Quantitative Evaluation of HIV-1 Coreceptor Use in the GHOST(3) Cell Assay

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The utility of the GHOST(3) cell assay has been evaluated for testing coreceptor use of primary human immunodeficiency virus type 1 (HIV-1) isolates. GHOST(3) cells were derived from the human osteosarcoma cell line, HOS, and have been engineered to stably express CD4 and one or another of the chemokine receptors CCR3, CCR5, CXCR4, Bonzo, or the orphan receptor BOB. The indicator cell line carries the HIV-2 long terminal repeat-driven green fluorescence protein (GFP) gene, which becomes activated upon infection with HIV or simian immunodeficiency virus. Viral entry is followed by Tat activation of transcription and GFP becomes expressed. Infected cells can be detected 2 or 3 days after infection by simple fluorescence microscopic observation. This simplicity is the main advantage of the GHOST(3) cell system and makes it particularly suitable for screening of a large number of isolates. In addition, the efficiency of coreceptor use can be accurately quantitated with flow cytometric analysis. Here, we evaluated the coreceptor use of 59 primary HIV-1 isolates of different subtypes. © 2001 Elsevier Science

Key Words: GHOST(3); GFP; coreceptor; quantitative assay; HIV; SIV.

INTRODUCTION

Human and simian immunodeficiency viruses (HIV and SIV) enter target cells using CD4, the primary cell surface receptor, and a secondary receptor (coreceptor) from the G-protein-coupled seven-transmembrane chemokine receptor family (Chen et al., 1997; Deng et al., 1996, 1997; Dragic et al., 1996; Edinger et al., 1997, 1998a,b; Farzan et al., 1997; Feng et al., 1996; Hoffman et al., 1998; Marcon et al., 1997). Biological features of HIV-1 correlate with the coreceptor used. CCR5 using (R5) viruses are isolated during the early, asymptomatic phase of infection, while CXCR4 using (X4) or dual-tropic R5X4 viruses can often be detected at the late phase of infection concomitant with development of AIDS (Björndal et al., 1997; Scarlatti et al., 1997). Based on their phenotype in cultures of peripheral blood mononuclear cells (PBMC), R5 viruses have previously been classified as slow/low or non-syncytium-inducing (NSI), whereas X4 viruses were designated rapid/high or syncytium-inducing (SI) (Fenyö et al., 1988; Tersmette et al., 1988, 1989). Viruses from the latter group were able to infect established cell lines of T-lymphoid or monocyteoid origin. While a clear relationship between HIV-1 biological phenotype and severity of infection could be established, the pattern of SIV coreceptor use in relation to pathogenesis remained unclear (Chen et al., 1997; Edinger et al., 1997, 1999; Rudensey et al., 1998).

For determination of HIV and SIV coreceptor use, different kinds of indicator cell lines have been utilized. These cell lines contain reporter genes such as the gene for chloramphenicol acetyltransferase (CAT) (Merzouki et al., 1995), β-galactosidase (β-gal) (Kimpton and Emerman, 1992), luciferase (luc) (Schwartz et al., 1990), or alkaline phosphatase (Means et al., 1997), under the control of HIV-1 or HIV-2 long terminal repeat (LTR). Upon infection the viral Tat protein increases transcription from the HIV LTR promoter, leading to high-level expression of the reporter gene (Dorsky et al., 1996). Detection of the reporter gene products, however, requires processing of the cells by lysis, by fixation, or by addition of a substrate. Here we describe an indicator cell system in which the readout is a simple microscopic observation, without cumbersome processing of cells. The GHOST cells (derived from the human osteosarcoma cell line, HOS) contain the gene of the green fluorescence protein (GFP) driven by the HIV-2 LTR LTR. The cells have been engineered to stably express CD4, the primary receptor used by HIV and SIV, and one of several coreceptors (Cecilia et al., 1998; Mörner et al., 1999; KewalRamani, unpublished data). Upon infection, viral entry is followed by Tat activation of transcription and GFP becomes expressed. Since these are early steps in the virus replication cycle, infected cells can be detected already 2 or 3 days after infection.
infection with the help of a fluorescence microscope. This simplicity is the main advantage of the GHOST cell system. In the present work, we used the GHOST cell system and obtained quantitative data by instrumental measurements using a flow cytometer (FACS). By measuring fluorescence intensity and the proportion of infected cells, the efficiency of viruses in using a certain type of coreceptor could be determined. In addition, productive infection was evaluated by measuring the HIV-1 p24 antigen content of culture supernatants by enzyme-linked immunosorbent assays (ELISA).

RESULTS

Evaluation of data obtained by flow cytometry

GHOST(3) cell lines expressing CD4 and one of each coreceptor CCR3, CCR5, CXCR4, BOB, or Bonzo were infected with 59 different HIV-1 isolates (Table 1). Infected cells were analyzed on a flow cytometer 3 days after infection. In order to find a quantitative way of evaluating the efficiency of infection we took into consideration both the percentage of fluorescence-positive cells (%) and the mean fluorescence intensity (FI) obtained for each virus–coreceptor combination. These two parameters were multiplied for each sample and the fold difference from uninfected control cells was calculated as

$$\text{RTCN} = \frac{\% \times \text{FI}_{\text{virus}}}{\% \times \text{FI}_{\text{control}}}$$

where RTCN (ratio to cell negative) gives a quantitative measure of the efficiency of infection. Controls were mock-infected cultures from the corresponding coreceptor-expressing cell lines. The FI cut-off level was set to 0.1% (0.08–0.13%) fluorescence-positive cells for the uninfected cultures. Thus the mean (% × FI/FI_{control}) values for the negative controls ranged from 0.14, for the parental and BOB- and Bonzo-expressing cell lines, to 0.17, for the CCR5-expressing cell line. The standard deviation did not exceed 0.04 for any of the cell lines (data based on 13 independent experiments). The close similarity of these values in the different negative control cultures allowed us to compare the efficiencies with which the different receptors are used by viruses.

We then made the assumption that RTCN values above 10 are positive, those below 5 are negative, and those between 5 and 10 are indeterminate. To see whether this assumption holds, we examined the relationship of the calculated RTCN values to the percentage fluorescence-positive cells and the fluorescence intensity (fold increase above the negative control) for each data point obtained on GHOST(3).CCR5 cells (Fig. 1). The data points enclosed in the area formed by 0.5% positive cells and FI/FI_{control} included the mock-infected cultures and viruses that were negative on U87.CD4–CCR5 cells. RTCN values for this on the GHOST(3).CCR5 cells were less than 5. The indeterminate data points (5 <

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* Tested on U87.CD4–CCR1, -CCR2b, -CCR3, -CCR5, and -CXCR4 cells (Tscherning-Casper et al., 2000a).

* Tested on GHOST(3) cells expressing CD4 and one of the CCR3, CCR5, CXCR4, BOB, or Bonzo receptors.

* A/J, A subtype by env and J by the protease sequences (Tscherning-Casper et al., 2000b). U/F, env subtype unclassified both by HMA and sequencing; gag subtype F.
RTCN < 10) within or immediately outside this area either were classified as X4 viruses on U87.CD4 cells (points C, E, F, G in Fig. 1) (Björndal et al., 1997; Tscherning-Casper et al., 2000a) or had R5 phenotype (points A, B, and D) but were used in very small amounts (\( \leq 5 \times 10^{-5} \), infectious dose-50, on PBMC) in the present experiments involving GHOST(3) cells. The viruses depicted in data points H and I had low but positive RTCN values (10.9 and 11.4, respectively). One of these viruses had the R5 phenotype (data point I) but was tested in small amounts (ID50 = 5); the other was of X4 phenotype (data point H) and infected GHOST(3).CCR5 cells due to the endogenous CXCR4 expression in these cells (see Discussion).

All other data points in Fig. 1 indicate that RTCN values represent an adequate measurement of the efficiency of coreceptor use and, in the following, will be applied to characterize the efficiency of virus–coreceptor interactions.

**Comparison of flow cytometry with microscopic observation and HIV-1 p24 antigen production**

Infected GHOST cells were observed in a fluorescence microscope 3 days after infection. The same day FACS analysis was carried out and RTCN values were calculated. Viral antigen production was determined in culture supernatants by ELISA 6 days after infection. Thus, for each culture, data were obtained by three different methods.

Results obtained by microscopic observation and flow cytometric analysis showed strict correlation on both CCR5- and CXCR4-expressing cells (Figs. 2A and 2B). Microscopic observation is thus sufficient and can be used alone for screening of a large amount of material.

For the comparison of RTCN values and HIV-1 p24 antigen production, viruses were divided into several groups: R5, X4 or R5X4, and Cuban isolates. This was necessary because of the known endogenous expression of CXCR4 on the GHOST(3) cells. The data obtained by the different methods were validated within each group.

In general, infection of the GHOST(3).CCR5 cells with R5 viruses showed that once RTCN was positive, p24 antigen could also be detected in culture supernatants (Fig. 3A). However, large quantitative variations in antigen production were present. In fact, in two cases (isolate 4164C and 1986B from Cameroon) culture supernatants remained antigen negative. Discordant RTCN and antigen values are explained by extensive cell death at the time of sampling for antigen production (day 6 postinfection). In contrast, CXCR4-expressing cells infected with R6 viruses remained negative by both parameters (Fig. 3B). Conversely, infection with X4 viruses clearly

**FIG. 1.** Correlation between the proportion of fluorescence-positive cells and the increase of fluorescence intensity above the negative control (FI/FIneg). Individual points represent RTCN values: • RTCN > 100; ■ 10 < RTCN < 100; △ 5 < RTCN < 10; ○ RTCN < 5. All tests done on the GHOST(3).CCR5 cells are included in this figure. The inset is a blow-up of the critical part of the diagram.
induced GFP and yielded antigen-producing GHOST(3).CXCR4 cultures. However, three of four X4 viruses gave low but definitely positive RTCN on GHOST(3) cells engineered to express CCR5. These cultures nevertheless remained antigen production negative. As expected, infections with R5X4 dual-tropic viruses led to strong fluorescence induction and high levels of antigen production in both cell types.

Only one Cuban HIV-1 isolate was classified as R5X4 by using the U87.CD4 cell system (Table 1). It was therefore surprising that these viruses induced GFP in CCR5-as well as CXCR4-expressing GHOST(3) cells. However, antigen production was highly variable in CCR5-expressing cells. In fact, the only virus that yielded large amounts of p24 antigen was the one classified as R5X4 dual-tropic on U87.CD4 cells. These results suggested that the Cuban viruses either used the endogenously expressed CXCR4 very efficiently or used yet another undefined receptor present on the GHOST(3) cells. To distinguish between these two possibilities, inhibition experiments were carried out with the specific CXCR4 antagonist AMD3100.

Inhibition of CXCR4-using viruses with the CXCR4 antagonist AMD3100

To be able to dissect the contribution of CXCR4 use on the evaluation of receptor use of CXCR4-using viruses, infection of GHOST(3) cells was performed in the presence or absence of the specific CXCR4 antagonist AMD3100 (Table 2). In all cases, whether the viruses were multi-, dual-, or mono-tropic (R3R5X4, R5X4 or X4, respectively), infection of the parental cells could be inhibited. Similarly, infection of CCR3-expressing cells could also be inhibited, except with the Cuban 95Cu132 virus and the Cameroonian 001A and 001E isolates known to use CCR3. As expected, infection of CCR5-expressing cells by R5X4 viruses could not be inhibited, whereas CXCR4 use was inhibited in all cases. This allows us to conclude that the receptor used by HIV-1 to enter the parental cells (or CCR3- or CCR5-expressing cells by viruses not using these receptors) is solely CXCR4. The results in the GHOST(3) cell system agree with those obtained on U87.CD4 cells (Table 1).

The influence of endogenous CXCR4 expression on the detection of coreceptor use

First, we examined infection of the parental GHOST(3) cells (Fig. 4A). None of the R5 viruses gave a positive RTCN value on the parental cells (mean RTCN is 1.6), whereas in all other groups, consisting of CXCR4-using viruses, at least some isolates induced GFP in the parental cells. Notably, dual- or multi-tropic R5X4 or R3R5X4 Cameroonian isolates and Cuban viruses were the most efficient in this respect. These cultures, however, produced much less viral antigen than the corresponding GHOST(3).CXCR4 cultures (data not shown). Similar results were obtained on CXCR4- or CCR3-expressing cells, in that R5 viruses were negative (mean RTCN is 0.6 and 1.3, respectively). However, 2 of 30 isolates in this group induced GFP in BOB-expressing cells, although cultures remained antigen production negative, while both GFP induction and antigen production characterized infection of the GHOST(3).CCR5 cells when infected with R5 viruses.

Such a clear distinction could not be found with CXCR4-using viruses. As expected, all of them efficiently infected CXCR4-expressing cells (Fig. 4D), but could also induce GFP to a variable extent in other GHOST(3) sublines. In this respect, X4 viruses appeared to be the most specific, whereas the dual-tropic R5X4 or R3X4 and the multi-tropic R3R5X4 HIV-1 isolates gave positive results on the GHOST(3) cells expressing CCR3 or BOB. The RTCN values obtained with these viruses on GHOST(3).CCR3 cells were as high as with virus 25.
FIG. 3. Comparison of flow cytometric measurement and viral antigen production. GHOST(3) cells expressing CCR5 (A) or CXCR4 (B) coreceptor were infected with HIV-1 isolates. Antigen production represented as OD_{490} values of the infected culture supernatants and GFP induction expressed as RTCN are shown. Viruses were tested one to three times, the data of one representative experiment are shown here. The R5 3344 and the multitropic 25 control viruses were included in each experiment along with an uninfected culture. Results obtained with these control viruses are shown as the average of all experiments.
which is known to use CCR3 (Björndal et al., 1997). However, productive infection was more pronounced in those cases when CCR3 usage could be demonstrated on U87.CD4–CCR3 cells.

### The effect of virus dose

Parental cells and CXCR4- or CCR5-expressing cells were repeatedly infected with the multitropic 25 virus. A higher dose of infectious virus (measured as ID50 on PBMC) gave higher RTCN values in all three cultures. With a lower virus dose the parental cells became negative.

To further examine the dilution effect we carried out experiments with serial virus dilutions (Fig. 5). RTCN values strictly correlated with the amount of infecting virus, indicating that GFP induction measures viral entry. Correlation was also seen between infectious virus dose and the amount of HIV-1 p24 antigen production 6 days after infection. With all three viruses and all coreceptors tested (CCR5, CXCR4, and Bonzo) 50 ID50 (measured on PBMC) appears to be sufficient for quantitative evaluation of coreceptor use in the GHOST(3) cell system. If infection is performed with a lower virus dose, the most efficiently used coreceptor(s) can still be identified but additional coreceptor use may be overlooked.

### Expression of CD4 and coreceptors on the GHOST(3) cells

An interesting observation was that RTCN values on the parental cells (or on CCR3- or CCR5-expressing cells when infected by viruses not using these receptors) were in all cases lower than on the CXCR4- (or CCR5-) transfected cells. This indicated more efficient infection of the cells engineered to express CXCR4 compared to cells expressing "background" levels of CXCR4. This prompted us to analyze receptor expression in the different GHOST(3) sublines.

The GHOST(3).CCR5 and -CCR3 cell lines were double-stained for CD4 and CCR5 or CCR3, respectively, and all GHOST(3) cell lines were double-stained for CXCR4 and CD4. As judged from the parallel dislocation of cell populations after staining with the specific monoclonal antibodies, CD4 and CXCR4 expression was present in all cells of each GHOST(3) subline (Fig. 6). The dislocation of cells stained for CD4 was more pronounced (fold increase of median values was 11.87–17.01) than those stained for CXCR4 (fold increase of median values was 1.73–2.87), suggesting higher levels of CD4 expression than of CXCR4 expression. The levels of CCR5 and CCR3 expression on the CCR5- and CCR3-transfected cells, respectively, were comparable to those of CXCR4 expression (fold increase of median values in populations stained with the specific monoclonal antibodies was 2.29 and 1.83, respectively). It is surprising that the GHOST(3) cell sublines, whether transfected with CXCR4 or not, express comparable levels of CXCR4 when visualized by staining with monoclonal antibody, yet the efficiency of infection by CXCR4-using viruses is lower when the endogenously expressed CXCR4 receptor is utilized for entry. If a part of the receptors detected by the antibody is not functional as coreceptors for HIV-1, such a result would be expected. It is therefore mandatory to use standard virus controls in each experiment, these will give a better estimate of coreceptor function.

### DISCUSSION

In the present work we describe a simple and sensitive assay for HIV-1 coreceptor use that gives quantitative results in 3 days. We used the GHOST(3) indicator cells, which express CD4 and different coreceptors for HIV and SIV and carry the gene for GFP driven by the HIV-2 LTR. GFP becomes activated upon infection and the read-out is optimally performed 3 days postinfection. Qualitative data can be obtained by simple microscopic observation. With the help of flow cytometric analysis the efficiency of coreceptor use can be quantitated. Here we show that by taking into consideration both the percentage of fluorescent cells and their fluorescence intensity and calculating the fold difference from the uninfected control, each virus–receptor combination can be quantitatively determined. With the use of 59 HIV-1 isolates and 6 sublines of the GHOST(3) cells (the parental cells and 5 cell lines transfected with different coreceptors) the assay has been validated. The same validation applies to SIV and the accompanying paper (Vödrös et al., 2001) describes the coreceptor use of 20 sequentially obtained SIVsm isolates.
R5 monotropic HIV-1 isolates were characteristically infecting CCR5-expressing cells only. CXCR4-using viruses could infect all GHOST(3) sublines although GFP induction was less efficient and was in most cases not followed by productive infection unless CXCR4-transfected cells were used. We show that these viruses enter through CXCR4 generally expressed on GHOST(3) cells, since infection could be inhibited with the specific CXCR4 antagonist, AMD3100. Whenever these viruses used CCR5 or CCR3 in addition to CXCR4, entry into the CCR5- or CCR3-expressing cells was not inhibited by AMD3100. The level of GFP induction in CCR5-expressing cells by the dual-tropic viruses was high and could easily be distinguished from that of the parental cells. However, in the case of the less efficiently used CCR3 receptor, CXCR4 use may overshadow CCR3 use. To verify CCR3 usage, the CXCR4 receptor must be blocked on the same cells. Alternatively, CXCR4-using viruses can be tested on U87.CD4 cells that lack endogenous CXCR4 expression.

We studied the effect of virus dose on the efficiency of infection and found that infection with a higher virus dose gives higher RTCN. Fifty ID_{50} was sufficient to reliably ensure detection of coreceptor usage patterns. Indeterminate or borderline results were obtained when <5 ID_{50} was tested or when CXCR4-using viruses used the endogenous CXCR4 receptor to enter cells. In the latter case, dilution of viruses decreased the effect of endogenously expressed CXCR4 receptor. This dependence on infectious virus dose demonstrates that GFP induction in the GHOST(3) cells measures viral entry. Thus the method is useful for quantifying HIV-1 and also SIV co-receptor use, as described in the accompanying paper (Vódros et al., 2001).

Our attempts to quantitate receptor expression on the cell surface showed that both CD4 and the coreceptors...
were expressed on all cells of a given population. Addition of the specific monoclonal antibodies resulted in a shift of the entire cell population. We expressed the magnitude of the shift as the difference in median values of stained/unstained populations, as suggested by Lee et al. (1999). In this way we found a large difference.

FIG. 5. Effect of virus dilution on the infection of the GHOST(3) cells. The CCR5- and Bonzo-using 2236B and R3R5X4 multitropic 001E and 001A isolates were tested in different dilutions on the GHOST(3) parental cell line (par) and cells expressing CCR5, CXCR4, or Bonzo, as indicated. Calculated RTCN values are shown as columns while antigen production of the infected cultures is represented by the lines. ID50 values of the infectious virus, titrated on PBMC, are also shown.

FIG. 6. Level of receptor expression on the GHOST(3) cells. The GHOST(3) cell lines were stained for CD4 and CXCR4. In addition, GHOST(3).CCR5 and -CCR3 cells were stained for CCR5 and CCR3, respectively, and the cell populations were analyzed by flow cytometry. Continuous lines represent samples without specific antibody added, dotted lines show distribution of cells with specific antibody added. The numbers in each histogram indicate the fold difference in median values of stained cells compared to the negative control samples without specific antibody added.
between CD4 and coreceptor expression in general, in that CD4 showed a higher level of expression than any other coreceptors. Edinger et al. (1998a) made a similar observation when comparing CD4 and CCR5 expression on 293T and GHOST cells. In our experiments not only CCR5, but also CXCR4 and CCR3 showed similar low levels of expression when cells were tested with the different specific monoclonal antibodies. The most surprising finding was that CXCR4 expression, as detected by the antibody, appeared to differ very little between parental cells and CXCR4-transfected cells, while virus infection was more efficient on the CXCR4-transfected cells than on parental cells. This indicates that the antibody binding assay does not give information on receptor function and the most important controls in the assay are standardized virus controls.

MATERIALS AND METHODS

Viruses

Viruses of different origins and subtypes were used to calibrate the GHOST cell system. HIV-1 isolates from Cameroonian pregnant women, mainly subtype A, were obtained within the framework of a European Network for “In Utero Transmission of HIV” (Tscherning-Casper et al., 2000a). HIV-1 subtype B isolates from Cuba and the TZ98010 and IN97003 isolates from Tanzania and India, respectively, were obtained within the framework of the UNAIDS Network for HIV Characterization. HIV-1 strain 25, known to use all the coreceptors tested, was included in all experiments as “positive control” virus (Björndal et al., 1997). Virus stocks were produced in human PBMC and cell-free supernatants were used to infect GHOST(3) cells. The characteristics of the viruses included in this study are shown in detail in Table 1.

Cell lines

The human osteosarcoma cell line, GHOST(3), was engineered to stably express CD4 and one or another of the chemokine receptors CCR3, CCR5, CXCR4, Bonzo, or the orphan receptor BOB (Cecilia et al., 1998; Mattloubian et al., 2000; Mörner et al., 1999; KewalRamani, unpublished data). The parental cell line has been engineered to express CD4 but none of the coreceptors. The cells were stably transfected with the GFP gene driven by the HIV-2rod LTR. In the case of infection when the virus enters the cells by using CD4 and the appropriate coreceptor, the viral Tat protein becomes expressed and transactivates the GFP gene by the LTR. GFP expression in infected cells is easily detected in a fluorescence microscope and by flow cytometry. The GHOST(3) cell lines were maintained in Dulbecco’s modified Eagle’s medium (Life Technologies, Paisley, Scotland) containing 7.5% fetal calf serum (FCS, Life Technologies, Karlsruhe, Germany) and antibiotics and incubated in a humidified atmosphere with 5% CO2 at 37°C. Cultures were split twice a week by use of 5 mM EDTA.

Infection of the GHOST cells

One day before infection 24-well plates were prepared with 2–3 × 10⁴ cells/well in 1 ml medium. Before infection, medium was replaced with 200 μl fresh medium and virus was added to duplicate wells in a volume of 300 μl/well. In parallel experiments, using PBMC, we determined the ID₅₀ for each virus (Weber et al., 1996). Two hours after infection, medium was added to 1 ml/well. After an overnight incubation, cells were washed with PBS (Life Technologies, Paisley, Scotland), 1 ml medium was added to each well, and the plates were further incubated. Three days after infection cultures were observed in a fluorescence microscope and cells from one of the parallel wells were prepared for flow cytometry by adding paraformaldehyde to a final concentration of 2% for at least 2 h. Remaining wells were split 1:5–1:10 and cultures were maintained for another 3 days for viral antigen detection by ELISA.

In one series of experiments the specific CXCR4 antagonist AMD3100 was used (Schols et al., 1997). AMD3100 was added to the cells prior to infection in 200 μl medium at a concentration of 1 μg/ml and virus was added 5 min later. Parallel wells without inhibitor were included in each experiment and treated in the same way.

Flow cytometric analysis

We selected the GHOST cell population on the side scatter–forward scatter diagram and measured the fluorescence intensity of 1–1.5 × 10⁴ cells (FACScan, Becton Dickinson, San Jose, CA). The percentage of fluorescence-positive cells and their fluorescence intensity were determined and further used to calculate a characteristic number to evaluate the efficiency of infection as detailed under Results.

Viral antigen detection

To detect the antigen produced by the infected cells, an in-house HIV-1 p24 antigen ELISA was used. The method has been described previously (Sundqvist et al., 1989). In brief, 100-μl aliquots of cell-free supernatants containing 0.5% Triton 100 were added to 96-well microtiter plates previously coated with rabbit anti-Gag sera. Antigen was allowed to bind during an overnight incubation at 4°C. Plates were then washed and a secondary antibody (anti-HIV-1 p24 mouse monoclonal IgG) conjugated with horseradish peroxidase was added. Following incubation for 2 h at 37°C, the plates were washed and the substrate, o-phenylenediamine-dichloride activated with H₂O₂, was added. The reaction was stopped with 2.5 M H₂SO₄ and the optical density of the product was measured spectrophotometrically at 490 nm (OD₄₉₀).
**ID₉₀ titration**

Viruses were titrated on PBMC in an ID₉₀ assay as previously described (Weber et al., 1996). Briefly, six fivefold dilutions were made, starting from 1:5. Seventy-five microliters of each virus dilution was added to five parallel wells in 96-well round-bottom microtiter plates (Nunc, Roskilde, Denmark). Thereafter 1 × 10⁵ phytohemagglutinin (Sigma) stimulated PBMC from two healthy donors in a 150-μl volume were added to each well and the plates were incubated for 6 days. At days 1 and 3 the plates were washed by centrifugation and change of 200 μl medium. At day 6, supernatants from each well were analyzed for the presence of viral antigen by ELISA. The ID₉₀ was defined as the reciprocal of the virus dilution resulting in 50% positive wells (Reed-Muench calculation).

**Receptor staining**

For staining, 1 × 10⁶ GHOST(3) cells were washed with staining buffer (SB: PBS, containing 2% FCS) and antibodies were added. For CXCXR labeling, 20 μl undiluted antibody (12G5; Endres et al., 1996) was added to the samples. For CCR5 and CCR3 labeling, the antibodies 2D7 and 7B11 (Heath et al., 2001) were added. For CXCR4 labeling, 20 μl of staining buffer was added. Double-staining for CD4 was performed at this step. For this we used 10 μl of the CD4/RPE-Cy5-conjugated antibody (Dako; Catalog No. C7069) according to the manufacturer's recommendation. Following incubation on ice for 30 min in the dark, the cells were washed twice with SB and fixed by addition of 300 μl of 4% paraformaldehyde. Analysis was then performed on a FACScan using CellQuest software.

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