



CC CKR5: A RANTES, MIP-1 α , MIP-1 β Receptor as a Fusion Cofactor for Macrophage-Tropic HIV-1

Author(s): Ghalib Alkhatib, Christophe Combadiere, Christopher C. Broder, Yu Feng, Paul E. Kennedy, Philip M. Murphy and Edward A. Berger

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−4.6 per mil ($n = 13$); for presumed predators (C group) $\delta^{15}\text{N} = -1.58$ per mil ($n = 9$). These differences in $\delta^{15}\text{N}$ between successive trophic levels of 4.51 per mil (G group versus the P group) and 3.02 per mil (C group versus the G group) are statistically significant [Tukey's honestly significant difference (hsd) test (18), $P < 0.01$] and within the range of 3 to 5 per mil expected.

Results for the C isotope data are less clear. Grazers (mean $\delta^{13}\text{C} = -40.66$ per mil, $n = 13$) are significantly (Tukey's hsd test, $P < 0.01$) enriched by 3.08 per mil in ^{13}C relative to the mat (mean $\delta^{13}\text{C} = -43.74$ per mil, $n = 8$), which is a greater degree of enrichment than expected (10). However, the mean $\delta^{13}\text{C} = -42.16$ per mil ($n = 9$) for predators is intermediate between and not significantly different from (Tukey's hsd, $P > 0.05$) the value for the mat and grazers, which is not as expected (10). Many cave-dwelling organisms are dietary generalists (3), and thus it is possible that these presumptive "predators" are in fact omnivores, perhaps including mat material in their diets. Were this the case, we would also expect the behavior to be reflected in the isotope data for N, which it is not. Cave-dwelling organisms also tend to have attenuated appendages and a general increase in surface-to-volume ratio (3). For invertebrates this can increase the proportion of exoskeleton and chitin in the individual. Chitin may be depleted in ^{13}C by ~2 per mil relative to the rest of the organism (19). Thus, our carbon values for predators may reflect this "chitin effect."

The stable isotope data show that the Movile Cave ecosystem derives its organic carbon from in situ chemoautotrophic production. As far as we know, all other limestone cave ecosystems that have been studied require allochthonous inputs of organic material of photosynthetic origin from the surface. In addition, the terrestrial community in Movile Cave, which accounts for over 60% of the animal species and over 70% of the endemic species in the system, appears to be a community with a chemoautotrophic energy base. To our knowledge, all other chemoautotrophically based communities have been described from marine (20) and freshwater (21) habitats. The Movile Cave system is similar to deep sea vents in having a chemoautotrophic food base and a diverse biota. However, the cave system appears to lack the symbioses between chemoautotrophic microbes and animals so characteristic of deep sea vent communities.

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11. Values of δ (per mil) are calculated as $X = [(R_{\text{sample}} - R_{\text{standard}})/R_{\text{standard}}] \times 10^3$, where X is $\delta^{13}\text{C}$ or $\delta^{15}\text{N}$ and R is $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$. Standards are Pee Dee belemnite for carbon and air for nitrogen. The precision of the measurements was 0.2 per mil or better.
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CC CKR5: A RANTES, MIP-1 α , MIP-1 β Receptor as a Fusion Cofactor for Macrophage-Tropic HIV-1

Ghalib Alkhatib, Christophe Combadiere,*
Christopher C. Broder,* Yu Feng,* Paul E. Kennedy,*
Philip M. Murphy,† Edward A. Berger†

Human immunodeficiency virus–type 1 (HIV-1) entry requires fusion cofactors on the CD4⁺ target cell. Fusin, a heterotrimeric GTP-binding protein (G protein)–coupled receptor, serves as a cofactor for T cell line–tropic isolates. The chemokines RANTES, MIP-1 α , and MIP-1 β , which suppress infection by macrophage-tropic isolates, selectively inhibited cell fusion mediated by the corresponding envelope glycoproteins (Envs). Recombinant CC CKR5, a G protein–coupled receptor for these chemokines, rendered CD4-expressing nonhuman cells fusion-competent preferentially with macrophage-tropic Envs. CC CKR5 messenger RNA was detected selectively in cell types susceptible to macrophage-tropic isolates. CC CKR5 is thus a fusion cofactor for macrophage-tropic HIV-1 strains.

Individual isolates of HIV-1 display markedly distinct tropisms for infection of primary macrophages as compared with CD4⁺

T cell lines (1–4). The viral determinants for this cytotropism reside in the Env (1, 2). Direct assays of fusion mediated by recombinant Envs suggest that the cytotropism of different isolates is largely a consequence of the inherent membrane fusion selectivities of the corresponding Envs for various CD4⁺ target cell types (5).

Insight into the cellular determinants of this fusion selectivity derives from the fact that CD4 must be expressed on a human

G. Alkhatib, C. C. Broder, Y. Feng, P. E. Kennedy, E. A. Berger, Laboratory of Viral Diseases, National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH), Bethesda, MD 20892, USA
C. Combadiere and P. M. Murphy, Laboratory of Host Defenses, NIAID, NIH, Bethesda, MD 20892, USA.

*These authors contributed equally to the research.

†To whom correspondence should be addressed.

cell type in order to mediate both virus-cell and cell-cell fusion (6–8). This restriction is due to the requirement for human-specific fusion cofactors on the CD4⁺ target cell (9–12). Moreover, recent studies with transient cell hybrids (13) support a proposed model (1) in which cytotropism results from the requirement of different Envs for distinct cofactors that are differentially expressed in various target cell types. Molecular identification of these fusion cofactors is critical for understanding HIV-1 pathogenesis and may be useful for designing therapeutic strategies to combat HIV infection.

We recently identified a molecule (designated fusin) that serves as a fusion and infection cofactor for T cell line-tropic HIV-1 isolates (14). Fusin is a putative member of the G protein-coupled receptor superfamily and has features suggestive of a chemokine receptor, although its natural ligand and normal function are presently unknown. The C-C chemokines

RANTES, MIP-1 α , and MIP-1 β were recently shown (15) and subsequently confirmed (16) to suppress HIV-1 infection, particularly for macrophage-tropic isolates; a related C-C chemokine, MCP-1, had no effect (15). Together, these findings led to the hypothesis that the suppressive C-C chemokines exert their infection-inhibiting activities by binding to a chemokine receptor that serves as a fusion cofactor for macrophage-tropic HIV-1 isolates, thereby inhibiting fusion mediated by the corresponding Envs (14, 16).

To test this hypothesis, we used a recombinant vaccinia-based system in which fusion between Env-expressing and CD4-expressing cells induces activation of the *Escherichia coli lacZ* gene; β -galactosidase (β -Gal) is produced selectively in fused cells (17). The precise relation between cell fusion and infection is controversial, and arguments for (2) and against (18) their correspondence have been put forth. As judged by diverse criteria, the specificity of

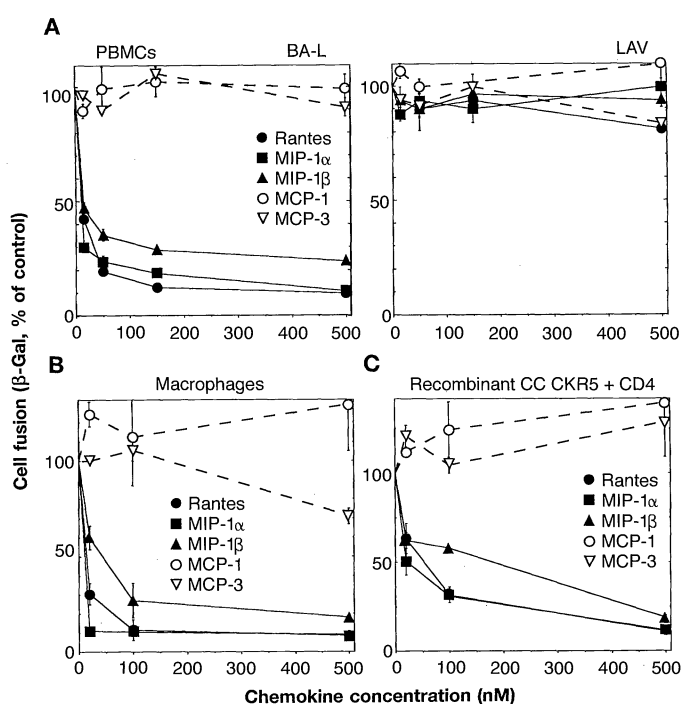
cell fusion measured with the vaccinia-based assay parallels the specificity of infection by HIV-1 virions (19). The successful application of this system in the identification of fusin, a cofactor implicated in both Env/CD4-mediated cell fusion and virus entry for T cell line-tropic isolates (14), validates its usefulness for identifying new fusion cofactors.

First, we examined the effects of C-C chemokines on the fusion reaction. Phytohemagglutinin (PHA)-activated peripheral blood mononuclear cells (PBMCs) were used as the CD4⁺ target cell type (Fig. 1A), and Envs from the prototypic macrophage-tropic Ba-L isolate (left) and T cell line-tropic LAV isolate (right) were compared. With the Ba-L Env, RANTES, MIP-1 α , and MIP-1 β each caused potent dose-dependent inhibition of fusion, whereas the related chemokines MCP-1 and MCP-3 had no effect. By contrast, with the LAV Env, no fusion inhibition was produced by any of these chemokines. We extended this analysis to other Envs of each class; RANTES, MIP-1 α , and MIP-1 β (but not MCP-1) inhibited fusion for Envs from all macrophage-tropic isolates examined (Ba-L, SF162, JR-FL, and ADA), whereas no inhibition occurred with Envs from T cell line-tropic isolates (LAV and IIIB) (20). We also analyzed chemokine effects with primary macrophages as the CD4⁺ target cell type using the Ba-L Env (Fig. 1B). The same specificity profile was observed: potent inhibition occurred with RANTES, MIP-1 α , and MIP-1 β , but little or no effect occurred with MCP-1 or MCP-3. Thus, the chemokine specificity for inhibition of Env/CD4-mediated cell fusion was similar to that reported for suppression of HIV-1 infection (15, 16): RANTES, MIP-1 α , and MIP-1 β (but not MCP-1 or MCP-3) were active, and the inhibition occurred preferentially with macrophage-tropic strains. We conclude that the suppressive effects of the C-C chemokines on HIV-1 infection are due, at least in part, to inhibition of the membrane fusion reactions involved in virus entry.

Six human C-C chemokine-restricted receptors have been identified to date; they bind overlapping but distinct subsets of C-C chemokines (21). Of these, only C-C chemokine receptor 5 (CC CKR5) has a chemokine specificity profile matching that for inhibition of HIV-1 infection (15, 16) and Env-mediated cell fusion (Fig. 1, A and B). Thus, RANTES, MIP-1 α , and MIP-1 β are all potent agonists for CC CKR5, but MCP-1 and MCP-3 are not (22, 23).

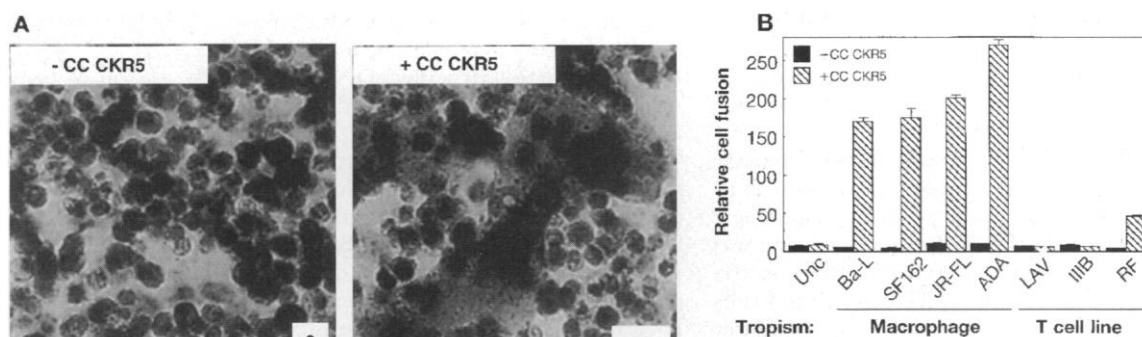
These parallel specificities prompted us to test whether recombinant CC CKR5 could act as a fusion cofactor for macro-

Fig. 1. Effects of chemokines on Env/CD4-mediated cell fusion. Recombinant proteins were produced by transfection with the indicated plasmids and infection with the indicated vaccinia recombinants. The multiplicity of infection (MOI) was 10 plaque-forming units per cell for each virus. After overnight incubation at 31°C and washing, the designated CD4-expressing target cells were preincubated with the indicated concentrations of recombinant chemokines (Peptrotech; Rocky Hill, New Jersey) for 1 hour at 37°C (1×10^5 cells per well in 80- μ l culture medium in 96-well flat-bottom plates). The Env-expressing cells (1×10^5 to 2×10^5 cells in 20- μ l culture medium) were added, and the cul-



tures were incubated for 2 hours. β -Gal activity was measured in detergent cell lysates (17), and the low background values obtained with vCB-16 encoding the Unc Env (5) were subtracted. Results with each chemokine are expressed as the percent β -Gal activity compared with that of the control samples with no chemokines added. (A) Fusion with PBMCs. PHA-activated PBMCs were infected with vCB21R-LacZ containing the *lacZ* gene linked to the T7 promoter (13). HeLa cells were infected in the presence of cytosine arabinoside (40 μ g/ml) with vTF7-3 encoding T7 RNA polymerase (29) and either vCB-43 encoding the Ba-L Env (left) or vCB-41 encoding the LAV env (right) (5). (B) Fusion with primary macrophages. Infections were performed in the presence of cytosine arabinoside (40 μ g/ml). Macrophages prepared from PBMCs by elutriation and differentiation (30) were infected with vTF7-3 encoding T7 RNA polymerase. HeLa cells were coinfecting with vCB43 encoding the Ba-L Env and vCB21R-LacZ. (C) Fusion with murine cells expressing vaccinia-encoded CD4 and CC CKR5. Infections were performed in the presence of cytosine arabinoside (40 μ g/ml). NIH 3T3 cells were transfected with plasmid pGA9-CKR5 containing the CC CKR5 cDNA and coinfecting with vCB-3 encoding CD4 (11) and vTF7-3 encoding T7 RNA polymerase. HeLa cells were coinfecting with vCB43 encoding the Ba-L Env and vCB21R-LacZ.

Fig. 2. CC CKR5 functions as a fusion cofactor for Envs from macrophage-tropic HIV-1 isolates. The designated cells types were transfected with either control plasmid pSC59 (28) lacking a cDNA insert or plasmid pGA9-CKR5 containing CC CKR5 cDNA; the cells were also coinfecting with vP11 gene1 (31) encoding T7 RNA polymerase plus vCB-3 (11) encoding CD4. As fusion partners, HeLa cells were coinfecting with vCB21R-LacZ plus vaccinia viruses encoding the indicated Envs: Ba-L, vCB-43 (5); SF162, vCB-32 (5); JR-FL, vCB-28 (5); ADA, vCB-39 (5); LAV, vCB-41 (5); IIIB, vCB-60 (28); RF, vCB-36 (5); and Unc, vCB-16 (5). Previous results documented comparable expression of each vaccinia-encoded Env (5). After overnight incubation at 31°C and washing, pairwise cell combinations were mixed in 96-well flat-bottom microtiter plates [1×10^5 cells per well for each cell type; total volume = 0.2 ml in culture medium plus cytosine arabinoside (40 μ g/ml)]. Cell fusion was analyzed by syncytium formation and by the quantitative reporter gene activation assay (17). **(A)** Fusion-promoting cofactor activity of CC CKR5. NIH 3T3 cells expressed vaccinia-encoded CD4, without (left) or with (right) CC CKR5. Fusion partner HeLa cells expressed the Ba-L Env. The fusion reaction was performed at 37°C on two separate



plates. One plate was incubated 5 hours for syncytium analysis; samples were fixed, stained with crystal violet, and photographed under a phase-contrast microscope. The second plate was incubated for 3 hours, and β -Gal activity in detergent cell lysates was measured. Each value (insets) represents absorbance per minute at 570 nm multiplied by 10^3 ; the low background value of 4 observed with the Unc Env has been subtracted to give the values shown. **(B)** CC CKR5 fusion cofactor activity for Envs from macrophage-tropic versus T cell line-tropic isolates. BS-C-1 cells expressed vaccinia-encoded CD4, without (filled bars) or with (cross-hatched bars) CC CKR5. Fusion partner cells expressed the indicated Env. Fusion was scored at 3 hours by measuring β -Gal activity in detergent cell lysate; each value represents absorbance per minute at 570 nm multiplied by 10^3 (mean of duplicate samples; error bars indicate sample standard deviations).

phage-tropic Envs (24). The vaccinia system was used in murine NIH 3T3 cells to express T7 RNA polymerase plus CD4, without or with CC CKR5 (Fig. 2A). These cells were mixed with HeLa cells containing the *lacZ* gene linked to the T7 promoter and expressing the Ba-L Env (5). Assay of both syncytium formation and β -Gal production demonstrated that coexpression of CD4 and CC CKR5 enabled the murine cells to undergo fusion; no fusion occurred without CC CKR5. Additional analyses demonstrated that fusion was absolutely dependent on coexpression of CD4 (20). Analysis of the fusion cofactor activity of CC CKR5 was extended to additional cell types expressing vaccinia-encoded CD4, and to Envs from other HIV-1 isolates. Figure 2B shows results with simian BS-C-1 cells expressing vaccinia-encoded T7 RNA polymerase plus CD4, without or with CC CKR5. These cells were mixed with HeLa cells containing *lacZ* and expressing Envs from different HIV-1 isolates having well-characterized tropisms for either macrophages or continuous T cell lines. CC CKR5 conferred fusion competence for Envs from all the macrophage-tropic isolates tested (Ba-L, SF162, JR-FL, and ADA) but much less efficiently for Envs from the T cell line-tropic isolates tested (LAV, IIIB, and RF). These results with CC CKR5 stand in marked contrast to those with fusin, which has much greater activity for Envs from T cell line-tropic isolates (14). We also observed that recombinant CC CKR5 enabled Envs from the macro-

phage-tropic isolates to mediate fusion with HeLa cells (20), a human cell line that is permissive for T cell line-tropic but not macrophage-tropic isolates. Certain Envs displayed some activity with both cofactors. For example, the Env from the T cell line-tropic RF isolate, previously shown to be strongly responsive to fusin (14), also responded slightly to CC CKR5. This finding is consistent with a report that macrophages infected with the RF strain form syncytia (25), as well as with our previous observation that the RF Env mediates low-level fusion activity with macrophages (5). Conversely, the ADA Env, strongly responsive to fusin (14), also shows a modest response to fusin (14).

To complement our finding that recombinant CC CKR5 allowed nonhuman cells to undergo fusion mediated by Envs from macrophage-tropic isolates, we tested the effects of C-C chemokines on fusion conferred by vaccinia-encoded CC CKR5 plus CD4 expressed on NIH 3T3 cells (Fig. 1C). Dose-dependent inhibition of Ba-L Env-mediated fusion occurred with RANTES, MIP-1 α , and MIP-1 β , but not with MCP-1 or MCP-3. Thus, there is a correspondence between the agonist selectivity of CC CKR5 (22, 23) and the specificity for chemokine inhibition of HIV infection (15, 16) and Env/CD4-mediated cell fusion with human PBMCs and macrophages expressing endogenous components (Fig. 1, A and B), or nonhuman cells coexpressing recombinant CC CKR5 and CD4 (Fig. 1C). Although the relative activities of the various

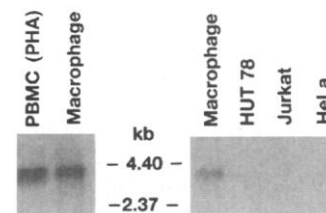


Fig. 3. Northern (RNA) blot analysis of CC CKR5 mRNA. Polyadenylated RNA was prepared from the indicated cell types with the Micro-Fast Track Kit (Invitrogen). Samples (0.5 μ g) were subjected to electrophoresis through 1% agarose-formaldehyde gels and transferred to a nitrocellulose membrane. The left and right panels represent separate gel analyses. A [α - 32 P]guanosine triphosphate-labeled hybridization probe was prepared from the full-length CC CKR5 cDNA template by random primer synthesis. Hybridizations were carried out with QuikHyb (Promega). The membranes were washed twice with 2% saline sodium citrate (SSC) and 0.1% SDS at room temperature for 30 min and once with 0.1% SSC and 0.1% SDS at 65°C for 1 hour. Radioactive bands were detected by autoradiography; no bands were observed outside the regions shown. Molecular sizes are indicated in kilobases.

C-C chemokines were qualitatively similar in each of these assays, the absolute potencies varied considerably. For example, the reported median inhibition concentration (IC_{50}) values for inhibition of infection by the Ba-L isolate were highly dependent on the target cell type (15); similarly, in our cell fusion assays, the potency of chemokine inhibition was greater for PBMCs and macrophages than for murine cells coexpressing

recombinant CC CKR5 and CD4. Differences in the expression levels of the relevant target cell molecules presumably make major contributions to these quantitative variations.

We tested directly whether recombinant CC CKR5 could render cells susceptible to productive infection by macrophage-tropic isolates. The CD4⁺ Jurkat T cell line was transfected with CC CKR5 cDNA in the expression plasmid pCDNA3; control cells were transfected with pCDNA3. After G418 selection, a CC CKR5 transformant colony was identified having strong cell fusion activity for the Ba-L Env. In initial infection experiments with Ba-L virus, the CC CKR5 transformant produced substantial amounts of p24 (150 ng/ml at day 13), whereas the control transformant produced no detectable p24 (<1 ng/ml); with LAV virus, comparable amounts of p24 were produced by the CC CKR5 and the control transformants (220 and 240 ng/ml at day 13, respectively).

When a CC CKR5 probe was used in Northern (RNA) blot analysis (Fig. 3), a 3.9-kb transcript was detected in PHA-activated PBMCs and primary macrophages. By contrast, CC CKR5 RNA was not detected in various human cell lines that are not susceptible to macrophage-tropic isolates, including the Hut78 and Jurkat T cell lines and HeLa cells. As a control for the integrity of the RNA preparations, we observed comparable levels of β -actin RNA for all cell types (20).

The present results complement our previous identification of fusin as a cofactor for T cell line-tropic strains (14). In our experimental approaches to identify fusion cofactors, we deliberately focused on HIV-1 isolates with marked selectivities for infection of either primary macrophages or continuous T cell lines. However, most isolates from infected patients display some capacity to infect either cell type (3, 4, 26). According to a simple model, CC CKR5 and fusin are the only fusion cofactors for HIV-1; the tropism spectrum for diverse isolates simply reflects the ability of the corresponding Envs to function with each cofactor. While the simplicity of the two-cofactor model is appealing, the situation may be more complex. A fraction of the Ba-L Env-mediated fusion was resistant to the C-C chemokines tested; perhaps other cofactors mediate the residual activity. Thus, a larger family of fusion cofactors may exist, each having different levels of activity for the diverse Envs within the evolving virus population. These cofactors might be differentially expressed

on various CD4⁺ cell types, and their production and function might be regulated in vivo. Numerous experimental variables might therefore influence detection of cofactor activity.

The finding of distinct fusion cofactors for HIV-1 isolates with different cytotropisms raises interesting questions about transmission and pathogenesis. Some individuals who remain uninfected despite repeated high-risk sexual exposure reportedly have CD4⁺ T lymphocytes that are relatively resistant to in vitro infection by macrophage-tropic isolates, but susceptible to T cell line-adapted variants (16). The viral determinants for this distinction mapped to Env. CD4⁺ T lymphocytes from exposed-uninfected individuals secreted greater amounts of RANTES, MIP-1 α , and MIP-1 β compared with nonexposed control subjects. Differences in putative second receptors have been proposed as one possible mechanism underlying this distinction (16). Our results suggest the importance of examining the expression levels and functionality of CC CKR5 in cells from such individuals. A similar concept applies in considering possible relations of fusion cofactors to long-term nonprogression of some HIV-infected individuals (27). Isolates with the capacity to infect T cell lines generally appear during the transition from the asymptomatic to the symptomatic stage, coincident with the decline of CD4⁺ T lymphocytes. We previously speculated that interaction of Env with CD4 and fusin might contribute to this decline by direct or indirect mechanisms (14); perhaps defects in the expression or activity of fusin (or related fusion cofactors) underlie the favorable disease course in some nonprogressors.

As previously noted for fusin (14), the identification of CC CKR5 suggests practical applications in the production of transgenic small animal models for HIV-1 infection, and possibly also in the design of strategies to treat HIV-1 infection. Indeed, the suppressive activity of RANTES, MIP-1 α , and MIP-1 β led to the proposal that these chemokines might have therapeutic benefits (15). Detailed understanding of the role of the various fusion cofactors in disease progression is required to assess the suitability of these molecules as therapeutic targets.

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