A Quantitative Affinity-Profiling System That Reveals Distinct CD4/CCR5 Usage Patterns among Human Immunodeficiency Virus Type 1 and Simian Immunodeficiency Virus Strains[▽]

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The affinity of human immunodeficiency virus (HIV) envelope for CD4 and CCR5 appears to be associated with aspects of R5 virus (virus using the CCR5 coreceptor) pathogenicity. However, entry efficiency results from complex interactions between the viral envelope glycoprotein and both CD4 and CCR5, which limits attempts to correlate viral pathogenicity with surrogate measures of envelope CD4 and CCR5 affinities. Here, we present a system that provides a quantitative and comprehensive characterization of viral entry efficiency as a direct interdependent function of both CD4 and CCR5 levels. This receptor affinity profiling system also revealed heretofore unappreciated complexities underlying CD4/CCR5 usage. We first developed a dually inducible cell line in which CD4 and CCR5 could be simultaneously and independently regulated within a physiologic range of surface expression. Infection by multiple HIV type 1 (HIV-1) and simian immunodeficiency virus isolates could be examined simultaneously for up to 48 different combinations of CD4/CCR5 expression levels, resulting in a distinct usage pattern for each virus. Thus, each virus generated a unique three-dimensional surface plot in which viral infectivity varied as a function of both CD4 and CCR5 expression. From this functional form, we obtained a sensitivity vector along with corresponding metrics that quantified an isolate's overall efficiency of CD4/CCR5 usage. When applied to viral isolates with well-characterized sensitivities to entry/fusion inhibitors, the vector metrics were able to encapsulate their known biological phenotypes. The application of the vector metrics also indicated that envelopes derived from elite suppressors had overall-reduced entry efficiencies compared to those of envelopes derived from chronically infected viremic progressors. Our affinity-profiling system may help to refine studies of R5 virus tropism and pathogenesis.

Human immunodeficiency virus (HIV) enters cells via engagement of its envelope glycoprotein with CD4 and a coreceptor (CCR5 or CXCR4), which induces fusion of the viral and target cell membranes (4). Although many chemokine receptors can serve as coreceptors for HIV in vitro, only CXCR4 and CCR5 have a major role in vivo (29). The majority of viruses transmitted use CCR5 as a coreceptor exclusively (R5 virus) (24, 43, 47). This is underscored by the observation that individuals homozygous for a 32-bp deletion in the CCR5 receptor gene are highly resistant to HIV infection and that heterozygous individuals have a delayed progression to disease (reviewed in reference 33).

While it is clear that the appearance of virus using the CXCR4 coreceptor correlates with progression to AIDS, many slow and rapid progressors harbor R5 virus throughout their clinical course (4, 29, 41, 46). Thus, viral tropism alone does not explain differences in disease progression among those

patients with R5 virus. There are many host and viral factors that account for the varied clinical outcomes of HIV-infected patients. Among viral factors, the role of coreceptor tropism in viral pathogenicity is complex. For clade B infections, up to half of patients develop CXCR4 (X4)-tropic HIV type 1 (HIV-1) variants prior to or during the onset of clinical AIDS (28, 30, 51); however, X4 tropism can be rare in other clades (e.g., clades A and C) that predominate in countries where patients still clearly progress to AIDS (3, 12). For patients with R5 viruses, HIV progression has been associated with enhanced macrophage tropism (1a, 22, 46), the increased ability to use low levels of CCR5 (11, 44), and an increasing replicative fitness (45) and relative entry efficiency of the infecting virus (26, 39). Neurovirulence is also correlated with an isolate's ability to use low levels of CD4 and/or CCR5 present on microglial cells (8, 10, 27). Furthermore, R5 viruses with increased fitness or derived from late as opposed to early disease show not only increased CCR5 usage but also greater resistance to inhibition by various CCR5 ligands or antagonists (11, 15, 17, 23, 31). Finally, in the simian immunodeficiency virus SIVmac model, R5 SIV strains can clearly become virulent without coreceptor switching (13, 14). Thus, it seems likely that the relative use/affinity of the CD4/CCR5 receptors during disease, rather than a simple switch from R5 to X4 coreceptor tropism, is a better predictor of viral pathogenicity.

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FIG. 1. Schema for generation of 293-Affinofile cells where CD4 and CCR5 expression can be simultaneously and independently induced and regulated. A dually inducible, quadruply stable cell line termed 293-Affinofile cells was generated as described in Materials and Methods. CD4 expression is under the control of the Trex (Tet-On) system, in which the addition of tetracycline or related analogs (e.g., minocycline) releases a repressor protein (expressed from pcDNA6/TR) (1) upstream of a Tet-O-CMV fusion promoter that controls CD4 expression (pcDNA5/TO-CD4) (3). This release allows CD4 expression to proceed. PonA regulates CCR5 expression by dimerizing two subunits of a functional ponasterone receptor expressed from its transactivator (expressed from pcVgRXR) (2). This allows them to bind a region upstream of the ponA-responsive promoter and activate CCR5 expression (pIND-R5) (4). CD4 and CCR5 expression can thus be independently regulated using minocycline and ponA, respectively. +/-Tet or +/-Pon, presence or absence of tetracycline or ponasterone, respectively. In the schematic for 293 affinofile cells, red circles correspond to CD4 immunoglobulin domains, and the green cartoon corresponds to CCR5.

To date, most attempts at determining the efficiency of CD4 and CCR5 usage have relied on indirect competition studies with soluble receptor, antibodies, or ligand. Some studies have used the clonal cell lines derived by the Kabat laboratory which express large or small amounts of CD4 or CCR5 (16, 34), resulting in useful but relatively binary information regarding whether a particular isolate can use high or low levels of CD4 and/or CCR5. Overall, the efficiency of HIV-1 entry into cells within the human host likely results from a complex interplay in the engagement of HIV-1 env glycoproteins with the CD4 and CCR5 receptors. The efficiency with which CCR5 is used for entry may depend on the level of CD4 present and vice versa.

Here, we developed a receptor affinity-profiling (Affinofile) system that can directly quantify the relative efficiencies of CD4 and CCR5 usage as an interdependent function of one another's expression levels. This Affinofile system relies on a dually inducible cell line with independent regulation of CD4 and CCR5 surface expression. The infection efficiency of any given isolate could be measured under multiple combinations of CD4/CCR5 expression levels. Mathematical transformation of the data and analysis of a "sensitivity vector" provided a quantitative, sensitive, and comprehensive measure of an isolate's CD4 and CCR5 usage efficiencies. The results of determining this sensitivity vector for viral isolates with well-characterized sensitivities to entry and fusion inhibitors suggest that our vector metrics not only reflected known biological phenotypes but also quantified complex biological properties that have not been hitherto appreciated. Further application of vector metrics indicated that envelope clones derived from elite suppressors (ES) had overall reduced entry efficiencies compared to those from chronically infected viremic progressors (CP). The use of the vector metrics was also able to cluster ES envs as being distinct from CP envs based on their entry

efficiency profiles in our Affinofile system. Our Affinofile system and vector metrics may help to refine how R5 virus tropism and pathogenesis are studied and defined.

MATERIALS AND METHODS

Viruses. 293T cells were used to make stocks of pseudotyped pNL-GFP SIV316 reporter viruses as previously described (2). Replication-competent stocks of 89.6 and Ba-L viruses were supplied by the Bryson laboratory. Stocks of both viruses were made via propagation in phytohemagglutinin-stimulated peripheral blood mononuclear cells (PBMCs), and viral supernatant was collected at day 7. Viral titers were determined by measuring viral capsid protein concentrations (p24) for PBMC-propagated viral stocks.

293-Affinofile cells. A quadruply stable cell line (HEK 293 background) expressing both the transactivators and the inducible promoters driving CD4 and CCR5 expression was made sequentially by using the selective reagents indicated in Fig. 1. The cell line was single cell cloned at each stage (labeled 1 to 4 sequentially in Fig. 1) to select for the clone with the best properties: a low basal level of expression and induced expression within the physiologic range of CD4 and CCR5 expression. A dually inducible cell line (293-Affinofile) was eventually generated in which the expression of CD4 and CCR5 was simultaneously and independently controlled with minocycline and ponasterone A (ponA), respectively. 293-Affinofile cells were maintained in Dulbecco's modified Eagle's medium–10% dialyzed fetal calf serum (D10F) supplemented with 50 μ g/ml blasticidin (D10F/B). Blasticidin was sufficient for maintaining short-term (<3 months), stable, inducible expression of both CD4 and CCR5.

PBMCs. Normal donor PBMCs stimulated with phytohemagglutinin were maintained in RPMI-1640 medium with 20% fetal bovine serum, 1% L-glutamine, 1% penicillin–streptomycin, and 10 units/ml interleukin-2 (RPMI growth medium).

Reagents and plasmids. Minocycline (Sigma Aldrich, St. Louis, MO) was dissolved in either dimethyl sulfoxide or sterile water to generate a stock concentration of 1 mg/ml. PonA (Invitrogen, Carlsbad, CA) was dissolved in 100% ethanol to generate a stock of 1 mM. Enfuvirtide, soluble CD4 (sCD4), TAK-779, and nevirapine were acquired from the AIDS Research and Reference Reagent Program. Blasticidin HCl (Invitrogen, Carlsbad, CA) was dissolved in sterile water to generate a stock solution of 5 mg/ml. pNL4-3-GFP was obtained from the NIH AIDS Research and Reference Reagent Program and contains green fluorescent protein (GFP) within the envelope gene reading frame. The SIV316 envelope plasmid was also obtained from the NIH AIDS Research and Reference Reagent Program.

FIG. 2. CD4 and CCR5 can be independently and simultaneously regulated in the dually inducible 293-Affinofile cell line. Minocycline (A) and PonA (B) induced CD4 and CCR5 expression without affecting one another. The dually inducible cells were seeded in 24-well plates 24 h before induction with the indicated concentrations of minocycline or ponA. At 18 h after induction, CD4 and CCR5 expression levels were determined by FACS analysis as described in Materials and Methods. Raw histogram data from a representative experiment are presented. (C) Quantification of CD4 and CCR5 expression. Data shown are nonlinear regression curves for CD4 and CCR5 expression levels over the indicated range of minocycline and ponA concentrations. Each minocycline and ponA data point is from six and five independent measurements, respectively. The average numbers of ABS/cell $\pm$ standard deviations are shown, although error bars are too small to be visible in some cases.

CD4 and CCR5 cell surface expression. Duplicate 24-well plates were seeded with 1.2×10^5 dually inducible 293-Affinofile cells/well, and expression of CD4 and CCR5 was induced the following day with minocycline and ponA, respectively. Cells were induced in a matrix format in serial dilutions from 0 to 5 ng/ml of minocycline (CD4) and 0 to 4 μM ponA (CCR5) and allowed to induce at 37°C for 18 h. Cells were then processed for quantitative fluorescence-activated cytometry (qFACS) analysis (21) by using either phycoerythrin-conjugated anti-human CD4 antibody (clone Q4120; Invitrogen, Carlsbad, CA) or phycoerythrin-conjugated mouse anti-human CCR5 antibody (clone 2D7; BD Biosciences, San Jose, CA). Receptor expression levels were quantified by using a QuantiBRITE fluorescence quantitation system (BD Biosciences, San Jose, CA) (20, 21). Regression curves were generated in Graphpad PRISM, where minocycline and ponA concentrations could be converted to their corresponding cell surface concentrations of CD4 and CCR5 in units of antibody binding sites per cell (ABS/cell $\times 10^3$).

Infection of 293-Affinofile cells. Twenty-four-well plates were seeded and induced as described above. Cells were then infected with 5 or 10 ng of p24 of each virus (SIV316, 89.6, or BaL) in the presence of 40 μg/ml DEAE-dextran. Infected cells were spinoculated for 2 h at 2,000 rpm and 37°C. The infection medium was then replaced with fresh D10F/B, and cells were incubated for 48 h at 37°C and 5% CO₂. Infection was assessed by intracellular p24 staining (KC57-RD1 monoclonal antibody) according to the manufacturer's instructions (Beckman Coulter, Fullerton, CA). Mock-infected cells with various concentrations of minocycline and ponA were used as negative controls. For each viral isolate used, additional wells were prepared as described above and infected in the presence of 50 μM TAK-779 to determine the specificity of CCR5-mediated infection.

Single-cycle assays with luciferase reporter viruses. Envelope-pseudotyped luciferase reporter viruses were generated by cotransfection of 293T cells with 1 μg of the luciferase-encoding pseudotyping vector pNL-Luc-AM (37) and 1 μg of envelope expression vector. Cells were washed after 24 h, and pseudoviruses were collected after a subsequent 48 h. The relative numbers of particles were determined by limiting-dilution reverse transcriptase (RT) assay (25). All pseudotyped reporter viruses were used within the linear range of the assay. Equivalent amounts (RT activity) of pseudotyped viruses were added to 293-Affinofile cells previously induced with the indicated matrix formulation of minocycline and ponA. Forty-eight hours later, cells were washed with phosphate-buffered saline and lysed with 100 μl of Glo lysis buffer (Promega, Mad-

ison, WI). Samples were assessed for luciferase activity on a Bio-Rad Lumimark-plus (Bio-Rad, Hercules, CA).

Mathematical analysis. Minocycline and ponA concentrations were rescaled according to

$$x = \frac{\ln \left[\frac{[\text{CD4}]}{[\text{CD4}_{\min}]} \right]}{\ln \left[\frac{[\text{CD4}_{\max}]}{[\text{CD4}_{\min}]} \right]} \quad \text{and} \quad y = \frac{\ln \left[\frac{[\text{CCR5}]}{[\text{CCR5}_{\min}]} \right]}{\ln \left[\frac{[\text{CCR5}_{\max}]}{[\text{CCR5}_{\min}]} \right]} \quad (1)$$

where [CD4] and [CCR5] are the corresponding surface receptor densities (ABS/cell) for each minocycline or ponA concentration. We indicate the minimum and maximum levels of the receptor CD4 as [CD4_{min}] and [CD4_{max}], respectively. Likewise, [CCR5_{min}] and [CCR5_{max}] indicate the minimum and maximum levels of coreceptor. For the experiments performed with SIV316, BaL, and 89.6, the averages (across all samples) of the minimum CD4 and CCR5 concentrations were independently measured to be [CD4_{min}] = 2,189 and [CCR5_{min}] = 1,199 ABS/cell, respectively. Similarly, the averages of the maximum levels of receptor and coreceptor densities across all samples were measured to be [CD4_{max}] = 92,715 and [CCR5_{max}] = 18,717. For all other experiments, we obtained minimum and maximum receptor and coreceptor levels by converting the minocycline and ponA concentrations according to the master regression curves shown in Fig. 2C. The results of all of these experiments were analyzed using [CD4_{min}] = 1,800, [CCR5_{min}] = 1,274, [CD4_{max}] = 113,952, and [CCR5_{max}] = 23,235. Using these values for the physiological ranges of receptor and coreceptor in the scaling defined in equation 1, the effective CD4 and CCR5 surface concentrations were represented by the variables x and y , respectively. The relevant ranges of x and y varied from approximately 0 for minimum surface concentrations to approximately 1 for maximum-level concentrations. With this rescaling, the viral infectivity as a function of [CD4] and [CCR5] levels can be expressed as a function of x and y , $F(x, y)$, with the common relevant range of surface concentrations defined by $x \in [0, 1]$ and $y \in [0, 1]$. We find numerical values of $F(x, y)$ for each viral envelope by a two-dimensional least squares fit to the polynomial

$$F(x, y) = a + bx + cy + dx^2 + ey^2 + fxy \quad (2)$$

with the additional constraint that $a \geq 0$. This assumed form imposes a positive

level of infectivity at the minimum physiologic concentrations $(x, y) = (0, 0)$. Each isolate results in a set of best-fit parameters (a, b, c, d, e , and f). Three-dimensional (3-D) surface plots representing the least squares fit to the rescaled data for each induction-infection experimental data set are independently described in Results and shown in Fig. 3 and 5. From the function $F(x, y)$, we find the relative sensitivity of a viral isolate's infectivity to variations in [CD4] and [CCR5] by first defining the normalized gradient in the x - y plane

$$\nabla F(x, y) = \hat{x} \frac{\partial F(x, y)}{\partial x} + \hat{y} \frac{\partial F(x, y)}{\partial y} \quad (3)$$

We define the "sensitivity vector," $\vec{s}$, by integrating the gradient over the relevant ranges of x and y as follows:

$$\vec{s} = \int_0^1 dx \int_0^1 dy \frac{\nabla F(x, y)}{|\nabla F(x, y)|} = S_x \hat{x} + S_y \hat{y} \quad (4)$$

The direction of $\vec{s}$ measures the relative sensitivity to changes in CD4 and CCR5 expression levels. For example, if $\vec{s}$ points predominantly in the $\hat{x}$ direction, viral entry is sensitive to changes in [CD4] but insensitive to variations in [CCR5]. The direction of $\vec{s}$ can be summarized by the angle

$$\theta = \tan^{-1} \left(\frac{S_y}{S_x} \right) \quad (5)$$

The sensitivity vectors $\vec{s}$ of the individual induction-infection experiments for various viral isolates could be represented on polar plots as described in the specific section in Results and shown in the figures indicated there. The vector end points corresponding to $\vec{s}$ were marked by circles of different colors, each color corresponding to a specific viral isolate. The vectors corresponding to each isolate may exhibit various magnitudes, indicating a varying absolute sensitivity to levels of surface CD4 or CCR5. This variation may arise from uncontrollable factors, such as heterogeneity in the susceptible cell surface area and, more likely, variations in the viral stock used. Note that the vector magnitudes for the cloned SIV316 envelope-pseudotyped GFP reporter virus stock were much less variable than those for the primary BaL and 89.6 stocks expanded in primary PBMCs. Thus, these variations can be minimized by using cloned envelopes and as more automated and high-throughput implementations of our assay provide larger numbers of measurements. Nonetheless, the relative sensitivities to changes in [CD4] and [CCR5] would be expected to vary less from sample to sample. This is demonstrated by the clustering of the sensitivity vector angles θ for the various viral isolates mentioned in Results.

Both the vector magnitudes and angles were generally more tightly clustered for the cloned envelopes pseudotyped onto an NL4-3 luciferase reporter backbone. Moreover, for these assays, we also determined the mean induction levels, M , (i.e., overall infectivity induced) for each sample as defined by

$$M = \int_0^1 dx \int_0^1 dy F(x, y) \quad (6)$$

The circles marking the endpoints of $\vec{s}$ are drawn with areas proportional to M (equation 6; see second polar plot in relevant Results section). Larger circles correspond to samples with an overall-higher level of induction. These luciferase-based measurements appear to be less noisy than the p24-based measurements; this is suggested not only by clustered magnitudes of $\vec{s}$ but also by consistency of M .

Automated computational analysis. The analysis described above was implemented via a user-friendly Web-based computing tool (VERSA [Viral Entry Receptor Sensitivity Analysis]) found at <http://versa.biomath.ucla.edu>. The Website accepts raw or normalized data as an array of infectivity values as a function of CD4 and CCR5 concentrations. The program automatically normalizes and rescales the CD4 and CCR5 concentrations to range between 0 and 1 and finds the best-fit coefficients (a, b, c, d, e , and f) of the surface $F(x, y)$. The mean induction, sensitivity vector magnitude, and direction (the angle θ) are also compiled.

RESULTS

CD4 and CCR5 can be independently and simultaneously regulated in a dually inducible cell line. In order to quantitatively assess the efficiency of CD4 and CCR5 usage, we first

generated a dually inducible cell line in which the expression of CD4 and CCR5 can be simultaneously and independently controlled with various concentrations of minocycline and ponA, respectively (Fig. 2A and B). We used qFACS to measure the number of ABS/cell and found that specific concentrations of minocycline and ponA reproducibly induced the same level of CD4 and CCR5 expression on these cells (Fig. 2C). Thus, absolute CD4 and CCR5 expression levels (ABS/cell) could be inferred from the concentrations of minocycline and ponA used (Fig. 2C). Minocycline induction of CD4 resulted in ~1,800 to 110,000 ABS/cell, while ponA induction of CCR5 resulted in ~1,200 to 23,000 ABS/cell (Fig. 2C). These levels are within the ranges of physiologic CD4 and CCR5 expression found on primary targets of HIV-1 infection (21), as CD4 on activated primary T cells can range upwards of 70,000 ABS/cell, whereas CCR5 on macrophages and T-cell subsets can range from ~1,000 to 12,000 ABS/cell.

HIV-1 and SIV isolates have differential patterns of CD4 and CCR5 usage that can be represented by distinct 3-D surface plots. Next, we sought to determine if our dually inducible cell lines could be used to reveal differences in the relative efficiency of CD4 and CCR5 usage among various R5 or R5X4 isolates. As proof of principle, we first examined three well-characterized SIV and HIV-1 isolates. Infection was performed using 20 to 24 different concentrations of minocycline and ponA, resulting in 20 to 24 distinct CD4 and CCR5 levels (ABS/cell) as measured by qFACS.

Infection with the "CD4-independent" SIV316 envelope pseudotyped on an NL4-3 backbone (Fig. 3A) resulted in a pattern of infection that was relatively insensitive to changes in CD4 levels. That is, increasing CD4 alone (0 to 2.5 ng/ml of minocycline) at any given level of CCR5 did not increase infection efficiency, whereas increasing CCR5 (0 to 4 μ M ponA) at any given level of CD4 markedly increased the percentage of infected cells (Fig. 3A). These findings are consistent with the known CD4 independence of SIV316 (5, 35) and indicate that our system can recapitulate known phenotypes.

The greatest variation for infectivity for differential expression of both CD4 and CCR5 was observed with BaL (Fig. 3B), a lung-derived macrophage R5-tropic HIV-1 virus. Infection with BaL increased as both CD4 and CCR5 levels were increased. However, note that at the lowest level of CCR5, no amount of CD4 could "rescue" infection, whereas at the lowest level of CD4, increasing CCR5 expression allowed for a moderate, two- to threefold increase in infection. This suggests that CCR5 levels are more limiting for virus entry and that low levels of CD4 (in comparison to CCR5) could be "scavenged" more efficiently by the infecting virus.

89.6 (Fig. 3C) is a blood-derived, dually tropic R5X4 virus. In direct contrast to SIV316 and BaL, at the lowest levels of CCR5 expression (0 μ M ponA), increasing CD4 expression (0 to 2.5 ng/ml minocycline) markedly increased infection efficiency. However, at the lowest levels of CD4, no amount of CCR5 could rescue infection. In effect, 89.6 could efficiently use the low basal levels of CCR5 (~1,200 ABS/cell) for infection but was critically dependent on the level of CD4 present. This was not due to the ability of 89.6 to use the low endogenous level of CXCR4 present on 293 cells, as infection by all three isolates tested could be inhibited by TAK779 (>80% inhibition) under all induction conditions (data not shown),

FIG. 3. HIV-1 and SIV isolates have differential patterns of CD4 and CCR5 usage that can be represented by distinct 3-D surface plots. The infectivity of pseudotyped SIV316 (A) or replication-competent clones of BaL (B) and 89.6 (C) was tested under 20 to 24 different induction conditions, as indicated. Infection was detected by intracellular p24 staining and quantified by FACS at 48 h postinfection. Data shown for each virus are from a single experiment with representative results. To ensure that p24-positive cells represented infection from one infectious virion, the titer of infectivity was determined so that maximum infection was less than $\sim 10\%$ p24-positive cells in a given experiment. On three independent occasions, 89.6 infections gave maximum infectivity of 2.4%, 4.4%, and 8.8%; BaL infections gave maximum infectivity of 3.5%, 9.3%, and 4.6%; and SIV316 gave maximum infectivity of 8.8%, 10.6%, and 8.5%. The patterns of CD4 and CCR5 usage, illustrated by the bar graphs (A to C) for each virus, can be mathematically transformed into corresponding 3-D surface plots (D to F) which describe the percentage of infected (p24 positive) cells (z axis) as a function of changes in CD4 (x axis) and CCR5 (y axis) levels (see equation 2 in Materials and Methods).

which underscores the CCR5-dependent behavior of these isolates in our system.

The distinct patterns of CD4 and CCR5 usage exhibited by each viral isolate (Fig. 3A to C) could be mathematically fitted to the corresponding 3-D surface plots shown in Fig. 3D to F. The efficiency of virus infection as a function of CD4 and CCR5 levels was described by the topology of the surface plot, which could be expressed as a least squares fit to the polynomial function $F(x, y) = a + bx + cy + dx^2 + ey^2 + fxy$, where x and y were mathematically rescaled quantities representing the absolute cell surface expression levels (ABS/cell) of CD4 and CCR5 (see Materials and Methods). The topology of the surface plot for each virus was relatively distinct and stable from experiment to experiment (see Fig. 4 below). This suggested that the fitting surfaces could serve as a “fingerprint” of each individual isolate’s pattern of CD4 and CCR5 usage.

Derivation of an angular metric that can quantify the efficiency of CD4 and CCR5 usage. In order to compare the mathematical surface plots associated with different isolates, we sought to quantify their gross features that can effectively

represent the biological properties of a viral isolate in terms of its efficiency of CD4 and CCR5 usage. To do this, we derived a vector-based metric that quantifies the relative sensitivity of a viral isolate’s infectivity to changes in CD4 and CCR5 levels from the function $F(x, y)$. For each unit gradient on the mathematical surface described by $F(x, y)$, we first define a vector in the x - y plane that describes whether the viral infectivity is more or less responsive to changes in CD4 (x axis) versus the response to changes in CCR5 (y axis) levels. We then derive an overall sensitivity vector, $\vec{s}$, by integrating the normalized gradient of the surface plot over the relevant ranges of x and y (see “Mathematical analysis”). The vector $\vec{s}$ is therefore the normalized average of the local gradient in $F(x, y)$ over the relevant area in the x - y plane; in other words, the direction of $\vec{s}$ measures the relative sensitivity of virus infectivity to changes in CD4 versus CCR5 levels within the range of expression defined by the data shown in Fig. 2C. For example, if $\vec{s}$ points predominantly in the y direction, the viral entry is more sensitive to changes in CCR5 than CD4 levels. This is further illustrated in Fig. 4.

Figure 4A shows a polar plot representing the sensitivity

FIG. 4. Sensitivity vector angles reflect distinct patterns of CD4 and CCR5 usage. (A) Polar plot of the sensitivity vectors derived from each surface plot shown in Fig. 3. The results of each independent induction-infection experiment resulting in a surface plot are represented by a circle, defining the end point of its sensitivity vector $\vec{s}$ (see text and “Mathematical analysis” for details). The vector end points corresponding to $\vec{s}$ are marked by colored circles, each color corresponding to a particular viral isolate. Each circle therefore represents the results of an independent induction-infection experiment consisting of one isolate infecting the cells at 20 to 24 different levels of CD4 and CCR5. The three viruses examined are clustered into three qualitatively distinct groups (indicated by different colors). (B) The CD4/CCR5 usage pattern for each isolate is reflected by its sensitivity angle, $\theta = \tan^{-1}(S_y/S_x)$. The angles for each isolate exhibit clustering and are significantly different from each other when plotted ($P < 0.0002$, one-way ANOVA).

vectors $\vec{s}$ for all viral isolates examined in Fig. 3. The angle θ of $\vec{s}$ is a scalar metric describing the relative sensitivity of virus entry to changes in CD4 (S_x) and CCR5 (S_y) levels. Note that the sensitivity vectors of the different viral isolates clustered into significantly different groups ($P < 0.0002$, one-way analysis of variance [ANOVA]) (Fig. 4A and B), suggesting that each independent induction gave a reproducible and quantifiable metric for a given virus. For example, Fig. 4B shows that the SIV316 vectors were clustered with θ close to 90° , suggesting that SIV316 infection, under our assay conditions, was much more sensitive to changes in [CCR5] than in [CD4]. On the other hand, the 89.6 vectors were clustered near the x axis ($\theta < 30^\circ$), indicating that 89.6 could scavenge the low basal levels of CCR5 in our system and that infection was sensitive to changes in CD4 but not CCR5 levels. The $\vec{s}$ for BaL had angles $50^\circ \leq \theta \leq 80^\circ$, indicating that BaL infection was slightly more sensitive to changes in CCR5 levels. Our results show that the angles θ captured the overall direction of the surface $F(x, y)$ and provided a quantitative measure to compare the efficiency of CD4 and CCR5 usage between viral isolates. Note that the SIV316 vectors clustered more tightly than the BaL or 89.6 vectors, as SIV316 infections were performed with cloned-envelope-pseudotyped NL4-3-GFP reporter viruses, while BaL and 89.6 were primary isolates expanded in primary PBMCs.

CD4 and CCR5 usage patterns reflect sensitivities to entry inhibitors. Our sensitivity vectors and angular metrics could also be used to predict the susceptibility of a given envelope glycoprotein to various entry inhibitors. To show this, we chose four HIV-1 envelopes (two paired sets) whose sensitivities to CCR5 inhibitors (TAK-779 and 2D7), enfuvirtide (a fusion

peptide inhibitor), and sCD4 have been carefully evaluated (23). To demonstrate the utility of our dually inducible cells for a higher-throughput characterization of CD4 and CCR5 usage patterns, we determined the relative infectivity of these HIV-1 envelope-pseudotyped luciferase reporter viruses using 48 different combinations of minocycline and ponA concentrations in a single experiment. The four envelopes used were (i) a primary clade B R5 HIV-1 isolate (B5), B5-91US056 [B5(YA)]; (ii) an isogenic B5 variant containing two V3 crown mutations ($Y_{318}A_{319}$ to $R_{318}T_{319}$) that increase susceptibility to entry inhibitors [B5(RT)]; (iii) a chimeric NL4-3 envelope with the V3 region from a primary clade A R5 HIV-1 isolate (NL4-3-A1), A1-92RW009 [NL43(YA)]; and (iv) the above-described homologous V3 crown mutations in this context [NL43(RT)] (23). The V3 crown mutations invariably rendered the envelope more susceptible to CCR5 and fusion peptide inhibitors (23 and data not shown).

For the B5 envelope, B5(YA) (Fig. 5A), there is a threshold level of CD4 that is required for efficient infection. Notably, at the lowest level of CD4 expression (0.16 ng/ml minocycline), no amount of CCR5 could rescue infection (Fig. 5A). However, above 0.31 ng/ml of minocycline, increasing CCR5 levels (ponA) resulted in a corresponding increase in infectivity. Indeed, at any given ponA concentration, increasing CD4 levels also increased infectivity. Primary envelopes like B5 have decreased susceptibility to sCD4 neutralization compared with the susceptibility of the chimeric NL4-3-A1 envelope (data not shown), suggesting a lower affinity for CD4 and, thus, a greater dependence on the level of cell surface CD4 for efficient infection. Both B5(YA) and B5(RT) exhibited the same CD4 usage pattern and sensitivity to sCD4 (Fig. 5A and B and data not shown).

In contrast, the chimeric NL43(YA) envelope shows a different pattern of infection with respect to CD4 usage (Fig. 5C). NL4(YA) contains the CD4 binding site derived from the T-cell line-adapted virus NL4-3, and consequently, these viruses are significantly neutralized by sCD4 (data not shown), suggesting a higher affinity for CD4. Consistent with this, infection with NL4-3-A1, even at the lowest CD4 level (0.16 ng/ml minocycline), was significantly enhanced as CCR5 levels (ponA) were increased (Fig. 5C). Note that the B5(YA) envelope exhibited no increase in infection under similar conditions (compare Fig. 5A and C). The higher affinity of the NL4-3-A1 envelope for CD4 is underscored by its ability to scavenge the low basal levels of CD4 in our system: above a minimal level of CD4 (0.31 ng/ml minocycline), the efficiency of infection depended almost exclusively on CCR5 expression levels. Again, the CD4 usage pattern was similar for both NL43(YA) and NL43(RT).

The $Y_{318}A_{319}$ -to- $R_{318}T_{319}$ V3 crown mutations rendered envelopes more susceptible to CCR5 and fusion peptide inhibitors (23), which was reflected in the pattern of CCR5 usage for both B5 and NL4-3-A1. B5(RT) exhibited a reduced efficiency of CCR5 usage compared to that of B5(YA). Notably, at the lowest level of CCR5 (0.015 μ M ponA), infection efficiency was much less enhanced for B5(RT) than for B5(YA) as CD4 levels were increased (compare Fig. 5A and B). Moreover, the half-maximal infection response was achieved at significantly lower CCR5 levels for B5(YA) than for B5(RT) (Fig. 5 and data not shown). Similar trends were observed for NL43(YA)

FIG. 5. CD4 and CCR5 usage patterns reflect sensitivities to entry inhibitors. Four HIV-1 envelopes (two paired sets) with well-characterized differential sensitivities to CCR5 inhibitors (TAK-779 and 2D7), enfuvirtide (a fusion peptide inhibitor), and sCD4 were chosen. Equivalent amounts (RT activity) of four HIV-1 envelope-pseudotyped luciferase reporter viruses, B5(YA) (A, E), B5(RT) (B, F), NL43(YA) (C, G), and NL43(RT) (D, H), were used to infect the dually inducible cells at 48 different combinations of minocycline (0.015 to 5.0 ng/ml) and ponA (0.015 to 2.0 μ M) concentrations. Each infection was performed in triplicate. Data shown are from one replicate; raw luciferase values (relative light units [RLU]) are shown to illustrate that the V3 $R_{318}T_{319}$ mutants reproducibly resulted in less-efficient viral entry at any given level of CD4/CCR5 compared to the viral entry of their V3 $Y_{318}A_{319}$ counterparts. This property was also captured by the radii of the circles at the end points of the sensitivity vectors in the polar plot shown in Fig. 6. The standard deviation for each data point was less than 10% of the value shown. The patterns of CD4 and CCR5 usage, illustrated by the 3-D bar graphs (A to D) for each virus, were mathematically transformed into corresponding 3-D surface plots (E to H) which describe the efficiency of virus infection as a function of changes in CD4 and CCR5 levels, $F(x, y)$.

versus NL43(RT), where the V3 RT mutant also showed a reduced efficiency of CCR5 usage compared to that of the YA counterpart (Fig. 5C and D). Thus, our Affinofile system provides direct evidence that increased sensitivity to CCR5 inhibitors could be due to a decreased efficiency of CCR5 usage.

Figure 5E to H shows the corresponding 3-D surface plots for the isolates examined in Fig. 5A to D. These were generated using the same function, $F(x, y)$, that was described for Fig. 3. In contrast to the distinct topologies exhibited for SIV316, BaL, and 89.6 in Fig. 3, the surface plot topologies for the B5 and NL4-3-A1 viruses were relatively similar. While this is consistent with their dependence on both CD4 and CCR5 levels, it does not immediately reveal their efficiency of CD4 and CCR5 usage. Thus, we derived the sensitivity vector for each surface plot for a more quantitative comparison.

Our sensitivity vector-based angular metric can quantify subtle differences in CD4 and CCR5 usage patterns. Fig. 6 shows a polar plot representing the sensitivity vectors $\vec{s}$ for the surface plots shown in Fig. 5E to H. As described for Fig. 4, the vector end points corresponding to $\vec{s}$ are marked by colored circles, with each color corresponding to a particular viral isolate. The induction-infection experiment was performed in triplicate. The sensitivity vectors for the different isolates were tightly clustered based on their angular metric. Both NL4-3-A1

isolates clustered farther away from the CD4 (S_x) axis ($\theta > 70^\circ$) than both B5 isolates ($\theta < 55^\circ$) (Fig. 6A and C), indicating that the infection efficiency of NL4-3-A1 isolates was less sensitive to changes in CD4 levels. This is consistent with the higher affinity of laboratory-adapted strains (NL4-3) for CD4 and their corresponding increase in susceptibility to sCD4 neutralization (36).

Furthermore, for both B5 and NL4-3-A1 viruses, the V3 RT crown mutants shifted their sensitivity angles toward the CCR5 (S_y) axis relative to those of their V3 YA counterparts (Fig. 6A and C, θ increases of 5 to 15°), indicating increased sensitivity to changes in [CCR5]. Thus, although overall CD4/CCR5 usage as represented by the surface plots was somewhat similar among the isolates, as shown in Fig. 5E to H, the sensitivity vectors were able to reveal clear differences among the strains that were consistent with their biological phenotypes. Of note, for the viruses examined here, both V3 RT mutants exhibited overall lower levels of viral entry ($M < 10\%$) than their V3 YA counterparts ($M > 25\%$) (Fig. 6B), as represented by the circle sizes at the vector end points (mean induction values; see equation 6 in “Mathematical analysis”) and consistent with their decreased replicative fitness (23). Thus, the sensitivity angles, θ , and the mean induction levels, M , as an overall measure of CD4/CCR5 usage efficiency, were clearly able to

FIG. 6. Sensitivity vector metrics can quantify and differentiate subtle differences in CD4 and CCR5 usage patterns. (a) Polar plot of the sensitivity vectors shown in Fig. 5 for each isolate. The radius of each circle is proportional to the mean induction level, M , of virus entry for each sample. See “Mathematical analysis” (equation 6) for the formula for M . Larger circles correspond to samples with an overall higher level of virus entry. (b) Circle radii, indicative of the overall level of infection (M) induced by changes in CD4/CCR5 expression levels, were plotted and compared for each isolate. M is expressed as a percentage of the induction level at the highest values of CD4 and CCR5. (c) Virus isolates were clustered by their sensitivity angles, $\theta = \tan^{-1}(S_y/S_x)$. θ values for each isolate are plotted and show robust clustering ($P < 0.01$, one-way ANOVA).

distinguish even between viral isolates that gave grossly similar surface plots.

Sensitivity vector analysis of envelope clones derived from ES versus CP. Recently, using the Affinofile cells, ES envelope clones were shown to have reduced entry efficiency at fixed (high and low) CD4 and CCR5 concentrations compared to that of CP clones (19). Curiously, due to the large variation in 50% inhibitory concentrations between individual env clones, the difference between envelopes from the ES and CP groups with regard to their susceptibility to inhibition by CCL5 (RANTES), TAK779, or Enfuvirtide appears less marked (19), suggesting that indirect or surrogate markers for entry receptor usage efficiencies may not always reveal underlying differences in their biological phenotypes. The study of Lassen et al. (19) evaluated the infectivity of more than 70 independent plasma virus envelope clones derived from seven ES and seven CP at 42 distinct CD4/CCR5 expression levels using six and seven different combinations of minocycline and ponA concentrations, respectively. In order to apply sensitivity vector analysis to this large data set, we developed a Web-based automated computational platform (<http://versa.biomath.ucla.edu>) known as VERSA (Viral Entry Receptor Sensitivity Analysis), where anyone using the Affinofile cells can upload raw or normalized infection data and obtain sensitivity vector metrics (vector angle, magnitude, and mean induction levels) (see Materials and Methods).

The VERSA results indicated that the vector angles (θ) and mean induction levels (M) were significantly lower in the ES than in the CP group (Fig. 7A and B, respectively). The lower mean induction (M) of ES clones is consistent with their overall reduced entry efficiency compared to that of CP clones, as described in Lassen et al. (19). However, the higher angle

FIG. 7. Sensitivity vector analysis reveals differences between envelope clones derived from ES and those from CP. Normalized infection data using ES- and CP-derived envelope clones were imported to VERSA according to the detailed instructions on the Website (<http://versa.biomath.ucla.edu>). Sensitivity vector angles and magnitudes and mean induction levels were obtained for each envelope clone. The number of independent envelope clones obtained for each patient ranged from 2 to 12. The vector metrics were first averaged for each individual, and the averaged metrics from the seven individuals in each group (CP or ES, $n = 14$) were then compared. The median values and interquartile ranges of the sensitivity vector angles (A), mean induction levels (B), and magnitudes (C) for the CP and ES groups are shown. The entire comparison was performed in duplicate. P values were generated by the nonparametric unpaired t test. NS, not significant.

values exhibited by the CP group (Fig. 7A) also indicated that the infection efficiencies of CP envs were more sensitive to CCR5 expression levels, and, in combination with their higher M , reflected the more efficient use of CCR5 at any given level of CD4 expression than was found for the ES envs. This is further illustrated by the color-coded surface area projection graphs shown in Fig. 8, which are the averaged induction-infection responses from the more than 70 envelopes for the CP versus the ES group. Note that at any given CD4 level, efficient (>60%) or moderate (40 to 60%) infectivity was achieved at lower CCR5 levels for the CP than for the ES envelope clones (Fig. 8A and B, respectively). In other words, in every row represented by a minocycline concentration, efficient and moderate infectivity results always occupied a proportionately greater area for the CP group than for the ES

FIG. 8. CP envelopes are more efficient than ES envelopes at using CCR5 for viral entry. Normalized viral infectivity data were obtained for CP and ES envelope clones, as described in Lassen et al. (19). The infectivity (relative light units) at the highest minocycline and ponA concentrations was set at 100%. The relative infectivity for each envelope clone was obtained at 42 different combinations of minocycline and ponA concentrations, and the infectivity averaged for all CP clones (A) versus ES clones (B). A 3-D surface plot of the relative infectivity at each minocycline ([CD4]) and ponA ([CCR5]) concentration was projected onto a 2-D surface using a standard Excel function, and the information on the z axis (relative infectivity, % maximal infection) was color coded as indicated. (C) The averaged infectivity responses for individual CP ($n = 7$) and ES ($n = 7$) are shown. Data are presented and color coded exactly as described for panels A and B.

group. This pattern also held true when envelope clones from each individual patient from the CP and ES groups were compared (Fig. 8C). This increased efficiency of CCR5 usage was not apparent from the results of competition studies with CCR5 inhibitors, as ES and CP clones did not show significant differences in their susceptibility to CCL5 (RANTES) and TAK779 (19).

DISCUSSION

Our Affinofile system provides a more comprehensive method to characterize the CD4 and CCR5 usage pattern of extant R5 isolates; in essence, their efficiency of CD4 and CCR5 usage generates a signature 3-D surface plot which can then be correlated with the clinical or pathogenic characteristics of the virus. We wish to emphasize that each virus always gave its signatory surface curve in multiple independent experiments and that its vector metrics, derived from the function $F(x,y)$ that describes the topology of the surface plot, can be used for quantitative comparisons of CD4 and CCR5 usage patterns. The robustness of the assay system and the mathematical transformation that results in the sensitivity vectors is underscored by the clustering of the θ values for each of the viral isolates examined (Fig. 4 and 6). We propose to call our system 293-Affinofile cells to reflect their ability to profile the receptor affinities of viral isolates.

Primary biological isolates from well-defined cohorts can be subjected to the same analysis, and their patterns of CD4 and CCR5 usage, reduced to the metrics whose results are shown in

Fig. 6 and 7, may be used to correlate the efficiency of CD4 and CCR5 usage with various aspects of viral pathogenicity. For example, the increased pathogenicity and/or fusogenicity of AIDS versus pre-AIDS R5 strains has been attributed to their increased efficiency of CD4 and/or CCR5 usage, as demonstrated by increased resistance to various entry inhibitors (31, 44). Conversely, acutely transmitted “founder” viruses, though R5 tropic, can grow in activated PBMCs but not primary macrophages (42). Indeed, increased macrophage tropism has been used as a surrogate marker for the ability to use low levels of CCR5 (7, 9). However, Goodenow and Collman (7) have cautioned that coreceptor preference can be distinct from target cell tropism. Thus, not all R5-tropic strains can use CCR5 on macrophages, and dually X4R5-tropic strains may use one coreceptor on one cell type and not the other. 293-Affinofile cells present a unique opportunity to directly examine the efficiency of CD4 and CCR5 usage of viral isolates over a wide range of expression levels; the ability to ascribe a quantitative metric that defines an isolate’s overall CD4/CCR5 usage efficiency may illuminate the complexities that underlie the biological phenotypes mentioned above.

Recently, entry inhibitors, such as enfuvirtide (T-20) and maraviroc (a CCR5 antagonist), have emerged as a new class of drugs to treat HIV. Not surprisingly, viral resistance mechanisms have already been identified (40, 49). Current CDC HIV treatment guidelines recommend the use of coreceptor tropism assays (Trophile) prior to starting maraviroc. The Trophile assay is also proscribed in the case of treatment failure while

on a regimen including an entry inhibitor (32). The use of 293-Affinofile cells may complement the Trofile assay to better predict the success of regimens that include entry inhibitors and may also provide better insights into mechanisms underlying CCR5 inhibitor resistance. For example, for CCR5 inhibitor-resistant isolates that have been shown to use the inhibitor-bound form of CCR5 (37, 48, 49), Moore and colleagues used our 293-Affinofile cells to show that the degree of resistance is inversely proportional to the level of CCR5 cell surface expression (38). In addition, the extent of inhibitor resistance, as measured by the plateau inhibitory effect (and represented by the maximal percent inhibition value) in the presence of a saturating amount of inhibitor (vicriviroc or maraviroc), can vary significantly between two different inhibitor-resistant isolates, especially when CCR5 levels are limiting (38). This nuance may not be appreciated when using the commercial Trofile assay to generate resistance profiles, as the Trofile assay relies on U87-CD4/CCR5 cells (50) that express very high levels of CCR5. It would be of interest to determine how inhibitor resistance phenotypes would be reflected in changes in sensitivity vector metrics.

In the case of the V3 RT isolates, the angular metric suggests that their infection efficiency is more sensitive to changes in CCR5 levels (θ shifts closer to 90°) and, thus, would be more sensitive to CCR5 inhibitors and, by extension, fusion peptide inhibitors. We have confirmed this experimentally (23 and data not shown). The decreased efficiency of CCR5 usage likely prolongs the target window of opportunity for CCR5 inhibitors or peptide inhibitors of six-helix bundle formation to work. However, sensitivity angles should only be interpreted in the context of their mean induction levels (M). For the V3 crown mutants, the YA-to-RT mutations also led to a decrease in M , indicating an overall decrease in infection efficiency for the RT mutants due to their dependence on higher levels of CCR5 for efficient infection. On the other hand, for the comparison of the CP versus ES clones, the increase in both sensitivity vector angles and mean induction levels (M) for the CP clones (Fig. 7) indicates that their overall increased infection efficiency is likely due to their more efficient usage of CCR5 (Fig. 8). Conversely, it implies that ES clones have reduced entry efficiencies and a reduced ability to use low levels of CCR5.

There is a growing body of evidence that the ability to use low levels of CD4 and coreceptor contributes to expanded cellular tropism and disease pathogenesis. Until now, studies have been limited by using surrogate or indirect assays for the efficiency of CD4 and CCR5 usage. Our 293-Affinofile cells can be used to directly measure and profile the CD4/CCR5 usage efficiency of any given viral isolate and provide novel quantitative metrics that can be used for multiple comparisons. We have automated the complex computational analysis required to derive the sensitivity vector metrics on a Web-based platform (<http://versa.biomath.ucla.edu>) so that anyone using our Affinofile cells can obtain the vector metrics by imputing the raw infectivity data as described above. In an accompanying study, Doms and colleagues used our Affinofile cells and VERSA, in part, to show that a V3 loop-truncated R5 virus envelope compensated for its inefficient usage of CCR5 by increasing its ability to use low levels of CD4 (1). Not only is our system a valuable tool for better understanding the relationship between viral pathogenesis and the efficiency of CD4/

CCR5 usage, but the application of sensitivity vector analysis to clinical isolates may have implications for guiding entry inhibitor use.

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