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The C-X-C chemokine IP-10 stimulates HIV-1 replication

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Abstract

Chemokines play critical roles in HIV-1 infection, serving both to modulate viral replication and to recruit target cells to sites of infection. Interferon-γ-inducible protein 10 (IP-10/CXCL10) is a C-X-C chemokine that acts specifically upon activated T cells and macrophages and attracts T cells into the cerebrospinal fluid (CSF) in HIV-associated neurological disease. We now demonstrate that IP-10 stimulates HIV-1 replication in monocyte-derived macrophages and peripheral blood lymphocytes. We further demonstrate that neutralization of endogenous IP-10 or blocking the function of its receptor, CXCR3, reduces HIV-1 replication in these same cells. Therefore, blocking the interaction between IP-10 and CXCR3 represents a possible new target for anti-retroviral therapy.

Introduction

The immune response to HIV-1 plays an important role in the development and progression of disease in infected individuals. HIV-1-induced cytokines exert protective and pathogenic effects on many cellular targets (Garzino-Demo et al., 2000; Hogan and Hammer, 2001; Kinter et al., 2000). Chemokines, in particular, have been demonstrated to play a central role in the immune response to HIV-1 infection (Garzino-Demo et al., 2000). Chemokines are a subset of the cytokine family that attracts and activates leukocytes via interactions with seven-transmembrane domain G protein-coupled receptors (GPCR) (Zlotnik et al., 1999). In the past few years, it has been shown that the ligands of the C-C chemokine receptor CCR5, MIP-1α/CCL3, MIP-1β/CCL4, and RANTES/CCL5 suppress HIV replication (Cocchi et al., 1995; Coffey et al., 1997), although under some circumstances these same chemokines can actually enhance HIV-1 replication (Dolei et al., 1998; Kelly et al., 1998; Kinter et al., 1998; Schmidtmayerova et al., 1996). Isolates of HIV-1 that utilize CCR5 as a coreceptor (R5 HIV) can infect both monocyte-derived macrophages (MDM) and $CD4^+$ T lymphocytes (Alkhatib et al., 1996; Choe et al., 1996; Deng et al., 1996; Doranz et al., 1996; Dragic et al., 1996). It has been demonstrated that individuals having mutations in both alleles for CCR5 are highly resistant to HIV-1 infection and that individuals that are heterozygous for the CCR5 Δ32 mutation have a delayed clinical course following HIV-1 infection (Dean et al., 1996; Liu et al., 1996; Samson et al., 1996; Smith et al., 1997).

During the course of infection, HIV-1 expands its coreceptor usage to include the C-X-C chemokine receptor CXCR4, as well as other chemokine receptors. CXCR4-using strains of HIV-1 (X4 HIV) are associated with disease progression, decline in peripheral $CD4^+$ T lymphocyte levels, and the onset of the clinical symptoms of AIDS (Connor et al., 1997).

SDF-1α/CXCL12, the ligand for CXCR4, has also been shown to inhibit HIV-1 replication (Bleul et al., 1996; Marechal et al., 1999; Oberlin et al., 1996), although under some conditions, it also can stimulate HIV-1 replication (Bleul et al., 1996; Marechal et al., 1999; Oberlin et al., 1996).

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The interferon-γ-inducible protein 10 (IP-10/CXCL10) is a C-X-C chemokine that has been shown to attract activated T lymphocytes and monocytes (Luster and Leder, 1993; Luster et al., 1985; Taub et al., 1993, 1996). IP-10 exerts its actions by binding to the chemokine receptor CXCR3, which also serves as the receptor for monokine induced by γ-interferon (MIG/CXCL9), and interferon-inducible T cell alpha chemoattractant (I-TAC/CXCL11) (Loetscher et al., 1996). The expression and function of IP-10, MIG, and I-TAC are differentially regulated, and they are believed to serve nonredundant functions in vivo (Farber, 1997).

Although CXCR3 is not known to function as a coreceptor for HIV (Zlotnik et al., 1999), the expression of CXCR3 and its ligands is induced in the context of HIV infection. Increased levels of IP-10 have been detected in the plasma of HIV-1-infected individuals compared with controls, and persistently elevated IP-10 has been correlated with immunological treatment failure following HAART (Stylianou et al., 2000). IP-10 mRNA has also been shown to be elevated within alveolar macrophages of HIV-seropositive individuals (Buhl et al., 1993), and IP-10 is induced following infection of MDM and peripheral blood mononuclear cells (PBMC) (Poluektova et al., 2001; Wetzel et al., 2002). In addition, IP-10 and CXCR3 have been detected in the brains of SIV-infected macaques with encephalitis and of HIV-1-infected individuals (Sanders et al., 1998; Sasseville et al., 1996; Westmoreland et al., 1998). IP-10 has also been shown to be present in the cerebrospinal fluid (CSF) of HIV-1-infected individuals and acts as the major T cell chemotactic factor in the CSF (Kolb et al., 1999). IP-10 is produced by astrocytes treated with HIV-1 Tat and gp120 (Asensio et al., 2001; Kutsch et al., 2000). We now demonstrate that IP-10 stimulates HIV-1 replication in MDM and peripheral blood lymphocytes (PBL) and that neutralization of endogenous IP-10 or blocking CXCR3 reduces HIV-1 replication in these same cells. Inhibition of IP-10 function may therefore represent a new target for anti-retroviral therapy.

Results

IP-10 stimulates HIV-1 replication in MDM and PBL

Many previous reports have indicated that certain cytokines, including members of the chemokine family, can affect the ability of HIV-1 to replicate within target cells (Garzino-Demo et al., 2000; Hogan and Hammer, 2001; Kinter et al., 2000). The chemokines RANTES, MIP-1α, MIP-1β, and SDF-1α are major HIV regulatory factors that suppress HIV replication under most circumstances and enhance replication under other select circumstances (Bleul et al., 1996; Cocchi et al., 1995; Kinter et al., 1998; Oberlin et al., 1996; Schmidtmayerova et al., 1996). We have also demonstrated that the C-X-C chemokines GRO-α and IL-8 participate in autocrine loops that enhance HIV-1 replication (Lane et al., 2001a,b). Since the ligands for CXCR1, CXCR2, and CXCR4 affect HIV-1 replication in MDM and PBL, we investigated the effect that CXCR3 ligands have on HIV-1 infection in primary human lymphocytes and macrophages.

Cultures of PBL and MDM were collected from healthy seronegative volunteers and infected with HIV-1. Recombinant human IP-10 was added prior to infection and replenished throughout the period of culture. Viral replication in MDM infected with the CCR5-using isolate HIV-1_{BaL} was assessed by measuring the amount of RT activity present in the cellular supernatants at several points in time after infection (Fig. 1A). In experiments with 10 different donors' cells, IP-10 stimulated HIV-1_{BaL} replication 8.2-fold on average (range: 1.5- to 29-fold). This increase was found to be statistically significant ($P < 0.03$), as determined by the Student's *t* test. IP-10 was also found to stimulate the replication of the X4 isolate HIV-1_{BRU} in PBL (Fig. 1B). Doses of IP-10 in the range of 5 to 125 ng/ml stimulated replication, with a mean increase of 1.4-fold for 5 ng/ml, 2.3-fold for 25 ng/ml, and 2.8-fold for 125 ng/ml, in five independent experiments. The increases with 25 and 125 ng/ml were statistically significant with *P* values of 0.033 and <0.01 by the Student's *t* test. IP-10 also stimulated the replication of several other isolates of HIV in PBL, including HIV-1_{BaL} (R5), HIV-1_{IIIB} (X4), and HIV-2_{CBL-20} (X4) (data not shown).

The CXCR3 ligands MIG and I-TAC also had a stimulatory effect on HIV-1_{BRU} replication in PBL (Figs. 1C and 1D). In five independent experiments, mean increases of 2.4-fold for 5 ng/ml, 2.4-fold for 25 ng/ml, and 2.0-fold for 125 ng/ml were observed with MIG. I-TAC stimulated HIV-1 replication 2.5-fold at 5 ng/ml, 2.5-fold at 25 ng/ml, and 3.1-fold at 125 ng/ml. All of these increases were found to be statistically significant ($P < 0.05$) by the Student's *t* test. No difference in cellular viability was observed following treatment of MDM or PBL with IP-10 (Table 1), or with MIG or I-TAC (data not shown). These data indicate that the effect of these chemokines upon HIV-1 replication is principally via an effect upon the process of viral replication and not an effect upon the activation, proliferation, or viability of the cells themselves.

IP-10 does not activate HIV-1 gene expression

Many cytokines are known to stimulate HIV-1 replication via effects on HIV-1 gene expression (Duh et al., 1989; Folks et al., 1987, 1989). For this reason, we employed a number of models to determine whether IP-10, similar to TNF-α and the phorbol ester PMA, could be shown to stimulate HIV-1 long terminal repeat (LTR)-mediated gene expression. We first looked at two commonly used, chronically infected cell lines in which HIV-1 can be induced from latency by a number of factors, including PMA. The cell line U1 is a derivative of the U937 monocytic cell line

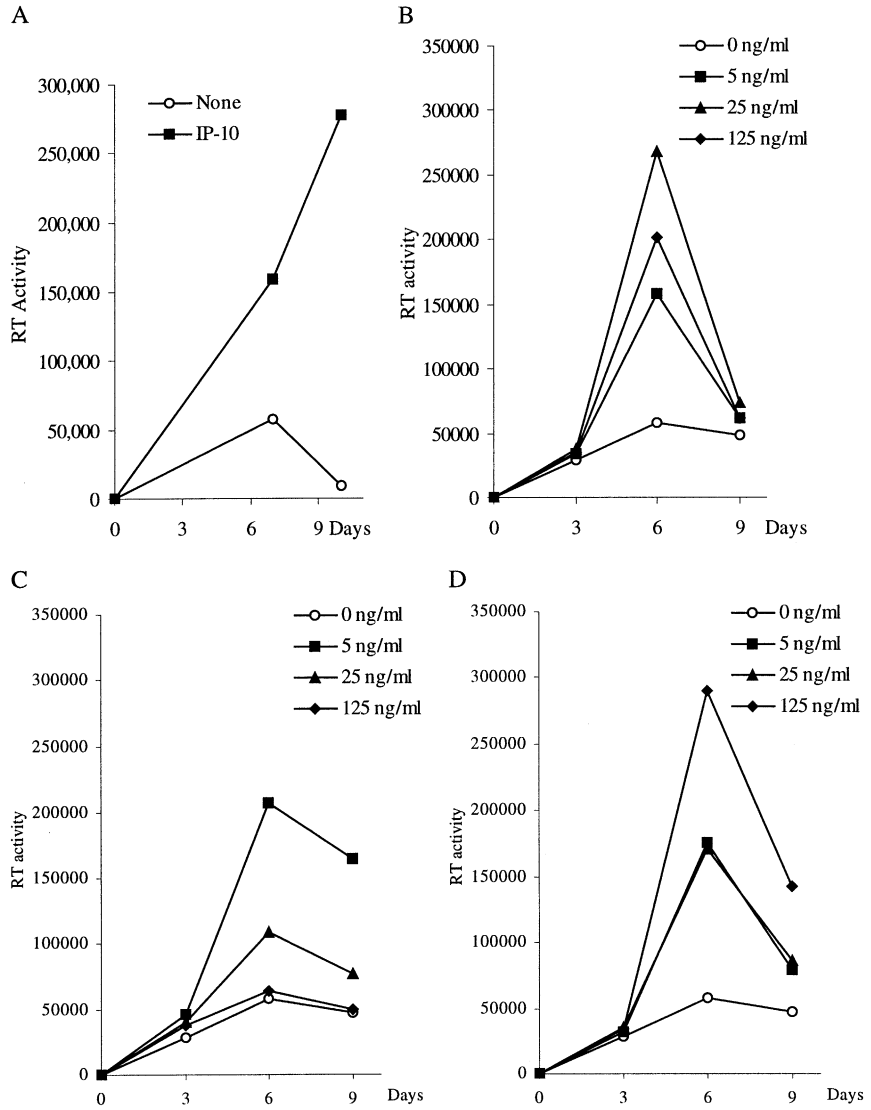

Fig. 1. The CXCR3 ligands stimulate HIV-1 replication. (A) MDM were treated with IP-10 (25 ng/ml) every 3 days, beginning 1 day before infection with the CCR5-using isolate HIV-1_{BaL}. Supernatants were analyzed for RT activity 7 and 10 days after infection. Data shown represent the average value of RT activity in triplicate wells, and this experiment is representative of 10 independent experiments. IP-10 (B), MIG (C), and I-TAC (D) stimulate replication of the CXCR4-using isolate HIV-1_{BRU} in PBL. PBL were treated with chemokines at a dose of 5, 25, or 125 ng/ml every 3 days beginning 1 day before infection. Viral replication was assessed on days 3, 6, and 9 by RT assay. Data shown represent the average value of RT activity in triplicate wells, and this experiment is representative of five independent experiments.

containing two integrated copies of provirus with *tat* mutations that maintain the virus in a state of postintegration latency (Emiliani et al., 1998). U1 cells have little baseline production of virus, but viral replication can be induced by PMA and inflammatory cytokines, such as TNF-α (Folks et al., 1987). Flow cytometric analysis of U1 cells revealed that these cells express CXCR3 on their cell surface (Fig. 2A). We found that, in contrast to treatment with PMA, treatment with IP-10 was unable to induce HIV-1 from latency in U1 cells (Fig. 2B). Similarly, while HIV replication can be induced in the chronically infected T cell line ACH2 by PMA and cytokines (Duh et al., 1989; Folks et al., 1989), we found that IP-10 had no effect (data not shown). Thus, unlike other cytokines, such as TNF-α and IL-1 (Duh et al., 1989), IP-10 does not exert its stimulatory effect on HIV-1 by enhancing LTR-mediated gene expression in these latent models of HIV-1 infection.

To determine whether IP-10 could induce gene expression in a model in which the Tat-TAR axis had not been disrupted, as it is in both the U1 and the ACH2 cell lines (Emiliani et al., 1996, 1998), we next studied the effect of IP-10 in Jurkat T cells. Jurkat cells previously have been shown to express CXCR3 (Loetscher et al., 1996). Jurkat cells were transfected with a CAT reporter gene under the control of the HIV-1 LTR (HIV-1 LTR-CAT) with or without a plasmid containing HIV-1 Tat under the control of the RSV promoter (RSV-Tat). After 16 h, Jurkat cells were treated with IP-10, treated with PMA as a positive control,

Table 1
Cellular viability is unaffected by treatment with IP-10

IP-10 (ng/ml)	MDM			PBL		
	Donor 1	Donor 2	Donor 3	Donor 1	Donor 2	Donor 3
0	0.108 ± 0.023	0.183 ± 0.016	0.264 ± 0.102	0.044 ± 0.001	0.062 ± 0.009	0.062 ± 0.016
5	0.088 ± 0.006	0.146 ± 0.023	0.344 ± 0.100	0.042 ± 0.004	0.050 ± 0.011	0.073 ± 0.005
25	0.125 ± 0.012	0.149 ± 0.022	0.305 ± 0.108	0.058 ± 0.031	0.065 ± 0.023	0.088 ± 0.012
125	0.115 ± 0.016	0.176 ± 0.028	0.258 ± 0.104	0.057 ± 0.037	0.042 ± 0.022	0.073 ± 0.023

Note. Cellular viability, proliferation, and activation were assessed by using an MTT-based colorimetric assay according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). The assay was performed on MDM 10 to 12 days after infection with HIV-1_{BaL} and on PBL 9 days after infection with HIV-1_{BRU}. Data shown are the average value and standard deviation of absorbance values (A_{570}–A_{650}) for triplicate wells for each of three donors. None of the differences were found to be significant by the Student's *t* test.

or subjected to no further treatment as a negative control. LTR-directed gene expression in the Jurkat cells was induced by both PMA and Tat, but IP-10 did not increase CAT activity in either the presence or the absence of HIV-1 Tat (Fig. 3A). We also examined the effect of IP-10 on 1G5 cells, a clonal cell line derived from Jurkat T cells that is stably transfected with the luciferase gene under the control of the HIV-1 LTR (HIV-1 LTR-luc). 1G5 cells have low basal luciferase activity, which can be induced 10- to 1000-fold by Tat and T cell activation signals (Aguilar-Cordova et al., 1994). Luciferase activity was stimulated by treatment of 1G5 cells with TNF-α or PMA, but not by treatment with IP-10 (Fig. 3B). Taken together, these experiments indicate that IP-10 does not stimulate HIV-1 LTR-mediated gene expression in the Jurkat T cell line.

To address whether IP-10 might affect HIV-1 gene expression in primary cells, we transiently transfected PBL with HIV-1 LTR-luc with or without RSV-Tat by an electroporation method. This method was determined to be effective for transfecting PBL, as luciferase activity was induced 100- to 1000-fold above background by PMA or HIV-1 Tat, which served as positive controls (Table 2). Following electroporation, PBL were separated into individual wells and treated with IP-10, PMA, or no treatment (Table 2). IP-10 did not have a consistent stimulatory effect on gene expression from the HIV-1 LTR in the absence of Tat and actually reduced Tat-mediated LTR activity 2.0- and 11.4-fold in the two donors (Table 2), making it unlikely that gene expression is responsible for the induction of HIV-1 replication seen in PBL. Taken together, these experiments suggest that the mechanism by which IP-10 stimulates HIV-1 replication is distinct from the mechanisms by which other cellular activation signals, such as TNF-α and PMA, have been shown to act.

IP-10 increases the accumulation of HIV-1 DNA in infected cells

To determine the step in the HIV-1 life cycle at which IP-10 acts, we next investigated the accumulation of the products of reverse transcription (RT) in cells infected with HIV-1 for less than 24 h. Primers to the U3 region and primer binding site (PBS) of the HIV-1 genome, and a probe recognizing the R/U5 region, were used in real-time quantitative polymerase chain reaction (PCR) reactions. This primer pair detects viral DNA sequences that are present after the second-strand transfer (Zack et al., 1990). Thus, this method allows us to assess whether the chemokine effect on HIV-1 replication takes place before the stage of viral transcription.

An increase in viral DNA was observed in MDM or PBL that had been pretreated with IP-10 for 24 h (Fig. 4). A similar increase was seen following treatment with GRO-α, which we have previously shown to also stimulate HIV-1 replication (Lane et al., 2001b). As we have observed previously with the effect of chemokine treatment on viral replication as determined by RT activity (Lane et al., 2001a,b), there was a good deal of donor-to-donor variability in the effect of IP-10 on the increase in the amount of viral DNA in infected cells. The range of increase in viral DNA following chemokine pretreatment was between 0.22- and 7600-fold (data not shown). However, the overall pattern clearly indicated that IP-10 augmented the amount of viral DNA detected following infection of MDM with HIV-1_{BaL} (five of seven donors) and in PBL infected by both HIV-1_{BaL} (three of four donors) and HIV-1_{BRU} (four of five). These data indicate that IP-10 stimulates HIV-1 replication largely by acting at a stage prior to viral transcription.

IP-10 decreases C-C chemokine production and has no effect on the surface expression of CD4, CCR5, or CXCR4

Several previous studies have demonstrated that the chemokines MIP-1α, MIP-1β, and RANTES can inhibit HIV entry into target cells (Cocchi et al., 1995; Coffey et al., 1997). We therefore examined whether IP-10 might be affecting viral entry by altering the expression of these inhibitory chemokines. Supernatants from PHA-activated PBL and MDM were collected after treatment with IP-10 and analyzed by ELISA for the presence of MIP-1α, MIP-1β, and RANTES. In experiments with five different donors' PBL, decreased amounts of chemokines were detected

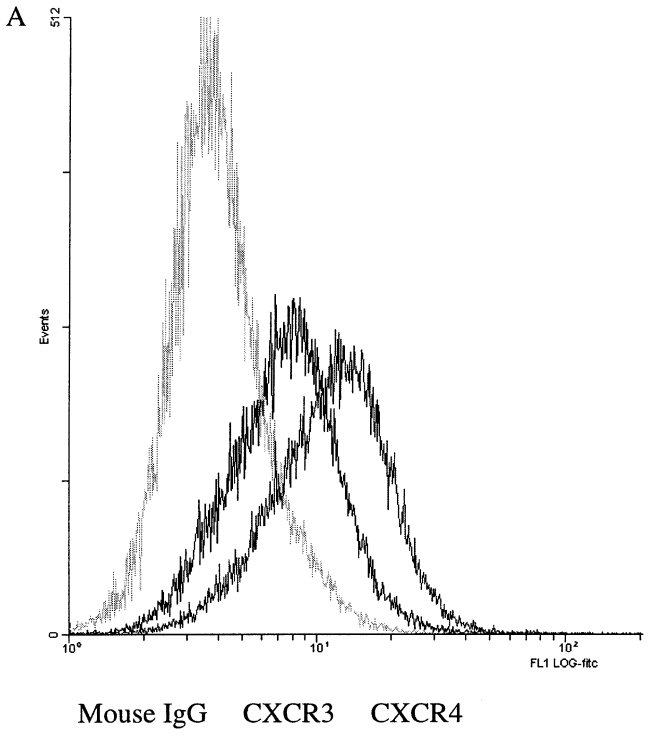

Fig. 2. IP-10 does not activate HIV-1 from latency in U1 cells. (A) The chronically infected monocytic cell line U1 expresses CXCR3. Surface receptors were analyzed by flow cytometric staining using a CXCR3-specific monoclonal antibody, a CXCR4-specific monoclonal antibody as a positive control, or mouse IgG as a negative control. Data shown are the overlaid histograms for these three antibodies. The *x*-axis indicates the FL1 signal intensity (log FITC) and the *y*-axis indicates the number of events. (B) IP-10 does not induce HIV-1 from latency in U1 cells. U1 cells were left untreated or treated with PMA (16 nM) or IP-10 (25 ng/ml). Supernatants were collected each day after infection for 4 days and analyzed for RT activity. Data shown are from triplicate wells, and this experiment is representative of three independent experiments.

24 h after treatment with IP-10 (25 ng/ml). Compared with untreated controls, MIP-1α levels were 89.9 $\pm$ 6.3%; MIP-1á levels were 79.9 $\pm$ 29.4%, and RANTES levels were 64.7 $\pm$ 24.6%. These decreases were small but statistically significant according to the Student's *t* test for MIP-1α ($P = 0.023$) and RANTES ($P = 0.032$), but can only be called a trend for MIP-1β ($P = 0.20$). Small decreases in MIP-1β and RANTES were also present in IP-10-treated PBL that had been infected with either HIV-1_{BRU} or HIV-1_{BaL} and cultured for up to 4 weeks. In three independent experiments, significant decreases in MIP-1α ($P < 0.05$) were found 4 weeks after infection with either HIV-1_{BRU} or HIV-1_{BaL}. The level of MIP-1α in HIV-1_{BRU}-infected PBL treated with IP-10 was 50.6 $\pm$ 20.0% of untreated, HIV-1_{BRU}-infected PBL. Similarly, the amount of MIP-1α in IP-10-treated, HIV-1_{BaL}-infected PBL was 67.3 $\pm$ 12.6% of that from the supernatants of untreated, HIV-1_{BaL}-infected PBL. In contrast to the results with PBL, IP-10 had no effect on MIP-1α, MIP-1β, and RANTES production by MDM (data not shown).

HIV entry can also be modulated by the pattern and levels of chemokine receptor expression (Kozak et al., 1997; Littman, 1998; Wu et al., 1997). The increase in the expression of the products of reverse transcription could be explained by a positive effect of IP-10 on HIV-1 receptor expression. We therefore examined whether treatment with IP-10 affected the surface expression of CD4, CCR5, or CXCR4 on peripheral blood monocytes (PBM) or PBL. We found that the mean fluorescent intensity of CCR5, CXCR4, and CD4 on PBL and PBM as measured by FACS analysis is not changed by a 24-h treatment with IP-10 (data not shown). In fact, the percentage of CCR5-expressing PBL following IP-10 treatment is decreased to 72 $\pm$ 11% of untreated controls ($n = 3$; $P = 0.05$). Taken together, these data indicate that the small decreases in the levels of MIP-1α, MIP-1β, and RANTES may be at least partly responsible for the effect of IP-10 on HIV-1 replication early in the viral life cycle, but that alterations in the level of expression of CD4, CCR5, or CXCR4 are unlikely to contribute.

Endogenous IP-10 and CXCR3 function to enhance HIV-1 replication in MDM

Having demonstrated that the addition of IP-10 stimulates HIV-1 replication in primary human cells, we next addressed whether endogenous CXCR3 and the CXCR3 ligands IP-10 and MIG are involved in HIV-1 replication as well. Examination of PHA-activated PBL following infection with HIV-1 revealed a small amount of IP-10 (<250 pg/ml) in the supernatants. MDM infected with viral stocks prepared from PHA-activated PBL produced between 0.1 and 10 ng/ml of IP-10, in a manner largely dependent on IFN-γ (data not shown). In contrast, MIG was undetectable in the supernatants of unexposed and HIV-1-exposed PBL and MDM (data not shown). Expression of I-TAC was not examined, as we are not aware of any antibodies that currently exist for use in ELISA for this chemokine.

Although IP-10 has been studied mainly as a T cell activator, it has also been shown to attract PBM (Luster and Leder, 1993; Luster et al., 1985; Taub et al., 1993, 1996). Even so, there has been some controversy in the literature

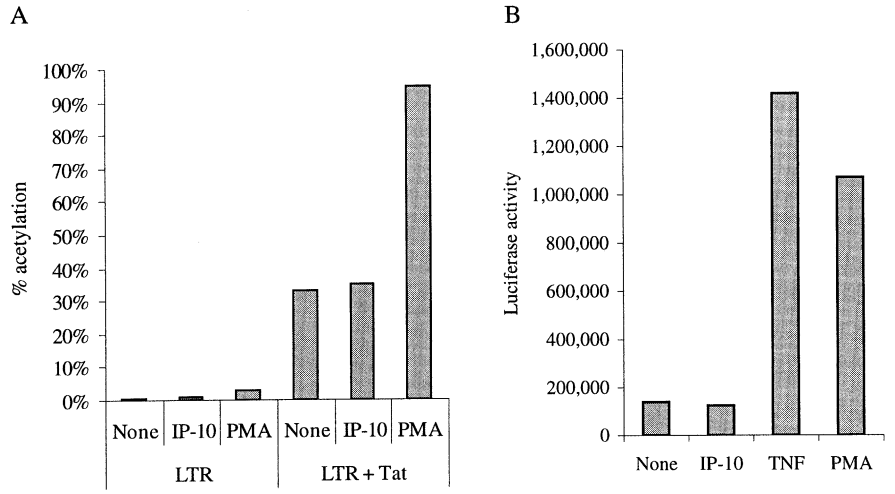

Fig. 3. HIV-1 gene expression is not altered by IP-10 treatment in Jurkat T cells. (A) Jurkat cells were transfected with HIV-1 LTR-CAT (10 mg), with or without RSV-Tat (1 μg). Transfected cells were then split into separate wells and stimulated 16 h later with IP-10 (25 ng/ml), PMA (16 nM), or no further treatment. CAT activity was evaluated 40 h after transfection. Data shown are representative of three independent experiments. (B) Jurkat cells stably expressing HIV-1 LTR-luciferase (1G5) were treated with IP-10 (50 ng/ml), TNF-α (50 ng/ml), or PMA (32 nM) for 48 h. Cell lysates were then analyzed for luciferase activity. Data shown are the average of duplicate measurements and are representative of three independent experiments.

about whether PBM express the IP-10 receptor CXCR3 (Loetscher et al., 1996). To determine whether endogenous IP-10 (or MIG or I-TAC) might act through CXCR3 to affect viral replication directly, we examined the expression of CXCR3 in PBL and PBM by flow cytometry. We found CXCR3 to be highly expressed on the cell surface of both PBL and PBM (data not shown). Thus, we conclude that signaling through CXCR3 has the potential to affect viral replication in each of these cell types.

To assess whether endogenous IP-10 can stimulate HIV-1 replication, as is the case with IL-8 and GRO-α (Lane et al., 2001a,b), we examined the effects of antibodies that neutralize IP-10 and CXCR3 function on HIV-1 replication. An IP-10-specific monoclonal antibody and a CXCR3-blocking antibody have previously been shown to prevent the migration of T cells in response to IP-10 present in cellular supernatants or added exogenously (Albanesi et al., 2000; Jinquan et al., 2000). We used these same antibodies to neutralize the activity of IP-10 in cultures of HIV-1-infected PBL and MDM. Viral replication in both PBL and MDM was inhibited by both anti-IP-10 and anti-CXCR3 antibodies (Fig. 5). In PBL, anti-IP-10 had less of an effect than that seen with anti-CXCR3 (Fig. 5A). This may indicate that the other CXCR3 ligands are contributing to HIV-1 replication in PBL, in agreement with our finding that MIG and I-TAC also stimulate HIV-1 replication in these same cells. In contrast, in HIV-1-infected MDM, in which IP-10 is the dominant CXCR3 ligand present in culture, treatment with either anti-IP-10 or anti-CXCR3 caused a significant reduction in HIV-1 replication (Figs. 5B and 5C). Neither antibody substantially affected cellular viability as determined by an MTT-based assay. Viability of MDM treated with anti-IP-10 was 89%, and with anti-CXCR3 was 84%, of untreated control cells in these six experiments. We therefore conclude that endogenous IP-10 and CXCR3 play a stimulatory role in regard to HIV-1 replication in PBL and MDM.

Table 2
IP-10 does not induce HIV-1-LTR-driven gene expression in PBL

Treatment	Donor 1	Donor 2
None	2,699,360	222,176
PMA	45,587,392	14,842,304
IP-10	601,312	283,040
HIV-1 Tat	17,852,768	3,062,944
HIV-1 Tat + IP-10	9,080,160	269,336

Note. PBL were transfected with HIV-1 LTR-luc (20 μg) with or without RSV-Tat (2 μg) by electroporation. Transfected PBL were then split into separate wells and either left untreated, treated with IP-10 (50 ng/ml), or stimulated with PMA (32 nM). Luciferase activity present in cellular lysates from two separate donors was measured 48 h after transfection. Data are shown as counts per minute following subtraction of background chemiluminescence.

Discussion

Cytokines play major roles in the pathogenesis of HIV disease (Hogan and Hammer, 2001; Kinter et al., 2000; Lahdevirta et al., 1988; Poli and Fauci, 1995). Chemokines, in particular, have been shown to inhibit viral replication by nature of their ability to compete with HIV-1 for binding to its coreceptors, but also have stimulatory effects on viral replication in certain settings. As evidence now indicates that the ligands for the C-X-C chemokine receptors CXCR1, CXCR2, and CXCR4 can all affect HIV-1 replication (Bleul et al., 1996; Lane et al., 2001a,b; Marechal et

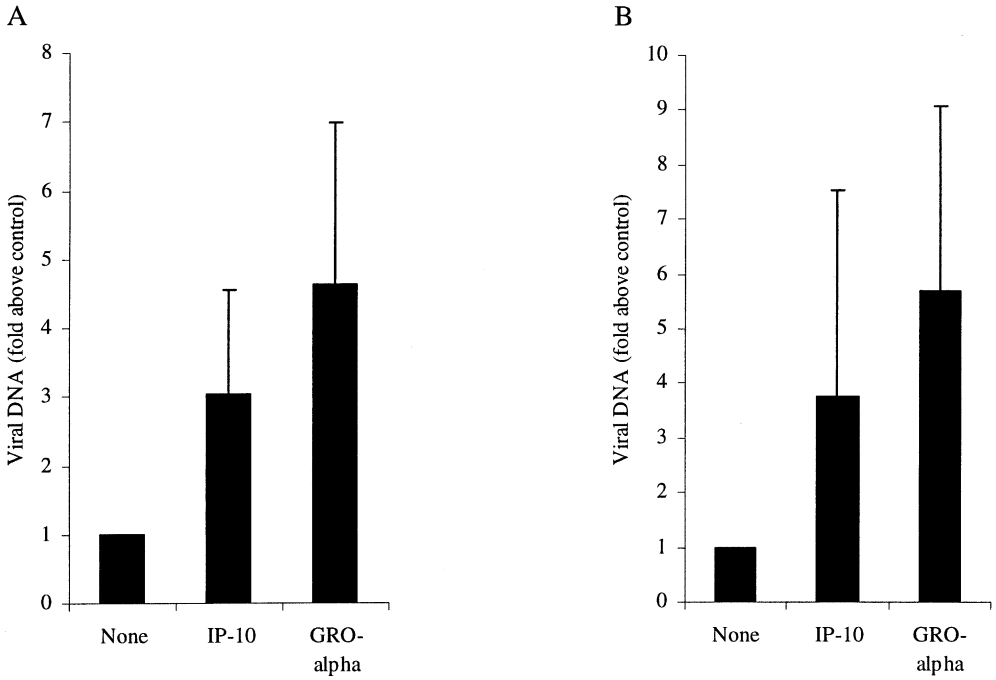

Fig. 4. IP-10 and GRO-α increase the accumulation of viral DNA in MDM and PBL. (A) MDM were treated with IP-10 or GRO-α (each at 25 ng/ml) for 24 h prior to infection with HIV-1_{BaL}. (B) Similarly, PHA-activated PBL were treated with IP-10 or GRO-α (each at 25 ng/ml) for 24 h prior to infection with HIV-1_{BRU}. Cells were harvested at less than 24 h after infection and cell lysates were used in real-time quantitative PCR as described under Materials and Methods. In these experiments, 10 to 1000 copies of viral DNA per 100,000 cells were detected in HIV-1-infected cells, while noninfected cells routinely expressed <1 copy of viral DNA per 100,000 cells. Data shown are the fold increase ($\pm$ standard deviation) in viral DNA expression relative to the untreated, HIV-1-infected cells. Shown in both panels are the mean values for all experiments in each of the two categories. IP-10 and GRO-α stimulated viral DNA accumulation in five of seven experiments with HIV-1_{BaL}-infected MDM, three of four experiments with HIV-1_{BRU}-infected PBL, and three of four experiments with HIV-1_{BaL}-infected PBL (not shown). The mean fold increases for IP-10 were 3.04, 3.75, and 13.6 for each of these experimental conditions, respectively, and mean fold increases for GRO-α were 4.62, 5.68, and 2.13.

al., 1999; Oberlin et al., 1996), we evaluated the ability of CXCR3 and its ligands to regulate HIV-1 replication.

While CXCR3 appears to function mainly on activated T cells, several reports have indicated that IP-10 also attracts PBM (Luster and Leder, 1993; Luster et al., 1985; Taub et al., 1993, 1996). We have found expression of CXCR3 on PBM as well as PBL and demonstrate here that IP-10 stimulates HIV-1 replication in both PBL and MDM and appears to act at an early step in the viral life cycle. Treatment with an IP-10-specific monoclonal antibody or a blocking antibody to CXCR3 reduces HIV-1 replication in MDM and PBL, demonstrating that endogenous IP-10 and CXCR3 can stimulate HIV-1 replication in these primary human cells.

We demonstrate here that IP-10, MIG, and I-TAC each can stimulate HIV-1 replication in PBL and have chosen to focus on IP-10 because of its greater relevance to HIV disease in vivo. IP-10 is active in the range of 5 to 125 ng/ml, which is likely to be physiologically relevant, as CSF levels of IP-10 up to 40 ng/ml have been detected in HIV-infected individuals (Stylianou et al., 2000). While IP-10 levels in the plasma reach only 0.5 ng/ml in HIV-infected individuals (Kolb et al., 1999), the amount of chemokine available in the tissues where viral replication occurs is likely to be much higher. PBMC and MDM have been shown to produce up to 50 ng/ml of IP-10 in culture (Poluektova et al., 2001; Wetzel et al., 2002). We found that MDM produced significant amounts of IP-10, up to 10 ng/ml, in a manner dependent on IFN-γ. We found lower levels of IP-10 in PBL cultures and no detectable MIG. The fact that anti-IP-10 had some effect in PBL, and anti-CXCR3 had a greater effect, suggests that endogenous I-TAC may also be involved in the stimulation of HIV-1 replication in PBL. IP-10 has previously been implicated as a downstream effector of IFN-mediated activity against recombinant vaccinia viruses (Mahalingam et al., 1999). As IP-10 production is induced by IFN-γ, IP-10 might act as a mediator of some of the pathogenic actions ascribed to IFN-γ in the context of HIV-1 infection (Cohen et al., 1997). IFN-γ has long been known to inhibit HIV-1 replication in MDM and does so via decreasing CD4 expression and viral entry (Dhawan et al., 1994, 1995). However, IFN-γ has also been shown to enhance the expression of several chemokine receptors, including CCR5, on monocytic cell lines (Zella et al., 1998). In any case, the inhibitory effects of IFN-γ on HIV-1 replication are not mediated by IP-10, which we show in this article to increase viral replication.

Several recent studies have shown that IP-10 plays a significant role in the immune response to viral and proto-

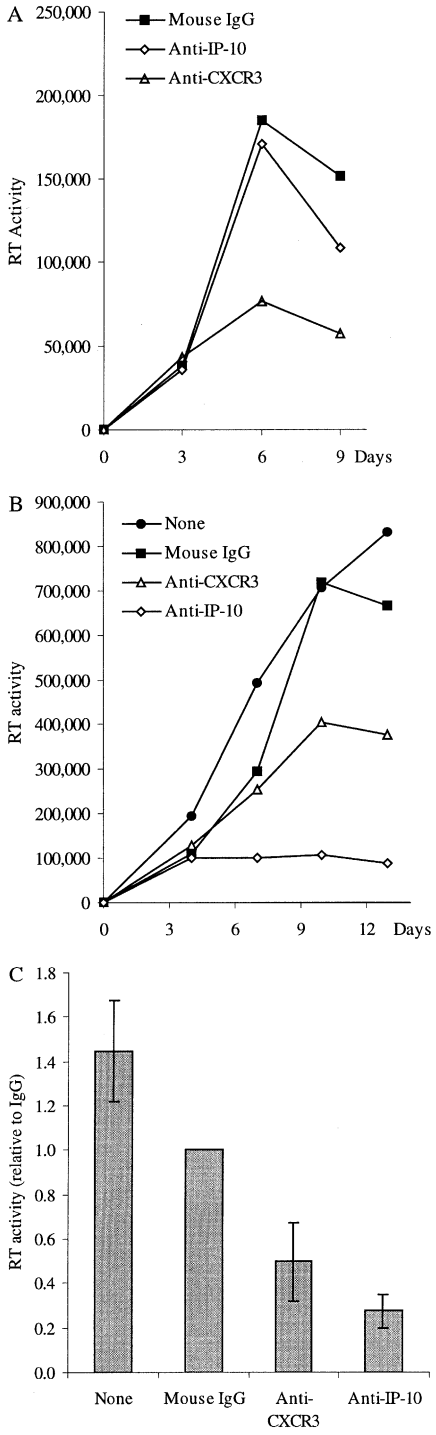

Fig. 5. Depletion of endogenous IP-10 or blocking CXCR3 inhibits HIV-1 replication in PBL and MDM. Cells were left untreated or treated with 20 mg/ml of either an isotype-matched control antibody (mouse IgG_1), an antibody that prevents interaction with CXCR3 (anti-CXCR3), or an antibody that neutralizes IP-10 activity (anti-IP-10) every 3 days, beginning 1 day before infection. Media was collected for the RT assay and replenished twice weekly. (A) PBL were infected with HIV-1_{BRU}. Data shown represent the average value of RT activity present in triplicate wells on days 3, 6, and 9 after infection. This experiment is representative of at least three experiments with each antibody. (B) MDM were infected with HIV-1_{BaL}. The average values of RT activity from triplicate wells of a representative experiment are shown over the course of infection. (C) RT data from six independent experiments in which MDM were infected with HIV-1_{BaL} are presented. Data shown are normalized to the mouse IgG_1 control on the day of peak viral replication (mean ± SEM). A significant reduction in HIV-1 replication relative to the no treatment and IgG control groups as determined by the Student's *t* test was observed for treatment with anti-IP-10 ($P = 0.005$, $P < 0.001$), and for treatment with anti-CXCR3 ($P = 0.016$, $P = 0.035$).

zoan infection (Amichay et al., 1996; Khan et al., 2000; Mahalingam et al., 1999; Ramshaw et al., 1997). One report indicates that IP-10 and MIG may participate in the host defense against HIV-1 as well, as these CXCR3 ligands contribute to the accumulation of HIV-specific cytotoxic T lymphocytes in the lung (Agostini et al., 2000). IP-10 may thus participate in the T cell mediated immune response to HIV-1. Ultimately, these T cell mediated responses do not usually control the spread of HIV-1, and so this action of IP-10 may be less important than the HIV-enhancing role we describe in this article. In addition, persistently elevated IP-10 expression has been correlated with immunological treatment failure following HAART (Stylianou et al., 2000), so reducing IP-10 expression and function may enhance the effectiveness of current anti-retroviral treatments.

Several lines of evidence suggest that treatments aimed at inhibiting IP-10 function may have therapeutic benefit in the context of HIV-1 infection. First, inhibition of IP-10 activity may decrease HIV-1 replication. We demonstrate here that exogenous IP-10 stimulates HIV-1 replication in PBL and MDM and that endogenous IP-10/CXCR3 signaling contributes to HIV-1 replication in these same cells. The mechanism by which IP-10 stimulates HIV-1 replication appears to be distinct from the mechanisms employed by other stimulatory factors, including TNF-α and SDF-1α. TNF-α is involved in a paracrine loop in which HIV-1 binding to CD4 induces TNF-α production, and TNF-α stimulates HIV-1 gene expression by an NF-κB-dependent pathway (reviewed in Poli and Fauci, 1995). SDF-1α has been shown to increase proviral gene expression by stimulating the ability of Tat to transactivate the LTR (Marechal et al., 1999). We have found that IP-10 does not affect HIV-1 LTR-mediated gene expression, but rather increases the accumulation of the products of reverse transcription in both infected T cells and macrophages. Thus, IP-10 must act at a step in the viral life cycle prior to viral transcription, such as reverse transcription or, more likely, viral entry. IP-10 decreases the production of MIP-1α, MIP-1β, and RANTES, which may be at least partially responsible for the increase in viral replication seen following treatment with IP-10.

The chemokines RANTES, MIP-1α, MIP-1β, MCP-2, and SDF-1α all have been shown previously to inhibit viral entry by interfering with CCR5 or CXCR4 coreceptor function (Bleul et al., 1996; Cocchi et al., 1995; Gong et al., 1998; Oberlin et al., 1996). As these chemokines exert strong effects on viral entry, we hypothesized that IP-10 might also act on viral entry, perhaps by inducing changes

in the expression of these chemokines or their receptors. Our findings indicate that IP-10 reduces the amount of MIP-1α, MIP-1β, and RANTES produced by activated PBL. We found no significant change in surface expression of CD4, CCR5, or CXCR4 in PBL or PBM, with only a slight decrease in the percentage of CCR5-expressing PBL. Thus, the effect of IP-10 on HIV-1 replication appears to be mediated at least in part by decreases in the expression of chemokines known to inhibit HIV-1 entry, but not by changes in receptor expression.

IP-10 plays a role in HIV-associated brain disease. IP-10 and CXCR3 have been detected in the brain and CSF of HIV-1-infected individuals (Kolb et al., 1999; Sanders et al., 1998), and IP-10 is produced by astrocytes treated with HIV-1 Tat and gp120 (Asensio et al., 2001; Kutsch et al., 2000). Further, as cells of the monocytic lineage are the major cell type infected with HIV-1 in the brain (Gartner, 2000; Gartner et al., 1986), monocytic cells produce IP-10, and IP-10 stimulates HIV-1 replication within these cells, inhibition of IP-10 activity may limit the brain pathology associated with AIDS.

IP-10 is a potent chemotactic factor for both activated T lymphocytes and PBM (Luster and Leder, 1993; Luster et al., 1985; Taub et al., 1993, 1996). Preventing chemotaxis of these cell types with inhibitors of IP-10 and CXCR3 function may reduce the spread of HIV-1 to uninfected target cells (Swingler et al., 1999). Further, since IP-10 has been demonstrated to function as the major T cell chemotactic factor in the brain during HIV encephalopathy, reducing IP-10 activity may control T cell migration into the CNS (Kolb et al., 1999).

In summary, we have demonstrated that IP-10 stimulates HIV-1 replication by enhancing early steps in the viral life cycle, likely including viral entry. We have also shown that neutralization of endogenous IP-10 or blocking CXCR3 reduces HIV-1 replication in MDM and PBL. As IP-10 is physiologically active in HIV-infected individuals both systemically and in the CNS, interventions targeting IP-10 or its receptor, CXCR3, may thus decrease HIV-1 replication and the pathogenic effects seen with HIV-1 infection and HIV-1-associated neurological disease.

Materials and Methods

Reagents

Recombinant human IP-10, MIG, and I-TAC, and monoclonal antibodies to human IP-10 (MAB266), were obtained from R&D Systems (Minneapolis, MN) and added as indicated in the figure legends. Cellular proliferation, viability, and activation were assayed by using an MTT-based colorimetric assay according to the manufacturer's instructions (Boehringer Mannheim, Mannheim, Germany). All other chemicals were obtained from Sigma (St. Louis, MO).

Cell lines

The latently HIV-1-infected subclone of U937 promonocytic cells (U1), the latently HIV-1-infected lymphocytic cell line ACH2, and a Jurkat cell line which contains a stably integrated HIV-1 LTR-luciferase construct (1G5) were obtained through the NIH AIDS Research and Reference Reagent Program. U1, ACH2, 1G5, and Jurkat cells were each cultured in RPMI 1640 supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete RPMI).

Isolation and preparation of PBMC, PBL, and PBM

Peripheral blood mononuclear cells were collected by venipuncture of healthy volunteers as described previously (Lane et al., 1999). PBMC contained approximately 20% $CD14^+$ monocytes as determined by flow cytometry. To separate the PBMC into subpopulations composed mainly of monocytes or lymphocytes, PBMC were subjected to a plate adherence step for 2 h. Adherent cells were consistently >90% peripheral blood monocytes as determined by Diff-Quik analysis and >85% $CD14^+$ by flow cytometric staining with a PE-conjugated mouse anti-human monoclonal antibody to CD14 (M5E2; PharMingen, San Diego, CA), as well as >99% viable as determined by trypan blue exclusion. PBM were differentiated into monocyte-derived macrophages by culture in DMEM supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete DMEM) for up to 2 weeks (3 days in most experiments) prior to infection.

The nonadherent cells following the plate adherence step were enriched for lymphocytes and contained less than 2% $CD14^+$ monocytes. These monocyte-depleted PBMC (PBL) were cultured at 1-2 $\times$ 10^6/ml in complete RPMI. PBL were stimulated with 5 μg/ml of phytohemagglutinin (PHA; Sigma) for 1 to 3 days and then maintained in IL-2 (40 U/ml; Hoffmann-La Roche, Nutley, NJ). In some experiments, PBL were also depleted of $CD8^+$ cells with magnetic Dynabeads M-450 CD8 according to the manufacturer's instructions (Dynal, Lake Success, NY).

Preparation of HIV-1 stocks

All of the HIV-1 isolates and infected cell lines used in this article were originally obtained from the NIH AIDS Research and Reference Reagent Program. Stocks of HIV-1_{BaL} were prepared by both infection of PHA-activated, CD8-depleted PBL and infection of HOS-CD4-CCR5 cells. Stocks of HIV-1_{BRU} were prepared by infection of both PHA-activated, CD8-depleted PBL and CEM-SS cells.

HIV-1 infection of MDM and PBL

For each experiment, multiple wells of MDM or PBL were infected with equal RT counts of HIV-1 (30-300 $\times$

10^6 CPM of RT used per 10^5 cells). This amount of CPM of RT activity per cell corresponds to a multiplicity of infection (m.o.i.) of between 0.01 and 0.1 as determined by titration on HOS-CD4-CCR5 and CEM-SS cell lines and quantitation of proviral DNA in PBMC 24 h after infection (data not shown). MDM were infected with HIV-1_{BaL} for 16 h, washed, and then cultured in complete DMEM. PBL were infected with HIV-1_{BRU} for 4 h, washed, and then incubated in complete RPMI + IL-2. A portion of the media (25%) was removed from the MDM and PBL cultures and replaced twice weekly.

Reverse transcriptase assay

HIV-1 replication was determined by quantification of the amount of RT activity present in the cellular supernatants at several points in time after infection using a poly(A)-oligo(dT) template primer as previously described (Potts et al., 1990). RT activity was assayed using ^{32}P-labeled deoxythymidine triphosphate incorporated in DNA bound to DE81 paper (Whatman) and quantified using a Series 400 PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA). RT activity is reported as PhosphorImager counts. The absolute values varied between experiments, but peak activity consistently occurred 4 to 7 days after infection of PBL and 7 to 14 days after infection of MDM.

Transfection of Jurkat cells

Jurkat cells were transfected by the DEAE-dextran method with 1 μg HIV-1 Tat (pCG-Tat1) or control vector (pCG) and 10 μg of a plasmid containing the chloramphenicol acetyl transferase (CAT) reporter gene downstream of the complete HIV-1 LTR. The cells were either left untreated or treated with IP-10 (25 ng/ml) or PMA (16 nM) at 16 h after transfection and harvested 40 h after transfection. CAT activity was quantified and normalized for amount of protein. Bars indicate the percentage conversion of chloramphenicol to its acetylated form. Approximately 7000 cpm of radioactivity were present in each sample.

Transfection of PBL

PBL were cultured in RPMI 1640 supplemented with 20% FBS, 1 μg/ml PHA, 100 U/ml IL-2, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin for 3 days. PBL were washed in serum-free media and 12×10^6 cells were transfected with 20 μg of a luciferase reporter gene under the control of the HIV-1 LTR (HIV-1 LTR-luc), and in some instances along with 2 μg HIV-1 Tat, in a volume of 400 μl. Cells were electroporated at 350 V in an Invitrogen (Carlsbad, CA) Electroporator II. Transfected cells were then split into separate wells (4×10^6/well) and either left untreated, stimulated with IP-10 (50 ng/ml), or stimulated with PMA (32 nM). Cells were harvested at 48 h after transfection. Cell lysates were collected and analyzed for luciferase activity according to the manufacturer's instructions (Promega, Madison, WI). Transfection yielded chemiluminescence readings 10- to 1000-fold above background, which was 25,184 and 20,576 in the two experiments performed.

ELISA

Cellular supernatants were collected after 19 to 48 h and stored at −70°C until analysis. Extracellular immunoreactive IP-10, MIG, MIP-1α, MIP-1β, and RANTES were measured using a sandwich-type immunoassay (ELISA) with capture and biotinylated detection antibodies according to the manufacturer's instructions (R&D Systems). The lower limits of detection for these assays were between 13 and 32 pg/ml.

Flow cytometry

Cell staining was performed using monoclonal antibodies to the human chemokine receptors CXCR2 (MAB331; R&D Systems), CXCR3 (MAB160; R&D Systems), CCR5 (2D7; LeukoSite, Cambridge, MA), or CXCR4 (12G5; PharMingen). PBMC or U1 cells were incubated in flow buffer (HBSS + 2% FBS + 0.05% sodium azide) with primary antibody for 30 min at 4°C. Cells were then washed with flow buffer and incubated in flow buffer with the secondary antibody (Biotin-conjugated goat anti-mouse IgG; Jackson ImmunoResearch Laboratories) for 30 min at 4°C. Cells were then washed with flow buffer and incubated in flow buffer with the staining reagent (fluorescein (DTAF)-conjugated Streptavidin; Jackson ImmunoResearch Laboratories) for 30 min at 4°C. In some experiments, cells were also stained with CyChrome-conjugated anti-human CD4 (PharMingen) during the incubation with DTAF-streptavidin. Background staining was determined by adding an isotype-matched control antibody (mouse IgG) during the first incubation. Cells were either analyzed immediately or fixed in PBS + 2% paraformaldehyde prior to analysis. Cell staining analysis was performed using an XL Z14107 cytometer. The PBL and PBM subpopulations were gated according to the pattern of forward scatter and side scatter and then analyzed for signal intensity in FL1. The viable population of U1 cells was gated and analyzed for FL1 signal intensity.

Real-time quantitative PCR for viral DNA

PBMC were collected by Ficoll–Hypaque density gradient centrifugation. PBM were adhered to plastic for 2 h, and nonadherent cells (PBL) were collected. PBL were then stimulated with PHA (5 μg/ml) in complete RPMI for 2 days. PBL were washed and then cultured in complete RPMI with IL-2 (40 U/ml) for two to four more days. PBL (2×10^6/ml) were then treated overnight with the following

chemokines at 25 ng/ml: IP-10, GRO-α, or none. Equal RT counts of HIV-1_{BRU} or HIV-1_{BaL} were added to PBL and cells were spin-infected for 3 h at 2500 rpm. The media were then replaced with fresh media containing the appropriate chemokine and IL-2. The next day, PBL were counted and serial dilutions of PBL (1 to 100,000 cells) were prepared. As a negative control, serial dilutions of uninfected PBL were also collected. (These latter cells routinely contained 0 copies of viral DNA.) PBL were then centrifuged, washed two times with PBS, and resuspended in lysis solution. The samples were then incubated for 16 h at 56°C and 4 h at 95°C and stored at −20°C until later use.

MDM were collected as described above and treated overnight with IP-10, GRO-α, or no chemokine prior to infection with HIV-1_{BaL} for 1 day. MDM were then treated again with the appropriate chemokine, harvested, counted, and lysed in the same manner as described for PBL.

The following primers and probe were used for real-time quantitative PCR (numbering of nucleotide positions corresponds to that for the HIV-1_{BRU} DNA sequence): for LTR U3/PBS: antisense primer (5′-CTT TCG CTT TCA AGT CCC TGT T-3′; nucleotides 666-645 in the primer binding site), sense primer (5′-CCC TCA GAT GCT GCA TAT AAG CA-3′; nucleotides 9487 to 9509 in U3); probe, 5′-FAM-CTC TCT GGT TAG ACC AGA TTT GAG CCT GG-TAMRA-3′; nucleotides 458 to 486 in U5). PCR reaction mixtures (50 μl volume) were set up containing 5 μl of lysate, 5 mM $MgCl_2$, 18 nM forward and reverse primer, 20 nM probe, 200 μM of each deoxynucleotide triphosphate, AmpErase UNG (0.5 μl), and AmpliTaq DNA polymerase (0.25 μl). DNA was amplified as follows: 50°C for 2 min, 95°C for 10 min (1 cycle), 95°C for 15 s, 59°C for 1 min (45-50 cycles), and 60°C for 1 min (1 cycle). The reaction was performed with an ABI PRISM 7700 Sequence Detection System (PE Applied Biosystems, Foster City, CA), in which the amount of PCR product can be assessed after each cycle. The number of copies of viral DNA was quantitated by comparing unknowns to the data obtained with a series of dilutions of a sample with known copy number (U1 or ACH2 cells) using Sequence Detector v1.6.3 software (PE Applied Biosystems).

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