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CXCR4 Mediates Entry and Productive Infection of Syncytia-Inducing (X4) HIV-1 Strains in Primary Macrophages

Antonio Valentin,[1] Harsha Trivedi, Wenhong Lu, Leondios G. Kostrikis,* and George N. Pavlakis[1]

*Human Retrovirus Section ABL-Basic Research Program, National Cancer Institute–Frederick Cancer Research and Development Center, Frederick, Maryland 21702-1201; and *Aaron Diamond AIDS Research Center, Rockefeller University, New York, New York 10016*

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CCR5 and CXCR4 are the main coreceptors for non-syncytia-inducing (NSI) and syncytia-inducing (SI) HIV-1 strains, respectively. NSI HIV-1 isolates do not infect either human lymphoid or monocytoid cell lines, and this inability correlates with the absence of CCR5 expression in these cell types. The ability of SI HIV-1 isolates to infect human primary macrophages has been disputed. Here, we report that CXCR4 is expressed in primary blood-derived human mononuclear phagocytes at all stages of differentiation, although the maturation process correlates with downregulation of CXCR4 mRNA. Infection experiments with the SI molecular clone NL4-3 tagged with a mutant of the green fluorescent protein established that both monocytes and attached macrophages are susceptible to infection with CXCR4-restricted HIV-1 strains. NL4-3 entry into primary macrophages could be blocked by SDF-1α in a dose-dependent manner, or by the anti-CXCR4 monoclonal antibody 12G5. HIV-1 entry led to productive infection. No evidence of postentry defects or nuclear import delay for CXCR4-restricted HIV-1 strains was detected using a quantitative real-time PCR assay measuring HIV-1 DNA entry into the nucleus. Macrophages infected by HIV-1 and expressing virus were maintained in culture for long periods of time (up to 5 months). These results demonstrate that CXCR4 is the main HIV-1 SI coreceptor in human primary macrophages and underline the importance of the macrophage as a long-living viral reservoir for HIV-1.

INTRODUCTION

Lentiviruses are characterized by their ability to infect nondividing cells, including cells of mononuclear phagocyte lineage. Infection of macrophages *in vivo* is considered essential for the course of lentiviral infection (Narayan *et al.,* 1989). Mononuclear phagocytes (blood monocytes, microglial cells, spinal cord macrophages, alveolar macrophages, lymph node macrophages) are targets for HIV-1 infection *in vivo* (Koenig *et al.,* 1986; Salahuddin *et al.,* 1986; Eilbott *et al.,* 1989; Innocenti *et al.,* 1992; Bagasra *et al.,* 1993; Orenstein *et al.,* 1997). Progression of HIV-1 disease correlates, in some cases, with the emergence of viral variants with new *in vitro* biological properties such as expanded tropism for CD4+ human cell lines and ability to induce syncytia. These variants have been termed T-cell line tropic. The term "macrophage tropic" has been used variably for HIV strains that efficiently infect macrophages, or, more commonly, for HIV strains that do not grow in T-cell lines. It has been previously reported that most or all HIV-1 isolates grow in primary macrophages although the extent of growth may vary depending on the virus strain and the source of macrophages (Gartner *et al.,* 1990; Olafsson *et al.,* 1991; Valentin *et al.,* 1994). Contrary to these reports, several groups have reported a complete inability to infect macrophages with syncytia-inducing (SI) or T-cell line tropic strains of HIV-1 (Gendelman *et al.,* 1990; Schuitemaker *et al.,* 1991). The discovery of CXCR4 and CCR5 as the major HIV-1 coreceptors essential for viral entry provided explanations for the different tropism of HIV strains (Alkhatib *et al.,* 1996; Choe *et al.,* 1996; Deng *et al.,* 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1996; Feng *et al.,* 1996). It was shown that the ability of different HIV-1 strains to infect primary cells and cell lines correlated with the levels of coreceptors expressed at the cell surface. The inability of NSI strains to infect human cell lines, either of lymphoid or myeloid origin, is due to the absence of CCR5 expression in those cells. Accordingly, the absence of syncytia formation in MT-2 cells inoculated with NSI HIV-1 strains is due to the inability of these strains to infect cells that do not express CCR5. It has been demonstrated that if CCR5 is present, NSI strains induce syncytia in CD4+ human cell lines (Bjorndal *et al.,* 1997).

The reported inability to infect macrophages using certain T-cell line tropic isolates indicated that a similar lack of CXCR4 expression on primary macrophages may be responsible for the restriction to infection with these HIV-1 strains. Prior to the identification of the HIV-1 coreceptors, it was reported that all HIV-1 isolates can enter and replicate in primary attached human macrophages (Valentin *et al.,* 1994), a finding that has recently been supported (Simmons *et al.,* 1996). To verify that this

[1] To whom correspondence and reprint requests should be addressed at ABL-Basic Research Program, Bldg. 535, Rm. 212, NCI-FCRDC, P.O. Box B, Frederick, MD 21702-1201. Fax: (301) 846-6368. E-mail: pavlakis@ncifcrf.gov, or valentina@ncifcrf.gov.

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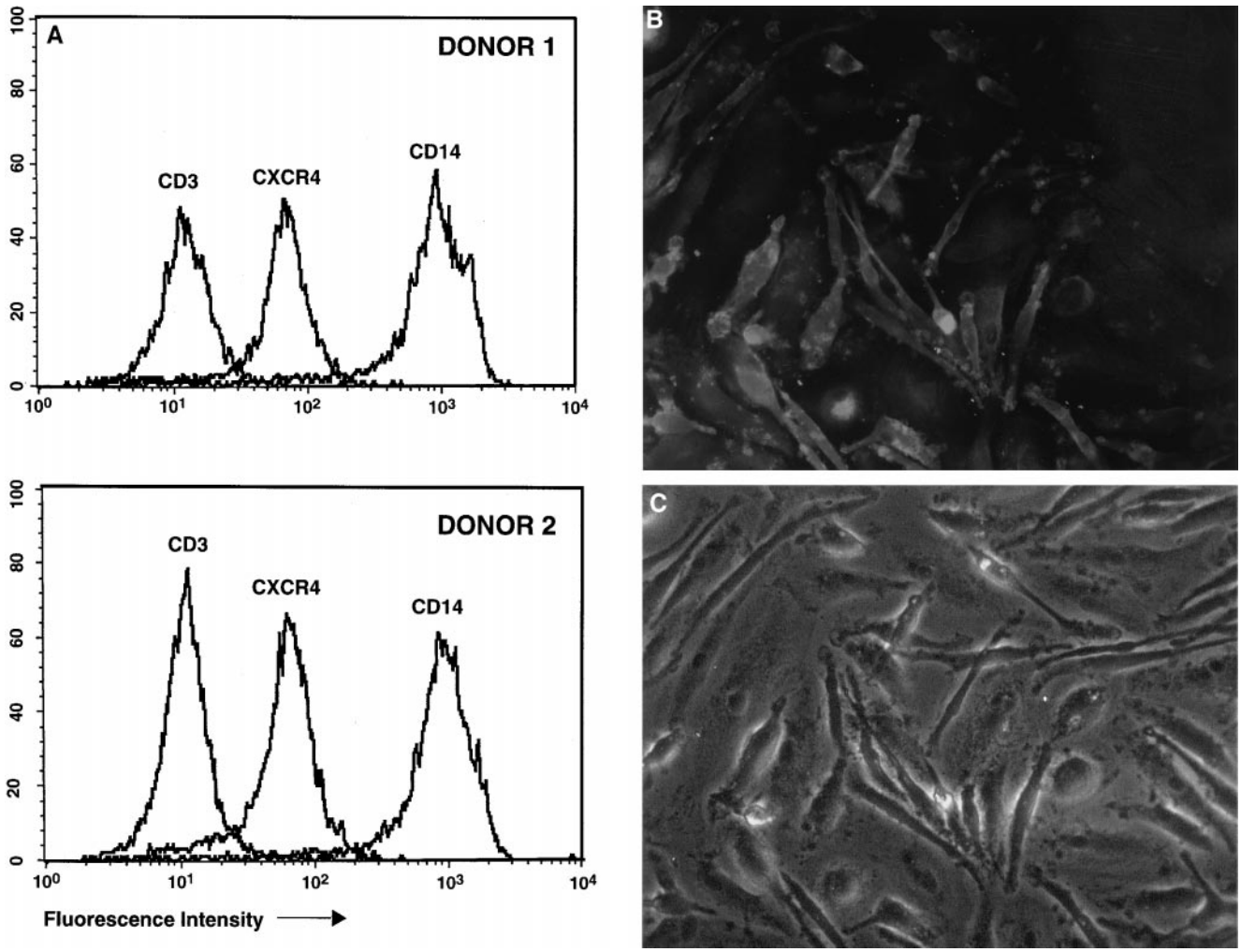

FIG. 1. Peripheral blood monocytes and mature macrophages express CXCR4. (A) FACS analysis of 24-h-old monocytes from two healthy blood donors purified by short-time adherence to plastic; the purified cells are CD14+ and CXCR4+ and do not express CD3 excluding any contamination with T-lymphocytes. (B) Immunostaining of 10-day-old attached macrophages with anti-CXCR4 mAb. (C) Phase image of the same field.

infection is mediated through the CXCR4 receptor, we used a molecular clone of HIV-1 (NL4-3) that uses exclusively CXCR4 among the known coreceptors. Analysis of infected cells showed that both monocytes and macrophages express CXCR4 and are infected by HIV-1 NL4-3. Infection could be blocked by the CXCR4 ligand SDF-1_ or by antibodies against CXCR4. Infected macrophages survived and expressed virus for months in culture, underscoring the importance of macrophage as an active virus reservoir.

RESULTS

CXCR4 expression decreases during mononuclear phagocyte maturation *in vitro*

HIV-1 strains that efficiently infect human T-cell lines use CXCR4 as coreceptor for viral entry into target cells. It has been reported that these type of viruses (SI, T-cell tropic) are unable to infect cells of the mononuclear phagocyte lineage, raising the question of whether these cells express CXCR4 on their surface. To further understand the interaction of HIV-1 with cells of the monocyte/macrophage lineage, we studied the levels of CXCR4 in freshly isolated blood monocytes and monocyte-derived macrophages. Monocytes purified by short-time adherence to plastic and stained with the anti-CXCR4 monoclonal antibody 12G5 were used in FACS analysis experiments. The purified cells were CD3 negative, demonstrating the absence of contaminating T-lymphocytes, and CXCR4 and CD14 positive (Fig. 1A). The differentiation of monocytes into macrophages is characterized by changes in the expression of several membrane antigens, including a potent downregulation of CD4, the main HIV-1 receptor. We also analyzed the pattern of CXCR4 expression in fully differentiated human macrophages. After *in vitro* maturation for 2–4 weeks, the adherent cells showed typical macrophage morphology and were still positive for CD14. Immunostaining of these adherent cells, followed by inspection under a fluorescence microscope, demonstrated that CXCR4 is ex-

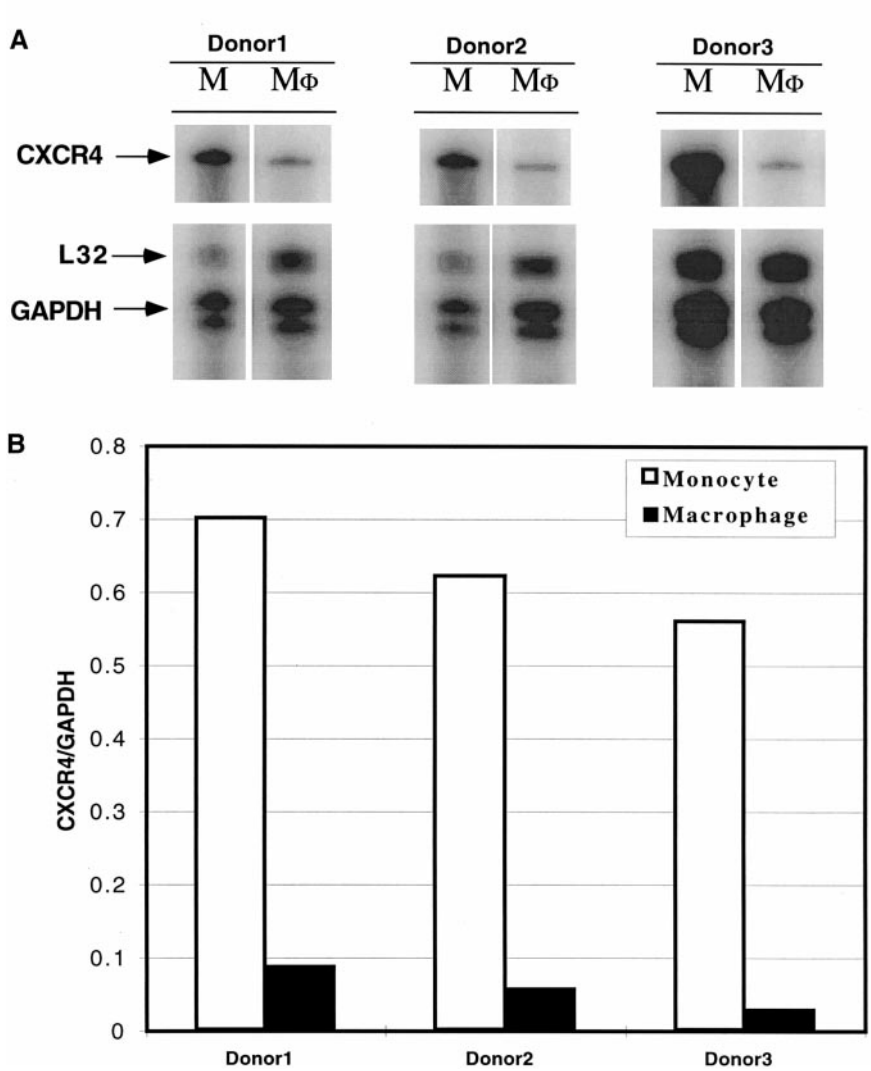

FIG. 2. CXCR4 mRNA is downregulated during monocyte maturation into macrophages. (A) RNase protection assay for CXCR4 mRNA in monocytes (M) and mature macrophages (Mo) from three healthy blood donors. Cellular transcripts GAPDH and L32 were used as internal controls. Monocytes were isolated by positive selection with magnetic beads coated with anti-CD14 mAb and lysed in RNAzol. (B) Quantification of CXCR4 mRNA expression. The amount of CXCR4 RNA was normalized to the control GAPDH by using the ImageQuant program. Similar results were obtained with L32 as internal standard.

pressed in fully differentiated macrophages (Fig. 1B). In addition, CXCR4 mRNA was measured in both fresh monocytes and mature attached macrophages by RNase protection as previously described (Valentin *et al.,* 1998). Comparison of CXCR4 mRNA to cellular mRNAs for GAPDH and L32 showed that CXCR4 expression is downregulated during the maturation of monocytes into macrophages (Fig. 2).

Monocytes are long-living targets in HIV-1-infected PBMC cultures

We examined whether mononuclear phagocytes are infectable by HIV-1 strains restricted to the use of CXCR4 as coreceptor for entry. For this, we used a virus stock produced after transfection of an infectious molecular clone, pNL43GFP11, into human 293 cells. Clone NL43GFP11 has been derived from NL4-3 (Adachi *et al.,* 1986), uses only CXCR4 among the known HIV-1 coreceptors, contains a mutant green fluorescent protein (GFP) gene within the nef region and expresses a Nef-GFP hybrid protein. Infection of primary cells with this virus stock leads to HIV-1 propagation and GFP expression, which can be detected by fluorescence microscopy and quantified by FACS analysis (Valentin *et al.,* 1998).

To study infection in nonattached monocytes, PHA-stimulated PBMC were inoculated with NL43GFP11 and the number of infected CD14+ cells was determined by FACS analysis at several time points postinfection. CD14+ cells were found to be HIV-1 infected in all the analyzed samples. The relative frequency of infected CD14 cells increased over time, and 3 weeks after infection approximately 20% of the infected cells were positive

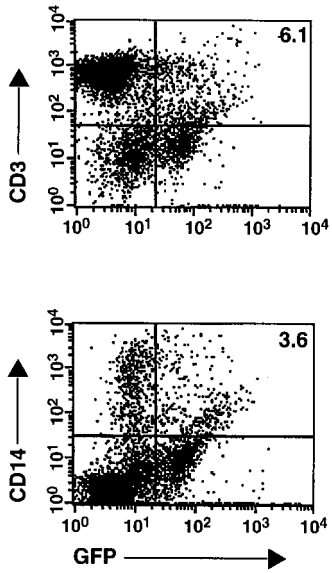

FIG. 3. FACS detection of CD3+ (upper panel) and CD14+ (lower pannel) cells infected with NL4-3GFP in PBMC samples 19 days postinfection. Quadrants were set according to the staining of control cells. Numbers indicate the percentage of positive cells in each quadrant.

for monocytic markers (Fig. 3). No differences in the GFP mean fluorescence intensity was found between CD14- or CD3-positive cells, suggesting that, at the single cell level, HIV-1 expression was similar in both cell types. A significant number of the GFP+ cells were negative for both CD3 and CD14 expression, suggesting either that HIV infection results in downmodulation of these antigens and/or that other cell types may be infected by HIV. Analysis of these GFP+CD3-CD14- cells showed that they are CD56+, which is a typical marker for natural killer (NK) cells (unpublished results).

Culture conditions for lymphocytes typically allowed the growth of these cells for about 3–5 weeks. After this period few T cells proliferate, and virtually no infected lymphocytes can be detected in the culture. In several experiments, we decided to culture the infected PBMC in tissue culture flasks kept horizontally to increase the culture area that could be used by the monocytes to attach. In this way, we were able to establish pure macrophage cultures from the HIV-1-infected PBMC. These adherent cells display typical macrophage morphology and can be kept in culture for several months (4–5 months) after the suspension lymphocytes vanish from the culture either because of the viral infection or due to the natural life span of the cells. These adherent macrophages are HIV-infected as judged by GFP expression in the majority of the cells (more than 50% infected cells in five independent experiments). Production of infectious HIV could be demonstrated by cell-free infection of Jurkat cells with supernatants collected from the macrophage cultures up to 20 weeks postinfection. Infection of Jurkat cells was monitored by GFP expression (data not shown).

Mature human macrophages can be infected with CXCR4-restricted virus

Although these experiments established that monocyte/macrophages can be infected with CXCR4-restricted HIV-1 molecular clones, it could be argued that cell to cell interactions in unfractionated PBMC may facilitate infection of monocytes by a fusion process that may be rather inefficient under other conditions. To address this question, we performed cell-free infections of fully differentiated macrophage cultures with NL43GFP11 or the macrophage tropic strain HIV-1BaL. Under these conditions, both viral stocks were able to infect human macrophages, although the extent of viral replication was lower for NL43GFP11, as measured by concentration of p24gag in culture supernatant (Fig. 4). To demonstrate that the NL43GFP11-infected cells were indeed attached macrophages, the cultures were inspected over time by microscopy using fluorescent light to excite GFP. This analysis demonstrated the presence of large adherent multinucleated macrophages positive for GFP expression (Fig. 5). The cells shown in Fig. 5 were cultured for more than 16 weeks in the absence of any exogenous cytokines, a period of time that does not allow survival of other cell types. These macrophage preparations are trypsin-resistant and were shown to be positive for the CD14 macrophage marker. Although infection with NL43GFP11 was positive in all the experiments, the percentage of GFP-positive cells varied dramatically (3–40%) among different macrophage preparations infected by cell-free virus preparations (data not shown).

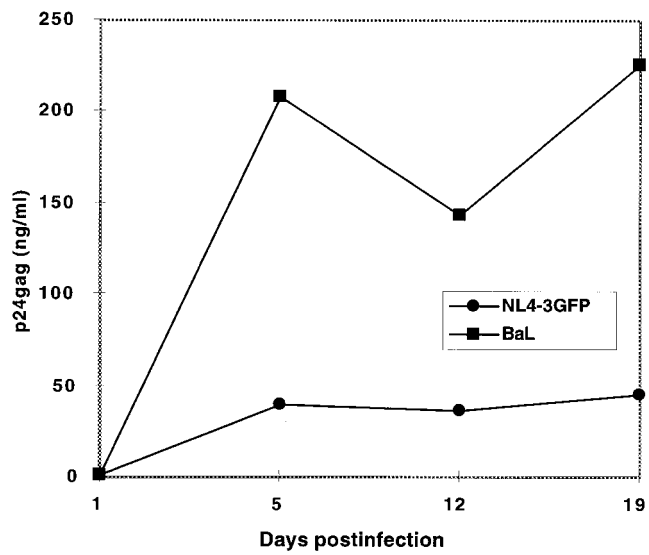

FIG. 4. Kinetics of HIV-1 expression in fully differentiated attached macrophages infected with the CCR5-restricted HIV-1BaL strain and the CXCR4-restricted NL4-3GFP molecular clone. Values represent p24gag concentration (ng/ml) in culture supernatant. Similar results were obtained in four additional experiments.

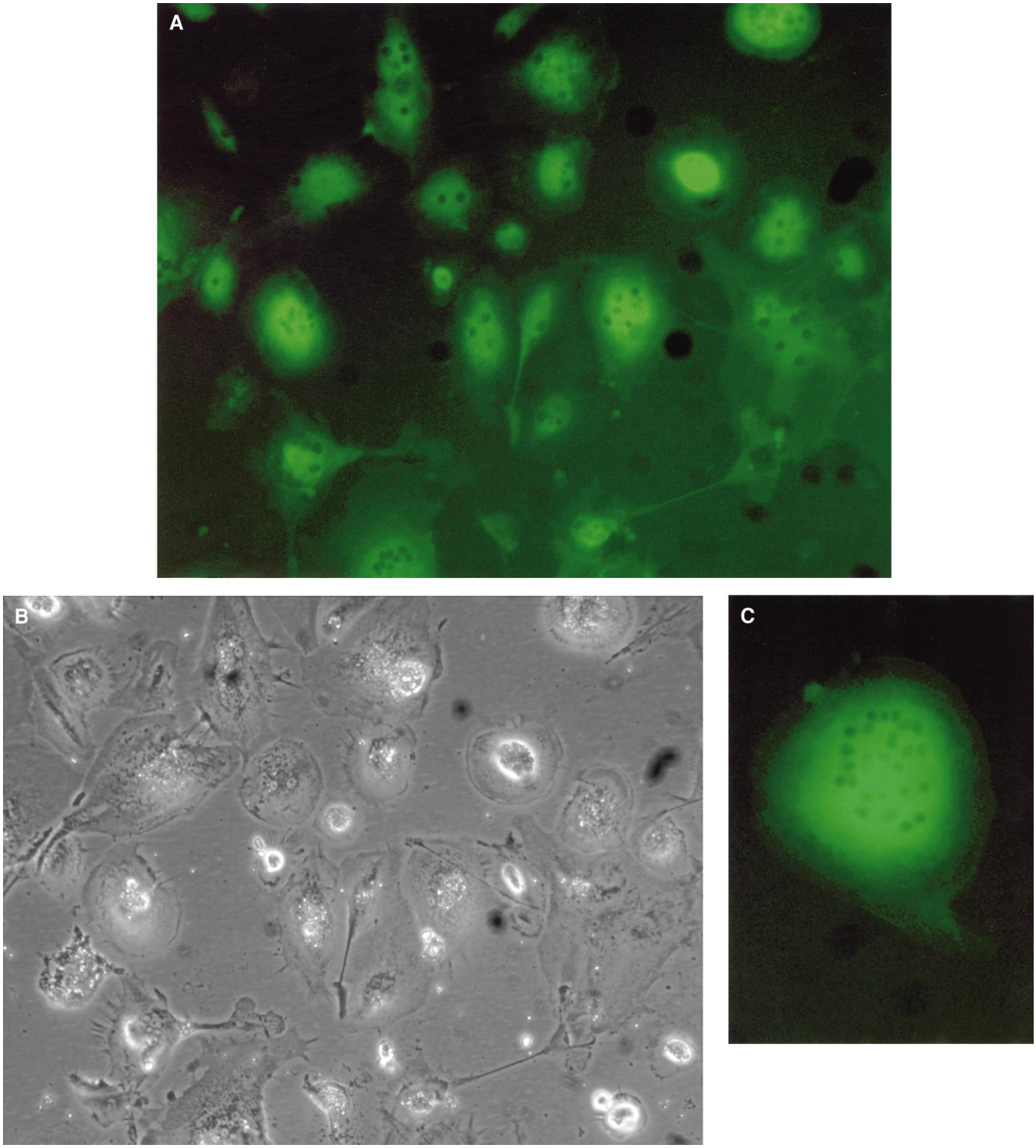

FIG. 5. Images of live primary macrophages infected with NL43GFP11. (A) Fluorescence image; (B) phase; (C) fluorescence image of a multinucleated macrophage at higher magnification. The cells were cultured for 16 weeks in the absence of any exogenous cytokines.

Quantification of macrophage infection by measuring 2LTR circles

Macrophage infection by the different viruses was verified and quantified by using a quantitative real-time PCR to measure 2LTR circles (Fig. 6). The number of circles reflects the number of reverse-transcribed genomes imported in the nucleus of infected cells. Primary macrophages were infected with stocks of JR-CSF or NL4-3 strains and 2 days postinfection cellular DNA was isolated. The number of two LTR circles was quantified

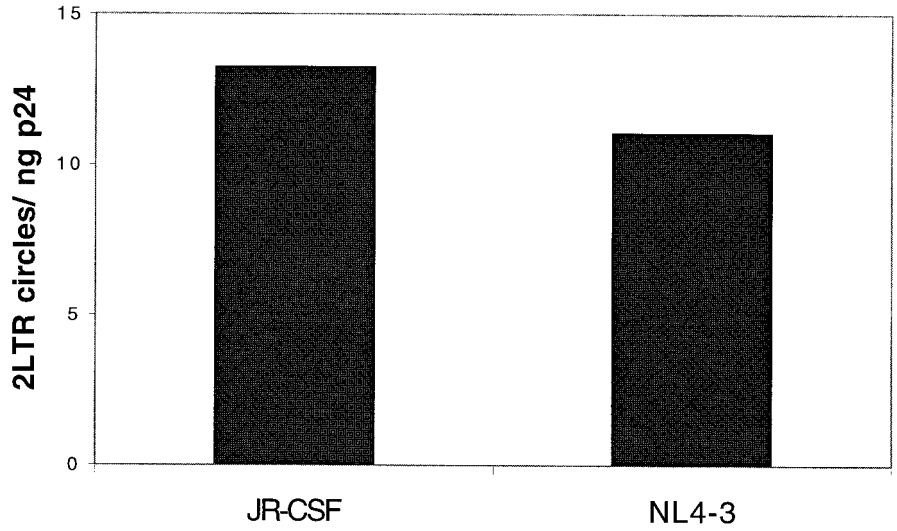

FIG. 6. Quantification of HIV-1 2LTR circles after infection of primary macrophages with two different virus stocks, JR-CSF (R5 using) and NL4-3 (X4 using). Two days postinfection DNA was isolated and the number of 2LTR circles were determined by quantitative real-time PCR.

by using a molecular beacon (Tyagi *et al.,* 1996; Kostrikis *et al.,* 1998) in combination with real-time PCR in a PE 7700 Taqman as described under Materials and Methods. The results were expressed as copies of 2LTR circles per nanogram of input virus p24 in 1 μg of cellular DNA. It was found that the number of the 2LTR circles generated after infection was similar for the two virus types, demonstrating that there are no differences in the postentry fate of the virus following utilization of CCR5 or CXCR4 coreceptors. These results are in contrast with the reported defect of X4-using HIV to enter the nucleus (Schmidtmayerova *et al.,* 1998).

CXCR4 is the main coreceptor for SI HIV-1 in primary macrophages

The previous experiments demonstrated that productive infection of macrophages by CXCR4-restricted HIV-1 clones is possible. To ascertain that the infection is in fact mediated by CXCR4 and not by any other unknown coreceptor, we performed blocking experiments using either an anti-CXCR4 monoclonal antibody or the natural ligand for CXCR4 stromal cell-derived factor-1a (SDF-1α). The ability to block CXCR4-dependent HIV entry of the monoclonal antibody (mAb) 12G5 has been demonstrated by several groups. Our blocking experiments were performed by treating the cells with the inhibitors for 30 min, prior to the addition of HIV. After a 1-h incubation with NL43GFP11 in the presence of different concentrations of either SDF-1α or mAb 12G5, both the virus and the inhibitors were washed out and the macrophages were cultured in the absence of any further treatment. HIV-1 infection was monitored by measuring intracellular p24gag 6 days postinfection. These results showed 90% inhibition of viral infection by the mAb 12G5 and dose-dependent inhibition by SDF-1α (up to 80% inhibition at a concentration of 500 nM) (Fig. 7). In contrast, under similar conditions, treatment with RANTES failed to inhibit macrophage infection with the X4-restricted HIV molecular clone (Fig. 7). In addition, an unrelated, isotype-matched antibody did not inhibit infection, indicating that inhibition by mAb 12G5 was specific.

DISCUSSION

The present study demonstrated that CXCR4 is expressed in both monocytes and mature macrophages. The levels of expression decrease upon *in vitro* differentiation of monocytes to mature attached macrophages. CXCR4-restricted HIV-1 variants were able to infect mononuclear phagocytes and this infection could be blocked by either SDF-1α or anti-CXCR4 mAb, indicating that CXCR4 functions as coreceptor in macrophages. In agreement with this study, other data indicate that mononuclear phagocytes express CXCR4 that can interact with HIV-1 env, although the exact mechanism of this interaction is still a matter of debate (Strizki *et al.,* 1997; Bazan *et al.,* 1998; Cho *et al.,* 1998; Schmidtmayerova *et al.,* 1998; Smyth *et al.,* 1998; Verani *et al.,* 1998; Yi *et al.,* 1998). Some investigators have suggested that CXCR4 using viruses either do not enter in macrophages or display delayed nuclear entry resulting in nonproductive infection. The quantitative 2LTR circle assay we applied here did not reveal any major differences in nuclear entry of the two virus types. In agreement with these results, study of Vpr translocation into the nucleus after infection with R5 or X4 strains also showed no differences (unpublished data). After the completion of this work, it was reported that macrophages can be infected by CXCR4-using isolates and that this infection is inhibited by AMD3100, an inhibitor of CXCR4-HIV interaction (Simmons *et al.,* 1998). In addition, it was reported that macrophages from CCR5-(Δ32/Δ32) homozygous individuals can be infected by X4 strains (Yi *et al.,* 1999).

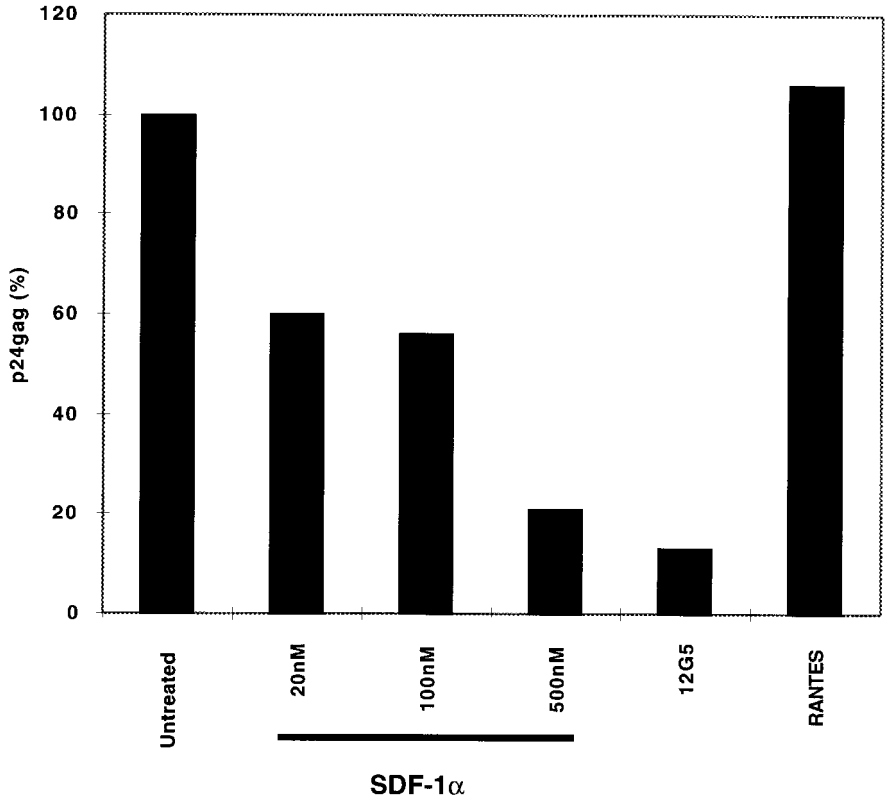

FIG. 7. Inhibition of NL4-3GFP infection of mature macrophages by SDF-1α or by anti-CXCR4 mAb. Macrophages were exposed to RANTES (500 nM), SDF-1α, or anti-CXCR4 mAb 30 min before and during the incubation with NL4-3GFP. Sixty minutes after viral adsorption, both the virus and the inhibitors were washed from the macrophage cultures. Values represent intracellular p24 (percentage as compared to untreated cultures) 6 days postinfection. Similar results were obtained in two independent experiments using macrophages from different individuals.

Our studies demonstrate not only that macrophages are infected with CXCR4-restricted viruses in unfractionated PBMC but also that pure macrophage cultures are targets for infection after exposure to cell-free stocks of CXCR4-restricted HIV molecular clones tagged with GFP. This technique allows detection and monitoring of infected cells by following green cells either by fluorescence microscopy or by FACS. FACS analysis allows in addition quantitation of GFP production at the single cell level. We found that the level of HIV-1 expression in monocyte/macrophages is similar to that of the infected lymphocytes. Because similar levels of GFP are produced in lymphocytes and macrophages from the same sample, we conclude that expression of HIV-1 variants with SI phenotype is not impaired in human macrophages. In addition, we demonstrated virus production of the GFP-expressing NL4-3 molecular clone by macrophages for long periods of time (months). Therefore, our results do not support the concept that CXCR4 using viruses are impaired at the level of nuclear translocation of viral DNA as recently suggested (Schmidtmayerova *et al.,* 1998). Many disagreements still remain among different laboratories on the infectivity of macrophages by HIV-1 strains. Several factors may be responsible for the reported discrepancies, such as different preparation protocols, purity of resulting populations, differentiation stage, and treatment with various growth factors. For example, very recently it was shown that GM-CSF, a frequently used cytokine in macrophage cultures, downregulates levels of CXCR4 in monocyte-derived macrophages (Lee *et al.,* 1999). Monocyte differentiation in the presence of autologous lymphocytes allows long-term culture of these cells by preventing the death of the monocytes *in vitro* (Lopez *et al.,* 1993). It was shown that this technique increases the susceptibility of the cells to infection with HIV and other human viruses (Ibanez *et al.,* 1991).

Monocyte/macrophage infection in unfractionated PBMC appears to be more efficient compared to infection of attached differentiated macrophages. This may reflect the more efficient cell to cell transmission (lymphocyte to macrophage) and/or the higher levels of CD4 and CXCR4 found at the earlier stages of differentiation of cells of mononuclear phagocyte lineage. This difference in transmission to macrophages is not limited to CXCR4-using isolates but it is also seen with CCR5-restricted HIV-1 isolates (not shown).

Several lines of evidence suggest that mononuclear phagocytes are infected by CXCR4-restricted HIV-1 strains *in vivo.* HIV was rescued from macrophages of a laboratory worker accidentally infected with the CXCR4-restricted IIIB strain (Popovic *et al.,* 1987). Several cases

of HIV-1 infection have already been documented in individuals homozygous for the CCR5Δ32 deletion; analysis of coreceptor usage revealed infection by CXCR4-using viruses in all cases (O'Brien *et al.,* 1997; Theodorou *et al.,* 1997). Analysis of the infected cells in these patients will definitively answer whether or not CXCR4-restricted viruses are transmitted into macrophages *in vivo.* Microglial cells are bone marrow-derived mononuclear phagocytes resident in the central nervous system and represent the main if not the only target for productive HIV-1 infection in neural tissues. In agreement with the results presented here, microglial cells have been shown to express CXCR4 that is fully functional in mediating virion-induced cell fusion leading to productive HIV-1 infection (He *et al.,* 1997).

It has been previously established that susceptibility of mature macrophages to HIV-1 infection is affected by the stage of differentiation, as well as by the tissue origin of the cells (Olafsson *et al.,* 1991; Valentin *et al.,* 1991). The plasticity of the mononuclear phagocyte system involves complex regulation of the chemokine receptors, including those mediating HIV-1 entry. Therefore, understanding the regulation of the HIV-1 coreceptors is important for understanding the interaction of these cells with HIV-1. HIV-1 infection of human macrophages is CD4-dependent (Collman *et al.,* 1990; Collin *et al.,* 1993). CD4 expression is highest in monocytes and decreases to almost undetectable levels in fully mature macrophages, while the susceptibility to HIV-1 infection increases during the first week of differentiation *in vitro* (Kazazi *et al.,* 1989; Valentin *et al.,* 1991). Monocyte susceptibility to infection is low and does not correlate with the level of CD4 expression. Like CD4, CXCR4 is downmodulated during the maturation of mononuclear phagocytes *in vitro,* although to a lesser extent (this work; Di Marzio *et al.,* 1998; Naif *et al.,* 1998). In contrast, it has been shown that CCR5 increases in mature macrophages (Di Marzio *et al.,* 1998; Naif *et al.,* 1998; Tuttle *et al.,* 1998). The complex and differential regulation of these three molecules suggests that the concentrations of CD4 and of the HIV-1 coreceptors required for HIV-1 infection are interdependent. Through the use of adherent human cell lines expressing different concentrations of either CD4 or CCR5, it was demonstrated that requirement for either of these molecules is increased when the other component is present in a limiting amount (Kozak *et al.,* 1997; Platt *et al.,* 1998). Because the binding of gp120 to CD4, an absolute requirement for HIV-1 infection, precedes the interaction with CXCR4, the strong CD4 downmodulation in terminally differentiated blood-derived macrophages can inhibit the entry process in both CXCR4- and CCR5-restricted HIV-1 isolates. In this context, the limiting factor for HIV-1 infection in mature macrophages may be CD4. Recent additional studies indicate an important role of CD4 levels for virus entry in macrophages (Dimitrov *et al.,* 1999; Pesenti *et al.,* 1999).

CXCR4-dependent HIV-1 strains appear during the symptomatic phase of the disease and their emergence correlates with faster progression to AIDS. The notion that CXCR4-restricted viruses could not infect macrophages implied that HIV evolution *in vivo* selects for variants restricted in their ability to infect mononuclear phagocytes. In contrast, we demonstrate that cells of the mononuclear phagocyte lineage are infectable also by CXCR4-restricted viruses. The mononuclear phagocyte is the cellular compartment that characterizes lentiviral disease and is likely essential for the generation of chronic active infection by HIV-1 and other lentiviruses. The fact that CXCR4-dependent HIV-1 strains are usually not transmitted led to the idea that the gates of entry for HIV-1 were cells resistant to infection with SI strains, due either to lack of CXCR4 expression on their surface or to other yet unidentified mechanisms. This, together with the resistance of macrophages to infection with SI HIV-1 isolates reported by several groups, led to the idea that, in sexually transmitted HIV-1, mucosal macrophages and/or Langerhans cells probably are the initial targets for infection. The results presented here do not support this hypothesis and demonstrate that CXCR4 is expressed and mediates HIV-1 entry in primary macrophages.

Our studies demonstrate not only that macrophages are infected with CXCR4-restricted viruses in unfractionated PBMC but also that it is possible to establish pure macrophage cultures that continue to produce infectious HIV for more than 4 months. The difference in the *in vitro* life span of infected lymphocytes versus macrophages is probably also true for tissue macrophages *in vivo.* These results emphasize the importance of macrophages as a reservoir of long-lived chronically infected cells able to maintain the infection during antiretroviral therapy.

In conclusion, our results demonstrate that CXCR4 is expressed in both fresh monocytes and terminally differentiated macrophages. CXCR4 is fully functional in mediating cell-free viral entry of CXCR4-restricted HIV-1 strains. In addition the strong inhibition mediated by 12G5 mAb suggests that CXCR4 is the main, if not the only, coreceptor for SI viruses in primary macrophages.

MATERIALS AND METHODS

Cells, molecular clones, and viral infections

Peripheral blood mononuclear cells (PBMC) from healthy, HIV-1-seronegative blood donors were purified by Histopaque gradient centrifugation (Sigma). The purified cells were stimulated for 3 days with PHA (5 μg/ml). Monocyte-derived macrophage cultures were established as previously described (Valentin *et al.,* 1994). Long-term macrophage cultures were established from HIV-1-infected PBMC, cultured in either 60-mm Petri dishes or tissue culture flasks. The flasks were kept horizontal to increase the area available for the monocytes to attach. Four to five weeks later, when no surviving lymphocytes were present in the culture, the remain-

ing adherent macrophages were washed once with PBS and cultured in RPMI supplemented with 10% FCS. These macrophage cultures can be kept *in vitro* for long periods of time (4–5 months) without any additional manipulation.

The infectious HIV-1 molecular clone pNL43GFP11 expresses the GFP mutant GFPsg11 (Palm *et al.,* 1997; Stauber *et al.,* 1998) as a fusion protein with the 24 N-terminal amino acids of Nef (Valentin *et al.,* 1998). For viral infections, 10^7 PHA-stimulated PBMC were incubated at 37°C with 1 ml of cell-free supernatants from HIV-1BaL-infected PBMC or 293 cells transfected with pNL43GFP11. After a 2-h incubation, the cells were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI supplemented with IL-2 (5 U/ml, Boehringer) at a density of 10^6 cells/ml. HIV-1 infection of pure adherent blood-derived macrophage cultures was performed as previously described (Valentin *et al.,* 1994). Viral replication was monitored by measuring p24gag in culture supernatants using a commercial ELISA kit (Cellular Products).

Immunostaining and FACS analysis

For indirect immunofluorescence, 10^6 cells were washed once with PBS containing 1% human serum and 0.1% sodium azide and incubated on ice for 30 min with the mouse anti-CXCR4 monoclonal antibody 12G5 (Endres *et al.,* 1996). After incubation, the cells were washed twice and resuspended in 100 μl of buffer containing FITC-conjugated F(ab′)2 goat anti-mouse IgG (Boehringer). After a 30-min incubation on ice, the cells were washed twice and analyzed by FACS (Becton-Dickinson). Dead cells were excluded by propidium iodide staining. Phycoerythrin (PE)-conjugated anti-CD3 and anti-CD14 mAbs (Pharmingen) were used for double staining experiments. FACS analysis of NL43GFP11-infected samples was performed in cells fixed with 2% paraformaldehyde. Primary macrophages attached to 35- or 60-mm plastic dishes (Becton-Dickinson) were stained under similar conditions.

RNase protection assay

Total RNA from primary monocytes positively selected by CD14-coated magnetic beads (Dynal) or from mature macrophages was extracted by the RNazol procedure. For CXCR4 mRNA analysis, 5 μg of total RNA was hybridized to radiolabeled probes produced by an *in vitro* transcription kit (Pharmingen) and digested with ribonuclease. Samples were electrophoresed on 5% acrylamide 7 M urea gels. Cellular transcripts GAPDH and L32 were used as internal controls. For quantification of CXCR4 mRNA expression, the amount of RNA per sample was normalized to the control GAPDH or L32 by using the ImageQuant program.

Real-time PCR quantification of 2LTR circles

Genomic DNA was isolated from HIV-1-infected primary macrophage cultures (QIAamp Blood Kit, Qiagen). HIV-1 DNA sequences encoding the R-U5-U3 region (approximately 200 nucleotides in size) of circularized HIV-1 genomes containing two long-terminal repeats (2LTR circle) were quantified by real-time PCR using an LTR-U5-specific molecular beacon (Tyagi *et al.,* 1996; Kostrikis *et al.,* 1998). The sequence of the molecular beacon was fluorescein-5′-GCGGGTTCTGAGGGATCTCTAGTTACCAGACCCGC-3′-DABCYL, where DABCYL is the quencher 4-(4′-dimethylaminophenylazo)benzoic acid, and underlined sequences indicate the complementary sequences forming the hairpin structure. The target recognition sequence for the molecular beacon was 5′-TCTGGTAACTAGAGATCCCTCAGA (at positions 9257 to 9281 of the LTR-U5 region of the LAI sequence). Primers used in the real-time PCR were LK163 (5′-GGTACTAGCTTGAAGCACCATCC at positions 8807 to 8829 of the LTR-U3 region) and LK164 (5′-GCCTCAATAAAGCTTGCCTTGAGTG at positions 9200 to 9224 of the LTR-R region). The PCR primers and the target recognition sequence of the molecular beacon were designed to hybridize on conserved regions from all the genetic subtypes within the M group based on a comprehensive DNA sequence alignment from published HIV-1 sequences. The amplicon was sequenced and found to contain the correct regions for a 2LTR circle that was formed after 5′- and 3′-end-end LTR ligation. Each 50 μl PCR contained 1 μg of genomic DNA, 0.5 μM of each primer, 0.25 μM of the LK161-fluorescein (FAM) molecular beacon, 0.2 mM of dATP, dGTP, dCTP, and dUTP, 2.5 units of AmpliTaq Gold DNA polymerase (Perkin–Elmer), 50 mM KCl, 3.5 mM of $MgCl_2$, 10 mM Tris-HCl (pH 8.3), and 0.2 units of UNG erase enzyme (Perkin-Elmer). Forty five cycles of amplification (94°C denaturation for 15 s, 60°C annealing for 1 min, and 72°C polymerization for 30 s) were performed in a spectrofluorometric thermal cycler (ABI 7700, Applied Biosystems, Foster City, CA). Fluorescence was monitored during every thermal cycle at 60°C and data collected by the computer were analyzed by the integrated software to obtain the LTR copy number in the sample based on a standard curve derived from known DNA standards.

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