



Review

Affinofile profiling: How efficiency of CD4/CCR5 usage impacts the biological and pathogenic phenotype of HIV

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ABSTRACT

HIV-1 envelope (Env) uses CD4 and a coreceptor (CCR5 and/or CXCR4) for viral entry. The efficiency of receptor/coreceptor mediated entry has important implications for HIV pathogenesis and transmission. The advent of CCR5 inhibitors in clinical use also underscores the need for quantitative and predictive tools that can guide therapeutic management. Historically, measuring the efficiency of CD4/CCR5 mediated HIV entry has relied on surrogate and relatively slow throughput assays that cannot adequately capture the full spectrum of Env phenotypes. In this review, we discuss the details of the Affinofile receptor affinity profiling system that has provided a quantitative and higher throughput method to characterize viral entry efficiency as a function of CD4 and CCR5 expression levels. We will then review how the Affinofile system has been used to reveal the distinct pathophysiological properties associated with Env entry phenotypes and discuss potential shortcomings of the current system.

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Introduction

Since human immunodeficiency virus type 1 (HIV-1) was discovered as the causative agent of acquired immune deficiency syndrome (AIDS), it has been estimated that > 40 million people have become infected with the virus and > 20 million have died of AIDS. Approximately 5 million new infections occur annually (UNAIDS, 2008). The overwhelming majority of these individuals live in third world countries with little or no access to antiretroviral therapies. Moreover, HIV-1 is predicted to become the leading burden of disease in middle and low income countries by 2015 (Colin and Dejan, 2006). Understanding how certain viral features affect HIV-1 pathogenesis and transmission remains essential to the development of more effective therapies, prevention strategies, and vaccines.

The process of HIV-1 entry into cells of the immune system begins with the viral gp120 envelope glycoprotein (Env) binding to cellular CD4 and then subsequently to a coreceptor, which is either of the chemokine receptors CCR5 or CXCR4. This initial binding of gp120 to CD4 promotes the exposure of the coreceptor binding site to facilitate CCR5 or CXCR4 binding. Upon coreceptor binding, the Env undergoes further conformational changes that reorient the gp41 glycoproteins to promote fusion between the viral and cellular membranes, facilitating the deposition of the viral contents into the cell cytoplasm. The principle steps of HIV-1 entry have been detailed in recent excellent review articles (Melikyan, 2008; Wilen et al., 2012b, 2012a).

There are a multitude of host and viral factors that contribute to the varied clinical outcomes of HIV-1-infected subjects. Among the viral factors, it is unlikely that co-receptor tropism *per se* accounts for viral pathogenicity. For subjects who harbor only CCR5-using (R5) viruses throughout their disease, a large body of evidence indicates the relative efficiency by which HIV-1 uses CD4 and CCR5 correlates with the pathogenic potential of the virus (Duenas-Decamp et al., 2010; Gorry and Ancuta, 2011). For patients with R5 viruses, HIV disease progression has been associated with enhanced macrophage (M)-tropism (Blaak et al., 2000; Li et al., 1999; Trkola et al., 1996), the increased ability to use low levels of CCR5 (Gray et al., 2005; Li et al., 1999; Smit et al., 2001; Tuttle et al., 2002), and the increasing relative entry efficiency of the infecting virus (Marozsan et al., 2005; Rangel et al., 2003). Neurovirulence is also correlated with an isolates ability to use low levels of CD4 and/or CCR5 present on microglial cells (Gorry et al., 2002; Smit et al., 2001). Furthermore, R5-viruses derived from late versus early disease not only show increased CCR5 usage but also greater sensitivity to inhibition by various ligands or antagonists of CCR5 (Gray et al., 2005; Koning et al., 2003; Kwa et al., 2003; Lobritz et al., 2007; Olivieri et al., 2007). It is possible, then, that a viral isolate capable of using minute amounts of CCR5 to infect may allow for expanded tropism of target cells, and therefore, increased pathogenicity (Dejuq et al., 1999; Pakarasang et al., 2006; Peters et al., 2007). Finally, in the SIVmac model, R5 SIV strains can clearly become virulent without coreceptor switching (Kimata et al., 1999a, 1999b). Thus, it seems likely that the relative efficiency of CD4 and CCR5 usage during disease rather than a simple switch from R5 to X4 co-receptor tropism is a better predictor of viral pathogenicity.

Until recently our ability to quantify the efficiency of CD4 and CCR5 usage has been limited by indirect and non-standardized measures such as competition with soluble CD4, specific antibodies, or chemokine receptor ligands. The development of new tools, such as the Affinofile system, provides an unprecedented ability to examine the mechanics and efficiency of CD4 and CCR5 mediated viral entry in greater detail, using a more quantitative methodology, and with a higher throughput format, than was previously possible. The Affinofile system, published in late 2009

(Johnston et al., 2009), has been used in a number of studies that have increased our understanding of how entry efficiency influences HIV-1 pathogenesis and impacts on the clinical management of disease using an evolving class of entry inhibitors.

Initial studies of HIV-1 entry examined the ability of laboratory adapted and primary viral isolates to grow on primary cell cultures and CD4+ T cell lines (Dalglish et al., 1984; Klatzmann et al., 1984a, 1984b). These early studies led to the classification of HIV-1 laboratory and primary isolates according to their ability to grow on primary macrophages (M-tropic), T cell lines (T-tropic) or both (Dual-tropic). Although CD4 was discovered as a critical receptor for HIV-1 entry soon after the discovery of HIV-1 as the causative agent of AIDS, it was soon realized that CD4 alone was not sufficient to support HIV-1 entry (Chesebro et al., 1990; Clapham et al., 1991; Kikukawa et al., 1986). A coreceptor was required. When CCR5 and CXCR4 were identified as bona fide coreceptors for HIV entry, they were initially thought to be the cognate coreceptors for all M- and T cell-tropic viruses, respectively, with the implication that dual-tropic isolates used both CCR5 and CXCR4. A new nomenclature re-designated M-, T-, or Dual-tropic HIV-1 strains as R5, X4 or R5X4 viruses based on their ability to enter cells expressing either or both coreceptors (Berger et al., 1998). Initial studies that examined the efficiency of HIV-1 entry principally employed soluble factors, proteins and antibodies as surrogate means to measure HIV-1 Env entry efficiency as a function of the ability of the viral Env to infect cells in the presence of each factor.

The study of HIV-1 entry efficiency in a more direct manner was facilitated by the engineering of a series of HeLa cell based lines by the Kabat laboratory (Kozak et al., 1997; Platt et al., 1998, 1997). A binary library of clonal cells was generated that expressed fixed amounts of CD4 and CCR5: all clones had either high (HI-J, 10^5) or low (HI-R, 10^4) amounts of CD4, but individual subclones within each CD4 library covered a spectrum of CCR5 expression levels. These cell lines provided a much needed tool to determine whether a viral isolate required high levels of CD4 or CCR5, or could scavenge relatively low levels of CD4 or CCR5 for entry, and were a significant improvement upon studies that used transient transfection to generate differing CD4 and CCR5 levels. Although these clonal cells have been used for multiple studies comparing viral isolates for differences in their relative ability to use CD4 and CCR5, most of the studies report relatively binary information regarding whether a particular isolate can use high or low levels of CD4 and/or CCR5. More importantly, the efficiency of HIV-1 entry into cells within the human host likely results from a complex interplay between the engagement of HIV-1 Env glycoproteins with the CD4 and CCR5 receptors, found at varying levels on the surface of susceptible cells. The efficiency at which CCR5 is used for entry may depend on the level of CD4 present and *vice versa*, and this interdependency may vary between different viral isolates from various cohorts.

Affinofile system

Our understanding of HIV-1 entry has undergone significant refinement in the past two decades. A growing body of evidence suggests that there are nuances of HIV-1 entry phenotypes that have gone unappreciated, largely due to the inherent limitations of tools available to quantify such differences. To better understand how CD4 and CCR5 expression levels influence HIV-1 infectivity, we created the Affinofile system (Johnston et al., 2009). This system consists of a CD4 and CCR5 dual-inducible cell line, and a mathematical approach to quantify the receptor usage pattern and entry efficiency of Env, as a function of CD4 and CCR5 expression. Together this system has provided a

quantitative tool to examine and compare HIV-1 entry efficiency in greater detail compared to previous methods. In this review, we will discuss the details of the Affinofile system, and better specify the biological meaning of the metrics used to quantify the entry phenotype of Env. We will then review how different groups have used the Affinofile system to reveal the distinct pathophysiological properties associated with particular Env entry phenotypes, discuss potential shortcomings of the current system, and offer our opinion as to which future studies could benefit from the Affinofile system.

293 Affinofile cell line

At the crux of the 293 Affinofile system is a quadruple stable cell line that can be effectively induced to express combinatorial amounts of CD4 and CCR5 receptor levels. We generated this cell line sequentially using the selective reagents and strategy indicated in Fig. 1 (see legend). The cell line was single-cell cloned at four different stages to select for a clone with low basal level of expression and inducible expression that covers the physiologic range of CD4 and CCR5 levels. Through this process, we eventually generated a dual inducible cell line where CD4 and CCR5 expression could be regulated independently and simultaneously by varying the concentration of tetracycline and Ponasterone A, respectively. In practice, we have found that minocycline or doxycycline offers a better degree of control over tetracycline itself. For ease of reference, we will simply refer to tetracycline induction unless otherwise stated. Ponasterone A is a potent inducer of the synthetic ecdysone-inducible mammalian expression system that is used in our Affinofile cells (Saez et al., 2000). Quantitative flow cytometry (qFACS) is used to determine the number of CD4 and CCR5 antibody binding sites (ABS)/cell. In the absence of any inducing reagent, the basal levels of CD4 expression can range from 1800 to 5000 ABS/cell (Agrawal-Gamse et al.,

2009; Johnston et al., 2009; Loftin et al., 2010; Tilton et al., 2010), whereas basal CCR5 levels range between 1000 and 8000 ABS/cell (Agrawal-Gamse et al., 2009; Johnston et al., 2009; Loftin et al., 2010; Roche et al., 2011a; Tilton et al., 2010). The published values for the maximally induced levels of CD4 and CCR5 from four to five independent labs are given in the inset tables in Fig. 1.

While there appears to be some variability in the range of CD4 and CCR5 levels that can be induced, we (and others) have found that the induced CD4 and CCR5 expression levels are generally reproducible within a lab. Nevertheless, the induction range can drift over time even in the same lab (compare Agrawal-Gamse et al., 2009 to Tilton et al., 2010). The variability can be due to procedural differences, cell passage number, and importantly, the nature or quality of the fetal calf serum (FCS) used. We have recommended the use of dialyzed FCS as the wide spread use of antibiotics (such as tetracyclines) in the agricultural industry might lead to trace amounts in the animal source used for FCS production. In addition, we cannot exclude the presence of cross-reactive small molecules in undialyzed fetal calf serum that might activate the synthetic VgRXR receptor used in the ecdysone-inducible system. Finally, we cannot exclude a systematic error inherent in the manufacturers standards provided by the different kits used for qFACS (see Lee and Doms, 2001 for a technical discussion of parameters that might affect the accuracy of qFACS measurements). Therefore, it is important that each operator conducts qFACS on the Affinofile cells prior to infection to insure consistent induction. However, Affinofile cells can be passaged for about 3 months before the CD4/CCR5 inducibility becomes unreliable. Regardless of the actual ABS/cell number that is obtained, the functional control for reproducible inducibility is whether a “standard” strain of R5 virus in each lab responds with the same infectivity profile as CD4 and CCR5 is induced. As will be discussed below, the infectivity profile of a given virus across a

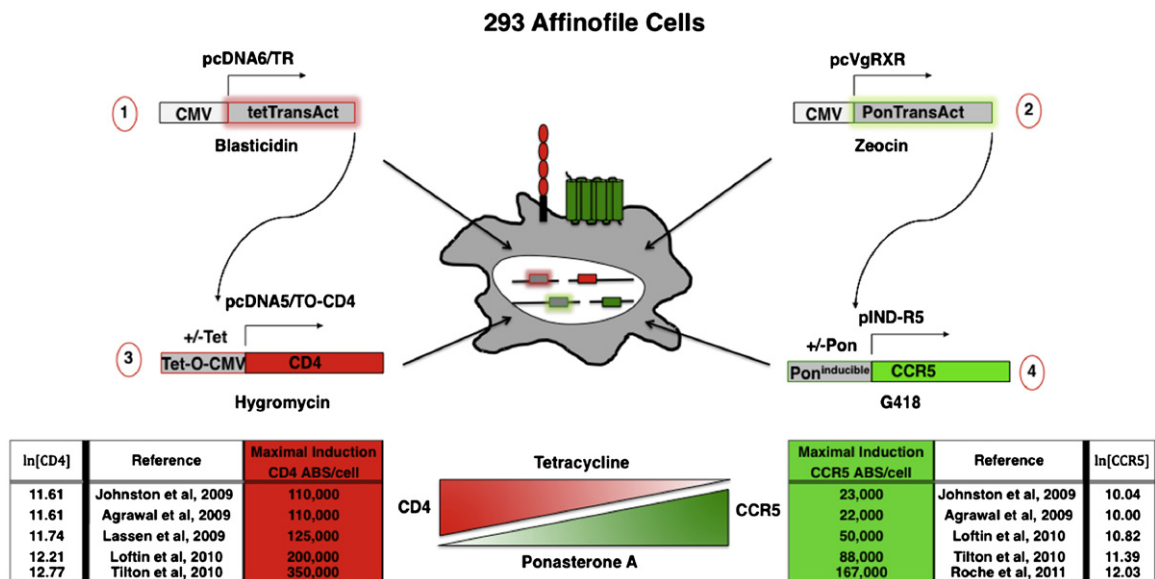


Fig. 1. CD4 expression is controlled by the Tet-On system in which constitutive expression of the tet transactivator from pcDNA/TR (1) represses expression from the Tet-O-CMV promoter (3) in the absence of tetracycline. In the presence of tetracycline, the tet transactivator is released from the Tet-O-CMV promoter on pcDNA5/TO-CD4 (3), allowing CMV driven expression of CD4. CCR5 expression is controlled by the synthetic ecdysone-inducible system in which the pon A transactivator, VgRXR (2), comprises of the modified heterodimeric subunits of the insect nuclear hormone receptors VgEcR and RXR. VgEcR and RXR are independently driven by constitutive promoters on the same plasmid (pcVgRXR) (2), but are shown simply as “ponTransAct” for clarity. In the presence of ponasterone A, the two subunits dimerize (VgRXR) and bind to the pon A inducible promoter on pIND-R5 (4) consisting of 5 × ecdysone/glucocorticoid response elements (5 × E/GREs), driving expression of CCR5. Thus, the addition of varying amounts of tetracycline and/or ponasterone A induces CD4 and/or CCR5 expression, respectively. The induced surface levels of CD4 and CCR5 are quantified by qFACS as described in the text. The inset represents the publications that specifically state the maximal number of CD4 (red) and/or CCR5 (green) antibody binding sites (ABS)/cell induced. The natural logarithms of CD4 and CCR5 cell surface concentrations (ABS)/cell are indicated here for convenience. We will refer to these numbers when discussing the analytic methods used to quantify the efficiency of CD4/CCR5 usage. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 1
Summary of publications using 293 Affinofile cells.

Category	Title	Env phenotype revealed by use of 293 Affinofile cells	Use of full induction matrix	Use of VERSA metrics	Reference
In vitro derived mutations	Adaptive mutations in a human immunodeficiency virus type 1 envelope protein with truncate V3 loop restore function by improving interaction with CD4	Lab adapted V3 truncation mutant compensates for inefficient CCR5 usage by efficient use of CD4; gains ability to enter cells at low levels of CD4	Yes	Yes	Agrawal-Gamse et al. (2009)
Cellular tropism	An altered and more efficient mechanism of CCR5 engagement contributes to macrophage tropism of Ccr5-using HIV-1 envelopes	Ability of Envs to enter low CCR5/moderate CD4 induced cells are positively correlated with macrophage tropism	No	No	Sterjovski et al. (2010)
Cellular tropism	Constrained use of CCR5 on CD4+ lymphocytes by R5X4 HIV-1: efficiency of Env-ccr5 interactions and low CCR5 expression determine a range of restricted Ccr5-mediated entry	Ability to use low levels of CCR5 on Affinofile cells is strongly correlated with R5-mediated entry into primary lymphocytes for R5X4 viruses	No	No	Loftin et al. (2010)
Cellular tropism	Macrophage-tropic HIV-1 variants from brain demonstrate alterations in the Way Gp120 engages both CD4 and CCR5	Affinofile cells reveal that brain R5 macrophage(M)-tropic Envs can use limiting levels of CD4 whereas non-M-tropic lymph node R5 Envs cannot; VERSA metrics reveal inter-subject phenotypic segregation of brain from LN-derived Envs	Yes	Yes	Salimi et al. (in press)
Pathogenesis	HIV-1 replication in the central nervous system occurs in two distinct cell types	R5 macrophage-tropic viruses derived from CSF have enhanced ability to enter CD4 ^{low} /CCR5 ^{high} Affinofile cells compared to R5 T-cell tropic viruses derived from the same compartment	No	No	Schnell et al. (2011)
Pathogenesis	Primary infection by a human immunodeficiency virus with atypical coreceptor tropism	Rare GPEK V3 crown motif found in T/F virus impairs efficiency of CCR5 usage in vitro, despite efficient replication in vivo; reversion to consensus GPGK motif restores ability to use low levels of CCR5 on Affinofile cells	Yes	Yes	Jiang et al. (2011)
Pathogenesis	Elite suppressor-derived HIV-1 envelope glycoproteins exhibit reduced entry efficiency and kinetics	Envs derived from elite suppressors (ES) use CD4 and CCR5 less efficiently compared to Envs derived from chronic progressors (CP)	Yes	No *	Lassen et al. (2009) *Phenotypes confirmed by VERSA metrics in Johnston et al. (2009)
Entry inhibitor	HIV-1 predisposed to acquiring resistance to maraviroc (MVC) and other CCR5 antagonists in vitro has an inherent, low-level ability to utilize Mvc-bound CCR5 for entry	Precursor MVC-sensitive Env that gave rise to many MVC-resistant Envs has low-level ability to use MVC bound form of CCR5, which is significantly revealed as levels of CCR5 is lowered on Affinofile cell line	No	No	Roche et al. (2011a)
Entry inhibitor	HIV-1 escape from the CCR5 antagonist maraviroc associated with an altered and less efficient mechanism Of Gp120 - CCR5 engagement that attenuates macrophage tropism	In presence of maraviroc, MVC resistant clone requires higher levels of CCR5 for efficient entry	Yes	Yes	Roche et al. (2011b)
Entry inhibitor	A maraviroc-resistant HIV-1 with narrow cross-resistance to other CCR5 antagonists depends on both N-terminal and extracellular loop domains of drug-bound CCR5	Rare in-vivo MVC resistant isolate can use CCR5 very efficiently, even in presence of inhibitor, provided that an adequate amount of CD4 is present	Yes	Yes	Tilton et al. (2010)
Entry inhibitor	HIV-1 resistance to CCR5 antagonists associated with highly efficient use of CCR5 and altered tropism on primarycd4+ T cells	Presence of inhibitor increases sensitivity of MVC-resistant Env to changes in CCR5 levels; this is reflected in altered vector metrics (increased θ and Δ) which corresponded with altered T cell tropism ($T_{EM} > T_{CM}$) in presence of inhibitor	Yes	Yes	Pfaff et al. (2010)
Entry inhibitor	Inefficient entry of vicriviroc-resistant HIV-1 via the inhibitor-ccr5 complex at low cell surface CCR5 densities	Expression levels of CCR5 influences entry efficiency of VVC-resistant Env in the presence of VVC; resistance is more apparent at high CCR5 levels	No	No	Pugach et al. (2009)
Pathogenesis/entry inhibitor	A quantitative affinity-profiling system that reveals distinct CD4/CCR5 usage patterns among human immunodeficiency virus type 1 and simian immunodeficiency virus strains	Original paper describing the Affinofile cell system and use of the VERSA metrics for profiling CD4/CCR5 usage efficiencies; VERSA metrics confirm and quantify the phenotypic differences between ES and CP Envs described in Lassen et al. (2009)	Yes	Yes	Johnston et al. (2009) → The definitions of the VERSA metrics in this review supercedes those given in the original paper
Gene therapy	Targeted transduction via CD4 by a lentiviral uses clathrin mediated entry pathway	Novel Sindbis/ab envelope transduction of cells is dependent on CD4 expression level	No	No	Liang et al. (2009)
Review	R5X4 HIV-1 coreceptor use in primary target cells: implications for coreceptor entry blocking strategies	Review	No	No	Loftin et al. (2010)

range of CD4 and CCR5 levels can be quantified by the viral entry receptor sensitivity analysis (VERSA) computational platform. We will show that our analytic method somewhat mitigates the variability inherent in our inducible biological system.

This dual inducible cell line was dubbed the 293 Affinofile cells to reflect its potential ability to measure the relative binding affinities of Env for CD4 and CCR5, and thus profile the CD4 and CCR5 usage efficiencies of HIV-1 (Johnston et al., 2009).

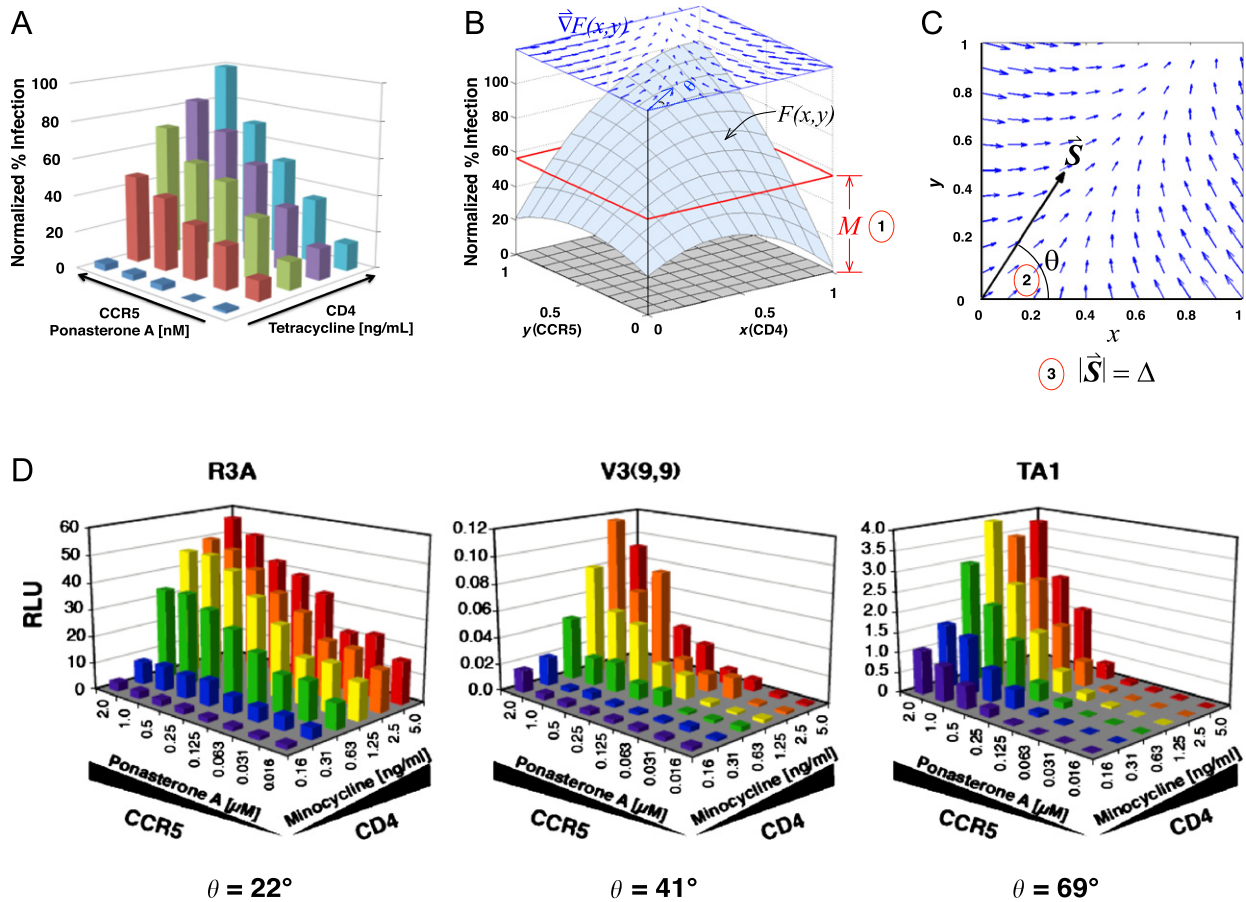


Fig. 2. (A) A prototypic R5 Env pseudotyped virus was used to infect Affinofile cells induced with varying concentration of tetracycline and ponasterone A. The infectivity profile generated in (A) can be mathematically fitted to the surface function $F(x,y)$ shown in (B), where x and y are the mathematically rescaled values for the relevant CD4 and CCR5 concentrations (see text for details). Essentially, the surface plot in (B) represents the infectivity response of Env as a function of CD4 and CCR5 expression level, which is described by the continuous polynomial function $F(x,y)$. As indicated in the text, three parameters can be found to represent the salient features of the surface function to capture the essential phenotypic behavior of Env in response to varying levels of CD4 and CCR5. The three metrics (1) mean infectivity M , (2) sensitivity vector angle θ , and (3) sensitivity vector amplitude Δ , are graphically represented in (B) and (C). The mathematical formulation of these metrics, their biophysical correlates, and how these metrics can be used to quantify the entry phenotype of Env are detailed in the text. (D) A reproduction of Fig. 5 from Agrawal-Gamse et al. (2009) illustrating how the nature of the infectivity plot is reflected in the angular metric. See text for details. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Viral entry receptor sensitivity analysis (VERSA)

Using 293 Affinofile cells, the infectivity of a given Env, in the context of a pseudotyped reporter virus, can be profiled across 24–48 distinct combinations of CD4 and CCR5 expression levels. These various studies are listed in Table 1. The infectivity profile of a typical R5 virus infection across 25 distinct levels of CD4/CCR5 expression levels is shown in Fig. 2A. To assist in describing and comparing the infectivity data associated with numerous viral Envs from various cohorts and research groups, we created an automated computational web-based tool: Viral Entry Receptor Sensitivity Analysis at versa.biomath.ucla.edu. For a given Env, the VERSA program permits the rapid distillation of the set of infectivity data points into three metrics that grossly describe the Env's CD4 and CCR5 usage pattern and entry efficiency. Below, we will first describe the analytical method used to determine these metrics, and then define the biological meaning of these metrics with respect to how they reflect the entry phenotype of Env. We will then review the studies summarized in Table 1 to illustrate how the Affinofile system, and the associated VERSA metrics, can help reveal the distinct pathophysiological Env phenotypes associated with differential CD4/CCR5 usage efficiencies.

Rescaling of CD4 and CCR5 expression levels: Since infectivities were measured across numerous (typically 25–48) combinations of CD4 and CCR5 concentrations, we sought to reduce the dimensionality of the data into a geometrically meaningful form. First, the experimentally relevant CD4 and CCR5 concentrations (ABS/cell) are rescaled according to $0 \leq x, y \leq 1$

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

where “min” and “max” refer to the common minimum and maximum receptor/coreceptor used across all measurements in a given experiment. The variability represented by the differential range of inducible CD4/CCR5 expression levels (when represented in units of ABS/cell) is somewhat dampened by this rescaling, where the minimum and maximum on both the x (CD4) and y (CCR5) axes are defined as 0 and 1, respectively. The use of natural logarithms for this rescaling also effectively reduces the magnitude of the variable expression levels (see inset tables in Fig. 1).

Transformation of the raw infectivity data: The infectivity profile exhibited by a given viral Env across a spectrum of CD4 and CCR5

expression levels, as illustrated by the 3-D column graph in Fig. 2A, can be mathematically fitted to a corresponding 3-D surface plot (Fig. 2B). The infectivity response as a function of CD4 and CCR5 expression level is described by the continuous polynomial function

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

which represents the surface of the plot indicated in Fig. 2B. The transformation of the raw infection data into the surface function $F(x,y)$ using normalized infectivity data and rescaled CD4 and CCR5 expression levels, allows us to define at least three parameters that quantify the fitted data surface $F(x,y)$. These three parameters capture the salient topographical features of the surface plot in Fig. 2B, which represents the phenotypic response of Env to varying levels of CD4 and CCR5. We will now describe how these three metrics are derived and computed in VERSA, describe their mechanistic interpretation, and specify how they are used to quantify the entry phenotype of Env.

VERSA metrics: The mean infectivity, M , provides a rough estimate of the overall efficiency of entry; it is the mean of the normalized function $F(x,y)$ across the entire range of CD4 (x) and CCR5 (y) surface expression levels represented by the x - y plane

$$M \equiv \int_0^1 \int_0^1 F(x,y) dx dy, \quad (3)$$

and is graphically represented by the height of the square plane (red) indicated in Fig. 2B.

Additional geometric features of the surface function can be easily quantified by defining a sensitivity vector

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

which represents the average, across the relevant CD4 and CCR5 levels, of the local gradient vector on the surface. The vector \vec{S} encodes the overall direction of the infectivity surface and its overall steepness (Fig. 2C). These can be represented by two metrics: the sensitivity vector angle, θ , and the vector amplitude, $\Delta \equiv |\vec{S}|$. Specifically, the overall direction of the sensitivity vector can be defined through the angle makes with the \hat{x} -axis

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

The angle θ is a measure of the relative infectivity response to changes in CD4 versus CCR5 levels. A virus that is predominantly sensitive to changes in CCR5 levels and not CD4 will have θ near 90° , while $\theta \sim 0^\circ$ for a virus that is only sensitive to changes in CD4 levels but not CCR5. A virus equally sensitive to changes in both CD4 and CCR5 levels would have $\theta \sim 45^\circ$.

The amplitude of the sensitivity vector $\Delta \equiv |\vec{S}|$ measures the overall “steepness” of the normalized infectivity function $F(x,y)$, averaged across the entire matrix of CD4 and CCR5 expression levels; it measures the combined rate of increase of infectivity as a function of CD4 and coreceptor concentrations. The vector amplitude Δ is graphically represented by the length of the sensitivity vector in Fig. 2C. This metric was previously termed the vector “magnitude” (Johnston et al., 2009), which has the unfortunate coincidence of starting with the letter “m”, and may lead to confusion with the mean infectivity metric M , which is clearly distinct. Here, we formally designate the three metrics described in Fig. 2B and C as mean infectivity M , sensitivity vector angle θ , and sensitivity vector amplitude Δ . These designations will be changed accordingly in the updated version of VERSA. Finally, note that M, θ , and Δ can all be explicitly written in terms of simple functions of the fitting parameters $\{a, b, c, d, e, f\}$, and thereby explicitly encodes the geometric features of the data function $F(x,y)$.

Inputting normalized or raw infection data

VERSA supports the input of normalized or raw infection data (e.g., RLU if using luciferase reporter viruses). Since our metrics $\{M, \theta, \Delta\}$ are all dimensionless, the actual type of infection data used (RLU, or %GFP+ cells, or %p24+ cells, etc.) to obtain the VERSA metrics is immaterial as long as the same type of data is consistently used between experiments for comparisons. Given this flexibility, it is important to understand the impacts or constraints that various kinds of input data may have on each metric. While the vector angle θ is insensitive to the use of raw or normalized infectivity data, this is not true for mean infectivity M and vector amplitude Δ . For example, since raw RLU data can have a large dynamic range (~ 3 – 5 logs), mean infectivity and vector amplitude differences between viral Envs can be magnified. In these cases, Affinofile assays can be sensitive and relative comparisons of Env phenotypes in the same lab are possible and probably meaningful. However, the numbers obtained have no independent meaning outside of a particular comparison cohort in a particular lab. This is because RLUs can be affected by arbitrary factors such as instrumentation sensitivity, integrated time of detection, concentration and quality of the substrate used, variations in room temperature, and a host of other systematic errors that may be particular to the experiment.

On the other hand, if input data are normalized to the maximum infectivity (set at 100%) obtained in each profiling experiment, comparisons between divergent cohorts and infectivity conditions may then be possible. However, normalization assumes that infectivity plateaus at maximally induced CD4 and CCR5 levels for a given Env, which is not always the case. Thus, the VERSA metrics obtained using normalized data may be less sensitive for detecting more subtle phenotypic differences in entry efficiencies. We will discuss specific instances where successful data interpretation has been obtained with one but not the other kind of input data.

Current publications using Affinofile system

Next, we review how the Affinofile system has been used to study (1) compensatory phenotypes mediated by unusual mutations and truncations in HIV-1 Env, (2) relationship between differential entry efficiencies and HIV disease pathogenesis, (3) the influence of entry inhibitors on CD4 and CCR5 usage patterns, and (4) the relationship between tropism and CD4/CCR5 usage. These studies are summarized in Table 1. In any given study, many lines of evidence are usually provided to support the authors' main conclusions. For the purposes of this review, we will mainly focus on the evidence provided by the Affinofile system. We do so in order to illustrate the phenotypic nuances that this system can reveal, and to highlight how the use of Affinofile cells can complement existing methodologies to gain a better understanding on R5 virus tropism and pathogenesis. It is not our intention to suggest that the evidence provided by the Affinofile system is of paramount importance in any given situation. We do, however, hope to show that the Affinofile system is a relatively convenient and high throughput way of evaluating HIV R5 Env phenotypes in a quantitative and reproducible fashion.

Results and discussion

Compensatory phenotypes mediated by unusual mutations and truncations in HIV-1 Env

The gp120 protein of HIV-1 Env includes five conserved (C1–C5) and variable (V1–V5) regions. The conserved regions,

found in inner domain of gp120, play a critical role in the function of gp120. The variable regions, found on the outer domain, act as an evolving shield for the virion against the immune response. Of all the variable regions, the V3 loop of HIV-1 gp120 has long been recognized as being particularly important due to its dual role in protecting the Env from neutralizing antibodies, as well as in determining coreceptor choice (Zolla-Pazner and Cardozo, 2010). Mutations and truncations in V3 usually result in an Env that is unable to promote entry, despite seemingly proper protein folding (Wyatt et al., 1993). In Agrawal-Gamse et al. (2009), the authors discovered that a dual tropic virus, termed R3A, could tolerate a deletion of 15 amino acids that removed the central portion of the V3 loop. This virus with the truncated Env, termed V3(9,9), was severely compromised in its ability to infect SupT1CCR5 cells compared to the parental R3A Env. Subsequent passage of the V3(9,9) virus on SupT1CCR5 cells, which express high levels of CCR5, partially restored the entry efficiency of the virus. Interestingly, the adapted virus, named TA1, retained the V3 truncation while gaining several other mutations and deletions in other areas of the Env.

In a vivid and elegant demonstration, the Doms lab used the Affinofile cells to profile the infectivity of the parental R3A virus, the initial V3(9,9) truncation mutant with compromised function, and the final TA1 virus adapted to grow well on SupT1-CCR5 cells. The infectivity plots (reproduced in Fig. 2D) reveal that the initial V3 truncation severely compromised R3A's ability to efficiently use low levels of CCR5. V3(9,9) no longer enter cells at low levels CCR5 (0.016–0.063 μM Pon A) no matter how much CD4 was provided. This defect was maintained in the adapted TA1 virus Env. Instead, the infectivity profile on Affinofile cells revealed that the partially restored function of the TA1 virus Env was due to an increased ability to use low levels CD4 (0.16–0.31 ng/ml minocycline), provided that a sufficient amount of CCR5 was present. The gestalt of the infectivity profiles between these three mutants is captured by angle metric θ . Thus, R3A, which responds more readily to changes in CD4 levels (at all levels of CCR5) has a θ of 22° , while the adapted TA1, which is now more responsive to changes in CCR5 levels has a θ of 69° . Note that an increase in the angular metric (towards 90°) simply means an increased infectivity response to changes in CCR5 levels (and vice versa for CD4). An increase or decrease in θ by itself says nothing about whether the change is due to more or less efficient usage of CCR5 or CD4. The angular metric needs to be interpreted in the context of the infectivity profile in order to make statements about receptor usage efficiencies. In this case, the authors rightfully point out that the scale on the z -axis, representing the efficiency of entry as reported by luciferase activity, clearly indicates that the adaptive mutations in TA1 increased its infection efficiency over V3(9,9) by about 10-fold, mainly by enhancing the its ability to use low levels of CD4. These Affinofile data are consistent with the cumulative results from the myriad other inhibition and time-of-addition experiments conducted in this study.

Relationship between differential entry efficiencies and HIV disease pathogenesis

Using the Affinofile cell line and system, several publications have discovered differences between HIV-1 pathogenic categories that can be ascribed to disparities in entry efficiencies; disparities that are not always revealed by conventional entry assays.

Elite suppressor vs. chronic progressor Envs

In Lassen et al. (2009), the authors used the Affinofile cells to examine a total of 70 Envs derived from chronic progressor (CP) and elite suppressor (ES) HIV+ subjects. The results of these

studies revealed that, on average, ES Envs are less efficient at using both CD4 and CCR5, when compared to CP Envs. Thus, when CCR5 levels were fixed, and CD4 levels varied or vice versa, ES Env pseudotypes achieved a moderately lower level of infection relative to CP Envs. Notably, due to the large variations in IC_{50} 's between individual Env clones, even among clones from the same patient, there was no significant difference between ES and CP Envs in their sensitivity to CCR5 antagonism (via CCL5 (RANTES) or TAK779) or fusion (Enfuvirtide) inhibition. However, when the infectivity data were reexamined using VERSA (Johnston et al., 2009), the mean infectivity M and vector angle θ were significantly higher for CP Envs compared to ES Envs. The data also indicate that the increased M values of the CP Envs were associated with their increased responsiveness to changes in CCR5 levels (higher θ values). Together, the Affinofile data (increased M with increased θ) suggest that the increased responsiveness of the CP Envs to changes in CCR5 levels was likely due to their increased efficiency of CCR5 usage. Inspection of the infectivity plots confirmed this interpretation. As mentioned earlier, this increased efficiency of CCR5 usage was not evident using conventional inhibitor assays, as ES and CP Envs did not show differential susceptibility to CCL5 (RANTES) or TAK779.

Central nervous system-derived Envs

The ability of the Affinofile system to phenotypically segregate potential pathogenic categories is supported by the following two studies. In Schnell et al. (2011), the authors demonstrated that R5 HIV-1 isolated from cerebral spinal fluid (CSF) of patients with or without HIV-1 associated dementia (HAD) segregated into two phenotypic categories: the ability or lack thereof to infect Affinofile cells induced at $\text{CD4}^{\text{low}}/\text{CCR5}^{\text{high}}$ levels. Viral isolates that can infect $\text{CD4}^{\text{low}}/\text{CCR5}^{\text{high}}$ Affinofile cells can also infect monocyte derived macrophages (MDM), and thus are R5 M-tropic. Alternatively, isolates that can only infect $\text{CD4}^{\text{high}}/\text{CCR5}^{\text{high}}$ but not $\text{CD4}^{\text{low}}/\text{CCR5}^{\text{high}}$ Affinofile cells, can also only infect activated CD4^+ T-cells, but not MDM, and thus are R5 T-tropic. The authors observed that CSF-derived viruses isolated from patients with HAD could be either R5 T cell-tropic and/or R5 M-tropic whereas viruses isolated from patients without HAD are invariably R5 T cell-tropic. Although the study is small and involved only eight HAD patients, at least for the cohort examined, it appears that the ability of infect $\text{CD4}^{\text{low}}/\text{CCR5}^{\text{high}}$ Affinofile cells at $> 10\%$ levels relative to $\text{CD4}^{\text{high}}/\text{CCR5}^{\text{high}}$ Affinofile cells can be as sensitive a marker for detecting R5 M-tropic viruses as using MDM infection itself. Interestingly, in longitudinal samples from one patient, the authors were able to show that CSF-derived R5 M-tropic Envs that could infect $\text{CD4}^{\text{low}}/\text{CCR5}^{\text{high}}$ Affinofile cells could be detected many months prior to subsequent HAD diagnosis.

More recently, Salimi et al. (in press) examined brain (BR) and lymph nodes (LN) Envs derived from patients that succumbed to HAD. Using the Affinofile system the authors reveal that R5 macrophage (M)-tropic BR Envs can use limiting levels of CD4 whereas R5 non-M-tropic LN Envs cannot. Furthermore, a combination of all three VERSA metrics clearly revealed inter-subject phenotypic segregation of BR- from LN-derived Envs, which occurred independent of genetic signature alterations. *Inter-subject genetic signature alterations associated with HIV-1 compartmentalization have not been identified in previous studies, principally because the functional affects of amino acid alterations in gp120 are usually strain-specific. However, the mathematical formulations and the normalization techniques that were used to derive the VERSA metrics serve to quantify some intrinsic functional properties of Env, which are more likely to be conserved among Envs derived from different tissue sources. These metrics can therefore be used to identify conserved phenotypic*

Env signatures based solely on the Env CD4 and CCR5 usage profiles.

Acute transmitted/founder virus Envs

However, the efficiency of CD4 and CCR5 usage, or the ability to use low levels of (co)-receptors, does not always correlate with the *in vivo* pathogenic potential of a viral isolate. In a recent study by Jiang et al. (2011), the authors isolated and examined an unusual R5 subtype B transmitter/founder (T/F) viral Env with a rare motif in the V3 crown. *In vivo*, this virus replicated very efficiently as shown by a high viral load during the time of acute primary infection ($> 10^7$ RNA copies/ml during Feinberg stage III). However, examination of the Env entry phenotype using the Affinofile system showed that this particular Env used CCR5 very inefficiently, requiring high levels of both CD4 and CCR5 for efficient entry (θ close to 45°). Additionally, mutating the rare GPEK V3 crown motif of the T/F Env to the consensus subtype B motif, GPGK, conferred greater ability to use CD4 and CCR5 on Affinofile cells, as well as other cell lines and primary cells. The reversion to the consensus V3 crown motif (GPEK→GPGK) was accompanied by a marked decrease in θ ($45^\circ \rightarrow 0^\circ$) and a 10-fold increase in Δ . Inspection of the infectivity profile (Fig. 10 of the paper) revealed that the GPGK reversion mutant is only sensitive to changes in CD4 levels (hence $\theta \sim 0^\circ$). The increase in Δ could be accounted for by the accumulated increase of the infectivity response at low levels of CCR5 (as CD4 levels are increased) where no infectivity response was previously present. The authors suggest the decrease in entry efficiency exhibited by this viral isolate may be due to usage of coreceptors other than CCR5 and CXCR4, or to abnormal coreceptor protein conformation, both of which are known to occur *in vitro* (Berro et al., 2011; Nedellec et al., 2009). This illustrates the possible dichotomy between *in vivo* and *in vitro* results.

Together, these studies demonstrate that the Affinofile system can be effectively used to examine, characterize and/or segregate the entry phenotypes of viruses that are associated with distinct pathophysiological properties *in vivo*.

Influence of entry inhibitors on CD4 and CCR5 usage

The clinical use of CCR5 antagonists was initially accompanied by concerns that resistance could easily occur by co-receptor switching of the virus to the more pathogenic CXCR4-using variants. These concerns were substantiated by the observation that *in vitro* co-receptor switching can occur due to mutations of as little as one amino acid (Pastore et al., 2004). Indeed, phenotypic and/or genotypic testing for coreceptor tropism is required before the use of maraviroc, currently the only FDA approved CCR5 antagonist, in combination antiretroviral therapy (Lin and Kuritzkes, 2009). In earlier clinical trials, the development of resistance to CCR5 antagonist was confounded by the relative insensitivity of the phenotypic coreceptor tropism test (trofile), which inadvertently allowed the outgrowth of pre-existing X4 or dual-tropic/mixed (D/M) strains (Lin and Kuritzkes, 2009; Wilkin and Gulick, 2012). In the absence of pre-existing X4 or D/M strains, the preferred pathway to resistance for R5 strains appears to be evolving the ability use the antagonist bound form of CCR5 rather than *bona fide* coreceptor switching (Westby, 2007). Several groups have used the Affinofile system to shed more light on how HIV-1 circumvents entry inhibition mediated by CCR5 antagonists.

MPI and CCR5 expression levels

In one of the first studies that used the Affinofile cell line (Pugach et al., 2009), the authors examined how the expression

levels of CCR5 can alter the maximum percent inhibition (MPI) of viral isolates resistant to the CCR5 antagonist vicriviroc (VVC). MPI indicates a plateau level of inhibition in the presence of excess of inhibitor, and is a mechanistically distinct from resistance that is attributed to increases in IC_{50} . A MPI effect is indicative that the virus is using the inhibitor-bound form of CCR5. The results of this study demonstrated that the CCR5 expression levels were inversely proportional to the resulting MPI, indicating that increasing levels of CCR5 can compensate for the inefficient usage of the inhibitor-bound form of CCR5 by VVC-resistant viruses. Interestingly, two different VVC-resistant clones independently derived from the drug sensitive parental clone (CC1/85) can exhibit differential levels of VVC-resistance that is revealed by the gradient of the MPI effect observed under varying levels of CCR5 in the Affinofile cells. The authors suggest that the CCR5 levels expressed on different cell lines or primary cells can lead to distinct MPI plateaus, thus altering ones interpretation of the degree of resistance.

These results were recently corroborated by Roche et al. (2011a), which reported that the commonly used “maraviroc (MVC) sensitive” CC1/85 Env (the parental derivative of many CCR5-inhibitor resistant clones) has an inherent, albeit low-level, ability to use the MVC-bound form of CCR5. The authors show that when using U87-CD4/CCR5, JC53, TZM-bl cells and PBMC, the MVC-sensitive CC1/85 virus is completely inhibited by saturating concentrations of MVC, hence the MPI is 100 in these cells. However, when the same experiment was done in NP2-CD4/CCR5 cells, which express comparatively higher levels of CCR5, or Affinofile cells induced to express high levels of CCR5, the MPI is 96.2% and 53%, respectively. Moreover, the authors show that systematically decreasing the levels of CCR5 on Affinofile cells gradually increases the MPI to the levels seen in the other cells, thus illustrating an inverse relationship between the MPI and CCR5 expression levels by this ostensibly “MVC-sensitive” CC1/85 Env. The authors suggest that this low level ability to use the MVC-bound form of CCR5, which is only revealed on cells capable of expressing high levels of CCR5, likely predisposes the CC1/85 Env to gaining (cross)-resistance to CCR5 antagonists. The robust inverse correlation of MPI with CCR5 induction levels suggests that the Affinofile cells may detect baseline resistance to CCR5 antagonists with greater sensitivity than the other commonly used cell lines. If validated, this would facilitate the clinical use of CCR5 inhibitors. The gradient of MPI observed as CCR5 is induced on the same cellular background is a particularly useful property. Conversely, U87-CD4/CCR5 cells that are commonly used for phenotypic testing of CCR5 inhibitor resistance may not be sensitive enough to detect the incipient development of CCR5 inhibitor resistance.

VERSA metrics reveal inefficient use of inhibitor-bound CCR5 by inhibitor resistant Envs

Using the Affinofile system, several groups have examined how resistance to CCR5 entry inhibitors influences viral usage of CD4 and CCR5. In Pfaff et al. (2010) and Roche et al. (2011b), the authors detailed how *in vivo* and *in vitro* derived Envs resistant to the CCR5 antagonists aplaviroc (APL) and MVC, respectively, have modified entry efficiencies in the presence of the inhibitor. In both studies, the authors showed that inhibitor sensitive and resistant Envs, in the absence of any inhibitor, had similar 3-D infection plots and vector metrics, highlighted by vector angles ranging from 10° to 17° , which implies a below average dependency on CCR5 expression levels. However, examination of the inhibitor resistant clones in the *presence* of inhibitor resulted in infectivity plots with an overall reduced infection, but more importantly the vector angles shifted to 23° – 30° . Additionally,

the presence of the CCR5 antagonist reduced the non-normalized “vector magnitude” (now termed vector amplitude Δ) of the resistant Env. Remarkably, in both cases, the presence of CCR5 inhibitor (APL or MVC) decreases the Δ values of the resistant Envs by about 30%: 6×10^6 to 4×10^6 in Pfaff et al. (2010), and 1.56×10^5 to 1.05×10^5 in Roche et al. (2011a, 2011b). Thus, an increase in vector angle θ combined with a corresponding decrease in absolute vector amplitude Δ indicates that the apparent increase in CCR5 dependency (increase in θ) is due to more inefficient use of CCR5, as revealed by the associated decrease in Δ (lower overall rate of increase in the infectivity response). These results together indicate that the resistant Envs have an overall inefficient usage of the inhibitor bound form of CCR5. In both cases the reduced entry efficiency calculated by the Affinofile cell system correlated with reduced and altered entry into primary cells; more specifically, the Pfaff study showed an altered tropism in CD4+ T cell subsets, while the Roche study showed an attenuated tropism for macrophages, both due to the reduced ability of resistant Envs to interact with drug-bound form of CCR5.

Finally, in a separate study, Tilton et al. (2010) examined CCR5 antagonist sensitive and resistant clones isolated from a patient who experienced virological failure on MVC. In the absence of inhibitor, the sensitive and resistant clones had vector angles that were similar, 6.7° and 7.6° , respectively. As expected, in the presence of inhibitor, infection with the sensitive clone was completely abrogated. However, unlike the previous reports, infection with the resistance clone was largely unaffected in the presence of inhibitor, as revealed by a similar vector angle of 7.2° , indicating a highly efficient usage of the drug-bound form of CCR5 by this particular resistant HIV-1 variant. Inspection of the infectivity plots confirmed that the MVC-resistant clone remained sensitive only to changes in CD4 levels. This latter study illustrates that CCR5 antagonist resistance can occur with diametrically opposed consequences on entry efficiency, as well as the power of the VERSA metrics to reveal such opposing differences.

Cellular tropism and efficiency of CD4/CCR5 usage

In Loftin et al. (2010), the authors showed that dual-tropic Envs have varying degrees of CCR5 usage in the absence of CXCR4 by infecting Affinofile cells expressing fixed levels of CD4, and varying levels of CCR5. Moreover, the authors show that the ability of dual-tropic Envs to mediate entry into CD4^{high}/CCR5^{low} induced Affinofile cells correlates with the efficiency of CCR5 mediated entry into lymphocytes. These results, coupled with other assays convincingly, demonstrate that the use of CCR5 on primary T-cells by dual-tropic Envs is positively correlated with their CCR5 usage efficiency in Affinofile cells. Similarly, in a separate study, Sterjovski et al. (2010) were able to show that the ability of R5 primary isolates to enter MDM correlates with the ability to enter Affinofile cells induced to express low levels of CCR5 and moderate levels of CD4. These results, as well as the reports discussed earlier, show that the Affinofile cell line may be useful as a surrogate to examine entry into primary cells as well as several other cell lines.

Limitations of 293 Affinofile system

Potential users should be aware of the inherent limitations and constraints on the use of the 293 Affinofile system. For example, we have determined that this cell line is not appropriate for conducting β -lactamase based virus–cell fusion assays because of the relatively high background levels (unpublished data). We suspect that the ampicillin resistance genes, on each plasmid used to make the quadruple stable cell line, collectively express

enough β -lactamase to increase the background level of CCF2-AM cleavage. Issues such as this should be considered before the using Affinofile cells outside designated parameters. As with many cell lines, Affinofile cells express a basal level of CXCR4 that comes from its HEK 293 parentage. Therefore, it is critical that the viral Env being examined is fully CCR5-dependent. This can be confirmed by the use of suitable CCR5 inhibitors; infection on the Affinofile cells should be abrogated by a known CCR5-inhibitor, or at least not affected by a known CXCR4-inhibitor. Alternatively, as done by previous authors, saturating amounts of CXCR4 entry inhibitor can be added to each well, preventing the use of CXCR4 for entry. However, if the objective of the experiment is to examine the efficiency of CD4 usage for CXCR4 viruses, the basal level of CXCR4 present in the cell will permit this, as the X4 Env HxB effectively gives infectivity plot with a vector angle close to zero degrees (unpublished data). Finally, to obtain VERSA metrics that meaningfully reflect the entry phenotype of a given Env, we recommend that the infectivity profile be determined across at least 24 distinct combinations of CD4/CCR5 expression levels.

Conclusions

We have reviewed all published studies that have used the Affinofile cells to reveal some new facet of the HIV entry process. We invite the reader to inspect the original articles summarized in Table 1. While expression levels of CD4 and CCR5 clearly impacts on the efficiency of R5 HIV-1 entry, they are clearly not the only host cell determinants that impact on the entry process. However, of the various soluble host cell factors that have been described to modulate HIV entry such as galectin-1 (Sato et al., 2012; St-Pierre et al., 2011, 2010), galectin-9 (Bi et al., 2011), and 25-hydroxycholesterol (Liu et al., in press), it would be of great interest to see if they do so in a manner dependent on CD4 and CCR5 expression levels. The VERSA website at versa.biomath.ucla.edu continues to accept suggestions as to further improvements, and we are working on developing more robust methods to facilitate more widespread usage of the Affinofile system. The Affinofile system will be deposited in the NIH AIDS Reagent Repository by early 2013.

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