HIV-1 exploits CCR5 conformational heterogeneity to escape inhibition by chemokines.

Philippe Colin, Yann Bénureau, Isabelle Staropoli, Yongjin Wang, Nuria Gonzalez, Jose Alcami, Oliver Hartley, Anne Brelot, Fernando Arenzana-Seisdedos, Bernard Lagane

To cite this version:


HAL Id: pasteur-00825629
https://hal-pasteur.archives-ouvertes.fr/pasteur-00825629
Submitted on 24 Nov 2013

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
HIV-1 EXPLOITS CCR5 CONFORMATIONAL HETEROGENEITY TO ESCAPE INHIBITION BY CHEMOKINES

Authors:
Philippe COLIN, Yann BENUREAU, Isabelle STAROPOLI, Yongjin WANG, Nuria Gonzalez, Jose ALCAMI, Oliver HARTLEY, Anne BRELOT, Fernando ARENZANA-SEISLEDOS, and Bernard LAGANE.

Author Affiliations:
* INSERM U819, Viral Pathogenesis Unit, Department of Virology, Institut Pasteur, 75015 Paris, France.
** Univ. Paris Diderot, Sorbonne Paris Cité, Cellule Pasteur, Rue du Docteur Roux, 75015 Paris, France.
§ Instituto de Salud Carlos III, 28220-Majadahonda, Madrid, Spain.
¶ Department of Pathology and Immunology, University of Geneva, Geneva, Switzerland.

Corresponding Author: Bernard Lagane, Institut Pasteur, 28 rue du docteur Roux, 75724, Paris cedex 15, France. Tel.:+33145688945. Fax:+33145688941. E-mail:bernard.lagane@pasteur.fr

§ PC and YB contributed equally to this work.
Abstract
CCR5 is a receptor for chemokines and the coreceptor for R5 HIV-1 entry into CD4+ T-lymphocytes. Chemokines exert anti-HIV-1 activity in vitro, both by displacing the viral envelope glycoprotein gp120 from binding to CCR5 and by promoting CCR5 endocytosis, suggesting that they play a protective role in HIV infection. However, we showed here that different CCR5 conformations at the cell surface are differentially engaged by chemokines and gp120, making chemokines weaker inhibitors of HIV infection than would be expected from their binding affinity constants for CCR5. These distinct CCR5 conformations rely on CCR5 coupling to nucleotide-free G-proteins (NF-G-proteins). While native CCR5 chemokines bind with subnanomolar affinity to NF-G-protein-coupled CCR5, gp120/HIV-1 does not discriminate between NF-G-protein-coupled and uncoupled CCR5. Interestingly, the antiviral activity of chemokines is G-protein independent, suggesting that ‘low-chemokine affinity’ NF-G-protein-uncoupled conformations of CCR5 represent a portal for viral entry. Furthermore, chemokines are weak inducers of CCR5 endocytosis, as is revealed by EC50 values for chemokine-mediated endocytosis reflecting their low-affinity constant value for NF-G-protein-uncoupled CCR5. Abolishing CCR5 interaction with NF-G-proteins eliminates high-affinity binding of CCR5 chemokines but preserves receptor endocytosis, indicating that chemokines preferentially endocytose low-affinity receptors. Finally, we evidenced that chemokine analogs achieve highly potent HIV-1 inhibition due to high-affinity interactions with internalizing and/or gp120-binding receptors. These data are consistent with HIV-1 evading chemokine inhibition by exploiting CCR5 conformational heterogeneity, shed new light into the inhibitory mechanisms of anti-HIV-1 chemokine analogs and provide insights for the development of new anti-HIV molecules.
Introduction

CCR5 is the principal coreceptor for entry of human immunodeficiency virus type-1 (HIV-1), used together with CD4 to enter and infect target cells (1), and a receptor for agonist (CCL3/MIP-1α, CCL4/MIP-1β, CCL5/RANTES) and antagonist/weak partial agonist (CCL7/MCP-3) chemokines (2, 3). The native agonist chemokine ligands of CCR5 induce conformational changes in the receptor that promote activation of pertussis toxin (PTX)-sensitive, heterotrimeric αβγ G-proteins (Gi/o-type G-proteins) by catalyzing an exchange of GTP for GDP on the Ga subunit. The GTP-bound Ga subunit and the Gβγ dimer then trigger intracellular signaling pathways involved in chemotaxis and activation of leukocytes (4).

Native CCR5 chemokines inhibit infection of R5-tropic HIV-1 in vitro. This occurs via two mechanisms: stERICally preventing the viral envelope gp120 from binding to the coreceptor and reducing cell surface coreceptor levels by inducing receptor downregulation (5-7). They are secreted by a number of cell types and in particular immune cells including R5 HIV-1 target cells (6, 8, 9). The potential role of native CCR5 chemokines in blocking HIV-1 transmission and progression has been extensively studied (9-12), but their efficacy as protective factors remains a matter of debate (13, 14). A major paradox relates to the observation that native CCR5 chemokines show lower antiviral potencies than would be expected based on their CCR5 binding affinity constants (15-18), which are in the subnanomolar range (2, 19, 20), much lower than the corresponding value for the HIV-1 envelope glycoprotein gp120, which is approximately 10 nM (19, 21).

A number of CCR5 chemokine analogs with improved antiviral potency have been identified, including N-terminally modified RANTES analogs with agonist (AOP-, PSC- or 6P4-RANTES) or antagonist features (5P12- or 2P3-RANTES), which represent promising molecules as topical microbicides (18, 22). While the enhanced potency of agonist analogs can be explained in terms of their increased capacity to induce CCR5 downregulation (23), the inhibitory mechanism of antagonist analogs, which neither activate G protein signaling nor induce receptor downregulation, is more elusive. It was speculated that it might involve increased steric blockade of CCR5, but competition binding assays using labeled CCL4 as a tracer did not show any significant increase in CCR5 binding affinity (22).

In this study, we present evidence that conformationally different CCR5 subpopulations with distinct chemokine binding capacities are present at the surface of HIV-1 target cells. In particular, a fraction of receptors shows strikingly low binding affinity for native CCR5 chemokines, providing an explanation for why native CCR5 chemokines have unexpectedly low anti-HIV-1 potencies. Our results also shed further light on the inhibitory mechanism of chemokine analogs, showing that they overcome the challenge of the chemokine low-affinity CCR5 population through (i) more efficient receptor downregulation and/or (ii) increased binding affinity for gp120-binding receptors. Overall, these findings explain how R5 HIV-1 could escape from inhibition by native CCR5 chemokines in the course of infection and provide clues for the development of new chemokine analogs as HIV inhibitors.

Results
Distinct CCR5 populations are differentially utilized by HIV-1 gp120 and chemokines. Chemokines and the CD4-bound form of HIV-1 envelope glycoprotein gp120 competitively bind to overlapping regions of CCR5 (24). Thus, to investigate whether distinct CCR5 populations interact differently with chemokines and gp120, we first tested unlabeled native chemokines (CCL-3, -4, -5 or -7) or chemokine analogs (AOP-, PSC-, 2P3-, 5P12-, or 6P4-RANTES) for their ability to inhibit binding of either $^{125}$I-labeled CCL3 ($^{125}$I-CCL3) or the $^{35}$S-labeled gp120 from the HIV-1 primary strain Bx08 ($^{35}$S-gp120$_{Bx08}$) on membranes from CCR5-expressing HEK 293T cells (HEK-R5 cells) (Fig. 1, Table 1). Using the data obtained, we calculated the affinity constant values ($K_i$) of the competing ligands for receptors using the Cheng and Prusoff equation (see SI materials and methods).

Except for CCL7, displacement of $^{125}$I-CCL3 binding revealed high affinities of competitors for CCR5, with $K_i$ values in the nM range or lower (Table 1). The $K_i$ values obtained for the native CCR5 agonists CCL-3, -4 and -5 are comparable to the $K_D$ values determined for these ligands in saturation binding assays (Table 1), consistent with binding of these chemokines to a similar class of high-affinity receptors in both competition and saturation assays. In contrast, CCL-3, -4 and -5, as well as the chemokine analogs AOP- and PSC-RANTES, only partly displaced $^{35}$S-gp120$_{Bx08}$ binding when used at a 100 nM concentration (Fig. 1B), suggesting that they have a lower affinity for $^{35}$S-gp120$_{Bx08}$-binding receptors as compared to $^{125}$I-CCL3-binding receptors. In contrast, the observation that $^{35}$S-gp120$_{Bx08}$ binds marginally to CCR5 in the presence of 100 nM 2P3-, 5P12- or 6P4-RANTES (Fig. 1B) suggests that these chemokine analogs preserve high affinity interactions with $^{35}$S-gp120$_{Bx08}$-binding CCR5.

In dose-response experiments, 2P3-, 5P12- or 6P4-RANTES and $^{35}$S-gp120$_{Bx08}$ competed for binding to an apparent single class of receptors, as is revealed by monophasic competitive binding curves (Fig. 1C-D). The $K_i$ values in the nM range calculated for these chemokine analogs confirm high-affinity interactions with the $^{35}$S-gp120$_{Bx08}$-binding receptors (Table 1). CCL7 similarly bound to a single class of $^{35}$S-gp120$_{Bx08}$-binding CCR5 for which the affinity of the chemokine was higher than that for $^{125}$I-CCL3-binding CCR5 ($K_i$ = 34 vs 119 nM). In contrast, displacements of $^{35}$S-gp120$_{Bx08}$ binding by the agonists CCL3 (Fig. 1C), CCL4 and PSC-RANTES (Fig. 1D) gave biphasic curves, consistent with the presence of two distinct $^{35}$S-gp120$_{Bx08}$ receptor populations, one with high affinity for these chemokines, the other with significantly lower affinity. CCL-3, -4 and PSC-RANTES had $K_i$ values for interaction with the ‘high-chemokine affinity’ receptor population similar to those determined in $^{125}$I-CCL3 displacement assays (Table 1), suggesting that the ‘high-chemokine affinity’ population of $^{35}$S-gp120$_{Bx08}$-binding receptors and $^{125}$I-CCL3-binding receptors represent the same receptors. Interestingly, the $K_i$ values obtained for the ‘low-chemokine affinity’ CCR5 population range from a few tens of nM up to more than $10^{-6}$ M (Table 1), thus exceeding the $K_D$ value for the interaction of $^{35}$S-gp120$_{Bx08}$ with CCR5 determined in saturation assays ($\approx$ 10 nM (19)). Hence, this fraction of CCR5 has a higher affinity for gp120 than for native CCR5 chemokines.

Poor ability of native CCR5 chemokines to displace gp120 binding to CCR5 correlates with low antiviral activity. We next investigated whether low anti-HIV potency of native chemokines is related to
low-affinity interactions with gp120-binding receptors. For that purpose, we infected activated CD4+ T-lymphocytes, which represent the major target cells for R5 HIV-1, and HeLa P4C5 cells with infectious Bx08Ren viruses expressing gp120 from the Bx08 strain in the presence of native CCR5 chemokines or chemokine analogs (Table 1, Fig. S1A and S1B). Except for the chemokine analog PSC-RANTES, the antiviral potencies of all other chemokines correlated better with their ability to displace the binding of 35S-gp120Bx08 than that of 125I-CCL3. In particular, the native CCR5 agonists (CCL-3, -4 and -5) that show low-affinity interactions with a proportion of gp120-binding receptors had much more weaker antiviral activities than chemokine analogs, even though both groups of molecules have comparable affinities for 125I-CCL3-binding CCR5 (Table 1). Similar results were obtained using five other NL4-3Ren-derived viruses expressing R5 gp120 sequences from laboratory-adapted (JRRen) as well as primary (25Ren, 34Ren, 50Ren or 58Ren viruses) viruses (Fig. S2).

The potent antiviral activities of the antagonist RANTES analogs 5P12 and 2P3, which do not downregulate CCR5 (Fig. 5B and ref. (22)), reflect their increased binding affinities detected using 35S-gp120Bx08 as a tracer (Fig. 1). The enhanced potency of PSC-RANTES, which occurs despite its relatively low capacity to compete with 35S-gp120Bx08 for binding to CCR5, is likely to be due to its enhanced capacity to induce CCR5 downregulation (23). To test this hypothesis, we performed infection inhibition experiments under conditions where receptor downregulation is suppressed. HeLa P4C5 cells were pre-incubated for 2 h with PSC-RANTES (40 nM), 5P12-RANTES (40 nM) or the CCR5 inverse agonist maraviroc (MVC, 20 μM) at either 37°C or at 4°C, a temperature at which receptor endocytosis does not occur. Bx08Ren virus was then added to the cells, which were incubated for a further 2 h at 4°C, then washed in cold PBS, warmed to 37 °C for 15 min to allow entry of attached viruses, trypsin-treated to remove residual viruses and incubated for 48 h at 37 °C (Fig. 2). Under conditions where CCR5 downregulation is suppressed, the antiviral activity of PSC-RANTES was almost completely abrogated, but the inhibitory potency of 5P12-RANTES or MVC was unaffected, in accordance with our results in Fig. 1 showing that PSC- and 5P12-RANTES are weak and potent inhibitors of gp120 binding to CCR5. Hence unlike 5P12-RANTES and maraviroc, PSC-RANTES owes a large part of its inhibitory activity to its capacity to induce CCR5 downregulation. Importantly, these results also validate the notion that the receptors interacting with monomeric gp120(soluble CD4 complexes in the binding assays presented here (Fig. 1B-D) and those that are used by infectious virus particles at the surface of intact cells in infection assays extensively overlap and would represent similar receptor populations.

CCR5 coupling to nucleotide-free G-proteins differentially regulates native agonist chemokine and gp120 binding. It has been established that conformations of GPCRs with high-affinity for agonists are stabilized by coupling to guanine nucleotide-free G-proteins (NFG-proteins), and that the receptors are induced to shift towards low-affinity conformations as soon as G-proteins are occupied by nucleotides (25). Similarly to what we and others showed in mammalian cell lines (19, 26), we observed that CCR5 coupling to NFG-proteins also stabilizes the receptor in a high affinity conformation for agonists in HIV-1 target cells.
Indeed, the non-hydrolysable GTP analogs GTPγS and Gpp(NH)p or PTX, which inactivates G_{i/o}-proteins, decreased ¹²⁵I-CCL3 binding to CCR5 expressed in human lymphoblastoid CD4⁺ T-cell lines (A3.01-R5 cells) or primary T-lymphocytes to levels approaching that of non-specific binding (Fig. 3A). Saturation binding of ¹²⁵I-CCL3 to membranes from HEK-R5 cells further revealed that Gpp(NH)p decreases the maximum number of binding sites for the chemokine (B_{max}) from 10.7±1.3 to 2.9±0.7 pmole/mg of protein (Fig. 3B) while only slightly affecting the K_{D} value from 0.25±0.05 to 0.46±0.08 nM, indicating that Gpp(NH)p reduces the amount of receptors that are of high affinity for ¹²⁵I-CCL3. In line with this, 0.1 nM ¹²⁵I-CCL3 showed only background levels of binding to membranes from HEK cells expressing the R126N-CCR5 mutant, which does not activate G-proteins (27) (Fig. 3D). Similarly to CCL3 and CCL4 (26), high-affinity binding of CCL5 also required CCR5 coupling to NFG-proteins (Fig. 3C).

In contrast to native CCR5 agonist chemokines, R5 HIV-1 gp120 acts as an antagonist/weak partial agonist for CCR5 as it does not discriminate between NFG-protein-coupled or uncoupled CCR5 and binds equally well to R126N-CCR5 and wild-type CCR5, both in the presence and absence of Gpp(NH)p (Fig. 3E). This led us to hypothesize that the biphasic competitive binding curves obtained with native CCR5 agonist chemokines using ³⁵S-gp120_{Bx08} as a tracer is a reflection of (i) the existence of populations of both NFG-protein-coupled and NFG-protein-uncoupled receptors with respectively high and low affinity for these chemokines, and (ii) the capacity of gp120 to bind indiscriminately to either population. We tested this hypothesis by repeating the competition experiments of ³⁵S-gp120_{Bx08} binding by CCL3, in the presence and absence of Gpp(NH)p (Fig. 3F). Treatment with Gpp(NH)p decreased the proportion of ‘high-chemokine affinity’ receptors versus ‘low-chemokine affinity’ receptors from 43% to 16% (p = 0.016 in unpaired, two-tailed student’s t test), without affecting the K_{i} value of the ‘low-chemokine affinity’ receptor population (Table 1, Fig. 3F). This result is consistent with our previous observation that Gpp(NH)p eliminates the fraction of ³⁵S-gp120_{Bx08}-binding CCR5 that binds CCL4 with high affinity (19).

Chemokine-mediated inhibition of HIV infection and CCR5 endocytosis are G-protein independent processes. Based on the observation that HIV envelope binds indiscriminately to ‘high-chemokine affinity’ NFG-protein-coupled CCR5 and ‘low-chemokine affinity’ NFG-protein-uncoupled receptors, we hypothesized that infection in the presence of chemokine ligands would be more likely to occur via the low-chemokine affinity NFG-protein-uncoupled receptors, and that the chemokine ligands would be required to engage this population of receptors in order to achieve inhibition of infection. This would explain why native CCR5 agonist chemokines have low potency as HIV inhibitors.

To test this hypothesis, we treated activated CD4⁺ T-cells, A3.01-R5 or HeLa P4C5 cells with PTX and then infected them with R5 HIV-1 in the presence of native CCR5 chemokines or chemokine analogs (Fig. 4, S2 and S3). PTX attenuated ¹²⁵I-CCL3 binding to target cells (Fig. S3A and 3A) and abrogated chemokine-induced chemotaxis (Fig. 4A), indicating that CCR5 coupling with G_{i/o}-proteins is required for both high-affinity binding of the ligands and signal transduction. In contrast, PTX changed neither viral infectivity (Fig. S2) nor the potency of chemokines to block infection (Fig. S2, S3B, 4B and Table 1). This suggests that
CCR5 engagement by HIV-1 is independent of G-proteins and that high-affinity binding of ligands to ^NF_G^protein-coupled CCR5 does not make a significant contribution to their capacity to inhibit infection.

These results also imply that CCR5 downregulation, which contributes to the anti-HIV activity of agonist chemokines, is not dependent on engagement of high-chemokine affinity ^NF_G^protein-coupled CCR5. To address this possibility, we tested the ability of CCL-3, -4, PSC-, 6P4- and 5P12-RANTES to downregulate FLAG-tagged CCR5 in HEK 293 cells (28) with and without PTX, which decreases high affinity binding of ^125^I-CCL3 by 87.4 ± 11.8 % (Fig. 5A-C, Table S1). PTX influenced neither the efficacy nor the potency (^EC_{50}^) of chemokines to downregulate CCR5 (Fig. 5A-B, Table S1). Moreover, the kinetic rates of CCR5 downregulation induced by PSC-RANTES and CCL4 were unchanged by PTX treatment (t_{1/2} (min) = 3.3 ± 0.6 vs 3.2 ± 0.9 and 8.9 ± 0.8 vs 8.7 ± 1.7 for PSC and CCL4 in the absence or in the presence of PTX, respectively) (Fig. 5C). Hence interaction with high-chemokine affinity ^NF_G^protein-coupled CCR5 is not a requirement for the induction of CCR5 downregulation by its ligands.

**CCR5 downregulation involves low-affinity interactions of native CCR5 agonists with internalizing receptors.** PSC- and 6P4-RANTES had nanomolar ^EC_{50}^ values for CCR5 downregulation (Table S1), while the ^EC_{50}^ values for CCL-3 and -4 exceeded by more than 2 or 3 orders of magnitude their ^K_i^ value for ^NF_G^protein-coupled CCR5 (Tables 1 and S1). The differential abilities of chemokines to downregulate CCR5 could be due to internalization-competent CCR5 that might represent a receptor subpopulation to which CCL-3 and -4, but not PSC- and 6P4-RANTES, bind with a low affinity. Alternatively, but not exclusively, RANTES analog-induced inhibition of receptor recycling could also contribute to their potent ability to downregulate CCR5, as previously suggested (23).

To assess these hypotheses, we compared the abilities of PSC-RANTES and CCL4 to downregulate WT-CCR5 or the 349-CCR5 mutant, which does not recycle back to the cell surface (28) (Fig. 5D). As compared to WT-CCR5, CCL4 downregulated 349-CCR5 with higher potency (6-fold, Table S1) and efficacy, confirming that receptor recycling interferes to some extent with the ability of CCL4 to downregulate CCR5. PSC-RANTES also downregulated 349-CCR5 more efficiently than WT-CCR5 (93.9 ± 1.6 % vs 72.6 ± 3.3 %, respectively), albeit with comparable potencies (Table S1), suggesting that PSC-RANTES slows down CCR5 recycling but does not prevent it. However, CCL4 induced endocytosis of 349-CCR5 with a 18-fold higher ^EC_{50}^ value as compared to PSC-RANTES, indicating that low potency of CCL4 in downregulating CCR5 is modestly due to its inability to prevent receptor recycling. Rather, this ^EC_{50}^ value for endocytosis of 349-CCR5 by CCL4 (47 nM) is similar to its ^K_i^ value for interaction with the ‘low-chemokine affinity’ ^NF_G^protein-uncoupled population of CCR5 (44.7 nM). This suggests that native CCR5 agonist chemokines have a low potency to downregulate CCR5 owing to their inability to prevent CCR5 recycling and, above all, to their low affinity for ^NF_G^protein uncoupled CCR5 undergoing endocytosis.

**Discussion**
Our findings indicate that inhibition by native CCR5 chemokines of HIV-1 infection is hindered by a proportion of receptors that exists in a low-chemokine affinity conformation at the target cell surface. This likely explains the discrepancy between the apparently high CCR5 affinities measured previously for native chemokine ligands (2, 20) and their relatively modest potency as entry inhibitors (15-18). Different CCR5 conformations with distinct pharmacological and antigenic properties have been described (27, 29). Here, we found that the apparent affinity of native chemokines and RANTES analogs for CCR5 varies depending on whether $^{125}$I-CCL3 or $^{35}$S-gp120 is used as a tracer in competition experiments (Fig. 1), identifying that distinct receptor populations interact with $^{125}$I-CCL3- and $^{35}$S-gp120-binding receptors. Indeed, we further showed that while high-affinity binding of $^{125}$I-CCL3 requires CCR5 to be coupled to NGF-proteins, $^{35}$S-gp120 binds with the same affinity to both high-chemokine affinity NGF-protein-coupled CCR5 and low-chemokine affinity NGF-protein-uncoupled CCR5. Although native CCR5 agonist chemokines interact with subnanomolar affinities with $^{125}$I-CCL3-binding receptors, they bind to the low-chemokine affinity population of $^{35}$S-gp120-binding receptors with affinities lower than those of primary gp120 (legend of Fig. 3E), thereby contributing to limiting their antiviral potency.

PTX treatments of HIV-1 target cells had no effect on virus entry and replication (Fig. S2), suggesting that similarly to gp120, HIV-1 attachment to CCR5 is independent of G-proteins, in agreement with our previous data showing that the non-G-protein coupling mutant receptor R126N-CCR5 supports HIV entry (30). The observations that CCR5 is constitutively active (19, 27) and that preformed receptor/G-protein complexes exist in living cells (31) suggest that an equilibrium may exist between NGF-protein coupled and uncoupled CCR5 in HIV target cells. On the other hand, NGF-proteins that stabilize high-agonist affinity conformations of CCR5 likely represent a minor fraction of total G-proteins in intact cells (25). In line with this, our observations that PTX does not change the anti-HIV potency of chemokines (Table 1) suggest that high-chemokine affinity NGF-protein-coupled receptors play a minor role in the antiviral activity of chemokines and that low-chemokine affinity NGF-protein-uncoupled CCR5 represent a portal for HIV entry into target cells. Interaction with NGF-protein-uncoupled CCR5 could allow HIV to evade inhibition by the chemokines secreted in the surrounding environment. At the same time, through high affinity interactions with receptors coupled to NGF-proteins, these chemokines would still be capable of activating target cells, facilitating viral replication (32), and recruiting target cells into sites of HIV replication.

While it is commonly accepted that coreceptor downregulation contributes to chemokine inhibition of HIV-1 infection (5-7), we showed that native CCR5 agonist chemokines exhibit a weak ability to downregulate CCR5, as is indicated by EC$_{50}$ values for CCR5 downregulation by the chemokines that are close to their K$_i$ values for interaction with the ‘low-chemokine affinity’ population of CCR5. Preventing CCR5 recycling only modestly increases the ability of CCL4 to downregulate CCR5, but several observations suggest that CCR5 downregulation involves low-affinity interactions of native chemokines with NGF-protein uncoupled CCR5. Indeed, we previously demonstrated that R126N-CCR5 does not trigger G-protein signaling but retains β-arrestin-dependent endocytosis, indicating that both processes are independent functions of CCR5 mediated by different receptor conformations (27). R126N-CCR5 is also altered in its
ability to bind $^{125}$I-CCL3 (Fig. 3D) and $^{125}$I-CCL4 (26), indicating that the $^{Nf}G$-protein-coupled conformation of CCR5 required for high affinity binding of agonist chemokines is distinct from the CCR5 conformation undergoing endocytosis. This conclusion agrees with our present results that PTX that inhibits CCR5/G-protein coupling and high affinity binding of native agonist chemokines preserves CCR5 endocytosis (Fig. 5). Overall, these data support the view that natural agonist chemokines engage low-affinity interactions with internalizing CCR5, hence explaining why they are weak inducers of CCR5 endocytosis and inhibitors of HIV infection.

Structurally different agonists can stabilize distinct receptor conformations with distinct signaling outcomes (33). In particular, ligands referred to as biased ligands differentially stimulate G-protein- and $\beta$-arrestin-dependent signaling pathways (34). Similarly, PSC-RANTES and CCL4 have comparable binding affinities for CCR5 (Fig. 1) and potencies for activating G-proteins in a $^{35}$S-GTP$_{\gamma}$S binding assay (EC$_{50}$ = 4.1±0.9 and 6.3±0.4 nM for CCL4 and PSC, respectively, n = 2), while PSC-RANTES is substantially more potent in internalizing CCR5 (Fig. 5), suggesting that the two ligands stabilize distinct CCR5 conformations. In fact, the EC$_{50}$ value for PSC-RANTES to downregulate CCR5 is roughly equal to its K$_i$ value for interaction with $^{Nf}G$-protein-coupled, $^{125}$I-CCL3-binding CCR5, suggesting that PSC-RANTES preserves high-affinity interactions with internalizing CCR5, despite the fact that these receptors are not coupled to $^{Nf}G$-proteins. It could be that PSC-RANTES stabilizes a $\beta$-arrestin-coupled conformation of CCR5 for which it maintains a high affinity, similarly to other receptors, which are in a high affinity state for agonists when complexed with arrestins (35). Finally, the robust CCR5 downregulation induced by PSC-RANTES explains why the molecule preserves a strong antiviral activity in spite of having a low affinity for gp120-binding receptors. Indeed, preventing CCR5 endocytosis virtually abrogates PSC-RANTES-mediated inhibition of HIV infection (Fig. 2), indicating that steric inhibition of gp120 binding to CCR5 plays a marginal role in the antiviral activity of PSC-RANTES.

The antagonists 5P12- and 2P3-RANTES appeared instead to act solely by potently blocking the interaction between gp120 and CCR5 (Fig. 1). Using these antagonists together with CCR5-internalizing molecules such as PSC-RANTES could in principle represent an interesting therapeutic perspective, albeit no studies have shown yet whether these different analogs have additive inhibitory effects in HIV infection. Interestingly however, we showed here that 6P4-RANTES resembles both 5P12- and PSC-RANTES in that it preserves high affinity for gp120 binding receptors (Fig. 1C) and downregulates CCR5 at nanomolar concentrations. Considered altogether, these results are consistent with 5P12-, 6P4- and PSC-RANTES stabilizing different CCR5 conformations. In line with this, mutations in the transmembrane domains of CCR5 were found to modulate in different ways their ability to inhibit HIV infection, indicating that they have different structural constraints for HIV-1 inhibition (36). Notably, these mutations did not change the ability of the RANTES analogs to inhibit $^{125}$I-CCL3 binding to CCR5 (36), again strengthening the notion that chemokines have different structural requirements for interacting with $^{Nf}G$-protein coupled, CCL3-binding receptors and inhibiting gp120 binding and HIV infection.
Overall, our findings document that both mechanisms whereby native CCR5 chemokines exert their anti-HIV activity, inhibition of gp120/CCR5 interactions and CCR5 downregulation, are strongly limited by virtue of their low-affinity interactions with a proportion of CCR5 conformations. Overcoming these limitations explains why RANTES analogs show improved antiviral potencies as compared to their natural counterparts and should help guide the development of new anti-HIV agents. Finally, these limitations could make it difficult to accomplish the blockade of R5 HIV-1 isolates by chemokines in vivo and contribute to their preferential transmission and propagation in the early stages of infection.

Materials and Methods
Information regarding materials (chemokines, HIV-1 glycoproteins, viruses and cells) and experimental procedures (radioligand binding, chemotaxis, receptor downregulation and infection inhibition assays) is provided in SI materials and Methods.

Acknowledgements
This work was supported by ANRS, SIDACTION, INSERM, Institut Pasteur, the French Government’s Investissement d’Avenir program, Laboratoire d’Excellence “Integrative Biology of Emerging Infectious diseases” (Grant n°ANR-10-LABX-62-IBEID) and the Spanish Ministry of Economy and Competitiveness (FIS PI 080752). OH acknowledges support from the Swiss National Science Foundation. YB, YW and NG were supported by fellowships from SIDACTION, ANRS and the Spanish AIDS Research Network (ISCIII-RETIC RD06/0006), respectively.

Author contributions
P.C., Y.B., I.S., Y.W. and N.G. performed experiments and analyzed data. O.H., J.A. and A.B. contributed materials and technical support. F.A.S. and B.L. designed research and analyzed data and B.L. wrote the manuscript. All authors reviewed and edited the manuscript.

References


## Table 1. CCR5 binding affinity constants of native chemokines and RANTES analogs and their half-maximal inhibitory concentrations (IC\textsubscript{50}) of HIV-1 infection in CD4\textsuperscript{+} T-cells and HeLa P4C5 cells

<table>
<thead>
<tr>
<th>Chemokine</th>
<th>K\textsubscript{0} (nM)</th>
<th>K\textsubscript{D} (nM)</th>
<th>K\textsubscript{i} (nM)</th>
<th>- Gpp(NH)p</th>
<th>+ Gpp(NH)p</th>
<th>T-CD4\textsuperscript{+}</th>
<th>+ PTX</th>
<th>+ PTX</th>
<th>+ PTX</th>
<th>+ PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCL3</td>
<td>0.25 ± 0.05</td>
<td>0.06 ± 0.00</td>
<td>0.58 ± 0.04</td>
<td>43 ± 0.75</td>
<td>106.9</td>
<td>143 &gt; 1000</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL4</td>
<td>0.37 ± 0.1</td>
<td>0.21 ± 0.1</td>
<td>4.5 ± 1.8</td>
<td>100 &gt; 1000</td>
<td>6.7 &gt; 1000</td>
<td>444 366</td>
<td>99 99</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL5</td>
<td>0.05 ± 0.15</td>
<td>3 ± 1.1</td>
<td>341 ± 8.2</td>
<td>1.13 ± 0.18</td>
<td>-</td>
<td>- &gt; 1000</td>
<td>&gt; 1000</td>
<td>&gt; 1000</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CCL7</td>
<td>-</td>
<td>- 31.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- 31.7</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AOP</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- 1.13</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PSC</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- 1.13</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2P3</td>
<td>-</td>
<td>- 0.31</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- 0.31</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5P12</td>
<td>-</td>
<td>- 0.03</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- 0.03</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6P4</td>
<td>-</td>
<td>- 0.055</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>- 0.055</td>
<td>-</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

K\textsubscript{D} and K\textsubscript{i} represent the equilibrium dissociation constants for interaction of chemokines with CCR5 determined in competition assays using either \textsuperscript{125}I-CCL3 or \textsuperscript{35}S-gp120 as a tracer, respectively. K\textsubscript{0} values are the equilibrium dissociation constants of radiolabeled chemokine-CCR5 complexes deduced from saturation binding experiments. (a) The reported K\textsubscript{D} value is from ref. (20). (b) The reported K\textsubscript{i} values for interaction of CCL4 with \textsuperscript{35}S-gp120-binding CCR5 in the presence of Gpp(NH)p is from ref. (19). Except for inhibition of infection of T-cells by CCL3 or 6P4 in the presence of PTX, which was performed once, values represent means ± SD of at least three independent determinations. The independent experiments in CD4\textsuperscript{+} T-cells represent experiments run in cells obtained from different donors. Und, undetectable.
Fig. 1. Chemokine- and gp120-binding CCR5 represent different receptor populations. Binding of 0.1 nM $^{125}$I-CCL3 (A) or 10 nM $^{35}$S-gp120$_\text{Ba08}$ (in the presence of 30 nM soluble CD4) (B, C and D) was displaced by increasing amounts (A, C and D) or a 100 nM concentration (B) of unlabeled chemokines. Results were normalized for non-specific binding (0%) and specific binding in the absence of competitors ($B_0$, 100%) and fitted according to a one-site (panel A, CCL7, 6P4, 2P3 and 5P12 in panels C and D) or a two-site ($16.2 < F$ value $< 93.3$ with $p < 0.0001$ for CCL3 in C and CCL4 and PSC in D) competitive binding model. In panel B, data points are means ± SEM of 5 independent determinations. Panels A, C and D show representative experiments out of 3-5 performed independently.
Fig. 2. Steric inhibition of gp120 binding to CCR5 does not contribute to the anti-HIV-1 activity of PSC-RANTES. HeLa P4C5 cells were incubated with MVC, 5P12-or PSC-RANTES at 37 or 4 °C before being infected by Bx08Ren viruses and treated as indicated in the text. Results represent the luciferase activity in the cell lysates, expressed as relative light units (RLU). A representative experiment out of 5 independent determinations is shown. Uninfected cells (NI) served as negative controls in those experiments.
**Fig. 3.** CCR5 coupling to $^\text{N}^\text{F}$-G-proteins differentially influences native agonist chemokine and gp120 binding.

(A) Total binding of 0.2 nM $^{125}$I-CCL3 to 5.10$^5$ A3.01-R5 cells (left panel) or membranes from CD4$^+$ T-cells (15 µg of proteins) (right panel) was measured in the presence or absence (control) of GTP$\gamma$S or Gpp(NH)p or after treatment of cells with PTX. Non specific binding was determined using the antagonist TAK779 or MVC. *, $p < 0.05$; **, $p < 0.01$ as compared to controls in unpaired two-tailed Student’s $t$ test. Panels (B) and (C) are saturation experiments of $^{125}$I-CCL3 and $^{125}$I-CCL5 binding to HEK-R5 cell membranes, respectively. Specific binding was measured in the presence or in the absence of Gpp(NH)p. Total binding of 0.1 nM $^{125}$I-CCL3 (D) or 10 nM $^{35}$S-gp120 from the HIV-1 strains 25, 34 or Bx08 in complex with sCD4 (E) to membranes from HEK 293T cells expressing WT-CCR5 or R126N-CCR5 (R/N) was measured in the presence or absence (control) of Gpp(NH)p and/or MVC (nonspecific binding). Equal amounts of WT-CCR5 and R126N-CCR5 at the cell surface were confirmed by flow cytometry. Saturation binding experiments of $^{35}$S-gp120/sCD4 complexes revealed $K_D$ values (in nM) of 7.5, 8.3 and 9.9 for gp120$_{25}$, gp120$_{34}$ and gp120$_{Bx08}$, respectively. (F) Displacement of $^{35}$S-gp120$_{Bx08}$ binding by CCL3 was measured in the absence or presence of Gpp(NH)p. Data were fitted according to a two-site competitive binding model ($F = 42$ with $p < 0.0001$ and $F = 5.5$ with $p = 0.0062$ for data in the absence and in the presence of Gpp(NH)p, respectively). Panels show representative experiments out of at least 3 performed independently.
Fig. 4. Chemokine-mediated inhibition of HIV-1 infection, but not chemotaxis, is independent of G-proteins. PTX-treatment impaired PSC-RANTES-mediated chemotaxis of A3.01-R5 cells (A) but not the ability of the chemokine analog to inhibit infection of these cells by the Bx08Ren viruses (B). Panels show representative experiments out of at least three independent experiments.
Fig. 5. Native chemokines and RANTES analogs with agonist activity induce CCR5 downregulation in a G-protein independent manner. Native chemokines have a low potency in internalizing CCR5. (A-D) Cell surface expression of FLAG-tagged receptors stably expressed in HEK 293 cells was detected by flow cytometry as indicated in SI Materials and Methods. (A) CCR5 cell surface expression level (in Mean Fluorescence Intensity) was measured after stimulation or not by 300 nM PSC-RANTES (90 min, 37 °C) of cells treated or not by PTX. In panels (B) and (C), CCR5 downregulation is expressed as percent of maximum effect induced by PSC- or 6P4-RANTES, which showed equal potency (Table 2) and efficacy (panel (A)) in the assay. In panel (B), cells were stimulated by chemokines for 90 min at 37 °C. Data were fitted to a sigmoidal dose-response model with a variable slope, with bottom and top values constrained to equal 0 % and 100 %, respectively. Panel (C) shows time-dependent downregulation of CCR5 induced by 100 nM PSC-RANTES or CCL4. Data were analyzed using a one-phase exponential association function. In panel (D), the expression level of either WT- or 349-CCR5 at the surface of cells stimulated by CCL4 or PSC-RANTES (90 min, 37 °C) is expressed as percent of receptor expression level at the surface of untreated cells (100 %). Results are representative of two (C) or three (A, B and D) independent experiments.
Supporting information

Colin et al.

SI Materials and Methods

**Ligands, Cells and Viruses.** Radioactive chemokines were from PerkinElmer Life Sciences. Dr F. Baleux (Institut Pasteur, Paris) provided CCL4. CCL5 and RANTES analogs were produced as described in ref. (22). CCL3 and CCL7 were purchased from R&D systems (Minneapolis, MN). Recombinant soluble human CD4 (sCD4) was from Protein Sciences Corp., Meriden, CT. Soluble, monomeric HIV-1 gp120 were produced using a Semliki forest virus (SFV) expression system and metabolically labeled with $^{35}$S-Met/Cys as in ref. (19), purified by affinity chromatography on Strep-Tactin columns (IBA, Gottingen, Germany) using the One-STrEP-tag fused to the gp120 C-terminus as a bait, and quantified by Coomassie blue staining using BSA as a standard. Gp120$_{Bx08}$ was previously described (19). The sequences coding for gp120$_{25}$, gp120$_{34}$, gp120$_{50}$ and gp120$_{58}$, cloned into the SFV-derived expression vector pSFV2 or the pNL4-3Ren plasmid (v,i), were isolated from biological virus clones (clones 341.14 5C6, 341.75 6C4, 1031.20 8C1 and 1031.65 9D8, respectively) obtained from PBMCs of two individuals (patients # 341 and 1031) of the Amsterdam Cohort studies on HIV-1 and AIDS (a gift from Dr H. Schuitemaker, University of Amsterdam, The Netherlands). PBMCs were collected 30 (gp120$_{25}$), 128 (gp120$_{34}$), 22 (gp120$_{50}$) and 91 (gp120$_{58}$) months after seroconversion at CD4$^+$ T-cell counts of 630, 90, 520 and 50 cells/$\mu$L, respectively.

A3.01.R5 cells and HEK cells stably expressing WT-CCR5 (HEK-R5 cells), R126N-CCR5, FLAG-tagged WT- or 349-CCR5 were described previously (19, 27, 28, 37). CD4- and CCR5-expressing HeLa P4C5 cells were cultured at 37 °C under CO$_2$ in DMEM supplemented with 10 % FCS, 100 U/mL penicillin, 100 µg/mL streptomycin, 1 mg/mL G418 and 300 µg/mL hygromycin B. Human CD4$^+$ T-lymphocytes were purified from PBMCs of healthy blood donors (Etablissement Français du sang, The French Official Blood Bank) by Ficoll centrifugation (PAA) followed by immunomagnetic selection (Miltenyi Biotec) and maintained in phytohemagglutinin (1 µg/mL)- and interleukin 2 (300 IU/mL)-containing RPMI-1640 medium at 37 °C under CO$_2$ before use.

The pBx08Ren and pJRRen plasmids were described previously (19, 38). The plasmids p25Ren, p34Ren, p50Ren and p58Ren were generated from the pNL4-3Ren plasmid previously described (38). Using the technique of overlapping PCR, the fragment from residue 34 to 480 in the NL4-3 gp120 was replaced by the corresponding region from gp120$_{25}$, gp120$_{34}$, gp120$_{50}$ or gp120$_{58}$. The sequences of primers used are available under request. Protocols for virus production in HEK 293T cells and quantification (p24) are described elsewhere (19).

In this work, guanosine 5’-0-(γ-thio)triphosphate (GTP$_{7}$S, from Sigma, St Louis, MO)) and Guanosine 5’-[$\beta,\gamma$-imido]triphosphate (Gpp(NH)p) were used at 200 and 100 µM, respectively. Treatments of cells with
the toxin from *Bordetella pertussis* (PTX) (Sigma) were carried out overnight at a 100 ng/mL concentration. The M2 anti-Flag monoclonal antibody is from Sigma (St Louis, MO).

**Radioligand Binding Assays.** Protocols for membrane preparations and $^{125}$I-CCL3 binding studies to cell membrane preparations or intact cells were described previously (19). $B_{\text{max}}$ determinations for $^{125}$I-CCL3 and $^{35}$S-gp120 in intact HEK-R5 cells and membrane preparations from these cells were previously carried out (19). For saturation binding of $^{125}$I-CCL5, CCR5-expressing membranes (1 µg of proteins/well) and the radioligand were incubated in 96-well basic Flashplates (PerkinElmer Life Sciences) for 90 min at room temperature, in the presence or in the absence of Gpp(NH)p and/or 10 µM MVC (from the AIDS Research and Reference Reagent Program) in a 0.1 mL final volume of binding buffer (19). Bound and unbound $^{125}$I-CCL5 were separated by centrifugation (800 x g, 10 min) at 4 °C and removal of supernatants. Displacement of $^{35}$S-gp120 in the presence of 30 nM sCD4 was performed as in ref. (19) except that incubations were done in Eppendorf tubes. To remove unbound $^{35}$S-gp120, membranes were pelleted at 4 °C (16,000 x g, 5 min) and then washed once with washing buffer (50 mM Hepes, pH 7.4, 5 mM MgCl$_2$, 1 mM CaCl$_2$ and 500 mM NaCl). Pellets were resuspended in Optiphase Supermix scintillation liquid and radioactivity was counted in a Wallac 1450 MicroBeta Trilux® (PerkinElmer Life Sciences).

Analysis of the binding data was made using the Prism software (GraphPad Software Inc., San Diego). The inhibition constants $K_i$ of the competing chemokines were calculated according to the Cheng and Prusoff equation $K_i = [IC_{50}/(1+L/K_D)]$, where $L$ is the concentration of the radioligand; $K_D$ is the dissociation constant of the radioligand-CCR5 complex, and $IC_{50}$ is the concentration of competing ligand displacing 50% of the specific binding of the radioligand (39). In competition experiments, an $F$ test was used to determine whether the experimental data fitted better to a one-site or a two-site competitive binding model.

**Chemotaxis assays.** A3.01.R5 cells (1.5x10$^5$) in 0.08 mL of assay medium (RPMI-1640 supplemented with 20 mM HEPES and 1 % human serum AB) prewarmed at 37 °C were added to the upper chambers of HTS-Transwell-96 Well Permeable Supports with polycarbonate membrane of 5 µm pore size (Corning Inc.), and 0.235 mL of the same medium with or without (spontaneous migration) PSC-RANTES was added to the lower chambers. Chemotaxis proceeded for 6 h at 37 °C in humidified air with 5 % CO$_2$. The number of cells migrating across the polycarbonate membrane was assessed by flow cytometry with a FACSCanto (BD Biosciences). Specific migration was calculated by subtracting spontaneous migration from the number of cells that migrated toward the chemokine.

**Receptor downregulation.** HEK cells stably expressing either WT-CCR5 or 349-CCR5 fused to a FLAG epitope at their N-terminal end, pretreated or not overnight with 100 ng/mL PTX, were detached from culture plates in EDTA-containing PBS, and then incubated at 37 °C in conical-bottom 96-well plates (5x10$^5$ cells/well) for the indicated periods of time in 0.25 mL final volume of assay medium (DMEM supplemented with 20 mM HEPES and 1 % BSA), in the presence or in the absence of the indicated concentrations of chemokines. Cells were then placed on ice, centrifuged (200 x g, 4 °C) and washed once in cold PBS
supplemented with 1% BSA and 0.1% sodium azide. Cells were then incubated for 1 h at 4°C in 0.2 mL of PBS/BSA buffer containing the anti-FLAG M2 monoclonal antibody (Sigma) at 2 µg/mL, washed once, and then further incubated with a horse anti-mouse secondary antibody conjugated to phycoerythrin (5 µg/mL) (Vector). We controlled that chemokines and M2 did not compete for binding to Flag-tagged CCR5. The amount of receptors remaining at the cell surface was assessed by flow cytometry using a FACS Canto (BD Biosciences) and expressed as indicated in the legend of the Fig. 5.

**Infection inhibition assays.** HeLa P4C5 cells were cultured in flat-bottom 96-well plates (1.5x10^4 cells/well) for 24 h and then treated, or not, overnight with PTX (100 ng/mL). Unless otherwise mentioned, cells were then infected with 7 ng of p24 of each virus in the presence or in the absence of chemokines and/or PTX at 10 ng/mL. Regarding activated CD4+ T-cells and A3.01.R5 cells, 2x10^5 cells treated, or not, overnight with PTX (100 ng/mL) were dispensed in round or conical-bottom 96-well plates and then incubated with the viruses (2 ng or 7 ng of p24 for A3.01.R5 cells and CD4+ T-cells, respectively) in the presence or in the absence of the indicated concentration of chemokines and/or PTX at 10 ng/mL. Incubation of CD4+ T-cells was carried out in the presence of IL-2 at 300 IU/mL. At 48 h post-infection, cells were lysed, and viral replication was assessed by measuring luciferase activity (PROMEGA, Madison, WI), using the 96-well plate lumi/fluorimeter Mithras LB940 (Berthold).

**SI References**

7. Cheng Y & Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* 22(23):3099-3108.
Table S1. Half-maximal effective concentrations (EC<sub>50</sub>) for chemokine-induced downregulation of WT- or 349-CCR5 in HEK 293 cells treated or not by PTX

<table>
<thead>
<tr>
<th></th>
<th>CCL3</th>
<th>CCL3+PTX</th>
<th>CCL4</th>
<th>CCL4+PTX</th>
<th>PSC</th>
<th>PSC+PTX</th>
<th>6P</th>
<th>6P+PTX</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-CCR5</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>306±229</td>
<td>386±112</td>
<td>3.9±1.3</td>
<td>3.3±0.7</td>
<td>3.9±0.02</td>
<td>4.5±0.5</td>
</tr>
<tr>
<td>349-CCR5</td>
<td>-</td>
<td>-</td>
<td>47±23</td>
<td>-</td>
<td>2.6±0.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. S1. Native chemokines show weak anti-HIV-1 activities. CD4⁺ T-lymphocytes (A) or HeLa P4C5 cells (B) were inoculated with Bx08Ren viruses in the presence or in the absence of increasing concentrations of the indicated native chemokines or chemokine analogs. Results are expressed as percent infection relative to control infection measured in the absence of chemokines (100%) and were fitted to a sigmoidal dose-response model with a variable slope. Representative experiments out of at least 3 independent experiments performed in triplicate are shown.
**Fig. S2.** PTX treatment of activated CD4$^+$ T-cells (A-C) and HeLa P4C5 (D-F) cells neither influences HIV-1 infectivity nor the anti-HIV-1 activity of chemokines. Cells treated (C and F) or not (B and E) by PTX were infected as in Fig. S1 using 10 ng of p24 of the Bx08Ren, JRRen, 25Ren, 34Ren, 50Ren or 58Ren viruses in the absence (A and D) or in the presence of 10 nM CCL4 or 5P12-RANTES (B-C and E-F). Panels A and D are representative experiments. Panels B, C, E and F represent means ± SEM of four independent experiments performed in triplicate. For experiments in CD4$^+$ T-cells, cells obtained from two different donors were used.
**Fig. S3.** G-protein dependent high-affinity binding of agonist chemokines to CCR5 is dispensable for chemokine-mediated inhibition of infection. PTX-treatment impaired specific binding of 0.1 nM $^{125}I$-CCL3 to HeLa P4C5 cells (A) but not the ability of chemokines to inhibit infection of these cells (B). In Panel A, results represent means ± SEM of eight independent experiments (***, $p < 0.001$ as compared to the untreated control in unpaired two-tailed Student’s $t$ test). The other panel shows representative experiments out of at least three independent experiments.