JOURNAL OF VIROLOGY, Feb. 2003, p. 2550–2558 Vol. 77, No. 4
0022-538X/03/$08.00+0 DOI: 10.1128/JVI.77.4.2550–2558.2003

G Protein-Dependent CCR5 Signaling Is Not Required for Efficient Infection of Primary T Lymphocytes and Macrophages by R5 Human Immunodeficiency Virus Type 1 Isolates

Ali Amara,[1]* Aurore Vidy,[1] Genevieve Boulla,[2] Karine Mollier,[3] Javier Garcia-Perez,[4] Jose Alcamí,[4] Cedric Blanpain,[5] Marc Parmentier,[5] Jean-Louis Virelizier,[1] Pierre Charneau,[3] and Fernando Arenzana-Seisdedos[1]

Unité d'Immunologie Virale[1] and Groupe de Virologie Moléculaire et Vectorologie,[3] Institut Pasteur, and CNRS UPR415,[2] Paris, France; AIDS Immunopathogenesis Unit, Centro Nacional de Microbiologia, Madrid, Spain[4]; and Institute of Interdisciplinary Research, Université Libre de Bruxelles, Brussels, Belgium[5]

Received 24 July 2002/Accepted 12 November 2002

The requirement of human immunodeficiency virus (HIV)-induced CCR5 activation for infection by R5 HIV type 1 (HIV-1) strains remains controversial. Ectopic CCR5 expression in $CD4^+$-transformed cells or pharmacological inhibition of $G_\alpha i$ proteins coupled to CCR5 left unsolved whether CCR5-dependent cell activation is necessary for the HIV life cycle. In this study, we investigated the role played by HIV-induced CCR5-dependent cell signaling during infection of primary CD4-expressing leukocytes. Using lentiviral vectors, we restored CCR5 expression in T lymphocytes and macrophages from individuals carrying the homozygous 32-bp deletion of the CCR5 gene (*ccr5* Δ*32*/Δ*32*). Expression of wild-type (wt) CCR5 in *ccr5* Δ*32*/Δ*32* cells permitted infection by R5 HIV isolates. We assessed the capacity of a CCR5 derivative carrying a mutated DRY motif (CCR5-R126N) in the second intracellular loop to work as an HIV-1 coreceptor. The R126N mutation is known to disable G protein coupling and agonist-induced signal transduction through CCR5 and other G protein-coupled receptors. Despite its inability to promote either intracellular calcium mobilization or cell chemotaxis, the inactive CCR5-R126N mutant provided full coreceptor function to several R5 HIV-1 isolates in primary cells as efficiently as wt CCR5. We conclude that in a primary, CCR5-reconstituted $CD4^+$ cell environment, G protein signaling is dispensable for R5 HIV-1 isolates to actively infect primary $CD4^+$ T lymphocytes or macrophages.

Human immunodeficiency virus (HIV) entry into host cells requires the interaction of the gp120 viral envelope (Env) glycoprotein subunit with CD4 plus a chemokine receptor that acts as a coreceptor (8). Conformational changes induced within the HIV Env gp120 surface subunit, upon binding to CD4, lead to the creation of a new coreceptor recognition site on gp120. Subsequent interaction between gp120 and the coreceptor is thought to activate the HIV Env glycoprotein transmembrane subunit gp41, leading to fusion between viral and target cell membranes, resulting in viral entry (8). CXCR4 and CCR5 are, respectively, the principal coreceptors involved in entry of the X4 and R5 HIV type 1 (HIV-1) strains into primary cells (8). The importance of CCR5 for HIV-1 transmission is highlighted by the fact that individuals carrying the 32-bp deletion in the CCR5 gene are highly resistant to HIV infection (32). In vitro, CD4 T cells and macrophages isolated from these individuals lack CCR5 expression and are selectively refractory to infection by R5 HIV-1 isolates (31).

CXCR4 and CCR5 belong to the family of G protein coupled receptors (GPCR), which transduce signals via heterotrimeric G protein (8). Engagement of these coreceptors by HIV Env gp120 during viral entry initiates G protein-dependent signal transduction pathways which lead to calcium mobilization, activation of the tyrosine kinase pyk2 and FAK, and cell chemotaxis (11, 14, 25, 30, 37). Such signals may provide a link between HIV binding to the coreceptors and the regulation of cell functions (e.g., growth, survival, or differentiation) and perhaps even cytoskeletal modifications that may facilitate further virus entry and subsequent propagation to uninfected cells (10, 19). Indeed, the requirement of CCR5 and CXCR4 for HIV infection raises the question of whether their role as viral coreceptors is merely passive or, in contrast, actively involves cell signaling stimuli that may influence virus entry and early, postfusion events of viral replication. To date, a number of studies have suggested that coreceptor-mediated signaling is not essential for HIV entry into target cells. Indeed, blockade of the $G_\alpha i$ subunit of heterotrimeric G proteins by pertussis toxin (PTX), truncation of the CCR5 intracytoplasmic C-terminal tail, or mutation of the highly conserved Asp-Arg-Tyr (DRY) motif in the second intracellular loop required for agonist-induced CCR5 activation does not alter the capacity of CCR5 to support entry of R5 HIV-1 isolates (2, 5, 12, 15, 17). Similar mutations in CXCR4 also fail to affect its coreceptor capacity in vitro (3, 22, 35). Together, these findings, which were obtained in transformed cell lines, lead to the concept that coreceptor-mediated signaling initiated by HIV is not required for viral entry into host cells. However, indirect evidence opposing these conclusions has suggested that corecep-

* Corresponding author. Mailing address: Unité d'Immunologie Virale, Institut Pasteur, F-75724 Paris Cedex 15, France. Phone: 33 (0)1 40 61 31 97. Fax: 33 (0)1 45 68 89 41. E-mail: aamara@pasteur.fr.

tor-mediated signaling may indeed modulate HIV infection in primary cells. Inhibition of F-actin polymerization by cytochalasin D prevents HIV entry into activated peripheral blood mononuclear cells (PBMCs) (19). Moreover, PTX- or protein kinase C-dependent desensitization of CCR5 prevents cell entry of R5 HIV-1 isolates into primary, activated T lymphocytes without disturbing CCR5 or CD4 cell surface expression (1, 18). Finally, in macrophages, the capacity of R5 HIV-1 to replicate correlates with the ability of the virus to signal through CCR5, leading to the hypothesis that coreceptor activation determines the outcome of HIV replication in these cells (6). Thus, controversy regarding the role of coreceptor-mediated signaling in HIV-cell fusion and early postentry events persists. The divergent conclusions raised by the authors cited above may be accounted for by the disparate origin, activation, and differentiation statuses of the cells used. Alternatively, they could be due to the use of drugs (PTX, oligomer B, cytochalasin, etc.) with wide-ranging and often unpredictable effects on the target cells.

In this study, we used a lentivirus-mediated gene transfer strategy to introduce wild-type (wt) or signaling-deficient CCR5 molecules into primary T cells and macrophages from *ccr5* Δ*32*/Δ*32* individuals in order to investigate the contribution of G protein signaling to HIV replication.

MATERIALS AND METHODS

DNA constructs. The wt CCR5 pcDNA3 vector (Invitrogen) was a gift from B. Moser (Theodor Koher, Bern, Switzerland). The CCR5-R126N mutant was constructed by site-directed mutagenesis (Quickchange kit; Stratagene, La Jolla, Calif.) and inserted into a pcDNA3 vector. This CCR5 mutant receptor carries a mutation affecting the highly conserved DRY motif located in the second, intracellular loop of CCR5. TRIP ΔU3 is an HIV-1-derived vector that was modified to include a 178-bp HIV-1 DNA fragment encompassing the central polypurine tract, the central termination *cis*-active sequence, the enhanced green fluorescent protein gene downstream of the cytomegalovirus (CMV) promoter, and a 400-bp deletion in the HIV-1 long terminal repeat U3 enhancer region (13). TRIP ΔU3-CMV vectors encoding CCR5 cDNAs were generated by replacing the *Bam*HI-*Xho*I fragment encoding EGFP with a PCR-generated *Bam*HI-*Xho*I fragment carrying either wt CCR5 or CCR5-R126N nucleotide sequence. All PCR products were sequenced using the dideoxy method.

Lentivirus vector production. 293T cells were maintained in Dulbecco modified Eagle medium (Life Technologies) supplemented with 10% fetal calf serum (FCS), 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Stocks of TRIP ΔU3-CCR5 infectious particles were prepared in 293T cells by transient cotransfection of the corresponding CCR5-expressing vector; an encapsidation plasmid lacking *vif*, *vpr*, *vpu*, and *nef* accessory HIV-1 genes (40); and a vesicular stomatitis virus G protein (VSV-G) Env expression plasmid (pHCMV-G) (39) as previously described (27). Culture supernatants were collected at 48 h after transfection and cleared of cellular debris by low-speed centrifugation. The amount of VSV-G-pseudotyped particles was evaluated by measuring HIV-1 Gag p24 with an antigen capture enzyme-linked immunosorbent assay (NEN, Life Science). Aliquots of vector stocks were stored at −80°C until needed.

Lentiviral transduction. The lymphoblastoid T-cell line MT4 was maintained in RPMI 1640 medium supplemented with 10% FCS, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. PBMCs from *ccr5* Δ*32*/Δ*32* individuals were isolated by centrifugation through a Ficoll-Hypaque gradient (Sigma Aldrich, St. Louis, Mo.) and activated for 2 days with 3 μg of phytohemagglutinin (PHA) per ml before transduction with lentiviral expression vectors. In some experiments, *ccr5* Δ*32*/Δ*32* PBMCs were depleted of monocytes and cultured for 5 days in complete medium supplemented with 10 ng of interleukin-2 (IL-2) and IL-15 (Peprotech Inc.) per ml. MT4 or *ccr5* Δ*32*/Δ*32* cells (10^6) were then incubated for 12 h with TRIP ΔU3-CCR5 vector particles at a concentration corresponding to 500 ng of Gag p24 per ml, washed, and cultured in vitro for 48 h to ensure optimal transgene expression. Macrophages were isolated from Ficoll-isolated PBMCs by plastic adherence in 48-well plates and cultured for 7 days in RPMI 1640 medium supplemented with 10% FCS, 10% human serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Thereafter, cells were transduced with TRIP ΔU3 encoding either wt CCR5 or CCR5-R126N (1000 ng of HIV-1 Gag p24/well) for 12 h, washed, and cultured for 4 days before HIV infection. To evaluate the cell surface expression of CCR5, cells (10^5) were incubated on ice for 20 min in phosphate-buffered saline containing 0.5% bovine serum albumin, labeled with phycoerythrin (PE)-conjugated anti-CCR5 2D7 monoclonal antibody (MAb) (Pharmingen), washed in phosphate-buffered saline, and analyzed by fluorescence-activated cell sorting (Becton Dickinson, Pont de Claix, France). Background fluorescence was measured by using an immunoglobulin isotype control antibody (Pharmingen).

HIV-1 infection assays. The JR-CSF luciferase (Luc) is an HIV-1 provirus which carries the firefly *luc* reporter gene in place of the HIV-1 *nef* gene and was a gift from V. Planelles, Rochester, N.Y. Replication-competent JR-CSF *luc* viruses were generated as described by Davis et al. (14). 293T cells were transfected by calcium phosphate coprecipitation with JR-CSF *luc* provirus. Viruses were harvested at 48 h after transfection, filtered through 0.45-μm-pore-size filters, and stored at −80°C until needed. For HIV infection, cells were incubated with JR-CSF *luc* viruses for 4 h at 37°C, washed, and cultured in their appropriate culture medium. Cell lysates were obtained at 3 days postinfection, and luciferase activity was determined as previously described (29). HIV-1–GFP reporter virions pseudotyped with Ba-L Env were generated as previously described by cotransfection of HIV-1 NL ΔEnv GFP provirus (a gift from D. R. Littman, Skirball Institute, New York, N.Y.) and a cDNA plasmid encoding R5 isolate Ba-L Env (a gift from D. R. Littman). Activated PBMCs were infected with pseudotyped virions for 4 h, washed, and cultured for 3 days. Cells were simultaneously stained with anti-CD4–allophycocyanin (APC) and anti-CD45RO–PE MAb (Pharmingen) and then fixed in 4% paraformaldehyde. HIV replication was assessed by detecting GFP by flow cytometry on gated CD4 T cells. In some experiments, PBMCs or macrophages were infected with the R5 HIV-1 Ba-L virions (generated in mitogen-stimulated PBMCs), and HIV-1 replication was determined by measuring the amount of HIV-1 Gag p24 in culture supernatants.

Calcium mobilization assay. The calcium mobilization assay was performed as previously described (28). Briefly, MT4 cells (2×10^6) were loaded with 1.5 μM fura-2-AM in culture medium. After a 20-min incubation at 37°C, cells were centrifuged and the pellet was resuspended in 1 ml of mammalian saline buffer (140 mM, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, 2g of glucose per liter [pH 7.4]). After 2 min of recording, MIP-1β (50 nM) (a gift from F. Baleux, Institut Pasteur, Paris, France) was added to the cell suspension. Ca^{2+} measurements were performed at 37°C by measuring the fluorescence emissions at 340 and 380 nm with a Perkin-Elmer LS-B luminescence spectrometer (Bois d'Arcy).

RESULTS AND DISCUSSION

Functional characterization of lentivirus-expressed wt CCR5 and CCR5-R126N in lymphoblastoid T cells. The high gene transduction efficiency of the TRIP ΔU3 vector used in this study is illustrated in Fig. 1A. In this experiment, MT4 cells or PHA-activated PBMCs were incubated with VSV-G Env-pseudotyped TRIP ΔU3-CMV-EGFP vector particles for 12 h and thereafter cultured in their appropriate medium for an additional 48 h to ensure optimal gene expression. Usually, GFP expression was detected in 100% of MT4 cells and in more than 80% of IL-2-expanded PBMCs (Fig. 1A).

TRIP ΔU3 vectors plasmids encoding either the wt CCR5 or a mutant receptor carrying a single mutation in the DRY motif (CCR5-R126N) were generated. It is well established that the DRY motif is critical for coupling of $G_\alpha i$ or $G_\alpha q$ to a number of GPCR (26) and for agonist-mediated activation of CCR5, leading to chemotaxis, intracellular calcium mobilization, or phosphatidyl inositol turnover (15, 17). A CCR5 molecule carrying combined D125N and R126N mutations was able to bind CCR5 ligands but failed to mobilize intracellular calcium (15). A CCR5 variant carrying the single substitution R126N bound MIP-1β with an affinity similar to that of its wt counterpart and was also unresponsive to agonist activation (15). The functional characteristics of both wt CCR5 and CCR5-R126N were evaluated in human lymphoblastoid $CD4^+$ MT4 cells, and

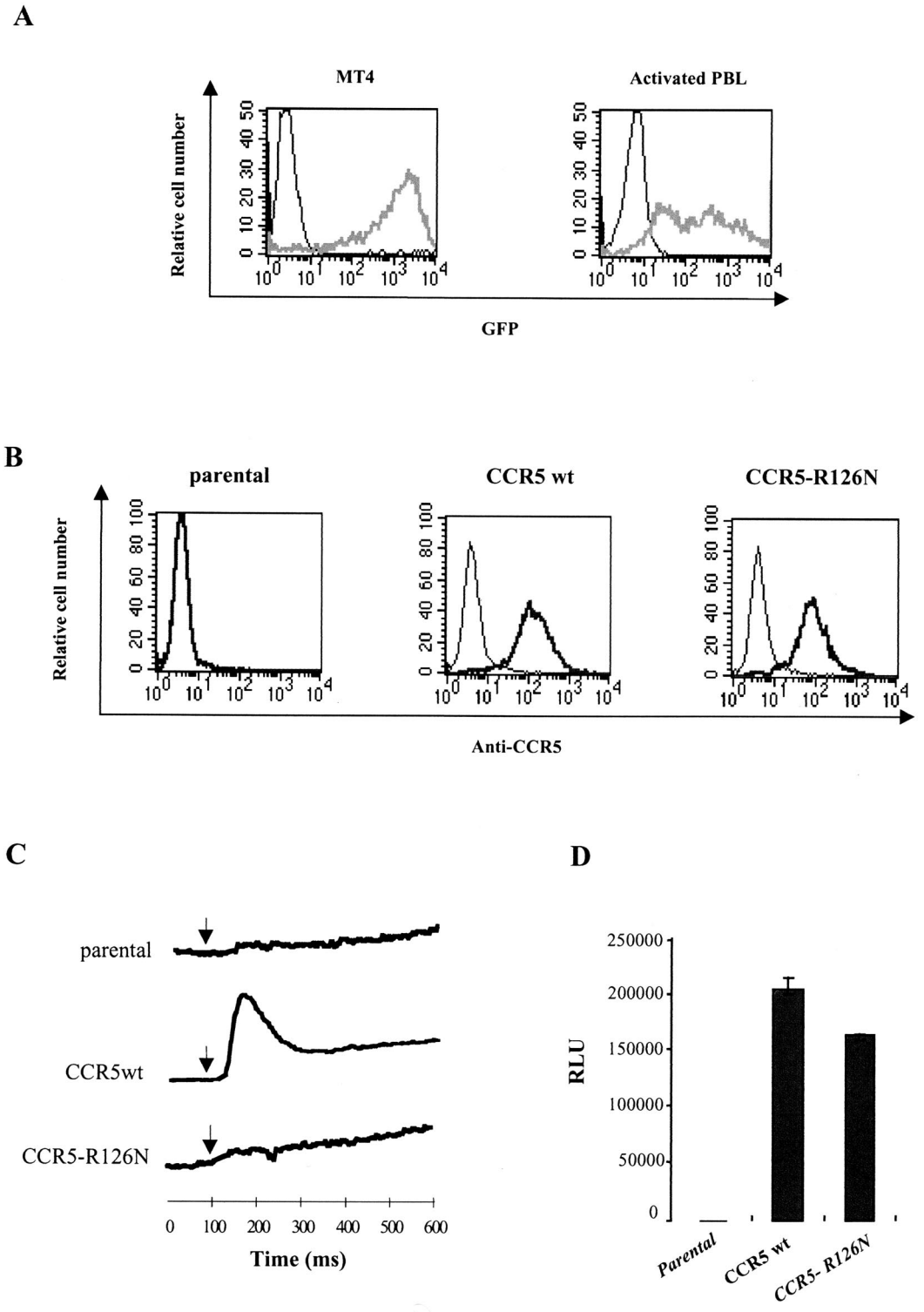


FIG. 1. Functional characterization of lentivirus-expressed wt CCR5 and CCR5-R126N in T-cell lines. (A) The transduction efficiency of the TRIP ΔU3 vector was evaluated in MT4 cells and PHA-activated PBMCs. Cells were exposed to VSV-G-pseudotyped TRIP ΔU3-CMV-EGFP vector particles for a 12-h period, washed, and cultured for 2 days to ensure optimal gene expression. GFP expression was analyzed by flow cytometry. (B) MT4 cells were incubated with wt TRIP ΔU3-CCR5 or TRIP ΔU3-CCR5-R126N vector particles for 12 h. CCR5 expression was evaluated 48 h later by flow cytometry with the anti-CCR5 2D7 MAb. (C) Calcium mobilization in response to MIP-1β. Parental MT4, MT4 wt CCR5, and MT4 CCR5-R126N cells were loaded with 1.5 μM fura-2-AM and stimulated with 50 nM MIP-1β at the time points indicated by the arrows. (D) Parental MT4, MT4 wt CCR5, and MT4 CCR5-R126N cells were infected with the replication-competent luciferase reporter HIV JR-CSF virions. Cell lysates were prepared at 72 h postinfection and analyzed for luciferase activity (in relative light units [RLU]). Results from one representative experiment out of two are shown. Error bars indicate standard deviations.

CCR5 expression was investigated by using the anti-CCR5 MAb 2D7, which recognizes a conformational epitope in the second, extracellular domain of the receptor (38). We observed that both the wt CCR5 and CCR5-R126N proteins were expressed at fairly comparable levels at the cell membrane (Fig. 1B). In both cases, the bulk of the transduced cells maintained the expression levels of wt CCR5 or CCR5-R126N protein, as well as CD4 and CXCR4 expression, for up to 4 months (data not shown). Using these stable transfectants, we tested whether wt CCR5 and CCR5-R126N were activated in response to MIP-1β stimulation. Agonist-dependent mobilization of calcium from intracellular stores was observed in MT4 cells expressing wt CCR5, while both the parental and CCR5-R126N-expressing cells remained unresponsive (Fig. 1C). This finding is in keeping with data from previous reports showing that activation of G protein-dependent pathways requires the integrity of the DRY motif in CCR5 (15, 17).

The capacities of wt CCR5 and CCR5-R126N to permit a full R5 HIV-1 replication cycle were investigated by using the JR-CSF *luc* reporter virus. Figure 1D shows that both wt CCR5 and CCR5-R126N allow HIV replication of R5 HIV-1 JR-CSF *luc* in MT4 cells. Similar results were obtained with R5 HIV-1 Ba-L and YU2 strains, as indicated by HIV-1 Gag p24 production in supernatants of infected cells (data not shown). Like the nontransduced MT4 cells, transfectants expressing either wt CCR5 or CCR5-R126N were susceptible to infection by X4 HIV-1 isolates (data not shown). These results show that the CCR5-R126N molecule allows R5 HIV-1 replication in MT4 cells despite its inability to support agonist-induced CCR5 activation. Overall, our findings show that, as in other transformed cell lines cells (15, 17), G protein signaling is not required for CCR5 coreceptor function and viral replication in lymphoblastoid T cells. Similar results were obtained for herpesvirus saimiri-immortalized *ccr5* Δ*32*/Δ*32* $CD4^+$ T lymphocytes. Gene transduction by VSV-G-pseudotyped TRIP ΔU3 vector particles enabled membrane expression of wt CCR5 or CCR5-R126N and, in both cases, rendered herpesvirus saimiri-immortalized *ccr5* Δ*32*/Δ*32* $CD4^+$ T lymphocytes susceptible to infection by R5 HIV strains (data not shown).

Next, we took advantage of the properties of the TRIP ΔU3-based transduction system to express wt or mutated CCR5 molecules in primary T lymphocytes or macrophages from CCR5-null individuals and studied their ability to support HIV-1 infection.

Role of G protein signaling in replication of R5 HIV strains in primary T cells and macrophages. The controversy regarding the HIV cell cycle and its dependency on coreceptor-mediated signaling relies basically on the presumed requirement of CCR5 activation by R5 HIV-1 Env to allow productive infection in primary, nontransformed CD4 T lymphocytes and macrophages (6, 18). Thus, we compared the capacities of wt CCR5 and CCR5-R126N mutants to support R5 HIV-1 infection when overexpressed in primary cells. PHA-activated PBMCs from a *ccr5* Δ*32*/Δ*32* individual were exposed to TRIP ΔU3-CCR5 vector particles for a 12-h period and were thereafter cultured for 4 days in IL-2-containing medium. As expected, we found that *ccr5* Δ*32*/Δ*32* cells did not express membrane CCR5. High levels of CCR5 expression were detected in *ccr5* Δ*32*/Δ*32* PBMCs after gene transduction, and wt CCR5 and CCR5-R126N were expressed at comparable levels in 100% of activated PBMCs (Fig. 2A). Parental, wt CCR5, and CCR5-R126N cells were then examined for their susceptibility to R5 HIV-1 infection. As shown in Fig. 2B and C, R5 viruses failed to replicate in *ccr5* Δ*32*/Δ*32* $CD4^+$ cells. Primary cells transduced with wt CCR5 or CCR5-R126N became highly susceptible to infection by the R5 HIV-1 isolates JR-CSF *luc* and Ba-L, as indicated, respectively, by the accumulation of luciferase activity in cell lysates (Fig. 2B) and HIV-1 Gag p24 production in the culture supernatants (Fig. 2C). Similar results were obtained with the R5 HIV-1 strains YU2 and ADA (data not shown). It should be noted that, reproducibly, CCR5-R126N supported infection by R5 HIV-1 strains more efficiently than its wt counterpart. The mechanisms accounting for this difference are unknown.

It was previously suggested that replication of R5 HIV-1 isolates occurs preferentially in memory $CD4^+$ T cells, as identified by the presence of $CD45RO^+$ antigen (9). To rule out the possibility that HIV-1-induced CCR5 activation occurring in other $CD4^+$ T-cell subpopulations could interfere in the assessment and interpretation of our findings, we selectively evaluated the replication of HIV-GFP reporter virions pseudotyped with Ba-L Env in $CD45RO^+$ $CD4^+$ T lymphocytes from *ccr5* Δ*32*/Δ*32* cells transduced with either wt CCR5 or CCR5-R126N vectors. As observed previously, in the bulk of $CD4^+$ T cells, the CCR5-R126N receptor supported R5 HIV-1 replication as efficiently as did its wt CCR5 counterpart in $CD45RO^+$ $CD4^+$ T lymphocytes (Fig. 2D).

We next investigated whether cell signaling induced by the potent polyclonal mitogen PHA could mask the requirement for CCR5 activation induced by HIV-1. For this purpose, we replaced PHA PBMC stimulation by either IL-2 or IL-15 cytokines. IL-2 and IL-15 are physiological activators of T lymphocytes and are known to promote efficient HIV-1 transcription in $CD4^+$ T lymphocytes in the absence of cell proliferation (36). Unlike cell activation induced by potent polyclonal mitogens or through the T-cell receptor, stimulation of resting T lymphocytes with cytokines does not change the state of cell differentiation, as indicated by the preservation of the naive phenotype of cells and the lack of activation markers such as HLA-DR (reference 36 and data not shown). PBMCs from a *ccr5* Δ*32*/Δ*32* individual were depleted of monocytes, stimulated with IL-2 and IL-15, and exposed to VSV-G envelope-pseudotyped TRIP-CCR5 vector particles for 12 h. After gene transduction, we found that wt CCR5 or CCR5-R126N was expressed in cytokine-activated *ccr5* Δ*32*/Δ*32* $CD3^+$ T cells. Reproducibly, the cell surface expression level of wt CCR5 was higher than that of CCR5-R126N (Fig. 3A). As previously observed in PHA-activated PBMCs, CCR5-R126N behaved as a more efficient coreceptor than wt CCR5 (Fig. 3B). These findings indicate that the independence of CCR5 activation shown by R5 HIV infection of T cells occurs whatever mechanism is used to activate T cells and support HIV replication.

The capacity of macrophages to support replication of R5 HIV or simian immunodeficiency virus isolates has been correlated with the capacity of their corresponding viral Env to signal through CCR5 (6). R5-dependent HIV-1 isolate 92MW959, which enters but does not replicate in macrophages, fails to trigger CCR5-dependent calcium mobilization. This contrasts with the capacity of R5 HIV-1 isolate Ba-L or JR-FL to both activate CCR5 and productively infect macro-

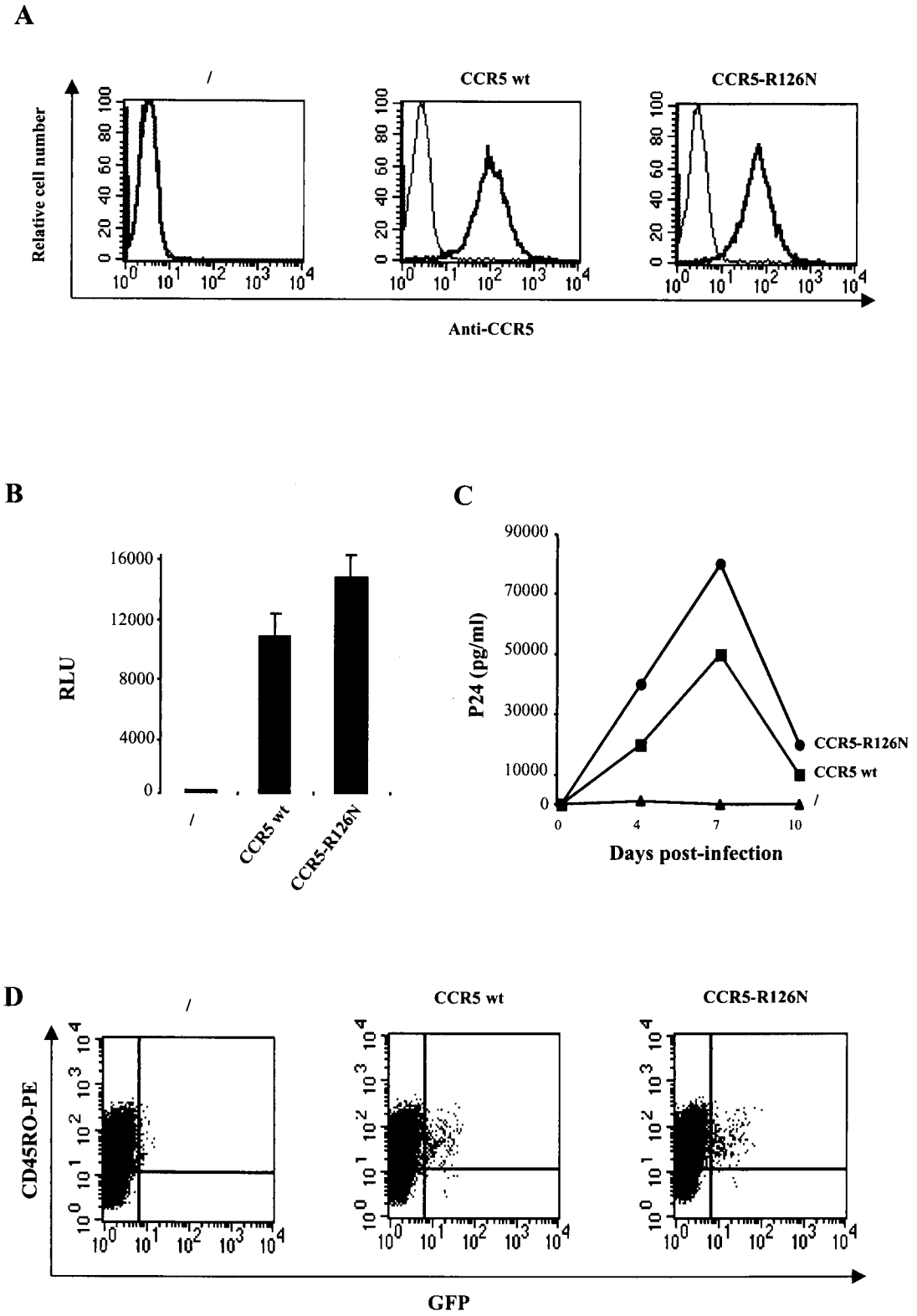
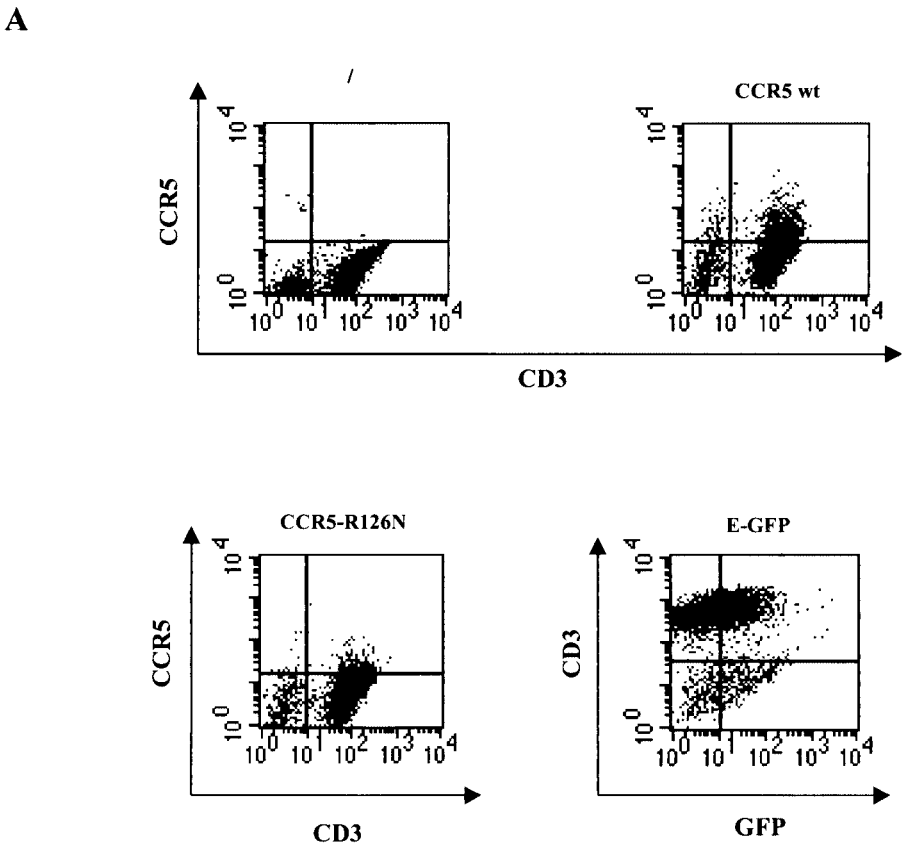


FIG. 2. wt CCR5 and CCR5-R126N HIV coreceptor function in PHA-activated PBMCs. (A) PHA activated-PBMCs from a *ccr5* Δ*32*/Δ*32* individual (donor A) were inoculated with wt TRIP ΔU3-CCR5 or TRIP ΔU3-CCR5-R126N vector particles. Cells were maintained in medium containing 20 ng of IL-2 per ml for 5 days, and CCR5 transgene expression was monitored by flow cytometry analysis with the PE-conjugated 2D7 anti-CCR5 MAb. (B to D) At day 5 posttransduction, parental (/) or wt CCR5- or CCR5-R126N-transduced PBMCs were infected with R5 HIV-1 JR-CSF *luc* reporter virus (B), R5 HIV-1 strain Ba-L (C), or HIV-GFP reporter virions pseudotyped with the R5 HIV-1 Ba-L envelope glycoprotein (D). In panels B and C, HIV replication was evaluated by measuring luciferase activity in cell lysates and p24 production in the culture supernatants, respectively. In panel D, infected cells were simultaneously stained with anti-CD4–APC and anti-CD45RO–PE MAbs. HIV replication was assessed by detection of GFP by flow cytometry on gated CD4 T cells. Data are representative of those from two independent experiments. RLU, relative light units. Error bars indicate standard deviations.



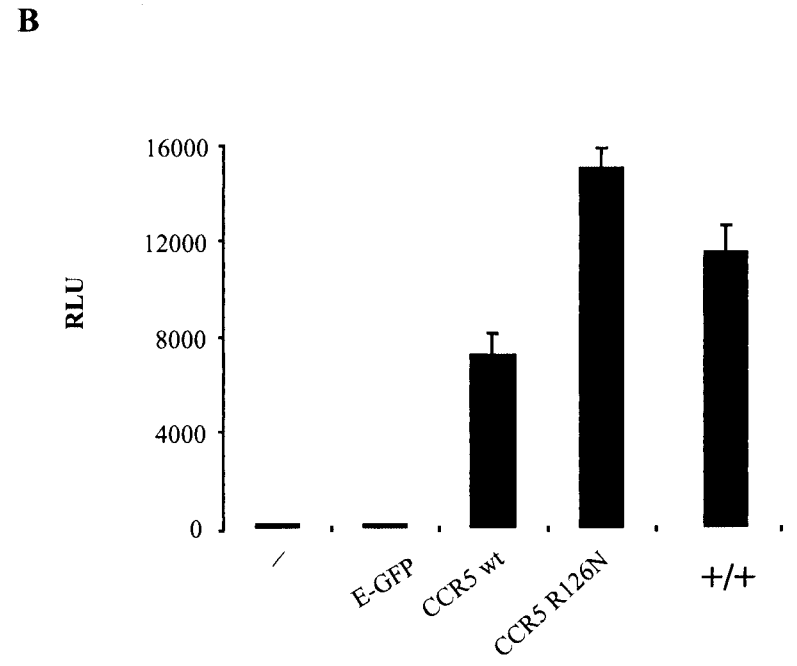


FIG. 3. PBMCs from a *ccr5* Δ*32*/Δ*32* individual (donor B) were depleted of monocytes and thereafter incubated with IL-15 and IL-2 for 5 days. Cytokine-treated cells were transduced with TRIP ΔU3 vector particles encoding EGFP, wt CCR5, or CCR5-R126N. (A) CCR5 and GFP expression in CD3 T cells from parental (/) and transduced cells was evaluated 4 days later by flow cytometry. (B) HIV infection was assessed as described for Fig. 2. +/+, PBMCs isolated from an individual carrying a wt CCR5 genotype, used as control. Data are representative of those from two independent experiments. RLU, relative light units. Error bars indicate standard deviations.

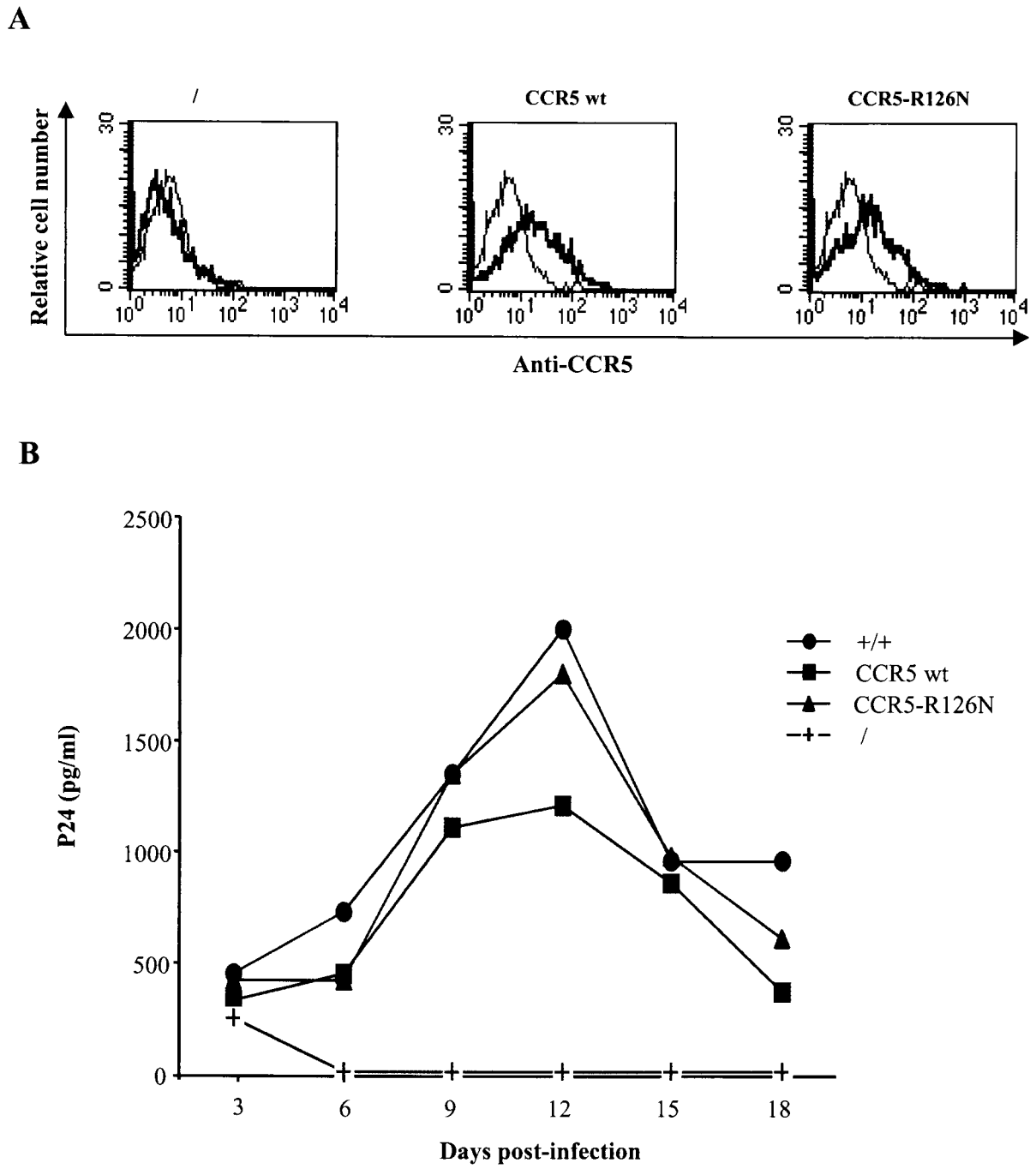


FIG. 4. Macrophages from a *ccr5* Δ*32*/Δ*32* individual (donor C) were inoculated with wt TRIP ΔU3-CCR5 or TRIP ΔU3-CCR5-R126N vector. (A) CCR5 expression was evaluated 4 days later as described for Fig. 1. (B) Transduced macrophages were infected with R5 HIV-1 Ba-L, and cell culture supernatants were sampled for detection of p24 production. Data shown are representative of those from two independent experiments (donors B and C). +/+, macrophages isolated from an individual carrying a wt CCR5 genotype, used as control.

phages (6). Macrophages inoculated with the 92MW959 strain support viral replication when costimulated with the CCR5 agonist MIP-1α, thus suggesting that activation of the HIV coreceptor provides the stimuli necessary for completion of the viral cell cycle (6). This situation may differ from the apparent independence shown by X4 HIV isolates regarding the activation of CXCR4. Indeed, overexpression of either wt CXCR4 or an SDF-1-noninducible (Δ3i mutant) CXCR4 in low-level CXCR4-expressing macrophages rescues infection by the X4 HIV-1 Lai isolate (35). However, although it is suggestive, this finding does not demonstrate the apparent CXCR4 signaling independence shown by X4 HIV isolates to infect macro-

phages, since these cells constitutively express CXCR4 coupled to a functional cellular transduction machinery.

To accurately assess whether replication of R5 HIV-1 isolates in macrophages relies on CCR5 activation by HIV-1 R5 Env, we transduced *ccr5* Δ*32*/Δ*32* macrophages with either wt CCR5 or CCR5-R126N vector particles, and thereafter we inoculated cell cultures with HIV-1 Ba-L. Similar to the case in primary leukocytes, lentiviral transduction of CCR5 restores both expression and HIV coreceptor function in *ccr5* Δ*32*/Δ*32* macrophages (Fig. 4). As noted before, CCR5-R126N showed a better capacity to permit HIV-1 Ba-L infection than wt CCR5 (Fig. 4B). We conclude that productive replication of HIV-1 R5 isolates can be dissociated from the activation of CCR5-coupled G protein transduction pathways. It should be noted that CCR5 activation independence of R5 HIV infection in macrophages is observed in the absence of exogenous stimulation. Thus, like in primary T lymphocytes, the activation status of the host cell does not seem to influence significantly the autonomy of R5 HIV strains to infect and replicate independently of CCR5 activation.

HIV-1 Env proteins, namely, those of R5 isolates, induce cell activation mechanisms characteristics of chemokine receptor ligation by their natural agonists (14). Indeed, R5 or X4 HIV-1 purified Env and, in some circumstances, HIV-1 particles induce chemotaxis, calcium mobilization, or Pyk2 phosphorylation, which involve activation of PTX-sensitive G proteins (14, 25, 37). The sensitivity of chemokine- or HIV-1 Env-induced cell signals to the inhibitory effect of PTX suggests that $G_\alpha i$ is the most prominent family of G_α proteins transducing HIV coreceptor-mediated cell activation. However, it is noteworthy that chemokine receptors can couple to $G_\alpha q$ as well as to $G_\alpha i$ (4, 21). Importantly, both families of G_α proteins have been shown to rely on the integrity of the DRY motif to bind to a number of GPCR (16, 34). This and the inability of the CCR5-R126N mutant to initiate the activation of classic GPCR second messengers upon agonist ligation make very unlikely the coupling of CCR5 to G_α proteins in the different T cells and macrophages used as experimental models in this work. It is striking that, despite the CCR5 agonist capacity of R5 HIV Env, we do not observe any evident effect of CCR5 ligation by R5 HIV regarding the viral life cycle. The inability of R5 HIV Env to influence HIV infection contrasts with the well-documented ability of chemokines to enhance in vitro X4 or R5 HIV-1 infection of various cells (6, 20, 23, 24). Thus, macrophage activation by RANTES or MIP-1α increases replication of a number of R5 HIV-1 isolates (6, 33). Moreover, chemokines that bind CCR5 have been found to enhance infection of T lymphocytes by X4 HIV isolates, an effect that is prevented by incubation with the $G_\alpha i$ inhibitor PTX (20). This characteristic is shared by SDF-1, which exerts a PTX-dependent stimulatory effect on the replication of infectious HIV-1 bearing R5 Env or a VSV-G Env protein (23). Similarly, aminooxypentane-RANTES, a potent inhibitor of R5 HIV strains, promotes, at high molar concentrations, a breakthrough of R5 HIV-1 isolate replication and activation of mitogen-activated protein kinase pathways, both of which are blocked by PTX (24). The disparate agonist capacity shown by CCR5 natural ligands and R5 HIV-1 Env, regarding their respective capacities to influence the HIV life cycle, could rely on differences in intensity and/or persistence of cell signaling induced by the virus compared to the CCR5-binding chemokines. Thus, in the report by Arthos et al. (6), the marked ability of MIP-1α to boost infection by the 92MW959 HIV-1 isolate, which fails to replicate spontaneously in macrophages, could be due to a CCR5-mediated cell activation capacity of the chemokine that is not shared by the viral particles. The effect of MIP-1α could be otherwise accounted for by the well-characterized capacity of the chemokine to bind and activate chemokine receptors other than CCR5 (7). Alternatively, the involvement of yet-uncharacterized mechanisms induced by the viral Env could repress CCR5-dependent activation pathways which lead to enhancement of HIV infectivity. The nature of the mechanisms underlying these divergent features remains unclear.

We conclude that the CCR5-induced G protein cell signaling is not required for efficient R5 HIV-1 infection and replication in both primary T lymphocytes and macrophages.

ACKNOWLEDGMENTS

We are grateful to Suzan Michelson for critical reading of and valuable suggestions on the manuscript. We are indebted to C. Gigi-Lévy for collecting of blood samples from donors.

This work was supported by the European Molecular Biology Organization, Ensemble contre le SIDA (France), Agence Nationale de Recherche contre le SIDA (ANRS), Ministerio de Ciencia y Tecnología (SAF00/0028), FIPSE, and Fundación Caja de Madrid (Spain). J.G.-P. was supported by a fellowship from the Instituto Carlos III (Madrid, Spain).

REFERENCES

1. **Alfano, M., H. Schmidtmayerova, C. A. Amella, T. Pushkarsky, and M. Bukrinsky.** 1999. The B-oligomer of pertussis toxin deactivates CC chemokine receptor 5 and blocks entry of M-tropic HIV-1 strains. J. Exp. Med. **190:**597–605.
2. **Alkhatib, G., M. Locati, P. E. Kennedy, P. M. Murphy, and E. A. Berger.** 1997. HIV-1 coreceptor activity of CCR5 and its inhibition by chemokines: independence from G protein signaling and importance of coreceptor downmodulation. Virology **234:**340–348.
3. **Amara, A., S. L. Gall, O. Schwartz, J. Salamero, M. Montes, P. Loetscher, M. Baggiolini, J. L. Virelizier, and F. Arenzana-Seisdedos.** 1997. HIV coreceptor downregulation as antiviral principle: SDF-1alpha-dependent internalization of the chemokine receptor CXCR4 contributes to inhibition of HIV replication. J. Exp. Med. **186:**139–146.
4. **Arai, H., and I. F. Charo.** 1996. Differential regulation of G-protein-mediated signaling by chemokine receptors. J. Biol. Chem. **271:**21814–21819.
5. **Aramori, I., S. S. Ferguson, P. D. Bieniasz, J. Zhang, B. Cullen, and M. G. Cullen.** 1997. Molecular mechanism of desensitization of the chemokine receptor CCR-5: receptor signaling and internalization are dissociable from its role as an HIV-1 co-receptor. EMBO J. **16:**4606–4616.
6. **Arthos, J., A. Rubbert, R. L. Rabin, C. Cicala, E. Machado, K. Wildt, M. Hanbach, T. D. Steenbeke, R. Swofford, J. M. Farber, and A. S. Fauci.** 2000. CCR5 signal transduction in macrophages by human immunodeficiency virus and simian immunodeficiency virus envelopes. J. Virol. **74:**6418–6424.
7. **Baggiolini, M., B. Dewald, and B. Moser.** 1997. Human chemokines: an update. Annu. Rev. Immunol. **15:**675–705.
8. **Berger, E. A., P. M. Murphy, and J. M. Farber.** 1999. Chemokine receptors as HIV-1 coreceptors: roles in viral entry, tropism, and disease. Annu. Rev. Immunol. **17:**657–700.
9. **Bleul, C. C., L. Wu, J. A. Hoxie, T. A. Springer, and C. R. Mackay.** 1997. The HIV coreceptors CXCR4 and CCR5 are differentially expressed and regulated on human T lymphocytes. Proc. Natl. Acad. Sci. USA **94:**1925–1930.
10. **Bukrinskaya, A., B. Brichacek, A. Mann, and M. Stevenson.** 1998. Establishment of a functional human immunodeficiency virus type 1 (HIV-1) reverse transcription complex involves the cytoskeleton. J. Exp. Med. **188:** 2113–2125.
11. **Cicala, C., J. Arthos, M. Ruiz, M. Vaccarezza, A. Rubbert, A. Riva, K. Wildt, O. Cohen, and A. S. Fauci.** 1999. Induction of phosphorylation and intracellular association of CC chemokine receptor 5 and focal adhesion kinase in primary human CD4+ T cells by macrophage-tropic HIV envelope. J. Immunol. **163:**420–426.
12. **Cocchi, F., A. L. DeVico, A. Garzino-Demo, A. Cara, R. C. Gallo, and P. Lusso.** 1996. The V3 domain of the HIV-1 gp120 envelope glycoprotein is critical for chemokine-mediated blockade of infection. Nat. Med. **2:**1244–1247.

13. **Dardalhon, V., B. Herpers, N. Noraz, F. Pflumio, D. Guetard, C. Leveau, A. Dubart-Kupperschmitt, P. Charneau, and N. Taylor.** 2001. Lentivirus-mediated gene transfer in primary T cells is enhanced by a central DNA flap. Gene Ther. **8:**190–198.
14. **Davis, C. B., I. Dikic, D. Unutmaz, C. M. Hill, J. Arthos, M. A. Siani, D. A. Thompson, J. Schlessinger, and D. R. Littman.** 1997. Signal transduction due to HIV-1 envelope interactions with chemokine receptors CXCR4 or CCR5. J. Exp. Med. **186:**1793–1798.
15. **Farzan, M., H. Choe, K. A. Martin, Y. Sun, M. Sidelko, C. R. Mackay, N. P. Gerard, J. Sodroski, and C. Gerard.** 1997. HIV-1 entry and macrophage inflammatory protein-1beta-mediated signaling are independent functions of the chemokine receptor CCR5. J. Biol. Chem. **272:**6854–6857.
16. **Fraser, C. M., C. D. Wang, D. A. Robinson, J. D. Gocayne, and J. C. Venter.** 1989. Site-directed mutagenesis of m1 muscarinic acetylcholine receptors: conserved aspartic acids play important roles in receptor function. Mol. Pharmacol. **36:**840–847.
17. **Gosling, J., F. S. Monteclaro, R. E. Atchison, H. Arai, C. L. Tsou, M. A. Goldsmith, and I. F. Charo.** 1997. Molecular uncoupling of C-C chemokine receptor 5-induced chemotaxis and signal transduction from HIV-1 coreceptor activity. Proc. Natl. Acad. Sci. USA **94:**5061–5066.
18. **Guntermann, C., B. J. Murphy, R. Zheng, A. Qureshi, P. A. Eagles, and K. E. Nye.** 1999. Human immunodeficiency virus-1 infection requires pertussis toxin sensitive G-protein-coupled signalling and mediates cAMP downregulation. Biochem. Biophys. Res. Commun. **256:**429–435.
19. **Iyengar, S., J. E. Hildreth, and D. H. Schwartz.** 1998. Actin-dependent receptor colocalization required for human immunodeficiency virus entry into host cells. J. Virol. **72:**5251–5255.
20. **Kinter, A., A. Catanzaro, J. Monaco, M. Ruiz, J. Justement, S. Moir, J. Arthos, A. Oliva, L. Ehler, S. Mizell, R. Jackson, M. Ostrowski, J. Hoxie, R. Offord, and A. S. Fauci.** 1998. CC-chemokines enhance the replication of T-tropic strains of HIV-1 in CD4(+) T cells: role of signal transduction. Proc. Natl. Acad. Sci. USA **95:**11880–11885.
21. **Kuang, Y., Y. Wu, H. Jiang, and D. Wu.** 1996. Selective G protein coupling by C-C chemokine receptors. J. Biol. Chem. **271:**3975–3978.
22. **Lu, Z., J. F. Berson, Y. Chen, J. D. Turner, T. Zhang, M. Sharron, M. H. Jenks, Z. Wang, J. Kim, J. Rucker, J. A. Hoxie, S. C. Peiper, and R. W. Doms.** 1997. Evolution of HIV-1 coreceptor usage through interactions with distinct CCR5 and CXCR4 domains. Proc. Natl. Acad. Sci. USA **94:**6426–6431.
23. **Marechal, V., F. Arenzana-Seisdedos, J. M. Heard, and O. Schwartz.** 1999. Opposite effects of SDF-1 on human immunodeficiency virus type 1 replication. J. Virol. **73:**3608–3615.
24. **Marozsan, A. J., V. S. Torre, M. Johnson, S. C. Ball, J. V. Cross, D. J. Templeton, M. E. Quinones-Mateu, R. E. Offord, and E. J. Arts.** 2001. Mechanisms involved in stimulation of human immunodeficiency virus type 1 replication by aminooxypentane RANTES. J. Virol. **75:**8624–8638.
25. **Misse, D., M. Cerutti, N. Noraz, P. Jourdan, J. Favero, G. Devauchelle, H. Yssel, N. Taylor, and F. Veas.** 1999. A CD4-independent interaction of human immunodeficiency virus-1 gp120 with CXCR4 induces their cointernalization, cell signaling, and T-cell chemotaxis. Blood **93:**2454–2462.
26. **Murphy, P. M.** 1994. The molecular biology of leukocyte chemoattractant receptors. Annu. Rev. Immunol. **12:**593–633.
27. **Naldini, L., U. Blomer, P. Gallay, D. Ory, R. Mulligan, F. H. Gage, I. M. Verma, and D. Trono.** 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272:**263–267.
28. **Oberlin, E., A. Amara, F. Bachelerie, C. Bessia, J. L. Virelizier, F. Arenzana-Seisdedos, O. Schwartz, J. M. Heard, I. Clark-Lewis, D. F. Legler, M. Loetscher, M. Baggiolini, and B. Moser.** 1996. The CXC chemokine SDF-1 is the ligand for LESTR/fusin and prevents infection by T-cell-line-adapted HIV-1. Nature **382:**833–835.
29. **Percherancier, Y., T. Planchenault, A. Valenzuela-Fernandez, J. L. Virelizier, F. Arenzana-Seisdedos, and F. Bachelerie.** 2001. Palmitoylation-dependent control of degradation, life span, and membrane expression of the CCR5 receptor. J. Biol. Chem. **276:**31936–31944.
30. **Popik, W., and P. M. Pitha.** 2000. Exploitation of cellular signaling by HIV-1: unwelcome guests with master keys that signal their entry. Virology **276:**1–6.
31. **Rana, S., G. Besson, D. G. Cook, J. Rucker, R. J. Smyth, Y. Yi, J. D. Turner, H. H. Guo, J. G. Du, S. C. Peiper, E. Lavi, M. Samson, F. Libert, C. Liesnard, G. Vassart, R. W. Doms, M. Parmentier, and R. G. Collman.** 1997. Role of CCR5 in infection of primary macrophages and lymphocytes by macrophage-tropic strains of human immunodeficiency virus: resistance to patient-derived and prototype isolates resulting from the Δ*ccr5* mutation. J. Virol. **71:**3219–3227.
32. **Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, G. Muyldermans, C. Verhofstede, G. Burtonboy, M. Georges, T. Imai, S. Rana, Y. Yi, R. J. Smyth, R. G. Collman, R. W. Doms, G. Vassart, and M. Parmentier.** 1996. Resistance to HIV-1 infection in Caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene Nature **382:**722–725.
33. **Schmidtmayerova, H., B. Sherry, and M. Bukrinsky.** 1996. Chemokines and HIV replication. Nature **382:**767.
34. **Shibata, T., C. Suzuki, J. Ohnishi, K. Murakami, and H. Miyazaki.** 1996. Identification of regions in the human angiotensin II receptor type 1 responsible for Gi and Gq coupling by mutagenesis study. Biochem. Biophys. Res. Commun. **218:**383–389.
35. **Tokunaga, K., M. L. Greenberg, M. A. Morse, R. I. Cumming, H. K. Lyerly, and B. R. Cullen.** 2001. Molecular basis for cell tropism of CXCR4-dependent human immunodeficiency virus type 1 isolates. J. Virol. **75:**6776–6785.
36. **Unutmaz, D., V. N. KewalRamani, S. Marmon, and D. R. Littman.** 1999. Cytokine signals are sufficient for HIV-1 infection of resting human T lymphocytes. J. Exp. Med. **189:**1735–1746.
37. **Weissman, D., R. L. Rabin, J. Arthos, A. Rubbert, M. Dybul, R. Swofford, S. Venkatesan, J. M. Farber, and A. S. Fauci.** 1997. Macrophage-tropic HIV and SIV envelope proteins induce a signal through the CCR5 chemokine receptor. Nature **389:**981–985.
38. **Wu, L., G. LaRosa, N. Kassam, C. J. Gordon, H. Heath, N. Ruffing, H. Chen, J. Humblias, M. Samson, M. Parmentier, J. P. Moore, and C. R. Mackay.** 1997. Interaction of chemokine receptor CCR5 with its ligands: multiple domains for HIV-1 gp120 binding and a single domain for chemokine binding. J. Exp. Med. **186:**1373–1381.
39. **Yee, J. K., T. Friedmann, and J. C. Burns.** 1994. Generation of high-titer pseudotyped retroviral vectors with very broad host range. Methods Cell Biol. **43:**99–112.
40. **Zufferey, R., D. Nagy, R. J. Mandel, L. Naldini, and D. Trono.** 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat. Biotechnol. **15:**871–875.