differences in the other parameters) is not specific to CSF-derived clones but rather is specific to the M-tropic clones generated from viruses being produced *in vivo* by long-lived cells. Also, we interpret these results to represent two distinct phenotypes for M-tropic and T cell-tropic viruses and not simply part of a continuum among isolates in the ability to use differing densities of CD4.

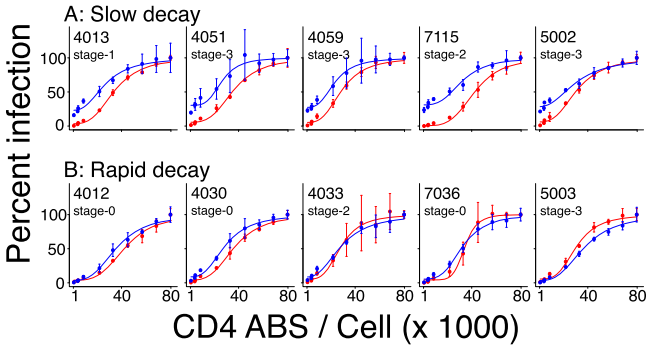

FIG 1 Dose-response curves for infectivity of M-tropic and T cell-tropic viruses to Affinofile cells expressing various densities of CD4. The sensitivity of pseudotyped viruses to CD4 levels was assessed by measuring their ability to infect Affinofile cells expressing 10 CD4 levels (1,425, 4,590, 4,981, 9,374, 22,667, 33,842, 46,204, 58,153, 69,897, and 81,649 CD4 ABS per cell), with CCR5 fully induced. (A) *env* genes were isolated from viruses in the blood and CSF of subjects who had rapid decay of virus in the blood but slow decay of virus in the CSF with the initiation of anti-HIV-1 therapy (23). These *env* genes were used in a transfection protocol to generate pseudotyped viruses that carried a reporter gene (luciferase). Equal amounts of virus were used at each CD4 level, and the level of infectivity at the highest level of CD4 was taken as 100%. The highest value was within the linear region of the dose-response curve for infectivity for each virus. The lines for each virus were generated by the R statistical package (drc) designed to analyze dose-response models (drm). Red lines represent viruses pseudotyped using *env* genes generated from viruses in the blood. Blue lines represent viruses pseudotyped using *env* genes generated from viruses in the CSF. (B) A similar analysis was done by comparing viruses from the blood and CSF of subjects who had rapid decay of virus in both the blood and CSF after the initiation of therapy (23). The stage designation for each subject refers to AIDS dementia complex staging, where 0 = neurologically asymptomatic, 1 = mild neurological impairment, and 2 and 3 = moderate to severe HIV-associated dementia (HAD) (23).

Titration of CD4 density on Affinofile cells mimics the densities of CD4 on $CD4^+$ T cells, macrophages, and monocytes. Given that Affinofile cells provide a clear phenotypic distinction between viruses produced in the central nervous system (CNS) from long-lived cells and viruses present in the blood (Fig. 1A), we next determined if the density of CD4 on Affinofile cells was similar to the density on the target cells for HIV-1 replication. We used an anti-CD4 antibody to stain $CD4^+$ T cells, monocytes, MDMs, and Affinofile cells either fully induced for CD4 expression or uninduced (Fig. 2A). The amount of antibody bound to each cell was estimated by creating a standard curve with a control set of fluorescent beads. We also estimated the sizes of the cells by using forward scatter and size standard beads. We found that T cells and macrophages had similar numbers of CD4 molecules on the surface but that $CD4^+$ T cells were significantly smaller than MDMs (Fig. 2B). Monocytes had a small number of CD4 mole-

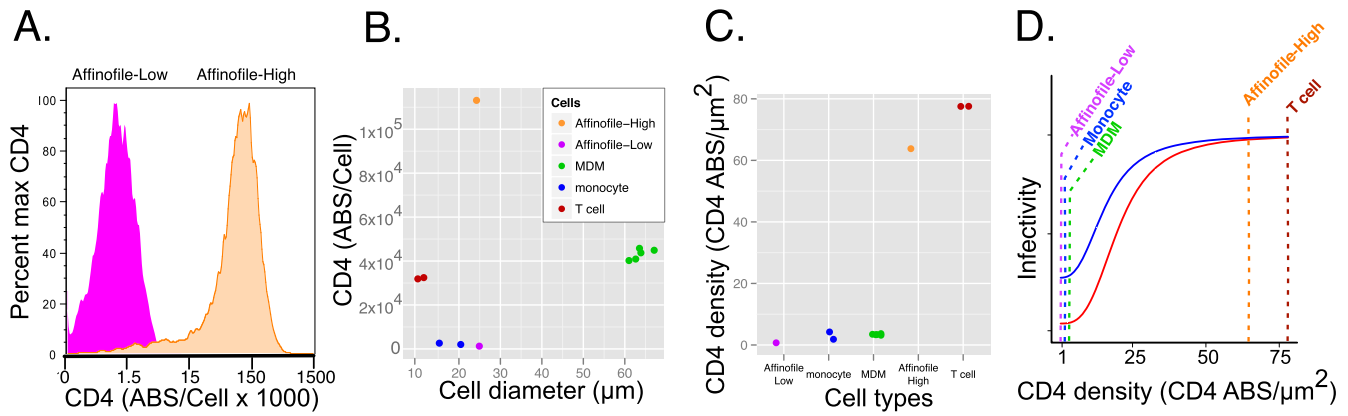

FIG 2 CD4 densities on Affinofile cells approximate the densities of CD4 on $CD4^+$ T cells, MDMs, and monocytes. (A) Affinofile cells were either uninduced or induced to express the highest level of CD4 on the cell surface. Data shown are flow analysis results for uninduced Affinofile cells and fully induced cells. CD4 was quantified as the number of ABS per cell, based on fluorescence calibration using a fluorescent bead standard curve. Cell size was also estimated based on forward scatter and compared to a bead standard curve. (B) Affinofile cells at the uninduced and induced levels, monocytes, MDMs, and $CD4^+$ T cells were compared by the number of CD4 molecules (ABS) per cell and the cell size, based on diameter. (C) Expression of CD4 as a function of density was plotted for different cell types (uninduced and induced Affinofile cells, $CD4^+$ T cells, monocytes, and MDMs). This analysis shows that uninduced Affinofile cells have a CD4 density that is slightly lower than those of MDMs and monocytes, while fully induced Affinofile cells have a CD4 density that is slightly lower than that of $CD4^+$ T cells. (D) Patterns of entry by M-tropic viruses (blue) and T cell-tropic viruses (red), combined from the data in Fig. 1 to generate average dose-response curves for infectivity as a function of CD4 density. The CD4 densities of monocytes, MDMs, and $CD4^+$ T cells are indicated, along with the extremes of CD4 densities on uninduced and fully induced Affinofile cells.

cules on the surface and also had a small cell diameter, and the Affinofile cells had a similarly small number of CD4 molecules on the cell surface in the uninduced state and much higher levels in the induced state. We normalized the number of CD4 molecules on the surface by using the surface area of the cell to obtain the density of CD4 per μm^2 (Fig. 2C). This showed that the uninduced Affinofile cells had a density of CD4 that was severalfold lower than that on monocytes or MDMs (0.7 versus 3.1 or 3.4 CD4 ABS/μm^2), and these three cell types were approximately 18- to 90-fold lower in CD4 density than the fully induced Affinofile cells (64 CD4 ABS/μm^2). Also, the CD4 density on fully induced Affinofile cells approximated but was still less than the density on $CD4^+$ T cells (78 CD4 ABS/μm^2). Figure 2D summarizes the CD4 densities on the different cell types and shows the approximate infectivity patterns for M-tropic and T cell-tropic viruses as a function of CD4 density. These measurements show that the titration of CD4 on Affinofile cells spans the range of CD4 densities found on relevant target cells for HIV-1 infection. Composite CD4 usage curves were generated by fitting a curve to the infectivity data for the five M-tropic clones and fitting a curve to the infectivity data for the paired, T cell-tropic clones.

Infection of MDMs varies between different MDM cultures. Infection of MDMs is one definition of macrophage tropism, and the ability to infect a cell with low levels of CD4 has been used by some investigators as a surrogate marker for macrophage tropism. Infectivity of MDMs can be variable, and typically, infections are repeated with at least two different preparations of MDMs to account for this variability, although how this variability is then reconciled is usually not discussed. In an effort to understand the biological basis for this confounding variability, we infected MDMs with pseudotyped viruses generated from 14 different *env* expression vectors and normalized their infectivity to the infectivity of the M-tropic virus Ba-L (Fig. 3). We infected a single preparation of MDMs from three donors (Fig. 3A) and three MDM preparations from one donor (Fig. 3B). As expected, the normalized infectivity of the prototypic R5 T cell-tropic virus JRCSF was extremely low (0.5 to 2% of that of the M-tropic clone). In contrast, some of the T cell-tropic viruses infected the MDMs quite well. Overall, normalized infectivity of the T cell-tropic viruses was much lower on MDMs from the donors with the lowest CD4 levels (donors 1 and 4) (Table 3) than on MDMs from the donor with the highest CD4 levels (donor 3) (Table 3; Fig. 3). Thus, infection of MDMs from some donors can give a discrepant entry phenotype compared to the definition obtained using Affinofile cells.

Macrophage-tropic viruses are able to use low levels of CD4 but are not CD4 independent. It is possible that the residual level of infectivity seen at the lowest density of CD4 on Affinofile cells is actually CD4-independent entry. To determine whether the Envs from the CSF-derived viruses were capable of CD4-independent entry, we evaluated the ability of these Envs to mediate fusion with cells expressing CD4 and CCR5 or CCR5 alone. In this assay, one cell type expresses the receptor and coreceptor, while another cell type expresses the viral Env protein. Upon mixing of the cell populations, the Env protein engages the receptor and coreceptor, thereby inducing fusion and providing a readout. As a positive control, we used a lab-derived CD4-independent variant of R3A, iR3A, that is capable of mediating CD4-independent fusion with cells expressing only CCR5. As expected, the Env protein from iR3A mediated fusion of cells expressing CCR5 with or without CD4, and the Env proteins from the T cell-tropic viruses isolated from plasma required both CCR5 and CD4 (Fig. 4). Like the Env proteins from the blood-derived viruses, all of the Env proteins from the CSF-derived M-tropic viruses required both CCR5 and CD4 to mediate fusion (Fig. 4). Thus, the residual infectivity at low CD4 density of the macrophage-tropic viruses is still CD4 dependent. It is worth noting that most of the Env proteins from the CSF-derived viruses consistently fused cells to a greater extent than their paired blood plasma-derived counterparts (Table 1) ($t_{paired} = -3.73$; df = 4; P value = 0.02). In a separate experiment

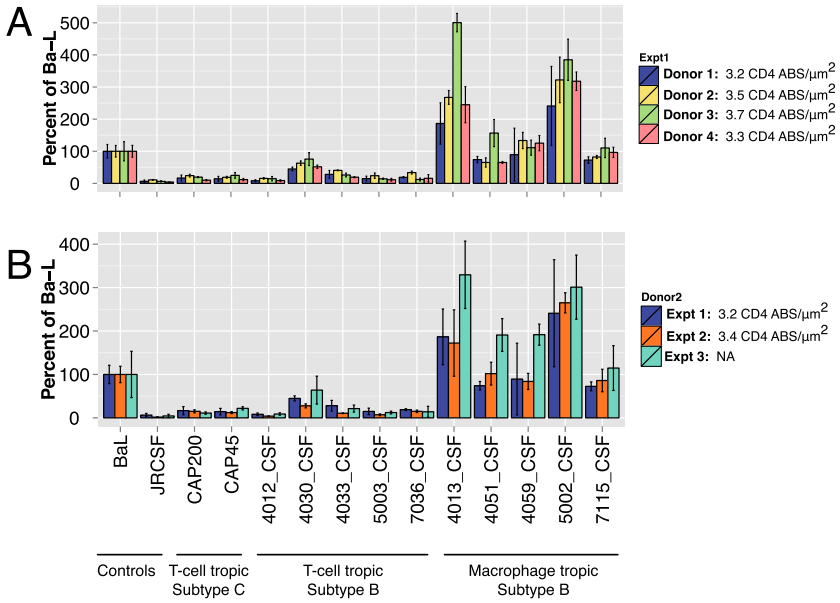

FIG 3 Pseudotyped virus infectivity of MDMs differs across both MDM donors and preparations. MDMs were infected with a panel of 14 pseudoviruses: a prototypic M-tropic virus (Ba-L), a prototypic R5 T cell-tropic virus (JRCSF), two R5 T cell-tropic viruses derived from the blood of individuals infected with HIV-1 subtype C (28), five R5 T cell-tropic viruses derived from the CSF of individuals infected with subtype B HIV-1 that decayed rapidly after initiation of antiretroviral therapy (23), and five M-tropic viruses derived from the CSF of individuals infected with subtype B HIV-1 that decayed slowly after initiation of antiretroviral therapy (23). These pseudotyped viruses were used to infect preparations of MDMs made from the blood of four different donors, drawn and processed in parallel on the same day (A), and preparations of MDMs prepared from the same donor compared over three different blood donations, each separated by several months (B). The amount of virus used in the infections was standardized to give the same level of infectivity on fully induced Affinofile cells. The level of infectivity with the virus pseudotyped with the Ba-L Env protein was used as 100% infectivity, and infectivities of all other viruses were recorded as percentages of this value.

(data not shown), we examined QT6 cells transfected with these constructs and found that they expressed very low CD4 densities (CD4 ABS/μm^2). Thus, the observation that CSF-derived Envs are more active in this fusion assay system is likely due to their ability to utilize low CD4 densities.

In order to confirm that virus entry into Affinofile cells is CD4 dependent, we performed an additional entry assay in the presence of an anti-CD4 antibody (leu3a). We treated Affinofile cells expressing the lowest (uninduced) level of CD4 with the antibody and found that it blocked both the very low levels of entry displayed by T cell-tropic viruses and the higher levels of entry displayed by macrophage-tropic viruses (data not shown). Thus, the enhanced ability of macrophage-tropic HIV-1 to enter cells expressing low levels of CD4 is not due to CD4-independent entry. In contrast, the antibody did not reduce entry of vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped HIV-1 (which does not require CD4 or a coreceptor) and did not completely block entry of a CD4-independent clone (iR3A) (which requires a coreceptor, but not CD4).

M-tropic and R5 T cell-tropic viruses do not differ in the ability to use low levels of CCR5. In a preliminary study, we examined whether M- and R5 T cell-tropic viruses differ in the ability to infect cells expressing a low level of CCR5. We did this by infecting Affinofile cells expressing high levels of CD4 ([Doxy] = 6 ng/ml; CD4 level = 85,055 ABS per cell) and either high levels of CCR5 ([Pon A] = 5 μM; CCR5 level = 30,472 ABS per cell) or low levels

TABLE 3 Receptor and coreceptor densities on MDMs

	Receptor/coreceptor density (ABS/μm^2)			
	Expt 1		Expt 2	
Donor	CD4	CCR5	CD4	CCR5
1	3.2	1.3	3.4	1.3
2	3.5	1.4		
3	3.7	1.5		
4	3.3	1.8		

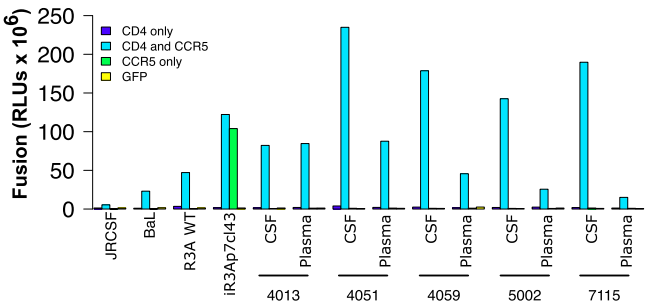

FIG 4 The evolution of macrophage tropism does not select for CD4-independent entry. QT6 cells were transfected with individual HIV-1 *env* expression vectors and infected with a vaccinia virus expressing T7 RNA polymerase. A second population of QT6 cells was transfected with vectors that express CCR5 and/or human CD4, or neither, and with a luciferase expression vector. The two types of cells were then mixed, and 8 h later, the level of luciferase was measured as an indication of cell fusion. Only the lab-derived positive control (iR3Ap7cl43) was able to fuse with cells lacking CD4.

TABLE 4 Parameters describing CCR5 usage when CCR5 levels are low[a]

Patient	Maraviroc sensitivity curve data											
	% Infectivity at lowest CCR5 concn			Maraviroc IC_{50} (nM)			Hill slope			Maraviroc resistance plateau		
	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference	Plasma clone (T-tropic)	CSF clone (M-tropic)	Difference
4013	86.5	96.0	−9.5	10.7	6.8	3.9	0.9	0.7	0.1	−1.6	−1.1	−0.5
4051	65.0	58.6	6.4	2.4	1.0	1.4	1.2	0.9	0.2	−0.8	1.2	−2.0
4059	132.3	99.6	32.7	10.7	2.8	7.9	0.9	0.8	0.1	−1.0	−1.5	0.5
5002		79.8			12.0			0.8			−2.4	
7115	66.7	59.7	7.0	52.4	17.5	34.9	1.3	0.9	0.4	0.0	−2.6	2.6

[a] See Fig. 5A.

of CCR5 ([Pon A] = 0 μM; CCR5 level = 2,691 ABS per cell) with our four paired, R5 M-tropic viruses and R5 T cell-tropic viruses. Reducing CCR5 expression to its uninduced level reduced infectivity by only 0 to 40% and did not reveal any consistent difference between M- and T cell-tropic viruses (Table 4) ($t_{paired} = -1.1$; df = 3; P value = 0.37).

In order to thoroughly evaluate the sensitivity to CCR5 expression levels, we chose to treat $CD4^{high}$ $CCR5^{low}$ Affinofile cells with the CCR5 antagonist maraviroc to titrate the available CCR5 coreceptor molecules and then infect the cells with M- and T cell-tropic pseudotyped viruses representing blood- and CSF-derived viruses from our five experimental subjects (Fig. 5A). We compared the maraviroc sensitivities of the four R5 T cell-tropic viruses to those of their paired M-tropic viruses (Table 4). Subject 5002 was excluded from this analysis because the T cell-tropic clone was previously shown to be a CXCR4-using virus (23). We observed that the remaining four pairs of M- and R5 T cell-tropic viruses did not differ in the 50% inhibitory concentrations (IC_{50}) of maraviroc ($t_{paired} = -1.55$; df = 3; P value = 0.22) or the slopes of their maraviroc resistance curves ($t_{paired} = 3.13$; df = 3; P value = 0.052).

We also examined whether the evolution of macrophage tropism selects for viruses capable of using a conformation of CCR5 that is insensitive to maraviroc (Fig. 5B and C). Use of this alternative conformation can be observed as partial resistance to maraviroc when CCR5 expression levels are high but not when they are

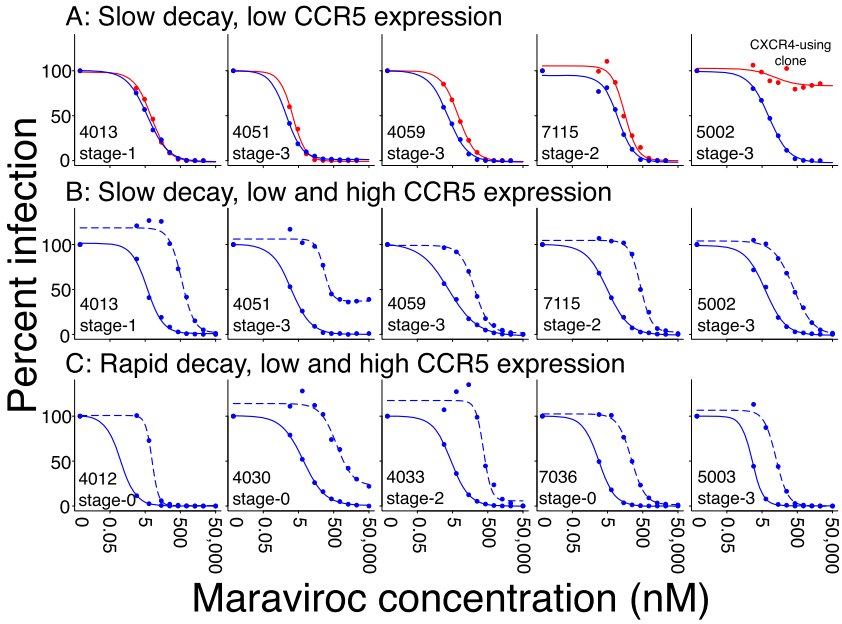

FIG 5 The evolution of macrophage tropism does not select for an increased ability to infect cells expressing low levels of CCR5 or the ability to use an alternative CCR5 conformation. (A) Affinofile cells expressing high CD4 and low/uninduced CCR5 were treated with 10 different concentrations of the CCR5 antagonist maraviroc (67, 68) and then infected with viruses pseudotyped using the five CSF-derived, M-tropic *env* clones (blue) and the paired blood-derived, T cell-tropic *env* clones (red) from subjects with slow decay of virus in the CSF with the initiation of therapy (as described in the legend to Fig. 1). Differences in sensitivity to maraviroc were observed only between the blood-derived, CXCR4-using virus from subject 5002 and the paired CSF-derived, CCR5-using virus. The lack of difference in maraviroc sensitivity between the M- and T cell-tropic viruses shows that the evolution to infect macrophages does not select for the ability to use lower levels of CCR5. (B and C) The same five R5 M-tropic pseudotyped viruses described for panel A (B) and five R5 T cell-tropic CSF-derived clones (C) were used to infect Affinofile cells expressing high CD4 and either low CCR5 (4,048 ABS per cell; solid blue lines) or high CCR5 (39,162 ABS per cell; dashed blue lines) in the presence of 10 different levels of maraviroc. We previously observed that expression of CCR5 at high levels on Affinofile cells results in a population of CCR5 molecules that can mediate entry for a subset of viruses even in the presence of maraviroc, giving rise to a residual plateau of infectivity in the presence of the inhibitor (28) and describing an alternative entry phenotype for these viruses. When we compared M-tropic (B) and R5 T cell-tropic (C) pseudotyped viruses with respect to the ability to use the alternative conformation of CCR5 for entry in the presence of maraviroc, we found no consistent difference among the two groups of viruses, as indicated by a plateau of resistance in only one M-tropic virus (4051) and one R5 T cell-tropic virus (4030).

low (28). This type of resistance cannot be explained by use of an alternative coreceptor (e.g., CXCR4) but rather is due to use of an alternative CCR5 conformation that is generated when CCR5 is expressed at high levels. We identified two clones with this type of resistance. These clones were partially resistant to maraviroc when infecting $CD4^{high}$ $CCR5^{high}$ Affinofile cells (CD4 level = 141,450 ABS per cell; CCR5 level = 39,162 ABS per cell) but were sensitive to maraviroc when infecting $CD4^{high}$ $CCR5^{low}$ Affinofile cells (CD4 level = 141,450 ABS per cell; CCR5 level = 4,048 ABS per cell). One resistant clone was M-tropic (CSF clone from subject 4051), and one was R5 T cell tropic (CSF clone from subject 4030). Thus, there is no consistent pattern that distinguishes R5 T cell-tropic viruses from M-tropic viruses in their ability to use this alternative form of CCR5.

DISCUSSION

Assessing the cellular tropism of HIV-1 is complicated by the fact that isolates are highly variable in their ability to infect target cells and target cells vary in their susceptibility to infection. Thus, most tropism assays do not yield easily interpretable, binary results. This is well illustrated by studies of macrophage tropism, which have revealed that viruses can vary up to 1,000-fold in the ability to infect MDMs (18), with most being capable of some level of infection (Fig. 3). In addition, our study and other studies have shown that MDMs are highly variable in their susceptibility to HIV-1 (Fig. 3) (34, 35). These sources of variation mean that assays that assess infection of MDMs do not yield unequivocal results that clearly separate HIV-1 variants adapted to growing in $CD4^+$ T cells, with their high levels of CD4, from viruses that have evolved the ability to grow in cells with low levels of CD4, such as macrophages. We have attempted to provide a more rigorous definition of both an entry assay that can identify the evolutionary step that allows entry using low levels of CD4 and examples of viruses that have undergone that evolutionary step to validate the assay.

MDMs themselves are only a representation of the heterogeneous populations of cells that collectively represent macrophage-like cells in the body. The vast majority of macrophages are found in tissue, where they differentiate either from progenitor cells that migrated into the tissue during embryonic development or from blood-derived monocytes (reviewed in reference 36). In the central nervous system, for example, microglia are the predominant macrophage-like cells in the brain parenchyma (37) and are derived from precursors that colonize the brain during embryonic development (38, 39). Their location allows microglia to survey the brain for pathogens and to promote brain development and homeostasis by performing tasks such as synaptic pruning (40) and phagocytosis of apoptotic neurons (41). In contrast, perivascular macrophages are located in the perivascular (Virchow-Robin) spaces of blood vessels that traverse the central nervous system and are derived from monocytes that migrate from the blood (42). Their position ensures that they are often the first immune cells that pathogens encounter after breaching the blood-brain barrier. As a result, they are exposed to more pathogens than cells elsewhere in the brain and may be more likely to be infected by HIV-1. Consistent with this possibility, a study of simian immunodeficiency virus (SIV)-infected brains found that perivascular macrophages are infected more often than microglia (43). Similarly, the liver has macrophage-like resident cells, i.e., Kupffer cells, that migrate into the liver during embryonic development (39, 44) and can have an influx of macrophages as the result of inflammation. These observations point out that ontogeny and anatomical location influence macrophage function and, potentially, relevance to infection by HIV-1.

Macrophage function is further affected by the activation state, which can change rapidly in response to changes in the signaling environment (reviewed in references 45 and 46). Macrophages have been classified as either M1 or M2, with activated M1 macrophages having proinflammatory, antimicrobial phenotypes and M2 macrophages having anti-inflammatory, wound-healing phenotypes (reviewed in reference 45). It is now appreciated that even this dichotomy does not capture the full continuum of macrophage phenotypes (47) and that additional macrophage diversity is generated from the complex signaling environment (47) and from inherent differences, such as basal gene expression (48), that alter how cells respond to signals. Together, cellular ontogeny, signaling environment, activation state, and basal gene expression generate diverse macrophage populations capable of performing a variety of functions.

The major function of CD4 is to act as a coreceptor with the T cell receptor in interactions with antigen-presenting major histocompatibility complex (MHC) class II complexes. Since macrophages are antigen-presenting cells, the low levels of CD4 that are expressed on macrophages cannot function in this role. Thus, it is not clear that the CD4 that is found on macrophages is biologically significant (for the macrophages), although it has been proposed (49) that CD4 in this context could serve an alternative function, as a coreceptor to and enhancer of signaling of Fcγ receptors. However, CD4 is not detected on macrophages of mice (50), suggesting that its presence is dispensable. There is no information concerning whether the different types of macrophages and macrophage-like cells in the body, or their different activation states, vary in the level of surface CD4 expression. Thus, the use of monocytes that are induced to differentiate in cell culture to measure viral entry phenotypes overly simplifies what is likely to be a much more complex interaction between HIV-1 and macrophages *in vivo*. In our own attempts to examine the levels of CD4 on macrophages, we have found that they can vary between donors (Fig. 3A; Table 3) and in the same donor at different collection times (Fig. 3B; Table 3). This makes it difficult to ascribe an entry phenotype consistent with macrophage tropism. Difficulty in assigning a clear "macrophage-tropic" phenotype has also been reviewed by Duncan and Sattentau (51).

How does the range of CD4 densities spanned in our tropism assay compare to CD4 densities expressed on MDMs and other potential target cells, at least as represented as primary cells in culture? A common misconception about macrophages is that they express fewer CD4 molecules per cell than do T cells, when in fact they express similar numbers of receptors per cell but express CD4 at much lower densities ($CD4/\mu m^2$). In this study, we confirmed (Fig. 2B) the earlier observation that when exposed to M-CSF, monocytes can differentiate into macrophages that express numbers of CD4 molecules similar to those expressed on T cells (52), but we observed that their large size causes MDMs to have CD4 densities that are approximately 20-fold lower than those of T cells (Fig. 2C). Furthermore, we showed that at their uninduced level, Affinofile cells express CD4 densities that are somewhat lower than those of monocytes and MDMs, while at their maximum induction level they express CD4 densities that are almost as high as that of T cells (Fig. 2C). Thus, our assay roughly spans the range of CD4 densities present on macro-

phages and T cells and reveals differences between viruses adapted to replication in cells that express very different CD4 densities (Fig. 2D).

While manipulating CD4 densities on Affinofile cells serves as a tractable system for testing entry phenotypes, accurately assessing those phenotypes also requires defining how true "macrophage-tropic" viruses perform within that system. Studies using other systems have shown that some brain-derived viruses are capable of replicating in macrophages and have an increased ability to enter (17, 19, 53) and/or fuse with (16, 53) cells expressing low levels of CD4. The ability to use low levels of CD4 for entry (17) has also been observed for a small number of prototypical macrophage-tropic viruses previously generated by coculturing lung tissue (BaL) (12), brain tissue (JR-FL) (20), or PBMCs (Ada) (54) with primary cells or by cloning viruses directly from brain tissue (YU-2) (55). It is worth considering, however, whether coculturing with MDMs or PBMCs allowed some of these viruses to evolve additional phenotypes that do not represent M-tropic viruses *in vivo*. We recently observed this for Ba-L, which has a neutralization-sensitive phenotype (K. T. Arrildt, unpublished data) that would likely be selected against *in vivo*. A common feature of most of these M-tropic clones is that they were not generated using endpoint dilution PCR to avoid PCR-mediated recombination of the viral genomes that can obscure their original sequence organization *in vivo*.

We previously generated a panel of *env* genes from viruses that were validated as being derived from macrophage-tropic variants by multiple *in vivo* and *in vitro* measures, including coming from a compartmentalized viral population in the CSF/CNS, being produced from long-lived cells, and being able to infect macrophages and cells with low levels of CD4 (23, 24). In addition, the *env* genes were generated by endpoint dilution to avoid PCR-mediated recombination, and in each case the CSF/CNS-derived viruses were paired with blood-derived viruses from the same subject. We believe that these viruses are well-validated examples of variants that can evolve within the CNS in late-stage infection. We found that these viruses largely fell into two distinct groups: T-tropic viruses with very low infectivities ($\leq$2%) when CD4 levels were low and M-tropic viruses that retained moderately high infectivities ($\geq$15%) even with low levels of CD4 (Fig. 1A; Table 1). Differences in infectivity remained pronounced for viruses infecting cells expressing all but the highest levels of CD4, as seen by the M-tropic viruses having significantly lower CD4 ED_{50} values (Table 1). Thus, our assay is a reliable and quantitative method for distinguishing M- and T-tropic viruses based on their capacity to infect cells expressing low CD4 densities. Using these types of assay parameters, we have been able to show that most isolates of HIV-1, including the transmitted virus (28), require high levels of CD4 for entry, indicating that most of the time HIV-1 is replicating in $CD4^+$ T cells, with their high densities of surface CD4, and that the virus does not have the entry properties of viruses that have evolved to infect macrophages. We have been able to identify rare examples of viruses with intermediate entry phenotypes (56), although we do not know if this represents phenotypic variation or evolutionary intermediates. Other investigators have also used Affinofile cells to identify a low-CD4-density entry phenotype (57).

We argue that the ability to enter cells with a low density of CD4 must be the first phenotypic change for a lineage of macrophage-tropic virus, because this mediates the initial step in the viral life cycle, i.e., entry. There may be other phenotypic adaptations to replication in macrophages, but these will appear linked on genomes that encode an Env protein that can enter cells with a low density of surface CD4. This reasoning leads to the conclusion that an entry phenotype using a low density of CD4 is a necessary feature of macrophage tropism, although it is not clear if this is the only viral function that must evolve to allow efficient replication in macrophages *in vivo*. Since macrophage-tropic lineages appear to evolve infrequently, at least with respect to the bulk of viral replication in $CD4^+$ T cells, the normal viral gene products are not selected for function in macrophages but rather for function in T cells. Conversely, the use of VSV-G pseudotypes to enhance entry into macrophages (or other cell types) to assess viral protein function obscures the fact that the protein being tested likely has not been selected for function in that cell type.

Do macrophage-tropic viruses ever appear in the blood? We failed to find macrophage-tropic variants in the blood of 62 subjects with intermediate levels of $CD4^+$ T cells (28). However, in macaques that were depleted of $CD4^+$ T cells at the time of infection, macrophages were easily detected as being infected, and these animals had relatively high viral loads (58). In macaques with end-stage disease that were infected with the X4 virus D12, there was clear evidence of infection of macrophages in the lymph nodes (59). In a study by Gray et al. (22), viruses isolated from the blood of patients late in infection (late R5 viruses) were found to infect macrophages better than viruses isolated early in infection (early R5 viruses). These late R5 viruses were found to have phenotypes similar to those of a prototypical macrophage-tropic virus (Ada) and were thus inferred to be macrophage tropic. However, as noted above, the use of MDMs can result in viruses being identified as macrophage tropic despite being poorly adapted to infecting macrophages *in vivo*. Thus, the extent to which macrophage-tropic viruses evolve outside the CNS and reach a significant fraction of the systemic viral load remains an important question about HIV-1 pathogenesis.

Do R5 T cell-tropic viruses ever infect macrophages? The fact that most HIV-1 lineages (in both the CNS and blood) are R5 T-cell tropic suggests that these lineages do not replicate predominantly in macrophages. However, this does not indicate that these viruses exclusively infect T cells. While macrophage-tropic HIV-1 is better overall at entering MDMs *in vitro* (Fig. 3), T cell-tropic viruses can enter MDMs at reduced levels. The effect that intermittent infection of macrophages by T cell-tropic viruses, if it occurs, may have on pathogenesis or latency is unknown.

We also examined several other entry-associated features of low-CD4-density entry. Using a cell-cell fusion assay, we were able to show that both M- and T cell-tropic clones still require CD4 and a coreceptor (CCR5 in this assay) for entry (Fig. 4), indicating that the entry activity of M-tropic viruses at low levels of CD4 does not represent CD4-independent entry. We also found that M-tropic and T cell-tropic viruses do not differ in the ability to infect cells expressing low levels of CCR5, their overall sensitivity to CCR5 density (Fig. 5A; Table 4), or their ability to utilize an alternative conformation of CCR5 (Fig. 5B and C). These findings are consistent with previous studies showing that macrophage tropism is not related to sensitivity to the CCR5 antagonist TAK-779 (16, 17, 60).

Having a more quantitative assay to define macrophage-tropic viruses will help to bring clarity to two other relevant issues. First, viruses with the ability to infect cells with low levels of CD4 create

independently replicating populations and potentially establish alternative types of latently infected cells. The observation that initiating therapy causes these $CD4^{low}$ viruses to slowly decay within the CSF of most subjects (24) indicates that they are produced from long-lived, but not immortal, cells within the CNS. However, it is not known if these cells (macrophages and/or microglia) can have latent infections that stochastically release virus over time. If this were a significant reservoir, either in subjects currently on therapy or after the introduction of purging strategies for latently infected $CD4^+$ T cells, then the rebound virus should be able to enter cells with low levels of CD4. Second, there are reports of infection of cells that have no detectable CD4, such as astrocytes (61) and renal tubular cells (62, 63). It is not clear if low-level infectivity of these cell types (or other cell types) occurs with M- or T-tropic HIV-1, although it has been reported that up to 10 to 20% of astrocytes are infected *in vivo* (61), despite there being no evidence that HIV can replicate in these cells (61, 64, 65). It is important to examine the entry phenotype of viruses reported to be in these cell types to be able to conceptualize a pathway that could account for viral entry in the absence of CD4. In this regard, a report that monocytes in the blood (with their low densities of CD4) were infected with a virus that was unable to infect T cells (66) also deserves careful examination, as this type of infection is the basis for the "Trojan horse" model for introduction of virus into the CNS.

The use of Affinofile cells to profile the dependence of viruses on CD4 density to define the entry phenotype will allow for a more rigorous identification of viral variants that have undergone the evolutionary step to be able to use low levels of CD4 in viral replication. Clarifying when and where such viruses evolve will allow them to be placed more accurately in the context of HIV-1 pathogenesis and latency. In addition, identifying the cell type in which a virus is replicating will also allow an appropriate link to be made between selection for a viral gene product function in the context of host cell interaction.

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REFERENCES

1. **Arrildt K, Joseph SB, Swanstrom R.** 2012. The HIV-1 ENV protein: a coat of many colors. Curr. HIV/AIDS Rep. **9:**52–63. http://dx.doi.org/10.1007/s11904-011-0107-3.
2. **Zamarchi R, Allavena P, Borsetti A, Stievano L, Tosello V, Marcato N, Esposito G, Roni V, Paganin C, Bianchi G, Titti F, Verani P, Gerosa G, Amadori A.** 2002. Expression and functional activity of CXCR-4 and CCR-5 chemokine receptors in human thymocytes. Clin. Exp. Immunol. **127:**321–330. http://dx.doi.org/10.1046/j.1365-2249.2002.01775.x.
3. **Pace MJ, Agosto L, O'Doherty U.** 2011. R5 HIV env and vesicular stomatitis virus G protein cooperate to mediate fusion to naive CD4(+) T cells. J. Virol. **85:**644–648. http://dx.doi.org/10.1128/JVI.01851-10.
4. **van't Wout AB, Kootstra NA, Mulderkampinga GA, Albrechtvanlent N, Scherpbier HJ, Veenstra J, Boer K, Coutinho RA, Miedema F, Schuitemaker H.** 1994. Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. J. Clin. Invest. **94:**2060–2067. http://dx.doi.org/10.1172/JCI117560.
5. **Zhu TF, Mo HM, Wang N, Nam DS, Cao YZ, Koup RA, Ho DD.** 1993. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science **261:**1179–1181. http://dx.doi.org/10.1126/science.8356453.
6. **Braathen LR, Ramirez G, Kunze ROF, Gelderblom H.** 1987. Langerhans cells as primary target cells for HIV infection. Lancet **ii:**1094.
7. **Palacio J, Souberbielle BE, Shattock RJ, Robinson G, Manyonda I, Griffin GE.** 1994. In vitro HIV1 infection of human cervical tissue. Res. Virol. **145:**155–161. http://dx.doi.org/10.1016/S0923-2516(07)80017-3.
8. **Li H, Bar KJ, Wang S, Decker JM, Chen Y, Sun C, Salazar-Gonzalez JF, Salazar MG, Learn GH, Morgan CJ, Schumacher JE, Hraber P, Giorgi EE, Bhattacharya T, Korber BT, Perelson AS, Eron JJ, Cohen MS, Hicks CB, Haynes BF, Markowitz M, Keele BF, Hahn BH, Shaw GM.** 2010. High multiplicity infection by HIV-1 in men who have sex with men. PLoS Pathog. **6:**e1000890. http://dx.doi.org/10.1371/journal.ppat.1000890.
9. **Ochsenbauer C, Edmonds TG, Ding HT, 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.
10. **Salazar-Gonzalez JF, Salazar MG, Keele BF, Learn GH, Giorgi EE, Li H, Decker JM, Wang SY, Baalwa J, Kraus MH, Parrish NF, Shaw KS, Guffey MB, Bar KJ, Davis KL, Ochsenbauer-Jambor C, Kappes JC, Saag MS, Cohen MS, Mulenga J, Derdeyn CA, Allen S, Hunter E, Markowitz M, Hraber P, Perelson AS, Bhattacharya T, Haynes BF, Korber BT, Hahn BH, Shaw GM.** 2009. Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection. J. Exp. Med. **206:**1273–1289. http://dx.doi.org/10.1084/jem.20090378.
11. **Isaacman-Beck J, Hermann EA, Yi YJ, Ratcliffe SJ, Mulenga J, Allen S, Hunter E, Derdeyn CA, Collman RG.** 2009. Heterosexual transmission of human immunodeficiency virus type 1 subtype C: macrophage tropism, alternative coreceptor use, and the molecular anatomy of CCR5 utilization. J. Virol. **83:**8208–8220. http://dx.doi.org/10.1128/JVI.00296-09.
12. **Gartner S, Markovits P, Markovitz DM, Kaplan MH, Gallo RC, Popovic M.** 1986. The role of mononuclear phagocytes in HTLV-III/LAV infection. Science **233:**215–219. http://dx.doi.org/10.1126/science.3014648.
13. **Dunfee R, Thomas ER, Gorry PR, Wang JB, Ancuta P, Gabuzda D.** 2006. Mechanisms of HIV-1 neurotropism. Curr. HIV Res. **4:**267–278. http://dx.doi.org/10.2174/157016206777709500.
14. **Duenas-Decamp MJ, Peters PJ, Burton D, Clapham PR.** 2009. Determinants flanking the CD4 binding loop modulate macrophage tropism of human immunodeficiency virus type 1 R5 envelopes. J. Virol. **83:**2575–2583. http://dx.doi.org/10.1128/JVI.02133-08.
15. **Gorry PR, Taylor J, Holm GH, Mehle A, Morgan T, Cayabyab M, Farzan M, Wang H, Bell JE, Kunstman K, Moore JP, Wolinsky SM, Gabuzda D.** 2002. Increased CCR5 affinity and reduced CCR5/CD4 dependence of a neurovirulent primary human immunodeficiency virus type 1 isolate. J. Virol. **76:**6277–6292. http://dx.doi.org/10.1128/JVI.76.12.6277-6292.2002.
16. **Martin-Garcia J, Cao W, Varela-Robena A, Plassmeyer ML, Gonzalez-Scarano F.** 2006. HIV-1 tropism for the central nervous system: brain-derived envelope glycoproteins with lower CD4 dependence and reduced sensitivity to a fusion inhibitor. Virology **346:**169–179. http://dx.doi.org/10.1016/j.virol.2005.10.031.
17. **Peters PJ, Bhattacharya J, Hibbitts S, Dittmar MT, Simmons G, Bell J, Simmonds P, Clapham PR.** 2004. Biological analysis of human immunodeficiency virus type 1 R5 envelopes amplified from brain and lymph node tissues of AIDS patients with neuropathology reveals two distinct tropism phenotypes and identifies envelopes in the brain that confer an enhanced tropism and fusigenicity for macrophages. J. Virol. **78:**6915–6926. http://dx.doi.org/10.1128/JVI.78.13.6915-6926.2004.
18. **Peters PJ, Sullivan WM, Duenas-Decamp MJ, Bhattacharya J, Ankghuambom C, Brown R, Luzuriaga K, Bell J, Simmonds P, Ball J, Clapham PR.** 2006. Non-macrophage-tropic human immunodeficiency virus type 1 R5 envelopes predominate in blood, lymph nodes, and semen: implications for transmission and pathogenesis. J. Virol. **80:**6324–6332. http://dx.doi.org/10.1128/JVI.02328-05.
19. **Gorry PR, Bristol G, Zack JA, Ritola K, Swanstrom R, Birch CJ, Bell JE, Bannert N, Crawford K, Wang H, Schols D, De Clercq E, Kunstman K, Wolinsky SM, Gabuzda D.** 2001. Macrophage tropism of human immunodeficiency virus type 1 isolates from brain and lymphoid tissues predicts

neurotropism independent of coreceptor specificity. J. Virol. **75:**10073–10089. http://dx.doi.org/10.1128/JVI.75.21.10073-10089.2001.

20. **Koyanagi Y, Miles S, Mitsuyasu RT, Merrill JE, Vinters HV, Chen ISY.** 1987. Dual infection of the central-nervous-system by AIDS viruses with distinct cellular tropisms. Science **236:**819–822. http://dx.doi.org/10.1126/science.3646751.
21. **Li S, Juarez J, Alali M, Dwyer D, Collman R, Cunningham A, Naif HM.** 1999. Persistent CCR5 utilization and enhanced macrophage tropism by primary blood human immunodeficiency virus type 1 isolates from advanced stages of disease and comparison to tissue-derived isolates. J. Virol. **73:**9741–9755.
22. **Gray L, Sterjovski J, Churchill M, Ellery P, Nasr N, Lewin SR, Crowe SM, Wesselingh SL, Cunningham AL, Gorry PR.** 2005. Uncoupling coreceptor usage of human immunodeficiency virus type 1 (HIV-1) from macrophage tropism reveals biological properties of CCR5-restricted HIV-1 isolates from patients with acquired immunodeficiency syndrome. Virology **337:**384–398. http://dx.doi.org/10.1016/j.virol.2005.04.034.
23. **Schnell G, Joseph S, Spudich S, Price RW, Swanstrom R.** 2011. HIV-1 replication in the central nervous system occurs in two distinct cell types. PLoS Pathog. **7:**e1002286. http://dx.doi.org/10.1371/journal.ppat.1002286.
24. **Schnell G, Spudich S, Harrington P, Price RW, Swanstrom R.** 2009. Compartmentalized human immunodeficiency virus type 1 originates from long-lived cells in some subjects with HIV-1-associated dementia. PLoS Pathog. **5:**e1000395. http://dx.doi.org/10.1371/journal.ppat.1000395.
25. **Churchill M, Nath A.** 2013. Where does HIV hide? A focus on the central nervous system. Curr. Opin. HIV AIDS **8:**165–169. http://dx.doi.org/10.1097/COH.0b013e32835fc601.
26. **Johnston SH, Lobritz MA, Nguyen S, Lassen K, Delair S, Posta F, Bryson YJ, Arts EJ, Chou T, Lee B.** 2009. A quantitative affinity-profiling system that reveals distinct CD4/CCR5 usage patterns among human immunodeficiency virus type 1 and simian immunodeficiency virus strains. J. Virol. **83:**11016–11026. http://dx.doi.org/10.1128/JVI.01242-09.
27. **Staprans S, Marlowe N, Glidden D, Novakovic-Agopian T, Grant RM, Heyes M, Aweeka F, Deeks S, Price RW.** 1999. Time course of cerebrospinal fluid responses to antiretroviral therapy: evidence for variable compartmentalization of infection. AIDS **13:**1051–1061. http://dx.doi.org/10.1097/00002030-199906180-00008.
28. **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.** 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.
29. **Abrahams MR, Anderson JA, Giorgi EE, Seoighe C, Mlisana K, Ping LH, Athreya GS, Treurnicht FK, Keele BF, Wood N, Salazar-Gonzalez JF, Bhattacharya T, Chu H, Hoffman I, Galvin S, Mapanje C, Kazembe P, Thebus R, Fiscus S, Hide W, Cohen MS, Karim SA, Haynes BF, Shaw GM, Hahn BH, Korber BT, Swanstrom R, Williamson C.** 2009. Quantitating the multiplicity of infection with human immunodeficiency virus type 1 subtype C reveals a non-Poisson distribution of transmitted variants. J. Virol. **83:**3556–3567. http://dx.doi.org/10.1128/JVI.02132-08.
30. **Keele BF, Giorgi EE, Salazar-Gonzalez JF, Decker JM, Pham KT, Salazar MG, Sun CX, Grayson T, Wang SY, Li H, Wei XP, Jiang CL, 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 characterisation 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.
31. **Salazar-Gonzalez JF, Bailes E, Pham KT, Salazar MG, Guffey MB, Keele BF, Derdeyn CA, Farmer P, Hunter E, Allen S, Manigart O, Mulenga J, Anderson JA, Swanstrom R, Haynes BF, Athreya GS, Korber BTM, Sharp PM, Shaw GM, Hahn BH.** 2008. Deciphering human immunodeficiency virus type 1 transmission and early envelope diversification by single-genome amplification and sequencing. J. Virol. **82:**3952–3970. http://dx.doi.org/10.1128/JVI.02660-07.
32. **Del Prete GQ, Haggarty B, Leslie GJ, Jordan APO, Romano J, Wang N, Wang JB, Holmes MC, Montefiori DC, Hoxie JA.** 2009. Derivation and characterization of a simian immunodeficiency virus SIVmac239 variant with tropism for CXCR4. J. Virol. **83:**9911–9922. http://dx.doi.org/10.1128/JVI.00533-09.
33. **Rucker J, Doranz BJ, Edinger AL, Long D, Berson JF, Doms RW.** 1997. Cell-cell fusion assay to study role of chemokine receptors in human immunodeficiency virus type 1 entry. Methods Enzymol. **288:**118–133.
34. **Naif HM, Li S, Alali M, Sloane A, Wu LJ, Kelly M, Lynch G, Lloyd A, Cunningham AL.** 1998. CCR5 expression correlates with susceptibility of maturing monocytes to human immunodeficiency virus type 1 infection. J. Virol. **72:**830–836.
35. **Sonza S, Maerz A, Uren S, Violo A, Hunter S, Boyle W, Crowe S.** 1995. Susceptibility of human monocytes to HIV type 1 infection in vitro is not dependent on their level of CD4 expression. AIDS Res. Hum. Retroviruses **11:**769–776. http://dx.doi.org/10.1089/aid.1995.11.769.
36. **Wynn TA, Chawla A, Pollard JW.** 2013. Macrophage biology in development, homeostasis and disease. Nature **496:**445–455. http://dx.doi.org/10.1038/nature12034.
37. **Polazzi E, Monti B.** 2010. Microglia and neuroprotection: from in vitro studies to therapeutic applications. Prog. Neurobiol. **92:**293–315. http://dx.doi.org/10.1016/j.pneurobio.2010.06.009.
38. **Ginhoux F, Greter M, Leboeuf M, Nandi S, See P, Gokhan S, Mehler MF, Conway SJ, Ng LG, Stanley ER, Samokhvalov IM, Merad M.** 2010. Fate mapping analysis reveals that adult microglia derive from primitive macrophages. Science **330:**841–845. http://dx.doi.org/10.1126/science.1194637.
39. **Schulz C, Perdiguero EG, Chorro L, Szabo-Rogers H, Cagnard N, Kierdorf K, Prinz M, Wu BS, Jacobsen SEW, Pollard JW, Frampton J, Liu KJ, Geissmann F.** 2012. A lineage of myeloid cells independent of Myb and hematopoietic stem cells. Science **336:**86–90. http://dx.doi.org/10.1126/science.1219179.
40. **Paolicelli RC, Bolasco G, Pagani F, Maggi L, Scianni M, Panzanelli P, Giustetto M, Ferreira TA, Guiducci E, Dumas L, Ragozzino D, Gross CT.** 2011. Synaptic pruning by microglia is necessary for normal brain development. Science **333:**1456–1458. http://dx.doi.org/10.1126/science.1202529.
41. **Sierra A, Encinas JM, Deudero JJP, Chancey JH, Enikolopov G, Overstreet-Wadiche LS, Tsirka SE, Maletic-Savatic M.** 2010. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. Cell Stem Cell **7:**483–495. http://dx.doi.org/10.1016/j.stem.2010.08.014.
42. **Unger ER, Sung JH, Manivel JC, Chenggis ML, Blazar BR, Krivit W.** 1993. Male donor-derived cells in the brains of female sex-mismatched bone-marrow transplant recipients—a Y-chromosome specific in-situ hybridization study. J. Neuropathol. Exp. Neurol. **52:**460–470. http://dx.doi.org/10.1097/00005072-199309000-00004.
43. **Williams KC, Corey S, Westmoreland SV, Pauley D, Knight H, deBakker C, Alvarez X, Lackner AA.** 2001. Perivascular macrophages are the primary cell type productively infected by simian immunodeficiency virus in the brains of macaques: implications for the neuropathogenesis of AIDS. J. Exp. Med. **193:**905–915. http://dx.doi.org/10.1084/jem.193.8.905.
44. **Klein I, Cornejo JC, Polakos NK, John B, Wuensch SA, Topham DJ, Pierce RH, Crispe IN.** 2007. Kupffer cell heterogeneity: functional properties of bone marrow-derived and sessile hepatic macrophages. Blood **110:**4077–4085. http://dx.doi.org/10.1182/blood-2007-02-073841.
45. **Murray PJ, Wynn TA.** 2011. Protective and pathogenic functions of macrophage subsets. Nat. Rev. Immunol. **11:**723–737. http://dx.doi.org/10.1038/nri3073.
46. **London A, Cohen M, Schwartz M.** 2013. Microglia and monocyte-derived macrophages: functionally distinct populations that act in concert in CNS plasticity and repair. Front. Cell Neurosci. **7:**34. http://dx.doi.org/10.3389/fncel.2013.00034.
47. **Mosser DM, Edwards JP.** 2008. Exploring the full spectrum of macrophage activation. Nat. Rev. Immunol. **8:**958–969. http://dx.doi.org/10.1038/nri2448.
48. **Crain JM, Nikodemova M, Watters JJ.** 2013. Microglia express distinct M1 and M2 phenotypic markers in the postnatal and adult central nervous system in male and female mice. J. Neurosci. Res. **91:**1143–1151. http://dx.doi.org/10.1002/jnr.23242.
49. **Gibbings D, Befus AD.** 2009. CD4 and CD8: an inside-out coreceptor model for innate immune cells. J. Leukoc. Biol. **86:**251–259. http://dx.doi.org/10.1189/jlb.0109040.
50. **Crocker PR, Jefferies WA, Clark SJ, Chung LP, Gordon S.** 1987. Species

heterogeneity in macrophage expression of the CD4 antigen. J. Exp. Med. **166:**613–618. http://dx.doi.org/10.1084/jem.166.2.613.
51. **Duncan CJA, Sattentau QJ.** 2011. Viral determinants of HIV-1 macrophage tropism. Viruses **3:**2255–2279. http://dx.doi.org/10.3390/v3112255.
52. **Lee B, Sharron M, Montaner LJ, Weissman D, Doms RW.** 1999. Quantification of CD4, CCR5, and CXCR4 levels on lymphocyte subsets, dendritic cells, and differentially conditioned monocyte-derived macrophages. Proc. Natl. Acad. Sci. U. S. A. **96:**5215–5220. http://dx.doi.org/10.1073/pnas.96.9.5215.
53. **Thomas ER, Dunfee RL, Stanton J, Bogdan D, Taylor J, Kunstman K, Bell JE, Wolinsky SM, Gabuzda D.** 2007. Macrophage entry mediated by HIV Envs from brain and lymphoid tissues is determined by the capacity to use low CD4 levels and overall efficiency of fusion. Virology **360:**105–119. http://dx.doi.org/10.1016/j.virol.2006.09.036.
54. **Gendelman HE, Orenstein JM, Martin MA, Ferrua C, Mitra R, Phipps T, Wahl LA, Lane HC, Fauci AS, Burke DS, Skillman D, Meltzer MS.** 1988. Efficient isolation and propagation of human immunodeficiency virus on recombinant colony-stimulating factor 1-treated monocytes. J. Exp. Med. **167:**1428–1441. http://dx.doi.org/10.1084/jem.167.4.1428.
55. **Li YX, Kappes JC, Conway JA, Price RW, Shaw GM, Hahn BH.** 1991. Molecular characterization of human immunodeficiency virus type 1 cloned directly from uncultured human brain tissue—identification of replication-competent and replication-defective viral genomes. J. Virol. **65:**3973–3985.
56. **Sturdevant CB, Dow A, Jabara CB, Joseph SB, Schnell G, Takamune N, Mallewa M, Heyderman RS, Van Rie A, Swanstrom R.** 2012. Central nervous system compartmentalization of HIV-1 subtype C variants early and late in infection in young children. PLoS Pathog. **8:**e1003094. http://dx.doi.org/10.1371/journal.ppat.1003094.
57. **Salimi H, Roche M, Webb N, Gray LR, Chikere K, Sterjovski J, Ellett A, Wesselingh SL, Ramsland PA, Lee B, Churchill MJ, Gorry PR.** 2013. Macrophage-tropic HIV-1 variants from brain demonstrate alterations in the way gp120 engages both CD4 and CCR5. J. Leukoc. Biol. **93:**113–126. http://dx.doi.org/10.1189/jlb.0612308.
58. **Ortiz AM, Klatt NR, Li B, Yi Y, Tabb B, Hao XP, Sternberg L, Lawson B, Carnathan PM, Cramer EM, Engram JC, Little DM, Ryzhova E, Gonzalez-Scarano F, Paiardini M, Ansari AA, Ratcliffe S, Else JG, Brenchley JM, Collman RG, Estes JD, Derdeyn CA, Silvestri G.** 2011. Depletion of CD4(+) T cells abrogates post-peak decline of viremia in SIV-infected rhesus macaques. J. Clin. Invest. **121:**4433–4445. http://dx.doi.org/10.1172/JCI46023.
59. **Igarashi T, Brown CR, Endo Y, Buckler-White A, Plishka R, Bischofberger N, Hirsch V, Martin MA.** 2001. Macrophage are the principal reservoir and sustain high virus loads in rhesus macaques after the depletion of CD4(+) T cells by a highly pathogenic simian immunodeficiency virus/HIV type 1 chimera (SHIV): implications for HIV-1 infections of humans. Proc. Natl. Acad. Sci. U. S. A. **98:**658–663. http://dx.doi.org/10.1073/pnas.98.2.658.
60. **Peters PJ, Duenas-Decamp MJ, Sullivan WM, Brown R, Ankghuambom C, Luzuriaga K, Robinson J, Burton DR, Bell J, Simmonds P, Ball J, Clapham PR.** 2008. Variation in HIV-1 R5 macrophage-tropism correlates with sensitivity to reagents that block envelope:CD4 interactions but not with sensitivity to other entry inhibitors. Retrovirology **5:**5. http://dx.doi.org/10.1186/1742-4690-5-5.
61. **Churchill MJ, Wesselingh SL, Cowley D, Pardo CA, McArthur JC, Brew BJ, Gorry PR.** 2009. Extensive astrocyte infection is prominent in human immunodeficiency virus-associated dementia. Ann. Neurol. **66:**253–258. http://dx.doi.org/10.1002/ana.21697.
62. **Bruggeman LA, Ross MD, Tanji N, Cara A, Dikman S, Gordon RE, Burns GC, D'Agati VD, Winston JA, Klotman ME, Klotman PE.** 2000. Renal epithelium is a previously unrecognized site of HIV-1 infection. J. Am. Soc. Nephrol. **11:**2079–2087.
63. **Marras D, Bruggeman LA, Gao F, Tanji N, Mansukhani MM, Cara A, Ross MD, Gusella GL, Benson G, D'Agati VD, Hahn BH, Klotman ME, Klotman PE.** 2002. Replication and compartmentalization of HIV-1 in kidney epithelium of patients with HIV-associated nephropathy. Nat. Med. **8:**522–526. http://dx.doi.org/10.1038/nm0502-522.
64. **Gorry PR, Ong C, Thorpe J, Bannwarth S, Thompson KA, Gatignol A, Wesselingh SL, Purcell DFJ.** 2003. Astrocyte infection by HIV-1: mechanisms of restricted virus replication, and role in the pathogenesis of HIV-1-associated dementia. Curr. HIV Res. **1:**463–473. http://dx.doi.org/10.2174/1570162033485122.
65. **Thompson KA, Churchill MJ, Gorry PR, Sterjovski J, Oelrichs RB, Wesselingh SL, McLean CA.** 2004. Astrocyte specific viral strains in HIV dementia. Ann. Neurol. **56:**873–877. http://dx.doi.org/10.1002/ana.20304.
66. **Zhu TF, Muthui D, Holte S, Nickle D, Feng F, Brodie S, Hwangbo Y, Mullins JI, Corey L.** 2002. Evidence for human immunodeficiency virus type 1 replication in vivo in CD14(+) monocytes and its potential role as a source of virus in patients on highly active antiretroviral therapy. J. Virol. **76:**707–716. http://dx.doi.org/10.1128/JVI.76.2.707-716.2002.
67. **Gulick RM, Lalezari J, Goodrich J, Clumeck N, DeJesus E, Horban A, Nadler J, Clotet B, Karlsson A, Wohlfeiler M, Montana JB, McHale M, Sullivan J, Ridgway C, Felstead S, Dunne MW, van der Ryst E, Mayer H.** 2008. Maraviroc for previously treated patients with R5 HIV-1 infection. N. Engl. J. Med. **359:**1429–1441. http://dx.doi.org/10.1056/NEJMoa0803152.
68. **Stephenson J.** 2007. Researchers buoyed by novel HIV drugs—will expand drug arsenal against resistant virus. JAMA **297:**1535–1536. http://dx.doi.org/10.1001/jama.297.14.1535.