INTRODUCTION

Acute human immunodeficiency virus type 1 (HIV-1) infection is occasionally symptomatic. Hepatopathy, associated with hepatomegaly or serum liver test abnormalities, has been reported in different studies as one of these uncommon symptoms (Kobessho et al., 2002; Molina et al., 1992; Niu et al., 1993). Moreover, since the widespread use of the highly active antiretroviral therapy (HAART), the incidence of liver disease during the chronic phase has increased dramatically to become a leading cause of death among HIV-1-infected individuals. It can be proposed that the ability of HIV-1 to infect hepatocytes could influence liver diseases. Although the presence of HIV-1 was identified in hepatocytes from HIV-1-seropositive patients, the susceptibility of hepatocytes to HIV-1 infection in vitro remains controversial. We present evidence here that human hepatoma cells are not productively infected with CD4-dependent HIV-1 strains because of inefficient fusion related to an absence of cell surface CD4 and CXCR4. However, these cells display an increased susceptibility to infection with a CD4-independent viral isolate through an interaction with galactosyl ceramide, an alternate receptor for HIV-1. This study provides further understanding of the susceptibility of human hepatocytes to HIV-1 infection. However, in vivo investigations are recommended to consolidate these data.

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Inefficient fusion due to a lack of attachment receptor/co-receptor restricts productive human immunodeficiency virus type 1 infection in human hepatoma Huh7.5 cells

Rémi Fromentin, Mélanie R. Tardif and Michel J. Tremblay

Centre de Recherche en Infectiologie, Centre Hospitalier Universitaire de Québec-CHUL, and Département de Microbiologie-Infectiologie et Immunologie, Faculté de Médecine, Université Laval, Québec, QC G1V 4G2, Canada

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In this study, we evaluated the susceptibility to HIV-1 infection of various human hepatoma cell lines including Huh7.5 because the latter is also a target cell line for HCV (Blight et al., 2002; Lindenbach et al., 2005). After finding a weak susceptibility of these human hepatoma cells to HIV-1 infection, which is not sufficient to lead to productive infection, we tried to analyze the mechanism(s) limiting infection and evaluated whether some HIV-1 strains could bypass these restrictions.

RESULTS

Human hepatoma cells are weakly susceptible to HIV-1 infection

A very faint virus gene expression was detected following infection of Huh7.5 cells with both X4- and R5-using single-cycle reporter virus preparations, which was abolished in the presence of the non-nucleoside reverse transcriptase inhibitor efavirenz (EFV) (Fig. 1). Similar observations were made when using two additional hepatoma cell lines, namely HepG2 and Huh7 (data not shown). Infection of the human T-cell line PM1 with similar amounts of reporter virus resulted in luciferase activity counts that were at least two to three orders of magnitude higher than those observed with Huh7.5 cells (data not shown). It can thus be concluded that human hepatoma cells display a limited sensitivity to HIV-1 infection.

A block in the early step(s) of the HIV-1 life cycle is responsible for the restricted virus infection in human hepatoma cells.

We next examined which stage(s) of the virus life cycle is restricted in this particular cell type. We initially studied the early events through the use of vesicular stomatitis virus G (VSV-G) pseudotyped virions because this strategy allows bypassing the natural mode of HIV-1 entry into target cells. As illustrated in Fig. 2(a), exposure of Huh7.5 cells to VSV-G pseudotypes yielded a much higher luciferase activity than when using reporter viruses bearing HIV-1 Env (i.e. at least 3 logs higher). Luciferase counts induced upon infection with HIV-1-based VSV-G pseudotypes was inhibited by EFV, therefore confirming that reporter gene activity truly reflects virus infection. Altogether these results suggest that intermediate and late stages of virus replication (i.e. reverse transcription, integration and gene expression) are occurring normally in Huh7.5 cells, whereas the limiting step(s) seems to take place at the level of virus attachment/entry.

To more precisely pinpoint the block to virus replication in Huh7.5 cells, we quantified integration events by using a previously described nested Alu PCR test (Suzuki et al., 2003). As shown in Fig. 2(b), a very efficient integration of VSV-G pseudotyped reporter virions within the host chromosome is occurring in Huh7.5 cells. The potent inhibition of integration observed after pre-treatment with EFV and the integrase inhibitor 118-D-24 (99.9 and 92.0 ± 1.6 %, respectively, inhibition calculated with data from two independent experiments) indicates that both reverse transcriptase and integrase processes are normally taking place in this cell type.

Next, we investigated whether the integrated proviral DNA can give rise to transcription and translation of functional structural viral proteins (e.g. CA, capsid). As depicted in Fig. 2(c) (left panel), cell-associated CA was detected up to 12 days post-infection. The EFV-mediated suppression of intracellular CA confirms that it is due to de novo protein synthesis, and not to the initial virus input. Not surprisingly, the kinetics of cell-associated CA synthesis exactly follows the reporter gene expression seen in Fig. 2(a). Knowing that the structural viral protein CA could be synthesized, we next verified that virions were able to egress from such virus-infected human hepatoma cells. As shown in Fig. 2(c) (right panel), CA is efficiently released into the supernatant following the kinetics previously observed for virus-driven reporter gene expression and intracellular CA protein synthesis (Fig. 2a, c).
The next step was to verify whether extracellular CA is indicative of fully competent HIV-1 particles. This goal was achieved by using the TZM-bl indicator cell line. Results displayed in Fig. 2(d) indicate that infection of Huh7.5 cells with both fully infectious NL4-3 (left panel) and NL4-3Balenv virions pseudotyped with VSV-G (right panel) leads to the production of infectious viral particles. However, no infectious progeny virus could be detected following infection of Huh7.5 cells with non-pseudotyped NL4-3 and NL4-3Balenv viruses. Thus, it can be concluded that a block at an early step(s) in the virus life cycle is responsible for the weak susceptibility of human hepatoma cells to infection with fully competent NL4-3 and NL4-3Balenv virions that are not pseudotyped with VSV-G Env.

**An inefficient fusion process mediated by HIV-1 Env glycoproteins leads to virus degradation**

We next focused our attention on early processes associated with viral infection, i.e. binding and internalization. We have already demonstrated that HIV-1 can efficiently bind to Huh7.5 cells, a phenomenon favouring...

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Fig. 2. An early restriction block is seen in Huh7.5 hepatoma cells. (a) Huh7.5 cells were first either left untreated or treated with EFV for 30 min before infection with VSV-G pseudotyped reporter viruses (NL4-3L E'R'/VSV-G). After 16 h, cells were washed extensively with PBS, trypsinized and left in culture. Every 4 days, cells were split and grown in new medium. The passage of cells allowed measuring luciferase activity in cell lysates. (b) Huh7.5 cells were first either left untreated or treated for 30 min with EFV or 118-D-24 (50 μM) and next incubated with VSV-G pseudotyped virions. After 16 h, cells were washed extensively with PBS and left in culture for an additional 8 h before extracting total DNA, which was used to detect and quantify integrated viral DNA by real-time PCR. The number of HIV-1 copies was determined by a standard curve prepared with the NL4-3 vector. (c) The same experimental procedure was performed as in (a), except that virus replication was monitored by quantifying either intracellular CA in cell lysates (left panel) or the CA content in cell-free supernatants (right panel). (d) Huh7.5 cells were first either left untreated or treated with EFV for 30 min before infection with fully competent NL4-3 (left panel) and NL4-3-Balenv (R5) (right panel) viruses pseudotyped with pan-tropic VSV-G Env. After 16 h, cells were washed extensively with PBS, trypsinized and left in culture. Every 4 days, cells were split and grown in new medium. The passage of cells allowed harvesting cell-free supernatants used to assess infectivity of progeny virus using the TZM-bl indicator cell line as described in Methods. Data shown are the means ± SEM of data from quadruplicate samples of two independent experiments.
an efficient viral transfer to permissive CD4+ T-cells (Fromentin et al., 2010). Therefore, HIV-1 attachment is not the viral cycle step limiting HIV-1 infection in hepatocytes. We also established that human hepatoma cells can internalize non-pseudotyped HIV-1 particles (i.e. NL4-3 and NL4-3Balenv) at a level comparable to that of VSV-G pseudotyped viruses (data not shown and Fromentin et al., 2010).

Noting efficient internalization of HIV-1 particles despite inefficient infection of Huh7.5 cells, we wanted to understand the behaviour of internalized viral particles. To determine whether the internalized virions are eventually degraded, Huh7.5 cells were inoculated with similar amounts of viruses (standardized in terms of p24) before estimating the cell-associated p24 content. As shown in Fig. 3(a), wild-type NL4-3 and NL4-3Balenv virions are rapidly degraded within Huh7.5 cells. Interestingly, VSV-G pseudotyping seems to allow virus escape from this degradation pathway.

To define whether the strong proportion of degraded virions could result from an ineffective fusion of viral and cellular membranes, we performed a fusion test as described previously (Cavrois et al., 2002). Data shown in Fig. 3(b) indicate that virions bearing X4 and R5 HIV-1 Env are unable to enter efficiently into Huh7.5 cells due to a block at the level of virus-mediated membrane fusion, which is in sharp contrast to the situation seen with VSV-G pseudotypes.

Inefficient fusion is due to a lack of cell surface receptors

Susceptibility to HIV-1 infection is firstly dictated by the expression on target cells of the primary cellular receptor CD4 and coreceptor (i.e. primarily CXCR4 or CCR5), which are considered as the main surface proteins allowing membrane fusion. While it is unlikely that CD4 is expressed in hepatocytes because this molecule is found primarily on immune cells, some authors have reported controversial results about the expression of the co-receptors on human hepatoma cell lines (Banerjee et al., 1992; Cao et al., 1990; Iser et al., 2010; Vlahakis et al., 2003). To assess whether the weak HIV-1 susceptibility displayed by Huh7.5 cells could be explained by the absence of CD4 and/or co-receptor on the plasma membrane, flow cytometry analysis was performed. Neither CD4, CXCR4 nor CCR5 expression was detected on Huh7.5 cells, unlike on TZM-bl cells that were used as a positive control (data not shown). Nevertheless, the co-receptors CXCR4 and CCR5 could be expressed at levels too low to be detected by our protocol. In fact, recently, Lin and co-workers reported the presence of CXCR4 and CCR5 in a human hepatoma cell line derived from Huh7.5. In this study, they demonstrated that engagement of CXCR4 or CCR5 by HIV-1 particles increased HCV replication (Lin et al., 2008).

We thus hypothesized that the lack of CD4 is a major restriction to HIV-1 infection of hepatocytes. To solve this issue, Huh7.5 cells were transiently transfected with an expression vector encoding human CD4. Next, CD4-expressing Huh7.5 cells were exposed to X4- and R5-tropic pseudotyped reporter viruses. Human hepatoma Huh7.5 cells expressing human CD4 were found to be much more susceptible to infection with R5-using than the CD4-negative parental cells (Fig. 4). Virus infection with recombinant R5 virions was inhibited by a fusion inhibitor (i.e. T-20) or an antagonist of CCR5 (i.e. TAK-779), thus suggesting that virus entry relies on endogenous CCR5. However, infection with X4-tropic reporter HIV-1 viruses was not enhanced in Huh7.5 cells transiently expressing human CD4 (data not shown). As expected, CD4 expression on Huh7.5 cells does not modify infection with VSV-G pseudotyped viruses (data not shown). Thereby, we have shown that the lack of CD4 constitutes the main

![Fig. 3. Fusion step is inefficient in Huh7.5 cells.](image-url)
cells were inoculated with JR-FL pseudotyped viruses. After

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Soluble CD4 (sCD4), AMD-3100 (CXCR4 antagonist) and

Importantly, infection of human hepatoma cells with NDK pseudotypes was reduced when using a blocking anti-GalCer antibody (Fig. 5d), whereas infection with HXB2 pseudotypes was not affected (data not shown).

**Fig. 4.** Inefficient virus–cell membrane fusion is due to a lack of

**Glycolipid galactosyl ceramide (GalCer) is an alternative receptor for HIV-1 infection of Huh7.5 cells**

Although the CD4 glycoprotein is documented as the main cellular receptor for HIV-1, several CD4-negative cell lines are susceptible to infection with some viral isolates. One of them is the Zairian strain NDK reported to infect CD4-negative epithelial cells (Fantini et al., 1991). We therefore assessed the susceptibility of Huh7.5, HepG2 and Huh7 hepatoma cells to infection with luciferase-encoding virions pseudotyped with either NDK (CD4-independent) or HXB2 Env (CD4-dependent). As shown in Fig. 5(a), NDK pseudotypes are more potent at infecting the three human hepatoma cell lines tested compared with HXB2 pseudotyped virions. Nevertheless, we were unable to detect productive infection of human hepatoma cells with wild-type NDK viruses as monitored by incubating cell-free supernatants with the indicator cell line TZM-bl (data not shown). Moreover, the increased susceptibility of Huh7.5 cells to infection with NDK pseudotypes is not mediated by CD4, CXCR4 or CCR5 as revealed when using soluble CD4 (sCD4), AMD-3100 (CXCR4 antagonist) and TAK-779 (CCR5 antagonist) (Fig. 5b).

Altogether these results suggest the existence of alternate modes of viral entry mediated by the NDK envelope, involving one or more receptor molecules. One of them, GalCer was described previously as an HIV-1 alternate receptor on neural cell lines (Harouse et al., 1991) and also on human colon epithelial cells (Yahi et al., 1992). The propensity of the NDK isolate to infect CD4-negative epithelial cells through the GalCer cell surface constituent was confirmed in another study (Hammache et al., 1998). In order to determine if this CD4-independent infection process can ensue in Huh7.5 cells, we first assessed surface expression of GalCer by flow cytometry. As shown in Fig. 5(c), GalCer is highly expressed on such human hepatoma cells. Comparable surface expression levels of GalCer were measured on HepG2 and Huh7 cells (data not shown).

**DISCUSSION**

A number of studies have described liver failure during acute HIV-1 infection. However, most liver diseases associated with HIV-1 infection occur during the chronic phase, representing the most frequent non-AIDS-related cause of death among HIV-1-infected individuals. Although chronic viral infections contribute greatly to this outcome, the exact mechanisms involved in liver diseases during HIV-1 infection or HIV-1/HCV co-infection are still obscure. Knowing that HIV-1 is found in the liver and can potentially interact with hepatocytes, we monitored the susceptibility of these cells to HIV-1 infection in an attempt to discover a mechanism for HIV-1-associated liver injury and increase of HCV replication. In this study, we report that the Huh7.5 hepatoma cell line is weakly susceptible to HIV-1 infection.

The results described herein with different human hepatoma cell lines indicate that there are no major blocks to HIV-1 infection in hepatocytes that cannot be bypassed by VSV-G pseudotyping. We provide evidence that an inefficient fusion is the major restriction factor that limits HIV-1 growth in hepatocytes. Indeed, although HIV-1 enters massively in hepatocytes, in the absence of fusion this internalization rapidly leads to virus degradation.

Knowing that hepatocytes are highly active metabolically, we verified that internalized particles were recycling to the cell surface. Only 1% of internalized viruses recycle to the top of hepatocytes as monitored by assessing the extracellular p24 content after pulsing Huh7.5 cells with fully infectious HIV-1 (data not shown).

Our results describing a very weak in vitro infection of human hepatoma cells may appear at odds with those previously reported in other studies (Banerjee et al., 1992; Cao et al., 1990; Iser et al., 2010; Xiao et al., 2008). For example, Banerjee and colleagues reported the presence of...
CD4 on human hepatoma HepG2 cells, which is in sharp contrast to the present study and other published results. The explanation for this discrepancy is currently unknown but could relate to a distinct cellular clone. A CD4-independent HIV-1 productive infection of primary human hepatocytes and established cell lines has been demonstrated previously (Banerjee et al., 1992; Cao et al., 1990; Iser et al., 2010; Xiao et al., 2008). However, it must be stated that some crucial controls were missing from the study by Cao and co-workers, such as inhibition of infection by HIV-1-specific inhibitors allowing discrimination between de novo particles production and recycling. Moreover, the authors used up to 100 times more virus than we did. Such significant differences in infection protocols probably explain contrasting results. Moreover, as mentioned above, there is some inconsistency with regard to surface expression levels of CXCR4 and CCR5 detected on human hepatocytes that could account for the different susceptibility to productive HIV-1 infection.

Usually, HIV-1 attachment relies on interactions between viral Env glycoproteins and CD4 followed by conformational changes that trigger an association with appropriate chemokine co-receptors. This sequence of events is the major determinant responsible for HIV-1 tropism and pathology (Clapham & McKnight, 2002). However, in

![Image](https://example.com/image.png)

**Fig. 5.** Use of GalCer by the CD4-independent strain NDK. (a) Huh7.5 (left panel), HepG2 (middle panel) and Huh7 cells (right panel) were infected with similar amounts of reporter virions pseudotyped with NDK or HXB2 Env (m.o.i. of 0.01). After 16 h, cells were washed extensively with PBS and left in culture for 3 days before assessing luciferase activity in cell lysates. Results shown are the means ± SD of data from quadruplicate samples from three independent experiments (*P<0.05). (b) Huh7.5 cells were first either left untreated or treated for 30 min with the listed entry inhibitors (i.e. sCD4/40 μg ml⁻¹, AMD-3100/5 μM and TAK-779/10 μM) before infection with NDK pseudotypes. After 16 h, cells were washed extensively with PBS and left in culture for 3 days before assessing luciferase activity in cell lysates. Data shown are the means ± SEM of data from two independent experiments. (c) GalCer expression on Huh7.5 cells was assessed by flow cytometry as described in Methods (isotype-matched irrelevant control antibody, grey zone under curve area; GalCer, black line). (d) Huh7.5 (left panel), HepG2 (middle panel) and Huh7 cells (right panel) were first either left untreated or treated for 30 min with a blocking anti-GalCer antibody (10 μg) or an appropriate isotype-matched irrelevant control antibody before infection with NDK Env pseudotypes. After 16 h, cells were washed extensively with PBS and left in culture for 3 days before assessing luciferase activity in cell lysates. Data shown are the means ± SEM of data from two independent experiments (*P<0.05; **P<0.1).
CD4-negative cells (e.g. epithelial cells and endothelial cells) and in cells expressing minimal levels of the primary receptor (e.g. macrophages and dendritic cells), other types of interactions between the virion and the cell surface have been shown to dictate the attachment process (Curtis et al., 1992; Fantini et al., 1993; Harouse et al., 1991; Lambert et al., 2008; Larkin et al., 1989; Mondor et al., 1998; Pöhlmann et al., 2001). One of them involves plasma membrane glycosphingolipids such as GalCer and its sulphated derivative expressed on macrophages (Seddiki et al., 1994), neural cells (Harouse et al., 1991), colon epithelial cell lines (Fantini et al., 1993) and, as demonstrated in this study, human hepatoma cells (Fig. 5a). Those molecules, on CD4-negative cells, are involved in HIV-1 attachment and infection processes (Harouse et al., 1991; Moore et al., 2002; Yahi et al., 1992), but it is still unclear whether they are able to promote conformational changes of Env glycoproteins necessary to allow viral fusion. Our results suggest that intrinsic features of the HIV-1 Env are necessary to use GalCer as a receptor on the surface of hepatocytes since pseudotyping with NDK Env results in a superior virus gene expression in Huh7.5 cells. Recently, the CD4-independent clinical HIV-1 strain SDA-1 has been shown to infect with some efficiency primary human hepatocytes (Xiao et al., 2008). It would be interesting to know whether the SDA-1 Env uses the GalCer-mediated alternate pathway to infect primary human hepatocytes. Importantly, it has been reported that 8 of 23 primary isolates of HIV-1 can achieve infection through a CD4-independent and GalCer-dependent process (Harouse & González-Scarano, 1996), therefore suggesting that this infection pathway is frequent.

As mentioned above, transient CD4 expression increases susceptibility of Huh7.5 cells to HIV-1 infection. A new provocative concept has been proposed where cell-to-cell contact between a CD4-positive cells and co-receptor-bearing CD4-negative cells (i.e. primary human fetal astrocytes) can result in the transfer in trans of the primary cellular receptor (Speck et al., 1999). This process has been proposed as a possible means of HIV-1 infection of CD4-negative cells. Such a mechanism based on physical transfer of a piece of membrane bearing proteins during cell-to-cell contact is also called trogocytosis and is known to be a mechanism of intercellular communication (Joly & Hudrisier, 2003; Niu et al., 2009). Knowing that close direct interactions between CD4-expressing T-cells and hepatocytes can occur in the liver (Warren et al., 2006), it can be postulated that a transfer of plasma membrane fragments containing CD4 can enhance susceptibility of hepatocytes to HIV-1 infection. Moreover, the intercellular exchanges of intact membrane patches can also take place through another intercellular communication mechanism relying on membrane vesicles either directly shed from the surface membranes or secreted from the endosomal compartment of a donor cell (Ratajczak et al., 2006). This possibility is supported by the previous published transfer of CCR5 (Mack et al., 2000) and CXCR4 (Rozmyslowicz et al., 2003) by microvesicles leading to HIV-1 infection of cells without endogenous co-receptor expression. Therefore, it might be interesting to investigate whether microvesicles from permissive neighbouring hepatic cells (i.e. Kupffer cells, sinusoid endothelial cells and CD4\(^+\) T-cells) (Housset et al., 1990; Schmitt et al., 1990; Steffan et al., 1992) enhance hepatocyte susceptibility to productive HIV-1 infection.

To conclude, the data presented in this study provide further understanding of the susceptibility of human hepatocytes to HIV-1 infection. Herein, we demonstrate that human hepatoma cells are not permissive to productive infection with CD4-dependent isolates of HIV-1 due to an absence of cell surface CD4 and CXCR4. Moreover, our results also suggest that GalCer can be used by a CD4-independent variant to gain entry into hepatic cells. Nevertheless, all fully competent HIV-1 isolates studied could not achieve productive infection of the three tested human hepatoma cell lines. Studies with primary human hepatocytes and in vivo investigations are needed to consolidate these data.

**METHODS**

**Cells.** Human hepatocellular carcinoma cell lines Huh7.5, HepG2 and HepH7 were maintained as a monolayer in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% heat-inactivated FBS, streptomycin (100 \(\mu\)g ml\(^{-1}\)), penicillin G (100 U ml\(^{-1}\)) and 0.1 mM of non-essential amino acids (Invitrogen). Human embryonic kidney 293T cells (ATCC) and the TZM-bl indicator cell line (obtained through the AIDS Repository Reagent Program, Germantown, MA) (Derdeyn et al., 2000; Platt et al., 1998; Takeuchi et al., 2008; Wei et al., 2002) were cultured in complete DMEM. The human T-lymphoid cell line PM1, which is susceptible to infection with both CXCR4- and CCR5-tropic isolates of HIV-1, was obtained from the AIDS Repository Reagent Program (Lusso et al., 1995).

**Antibodies and reagents.** FITC-conjugated anti-CD4 mAb (clone RPA-T4) was purchased from eBioscience, PE-conjugated anti-CXCR4 mAb (clone 12G5) and PE-conjugated anti-CCR5 mAb (clone 2D7) from BD Biosciences, and the anti-GalCer mAb from Millipore. The following reagents were obtained through the AIDS Repository Reagent Program: EFV, a non-nucleoside reverse transcriptase inhibitor of HIV-1; 118-D-2A, an integrase inhibitor (Sarovkskaia et al., 2004); T-20, a fusion inhibitor; AMD-3100, an antagonist of CXCR4 (Bridger et al., 1995; De Clercq et al., 1994; Hendrix et al., 2000); and TAK-779, an antagonist of CCR5 (Baba et al., 1999).

**Plasmids.** pNL4-3 (X4-tropic) (Adachi et al., 1986), pNL4-3Balenv (R5-tropic) (Dornadula et al., 1999) and pNDR (Dumonceaux et al., 1998) are three full-length infectious molecular clones of HIV-1. The HIV-1 NDK isolate possesses a CD4-independent entry phenotype (Dumonceaux et al., 1998). The pNL4-3 Env vector (called NL4-3E\(^+\)) encodes a complete HIV-1 genome containing a 1 frameshift in the envelope (Env) precursor and was used to produce Env-deficient viruses (provided by D. E. Ott, National Cancer Institute, Frederick, MD, USA). The pNL4-3Luc Env (called pNL4-3L\(^{\text{Luc}}\) Env) vector encodes the complete HIV-1 genome in which the env gene has been replaced with the luciferase reporter gene (provided by N. R. Landau, The Salk Institute for Biological Studies, La Jolla, CA, USA).**
viruses. pCMV-BlaM-Vpr codes for a CA, USA) were used to produce single-cycle pseudotyped reporter and HCMV-G (codes for pan-tropic vesicular stomatitis virus-G Env) (50 nM) or 118-D-24 (50 μg) for 4 h at 37 °C and the intracellular cleavage of the CCF2/AM dye was assessed with a flow cytometer.

Flow cytometry. Subconfluent hepatoma cells were removed by 5 mM EDTA-PBS to perform flow cytometry analyses. Briefly, cells (1 × 10⁶) were incubated with 100 μM 10 % PBS-PBS containing a saturating amount of anti-CD4-FITC, anti-CXCR4-PE, anti-CCR5-PE, anti-GalCer, or an appropriate isotype-matched irrelevant control mAb (for non-specific staining) for 30 min on ice. Cells were next washed with PBS, and then incubated when appropriate with 100 μg of a saturating amount of R-PE-conjugated goat anti-mouse immunoglobulin G (Invitrogen) for 30 min on ice. After two washes with PBS, cells were fixed in 2 % paraformaldehyde and analysed on a cytofluorimeter (EPICS XL, Coulter Corp.).

Transient transfection of Huh7.5 cells with a CD4 expression vector. Huh7.5 cells were transiently transfected with either an empty control vector or a human CD4 expressing vector using FuGENE HD as described by the manufacturer (Roche). Transfected cells were trypsinized after 16 h of transfection to be plated at 2.5 × 10⁴ cells per well in a 24-well plate to perform infection as described previously. The efficiency of transfection was analysed in parallel by measuring CD4 expression by flow cytometry as described above.

Statistical analysis. Analysis was performed by matched-paired t test or by one-way ANOVA test followed by a Tukey multiple comparison post-test and performed by using Prism 4 software (GraphPad).

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REFERENCES


Susceptibility of hepatoma cells to HIV infection


and underappreciated mediators of cell-to-cell communication. Leukemia 20, 1487–1495.

Rozmysłowicz, T., Majka, M., Kijowski, J., Murphy, S. L., Conover, D. O., Poncz, M., Ratajczak, J., Gauton, G. N. & Ratajczak, M. Z. (2003). Platelet- and megakaryocyte-derived microparticles transfer CXCR4 receptor to CXCR4-null cells and make them susceptible to infection by X4-HIV. AIDS 17, 33–42.


