

# Use of a dual-inducible cell line and a vector-based angular metric to quantify distinct patterns of CD4/CCR5 usage exhibited by various HIV-1 and SIV strains

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## ABSTRACT

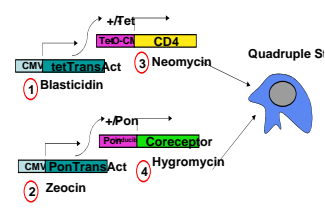
**Background:** HIV-1 entry is mediated by CD4 and one of two co-receptors, CCR5 or CXCR4. The ability of an HIV-1 strain to use differing levels of CD4/CCR5 reflects its affinity for CD4 and co-receptor, and is one factor associated with the overall viral infectivity and pathogenicity. We have designed a system to quantitatively describe the efficiency of CD4/CCR5 usage, and used it to examine SIV, HIV-1 strains, and primary isolates from infants with rapid- and slow-progressing (RP vs SP) disease.

**Methods:** An inducible cell line was created where CD4 and CCR5 expression can be simultaneously and independently controlled with tetracycline and ponasteroneA, respectively. CD4 and CCR5 levels can be induced and controlled within the range of expression found on primary cell targets of HIV-1. Infection was quantified by intracellular p24 staining, or GFP/luciferase activity when using pseudotyped reporter viruses.

**Results:** Infection of the cells with SIV-316 pseudotyped virus showed dependence on CCR5 but not CD4 concentration, consistent with the known CD4 independence of the virus. Infection with R5 viruses, Bal or YU2, increased with increasing concentrations of both CD4 and CCR5. Infection with 89.6 showed marked dependence on CD4 levels, regardless of the amount of CCR5 present. Infection with primary Clade B and A strains with previously defined high and low affinity for CCR5 and CD4, showed that each virus generates a distinct 3-D surface curve that represents their efficiency of CD4 and CCR5 usage. Mathematical modeling provided a scalar metric that clusters each isolate distinctly on a polar plot, and quantitatively describes their CD4/CCR5 usage pattern. Finally, at maximal CD4/CCR5 induction, infection with isolates from 3 RP and 4 SP resulted in a significantly higher percentage of infected cells from the RP isolates (12.11-18.99 % p24+ cells for RP vs 0.38-9.1% p24+ cells for SP isolates;  $p < 0.01$ ).

**Conclusions:** Various SIV and HIV-1 strains exhibit different patterns of CD4 and CCR5 usage, as shown by distinct 3-D surface curves, which can be clustered distinctly in a scalar polar plot. Also, viruses from RP are more efficient in using CD4 and CCR5 than those from SP. This assay can directly quantify CD4/CCR5 usage by primary HIV isolates, and can be used to study the correlation between CD4/CCR5 usage and viral pathogenicity, and potentially, to monitor the changes in CD4/CCR5 affinity that may occur with the clinical use of CCR5 inhibitors.

## RESULTS

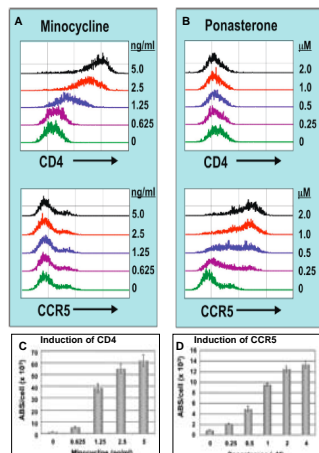


**Figure 1. Schema for generation of quadruple stable cell line in which CD4 and CCR5 expression can be induced and regulated.**

A quadruple stable cell line (HEK 293 background) expressing both the transactivators and the inducible promoters driving CD4 and CCR5 expression was made sequentially using the selective reagents indicated in the figure. The cell line was single-cell cloned at each stage (labeled sequentially 1 to 4 in figure) to select for the clone with the best properties.

• CD4 expression is under the control of the Trex system, in which addition of tetracycline releases a repressor protein upstream of a CMV promoter that controls CD4 expression. This release allows CD4 expression to proceed. Minocycline is within the same family of antibiotics and can be used in lieu of tetracycline.

• Ponasterone A regulates CCR5 expression by dimerizing 2 subunits of a functional ponasterone receptor expressed from its transactivator. This allows them to bind a region upstream of the promoter and activate CCR5 expression.



**Figure 2. CD4 and CCR5 can be independently and simultaneously induced.**

Expression of CD4 and CCR5 was induced with different concentrations of minocycline (CD4) and ponasterone A (CCR5) resulting in at least 20 distinct combinations of CD4 and CCR5 levels. 18 hours post-induction, receptor levels were measured by QFACS. QuantiBRITE Fluorescence Quantitation system (BD Biosciences) was used to quantify antibody binding sites per cell.

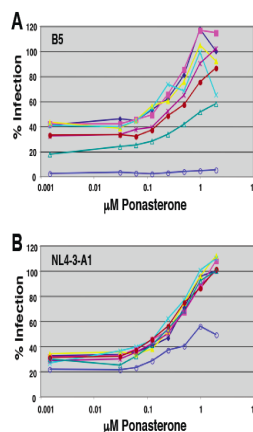
**A.** Induction of cells with minocycline showed a rise in CD4 expression, which did not affect CCR5 expression.

**B.** Induction of cells with ponasterone A showed a rise in CCR5 expression independent of CD4 expression

**C.** CD4 induction resulted in 2,000 to 60,000 ABS/cell. (Antibody=Leu3A)

**D.** CCR5 induction resulted in 800 to 12,000 ABS/cell. (Antibody=2D7)

## RESULTS



**Figure 3. R5 isolates have differential patterns of CD4 and CCR5 usage.**

**B5-primary Clade B env (B5-91U5096); NL4-3-A1=chimeric NL4-3 env** with the V3 region from a primary clade A R5 HIV-1 isolate, A1-92RW009.

(A) B5 and (B) NL4-3-A1, were used to infect dual-inducible cells at 64 different combinations of minocycline and ponasterone concentrations. Infection was detected by lysing cells and detecting luciferase activity 48 hours post-infection. Data are normalized to infection obtained at highest CD4/CCR5 induction.

• HIV-1 B5 env have decreased susceptibility to sCD4 neutralization compared with the chimeric envelope NL4-3-A1 (data not shown).

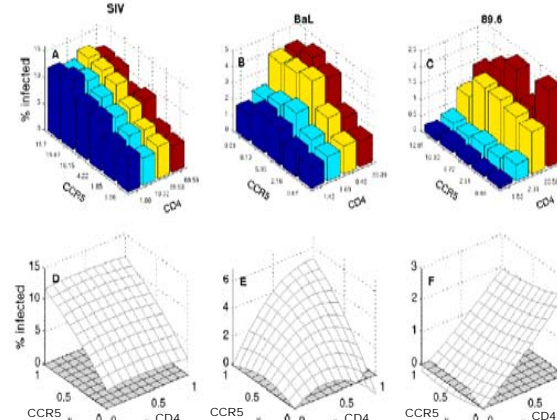
**A.** For the **B5 envelope**, at the lowest level of CD4 expression (0.015 ng/ml minocycline), no amount of CCR5 could rescue infection (blue line with open circles).

• However, at or above 0.3125 ng/ml of minocycline, increasing CCR5 levels (ponasterone) resulted in a corresponding increase in infectivity.

**B.** In contrast, **NL4-3-A1** infected even at 0.015ng/ml minocycline (20-55%). Above a minimal level of CD4 (0.3125 ng/ml minocycline), the efficiency of infection depended almost exclusively on CCR5 expression levels.

**Figure 4. The differential efficiency of CD4 and CCR5 usage can be represented by distinct 3-D surface plots.**

Infectivity of (A) pseudotyped SIV316, or replication competent clones of (B) Bal, and (C) 89.6 was tested at 20 different induction conditions resulting in 20 distinct combinations of CD4 and CCR5 expression levels. Infection was detected by intracellular p24 staining and quantified by flow cytometry 48 hours post-infection.



**A. SIV316** shows a pattern of infection relatively independent of CD4; regardless of cell surface CD4 levels, infection efficiency depends only on increasing CCR5 levels, consistent with known characteristics of SIV316 entry.

**B. Bal** shows moderate levels of infection when CD4 and CCR5 are limiting; infection efficiency increases as both CD4 and CCR5 levels are increased.

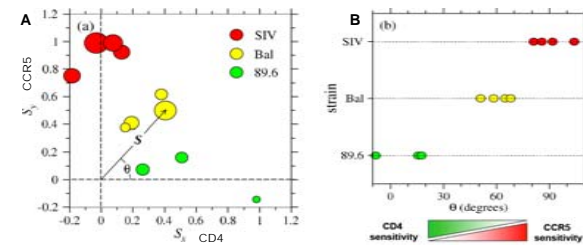
**C. 89.6** is a dual-tropic R5X4 virus. In contrast to SIV316, increasing CD4 expression can markedly increase infection efficiency to the same extent regardless of CCR5 expression levels. All infection was completely inhibited by TAK779, suggesting that 89.6 can efficiently use the low basal amounts of CCR5 present in our system.

The distinct patterns of CD4 and CCR5 usage exhibited by each viral isolate, and illustrated by the bar graphs in Fig. 4A-C, can be mathematically transformed into corresponding 3-D surface plots in Fig. 4E-F. The efficiency of infection as a function of CD4 and CCR5 levels is described by the topology of the surface curve, which can be expressed as a least squares fit polynomial function  $F(x,y) = ax + by + cx^2 + dy^2 + exy$  where  $x$  and  $y$  are mathematically rescaled quantities representing absolute CD4 and CCR5 cell surface concentrations (ABS/cell).

## RESULTS

**Figure 5. A vector-based angular metric can quantify the efficiency of CD4 and CCR5 usage.**

From the function  $F(x,y)$  that describes the surface curves in Figure 4, we can find the relative sensitivity of a viral isolate's infectivity to changes in CD4 and CCR5 levels by integrating the normalized gradient (of the surface curve) over the relevant ranges of  $x$  and  $y$ . From this, we can derive a sensitivity vector, the direction of the vector (measured by the angle,  $\theta$ , in the polar plot) that measures the relative sensitivity of virus infectivity to changes in CD4 ( $S_x$ -axis) versus CCR5 ( $S_y$ -axis) levels. The sensitivity vectors for the viruses examined are shown as a polar plot where the vector end-points are marked by each circle. Each circle is an independent experiment.



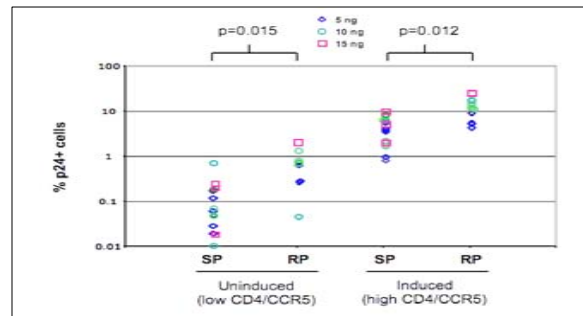
**A.** The sensitivity vectors for each isolate cluster. Thetas (angles) for SIV316 cluster near 90, whereas that for 89.6 cluster near zero degrees. Bal clusters at  $\sim 60$  degrees, consistent with dependency on both CD4 and CCR5.

**B.** Graphical comparison of the sensitivity vector angles for all viruses examined. The sensitivity angles are a quantitative measure of the overall efficiency of CD4 and CCR5 usage, and the viruses clearly cluster into distinct groups based on this metric ( $p < 0.00002$ , ANOVA for all pairwise comparisons).

• This sensitivity angular metric essentially describes the qualitative topology of the 3-D surface plots in Fig. 4, and provides a quantitative measure to compare the efficiency of CD4 and CCR5 usage between viral isolates.

**Figure 6. Use of dual inducible cell line reveals differential efficiency of CD4/CCR5 usage by perinatally infected from slow and rapid progressors.**

10 primary viral isolates obtained from 7 infants (3 rapid progressors (RP) and 4 slow progressors (SP)) with R5-tropic virus were used to infect the dual inducible cells.



• At background and maximal CD4/CCR5 induction, isolates from RP resulted in a higher percentage of infected cells than virus from SP. This difference was statistically significant over a range of viral inputs (cumulative infections at 5, 10 and 15 ng equivalents of p24). Note that the y-axis is on a log scale

## CONCLUSIONS

• We have generated a dual inducible HEK 293-based cell line that stably expresses CD4 and CCR5 upon induction with minocycline and ponasterone A, respectively. Induction of CD4 and CCR5 is within the physiologic range of cell surface molecules expressed on macrophages, dendritic cells and CD4+ T lymphocytes, allowing use of these cells to reflect *in vivo* receptor expression levels.

• Different laboratory isolates showed unique patterns of CD4 and CCR5 usage that were mathematically transformed into a quantitative measure of an isolate's relative sensitivity to CD4 or CCR5 levels for viral entry.

• Preliminary examination of primary isolates obtained from perinatally infected infants and children suggest that SP vs RP isolates may exhibit differential efficiency of CD4 and/or CCR5 usage during viral entry. We hope to use this system to further characterize and quantify CD4 and CCR5 usage of primary isolates and determine correlates with disease progression or viral pathogenicity in infants.

• This system may offer a standardized way of measuring and quantifying the inter-dependent efficiency of CD4 and CCR5 usage for any viral isolate. Such information may be clinically relevant for monitoring the quantitative changes that can occur with the use of entry inhibitors.